ACTIVE POLYPEPTIDES OF THE AMPHIBIAN SKIN AND THEIR SYNTHETIC ANALOGUES

V. ERSPAMER and P. MELCHIORRI

Istituto di Farmacologia Medica I, Università di Roma, Città universitaria, I-00185 Roma, Italy

ABSTRACT

Amphibian skin represents an enormous store-house of biogenic amines and active peptides. So far four groups of peptides have been identified:

(a) *physalaemin-like peptides* (physalaemin, phyllomedusin and uperolein) possessing an intense action on vascular and extravascular smooth muscle as well as a potent action on lachrymal and salivary glands. Physalaemin is the most potent hypotensive agent so far described.

(b) *bradykinin-like peptides* (authentic bradykinin, phyllokinin, Val^1-Thr^5-bradykinin) displaying the well known effects on calibre and permeability of the capillaries.

(c) *caerulein-like peptides* (caerulein, phyllocaerulein) reproducing on the smooth muscle of the gall bladder and the gut, and on the exocrine secretions of the stomach, the pancreas, and the liver, all the actions of the intestinal hormone cholecystokinin-pancreozymin. Like this hormone, caerulein-like peptides also stimulate the secretion of insulin, glucagon and calcitonin.

(d) *bombesin-like peptides* (bombesin, alytesin, ranatensin) possessing a broad spectrum of activity on vascular and extravascular smooth muscle, on gastric acid secretion, and on the kidney, with potent activation of the renin–angiotensin system and stimulation of erythropoietin release.

With the exception of uperolein and phyllomedusin all the above peptides have been reproduced by synthesis together with a number of peptides or peptide fragments similar to the natural models. This has permitted some conclusions concerning the problem of structure–activity relationships.

Peptides of the amphibian skin are very similar or identical in their structure to active peptides occurring in mammalian tissues (substance P, bradykinins, cholecystokinin, gastrins). Hence their conspicuous interest, transcending the field of comparative pharmacology and biochemistry. It is possible that active amino acid sequences first discovered in amphibian skin may lead to the discovery of similar sequences, i.e. of new biochemical messengers, in mammalian tissues.

INTRODUCTION

The amphibian skin may be considered, as repeatedly stated[1], a store-house of biogenic amines and active polypeptides.

From the studies carried out by our research group during the past fifteen years on this exceptionally interesting and rich material some fundamental facts have emerged, which may be now regarded as firmly established.

An observation of substantial value is that all or nearly all amines and peptides found in amphibian skin have their counterpart in mammalian tissues, where they usually occur in a much lesser variety and concentration. Hence results obtained in the study of amphibian skin are of an interest transcending comparative pharmacology and biochemistry, as they may substantially contribute to the understanding and interpretation of facts assessed in mammals and may offer the basis for new research trends in higher vertebrates.

The active polypeptides so far detected in the amphibian skin may be divided into five groups characterized by distinctive features: physalaemin-like polypeptides or tachykinins, bradykinin-like polypeptides or bradykinins, caerulein-like polypeptides, bombesin-like polypeptides, and finally, miscellaneous polypeptides, a residual group in which those peptides are provisionally placed which still await elucidation of their structure or a sufficiently complete pharmacological study.

PHYSALAEMIN-LIKE PEPTIDES

At present, this polypeptide group is represented in the amphibian skin by the following three members:

(i) physalaemin, the prototype of the group, first isolated in a pure form from methanol extracts of the skin of *Physalaemus bigilonigerus* (*fuscumaculatus*) and present in skin extracts of other *Physalaemus* species as well (*Physalaemus centralis*, *Physalaemus bresslaui*). The content of physalaemin ranged between 370 and 700 μg per g dry skin[2, 3].

(ii) phyllomedusin, isolated from methanol extracts of the skin of the Amazonian hylid frog *Phyllomedusa bicolor* (1100 μg per g fresh skin)[4].

(iii) uperolein, found in the skin of Australian amphibians belonging to the genera *Uperoleia* and probably also *Taudactylus*. The elucidation of the structure of uperolein is in progress[5].

Two important physalaemin-like peptides occur outside the amphibian skin: eledoisin and substance P. The first is present, in large amounts (100 μg per g fresh tissue), in the posterior salivary glands of the Mediterranean octopod *Eledone moschata*[6]; the second is a polypeptide or, more likely, a family of polypeptides occurring in the brain and intestinal wall of vertebrates.

One substance P has been recently isolated from the bovine hypothalamus and, after elucidation of its structure[7], it has been reproduced by synthesis[8]. Formulae for these peptides are presented below.

Physalaemin	Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2
Phyllomedusin	Pyr——Asn–Pro–Asn–Arg–Phe–Ile–Gly–Leu–Met—NH_2
Eledoisin	Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met—NH_2
Substance P	Arg–Pro–Lys–Pro–Gln–Arg–Phe–Phe–Gly–Leu–Met–NH_2

From the amino acid sequences reported above it may be seen that all the four physalaemin-like peptides so far isolated in a pure form have in common the C-terminal tripeptide and the phenylalanine residue in position 5 from the C-terminus.

The promptness of their stimulant action on smooth muscle, has suggested the denomination of *tachykinins* for this polypeptide group, as opposed to the group of slow-acting kinins, the true bradykinins[1].

The tachykinins more thoroughly studied from a pharmacological point of view are eledoisin and physalaemin, which display the following fundamental actions:

(a) potent vasodilating and hypotensive action in most animal species, including man. The action is a direct one on the vascular smooth muscle. Threshold intravenous doses of physalaemin in the dog were of the order of 0.1 to 0.5 ng kg^{-1}, and a dose one million times greater could be tolerated by the animal with full recovery after 5 to 6 hours, which seems to be a unique example of tolerability. The polypeptide was very effective in antagonizing the pressor effects of catechol amines, nicotine and angiotensin in the dog. When given in the same quick intravenous injection 0.5 μg physalaemin completely abolished the hypertensive effect of 50–75 μg L-noradrenaline, 2 mg nicotine bitartrate and 10 μg angiotensin[9].

The vascular beds most sensitive to physalaemin and eledoisin in the dog were those of the hind limb musculature (minimal dose active by close intra-arterial injection < 1 pg), and the coronaries (threshold 10 pg)[10,11]. Eledoisin infused locally into a branch of the left coronary artery of the dog at a rate of 8 ng kg^{-1} min^{-1} increased coronary sinus outflow by 20 per cent, coronary sinus oxygen tension by 10 per cent, and also increased stroke flow and cardiac oxygen consumption without affecting mean blood pressure and heart rate. Intravenous infusions of eledoisin were almost as effective in decreasing coronary vascular resistance, as were intracoronary infusions[12].

The blood vessels of the skin were less reactive than blood vessels in muscle. Renal and mesenteric vascular beds did not respond significantly to the tachykinins[10].

The following percentage changes in a number of cardiovascular parameters following intravenous injection of 4 ng kg^{-1} of physalaemin in the dog have been reported by Nakano, Darrow and McCurdy[13]: heart rate + 17.8, mean systemic arterial pressure −22.5, mean pulmonary arterial pressure +1·8, mean left atrial pressure −1, mean right atrial pressure +0.5, myocardial contractile force + 17.5, cardiac output + 52, total peripheral resistance −65.2, pulmonary vascular resistance −35.4.

Infusions of 0.6 μg min^{-1} of eledoisin in normal human subjects produced a transient fall in mean arterial blood pressure, tachycardia, marked increase in cardiac index, increase in stroke volume and fall in systemic vascular resistance. Blood flow to hand and forearm increased. More marked hypotension and tachycardia and a more pronounced increase in hand and forearm blood flows were observed during infusions of 2 to 5 μg min^{-1} of eledoisin. Intravenous infusions of 0.6 μg min^{-1} of eledoisin could be tolerated easily by all subjects studied, in spite of the occurrence of generalized intense erythema, burning of the eyes, throbbing in the head and dizziness. These results indicate that even in man eledoisin is a powerful dilator of vessels in skin and skeletal muscle and quite probably of vessels in other vascular areas[14].

(b) powerful stimulation of the salivary and lachrymal secretions in the rat, dog, man and hen by a direct effect on the secretory cells[15,16]. Response

by the lachrymal glands could be obtained not only by systemic administration but also by instillation of the polypeptide into the conjunctival sac[17].

In the salivary glands of the dog physalaemin, besides stimulating the acinous secretory cells (threshold by intravenous injection 0.5 to 1.75 μg kg^{-1}), potently stimulated myoepithelial cells in the salivary ducts. Intravenous doses of physalaemin necessary to elicit a pressure rise in the submaxillary and parotid ducts of dogs were 100 times less than those active on salivary secretion[18].

Electrolyte and amylase concentrations in rat saliva which was evoked by physalaemin were virtually identical to those found in saliva evoked by stimulation of post-ganglionic nerve fibres[19].

The secretagogue effect of physalaemin in the dog pancreas was barely 1 per cent of that of caerulein[20].

(c) intense spasmogenic action on a number of isolated preparations of extravascular smooth muscle, among which the rabbit large intestine (threshold 0.2 to 1 ng ml^{-1}), the guinea-pig ileum (0.5 to 2 ng ml^{-1}), the human Fallopian tube (10 to 20 ng ml^{-1}), the rat urinary bladder (0.5 to 2 ng ml^{-1})[3, 21, 22].

On the *in situ* jejunal loops of the anaesthetized dog, physalaemin was twice as potent as cholecystokinin, on a molar basis, 15 times as potent as human gastrin I, 50 to 100 times as potent as either bradykinin or carbachol, and more than 300 times as potent as acetylcholine, eserine, histamine, vasopressin and 5-HT. Only caerulein overcame physalaemin in its stimulant effect, by three times[23].

Other extravascular smooth muscle preparations were poorly sensitive to the polypeptide: rat uterus, rat colon, dog, cat and rabbit urinary bladder[3, 22].

(d) positive action on capillary permeability in the guinea-pig, rat and man[24, 25]. By intradermal administration eledoisin caused in man pain, local oedema and erythema at doses above 1 ng. However, the polypeptide failed to elicit any pain response when injected intraperitoneally, subcutaneously or intramuscularly into human subjects at doses of 17 μg, 34 μg and 50 μg, respectively[26].

Eledoisin was removed from the circulation mainly by the kidney. The half-life of the polypeptide in the circulation was calculated to be less than 30 seconds. However it seemed that eledoisin was being bound to some constituents of the tissues, without actual destruction, and after the infusion of eledoisin stopped, it was being leached into the circulation again, thereby maintaining the blood levels for a longer time than expected[27].

Eledoisin has been reported to increase blood flow and lower peripheral resistance in a number of patients suffering from peripheral vascular diseases[28–31]. Physalaemin, in its turn, gave apparently satisfactory results in the treatment of the Sjögren syndrome and similar morbid conditions, characterized by a defect in lachrymal and salivary secretions[32].

The relative potency, on several preparations, of the natural tachykinins physalaemin, phyllomedusin and eledoisin is shown in *Table 1*.

It may be seen that whereas distinction, by parallel bioassay, of physalaemin from phyllomedusin was difficult or even virtually impossible (dog urinary bladder?), the distinction of physalaemin from eledoisin was rather

Table 1. The relative potency, on twelve preparations, of physalaemin, phyllomedusin and eledoisin (physalaemin = 100)

Test preparation	Phyllomedusin	Eledoisin
Dog blood pressure	40–70	25–30
Rat salivary secretion	100–200	not tested
Rabbit large intestine	60–150	30–75
Guinea-pig ileum	30–80	30–70
Rat duodenum	70–150	1 000
Rat colon	100–150	1 000–3 000
Rabbit uterus	100–350	1 000
Hamster urinary bladder	200	10 000
Rat urinary bladder	80–120	30–100
Dog urinary bladder	600–800	5 000
Monkey urinary bladder	100	3 000–5 000

easy, because some indexes of discrimination between the two peptides were very high[33].

More than 150 physalaemin- and eledoisin-like peptides have been synthesized in an attempt to elucidate the problem of the relationship between chemical structure and biological activity, and to dissociate the main pharmacological actions peculiar to the tachykinins[34–42].

Table 2 illustrates the relative biological activity of some physalaemin-like and eledoisin-like peptides. The activity of physalaemin was considered equal to 100 and that of the other compounds was expressed in terms of this activity.

From the data shown in the *Table* and from the more numerous data reported in the pertinent publications, the following conclusions may be drawn:

(i) By means of a progressive elimination of the N-terminal amino acid residue up to the C-terminal hexapeptide it was possible to reduce considerably the size of the physalaemin molecule, without consistently reducing the hypotensive action. The spasmogenic effect on the rabbit and guinea-pig intestine could even be conspicuously increased. A minimum of five amino acid residues was necessary in order to have an appreciable activity (0.3 to 1 per cent). The C-terminal hexapeptide of physalaemin had 50 per cent of the hypotensive action of the parent polypeptide, and approximately the same spasmogenic action on the guinea-pig ileum. However, the sialagogue activity was barely 3 per cent of that of physalaemin, and even the C-terminal octapeptide had only 30 per cent of the sialagogue activity of the parent endecapeptide[42].

(ii) The terminal amide group was apparently not essential for biological activity. In the C-terminal hexapeptide of eledoisin methionine nitrile could replace the methioninamide residue with no loss of hypotensive activity. However Met-NH_2 could not be replaced by Met-$N(CH_3)_2$.

(iii) Whereas methioninamide could not be replaced by other naturally occurring amino acids, it could be substituted, even with advantage, by non-natural synthetic sulphur-containing amino acids, such as ethioninamide and a variety of alkylhomocysteinamide residues.

Table 2. Relative potency of physalaemin-like peptides, on a weight basis (physalaemin = 100)

	Peptide	Test preparation		
		Dog blood pressure	Rabbit large intestine	Guinea-pig ileum
1	Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2 Physalaemin	100	100	100
2	Pyr–Pro– Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2 Eledoisin	22	60	50
3	Pyr——— Asn–Pro–Ans–Arg–Phe–Ile–Gly-Leu–Met–NH_2 Phyllomedusin	40–70	60–150	30–80
4	Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	70	125–270	80
5	Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	35	60	70
6	Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	80–85	100	n.t.
7	Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	15	70	35
8	Lys–Phe–Tyr–Gly–Leu–Met–NH_2	50–85	500–750	130
9	Phe–Tyr–Gly–Leu–Met–NH_2	0.3	1.2	0.5
10	Orn–Phe–Tyr–Gly–Leu–Met–NH_2	45	400	80
11	His–Phe–Tyr–Gly–Leu–Met–NH_2	18	55	25
12	Arg–Phe–Tyr–Gly–Leu–Met–NH_2	45	n.t.	50
13	Ala–Phe–Tyr–Gly–Leu–Met–NH_2	5	50–100	20
14	Lys–Phe–Phe–Gly–Leu–Met–NH_2	50	n.t.	50
15	Lys–Phe–*m*-Tyr–Gly–Leu–Met–NH_2	45	900	105
16	Lys–Phe–Tyr(OMet)–Gly–Leu–Met–NH_2	55	250	130
17	Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Ile–Gly–Leu–Met–NH_2	20	80	50
18	Ala–Asp–Pro–Asn–Lys–Phe–Ile–Gly–Leu–Met–NH_2	55	120	75
19	Pro–Asp–Lys–Phe–Ile–Gly–Leu–Met–NH_2	80	330	115
20	Lys–Phe–Ile–Gly–Leu–Eti–NH_2	45–90	380	155
21	Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2	6–7	35	18
22	Ala–Phe–Ile–Gly–Leu–Met–NH_2	3–4	18	7
23	Ser–Phe–Ile–Gly–Leu–Met–NH_2	3–4	33	12
24	Phe–Phe–Ile–Gly–Leu–Met–NH_2	4–5	35	30
25	Pro–Phe–Ile–Gly–Leu–Met–NH_2	10	35	10
26	Met–Phe–Ile–Gly–Leu–Met–NH_2	4–5	55	30
27	Ala–Trp–Ile–Gly–Leu–Met–NH_2	<0.5	2.5	1.5
28	Ala–Ala–Ile–Gly–Leu–Met–NH_2	<0.2	<0.2	<0.01
29	Ala–Phe–Ile–Ala–Leu–Met–NH_2	4.5	40	22
30	Ala–Phe–Ile–Phe–Leu–Met–NH_2	<0.1	<0.3	<0.25
31	Ala–Phe–Ile–Sar–Leu–Met–NH_2	5	35	28
32	Ala–Phe–Ile–Gly–Ile–Met–NH_2	<0.02	<0.1	<0.05
33	Ala–Phe–Ile–Gly–Ser–Met–NH_2	<0.04	<0.15	<0.05
34	Ala–Phe–Ile–Gly–Ala–Met–NH_2	<0.05	2	<0.05
35	Ala–Phe–Ile–Gly–Leu–(*S*-methyl)Hcys–NH_2	3	18	8
36	Ala–Phe–Ile–Gly–Leu–(*S*-ethyl)Hcys–NH_2	18	130	40
37	Ala–Phe–Ile–Gly–Leu–(*S*-propyl)Hcys–NH_2	20	180	30
38	Ala–Phe–Ile–Gly–Leu–(*S*-benzyl)Hcys–NH_2	5	65	40
39	Ala–Phe–Ile–Gly–Leu–(*S*-methyl)Cys–NH_2	<0.02	<0.1	<0.05
40	Ala–Phe–Ile–Gly–Leu–(*S*-ethyl)Cys–NH_2	<0.2	3	1
41	Ala–Phe–Ile–Gly–Leu–(*S*-benzyl)Cys–NH_2	<0.2	3	<0.5
42	Ala–Phe–Ile–Gly–Leu–Eti–NH_2	15–22	130	45
43	Lys–Phe–Ile–Gly–Leu–Eti–NH_2	45	360	80
44	Ala–Phe–Ile–Gly–Leu–Met–$N(CH_3)_2$	<0.05	<0.1	<0.05
45	Asp–Ala–Phe–Ile–Gly–Leu–Ala–NH_2	n.t.	n.t.	2

Table 2.—continued

	Peptide	Test preparation Dog blood pressure	Rabbit large intestine	Guinea-pig ileum
46	Asn–Ala–Phe–Ile–Gly–Leu–Ala–NH_2	n.t.	n.t.	1
47	Asn–Lys–Phe–Tyr–Gly–Leu–Gln–NH_2	n.t.	n.t.	0.3
48	BOC–Ala–Phe–Ile–Gly–Leu–Met–NH_2	5	110	80
49	BOC–Asn–Phe–Ile–Gly–Leu–Met–NH_2	3	65	25
50	(D)Asn–Phe–Ile–Gly–Leu–Met–NH_2	5	40	25
51	Ala–(D)Phe–Ile–Gly–Leu–Met–NH_2	<0.05	<0.1	<0.05
52	Ala–Phe–Ile–Gly–Leu–(D)Met–NH_2	<0.02	<0.1	<0.05

Eti, ethionine; Hcys, homocysteine; Pyr, pyroglutamic acid; *m*-Tyr, meta-tyrosine; Tyr(OMet) tyrosine methyl ether; BOC, *tert*-butyloxycarbonyl; n.t., not tested

(iv) Substitution of the leucine or phenylalanine residues in the hexapeptide of either physalaemin or eledoisin produced a tremendous decay in the specific biological activity.

(v) Changes in biological activity produced by substitution of one of the three remaining amino acid residues were more irregular and apparently unpredictable. A high degree of activity was retained in hexapeptides where tyrosine (position 4 from the C-terminus) was replaced by valine, phenylalanine and isoleucine. However, hexapeptides in which tyrosine was replaced by leucine or alanine were practically devoid of activity. Likewise substitution of glycine (position 3) furnished in some cases active compounds, in other cases inactive compounds. Finally, lysine (position 6) could be replaced, with good preservation of activity, by a number of amino acids.

(vi) No significant changes in activity were noted when the N-terminal residue was protected by the *tert*-butyloxycarbonyl group.

(vii) The all D-enantiomer of the eledoisin hexapeptide was devoid of activity and did not antagonize the L-enantiomer of eledoisin. Similarly, replacement of an L-amino acid residue in the C-terminal pentapeptide with the corresponding D-amino acid residue caused a profound reduction of activity. However, the L-alanine in position 6 from the C-terminus could be substituted either by D-alanine or by D-asparagine with no important changes in activity.

(viii) Asn^5–eledoisin, Gly^5–Val^8–eledoisin and Asn^5–Val^8–eledoisin possessed 150, 80 and 60 per cent, respectively, of the activity of eledoisin.

It is evident that the results of these studies will gain a renewed attention after the elucidation of the structure of substance P which is no longer a humble polypeptide of the amphibian skin, but a noble constituent of the encephalic gray matter in all vertebrates, including man. All the above conclusions concerning relationship between chemical structure and biological activity are valid not only for the amphibian tachykinins but also for substance P.

BRADYKININ-LIKE PEPTIDES

In addition to authentic bradykinin (I), seven natural bradykinin-like polypeptides have been so far isolated in a pure form: kallidin or lysyl–bradykinin (II), methionyl–lysyl–bradykinin or methionyl–kallidin (III), Thr^6–bradykinin (IV), glycyl–bradykinin (V), polystes–kinin (VI), bradykinyl–isoleucyl–*O*-sulphate or phyllokinin (VII), and finally Val^1–Thr^6–bradykinin (VIII).

(I)	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(II)	Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(III)	Met–Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(IV)	Arg–Pro–Pro–Gly–Phe–Thr–Pro–Phe–Arg
(V)	Gly–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(VI)	Pyr–Thr–Asp–Lys–Lys–Leu–Arg–Gly–Bradykinin
(VII)	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–Ile–Tyr(SO_3H)
(VIII)	Val–Pro–Pro–Gly–Phe–Thr–Pro–Phe–Arg

Bradykinins (I), (II) and (III) occur in mammalian plasma, bradykinin (IV) in turtle plasma[43], bradykinins (V) and (VI) in the venom of *Polystes* wasps[44], and finally bradykinins (I), (VII) and (VIII) in the amphibian skin, together with the peptide Bradykinyl–Val–Ala–Pro–Ala–Ser (IX) which may be considered a bradykinin precursor.

Authentic bradykinin (I) has been isolated from the skin of the common European brown frog *Rana temporaria*, where it is present in amounts as high as 200 to 250 μg g^{-1} fresh tissue. Extracts of the skin of the European green frog *Rana esculenta* displayed a bradykinin-like activity corresponding to 10–25 μg bradykinin g^{-1} fresh skin[45].

Phyllokinin (VII) has been prepared in a pure form from skin extracts of the Brazilian frog *Phyllomedusa rohdei* and is probably present also in *Phyllomedusa bicolor* and in other *Phyllomedusa* species as well. It is the first and so far the only active natural bradykinin-like peptide with amino acid residues attached at the C-terminus of the bradykinin molecule. Trypsin digestion produced a splitting-off of the C-terminal dipeptide, giving rise to bradykinin which is known to be resistant to trypsin[46].

Desulphated phyllokinin, i.e. bradykinyl–isoleucyl–tyrosine, was consistently less active than phyllokinin on all tested preparations. The relative activity, on a molar basis, of phyllokinin and bradykinyl–isoleucyl–tyrosine in respect to that of bradykinin is shown in *Table 3*[47].

Val^1–Thr^6–bradykinin (VIII) has been isolated from extracts of the skin of the Japanese frog *Rana nigromaculosa*, together with authentic bradykinin and polypeptide (IX). Its stimulant activity on the rat uterus was about 20 per cent of that of bradykinin[48].

Since amphibian bradykinins display the same general activities as does bradykinin, these activities will not be discussed in detail. It will be sufficient to remember here some effects of the bradykinins which may be useful for their characterization and their distinction, in parallel bioassay, from other categories of active peptides.

The bradykinins are characterized by a remarkable but not exceptionally intense vasodilating and hypotensive action in the dog, rabbit and cat; by a

Table 3. The relative potency, on a molar basis, of bradykinin, phyllokinin and bradykinyl-isoleucyl-tyrosine (bradykinin = 100).

Test preparation	Phyllokinin	Bradykinyl-isoleucyl-tyrosine
Dog blood pressure	270–340	45–80
Rabbit blood pressure	40–90	15–30
Guinea-pig ileum	25–40	15–20
Rat uterus	30–40	20–25
Rat duodenum	7–9	n.t.
Human skin capillaries	130	n.t.
Rabbit urinary bladder	10–70	n.t.
Dog urinary bladder	40	n.t.
Cat urinary bladder	20	n.t.

n.t., not tested

potent stimulant action on the isolated guinea-pig ileum, cat small intestine and cat, dog and rabbit urinary bladder; and by a striking stimulant action on the oestrous uterus of the rat. They have a poor stimulant action on the rabbit and rat colon and display an inhibitory action on the rat duodenum. Bradykinin-like polypeptides effectively increase capillary permeability in man and experimental animals and cause pain when administered intra-arterially and intraperitoneally. All the known bradykinins are completely inactivated by incubation with chymotrypsin but are resistant to trypsin.

Bradykinins represent a polypeptide family having a widespread distribution in the amphibian skin. In fact bradykinin-like peptides occur, outside the species belonging to the genera *Phyllomedusa* and *Rana*, in species of other genera as well, among which are *Ascaphus truei*[49], *Physalaemus*, *Taudactylus* and *Litoria*.

Two recent localizations of kinin-like peptides outside the amphibian skin deserve particular mention. The first is that of bufokinin, present in methanol extracts of the urinary bladder of the toad *Bufo marinus paracnemis*[50], the second that of one or two bradykinin-like peptides in the skin of the lamprey *Eudontomyzon danforti vladykovi*[51].

Bufokinin is a negatively charged peptide, having a molecular weight of 1000 or slightly above, displaying the characteristic actions of bradykinin on isolated smooth muscle preparations and on blood pressure in the dog. Moreover, the peptide, like other bradykinins, when applied to the isolated toad bladder markedly inhibited the increase in the transepithelial osmotic water flux evoked by neurohypophysial hormones.

The lamprey in its turn contained in the skin small amounts of one or two biologically active bradykinin-like peptides. In contrast to authentic bradykinin they were inactivated not only by chymotrypsin but also by trypsin.

CAERULEIN-LIKE PEPTIDES

Caerulein is a decapeptide first isolated from methanol extracts of the skin of the Australian hylid frog *Litoria* (*Hyla*) *caerulea*, where it was present in concentrations of 100 to 1000 $\mu g\,g^{-1}$ fresh skin. The thick dorsal skin con-

tained 8 to 10 times more caerulein than the thinner ventral skin. Moderate losses of caerulein (20 to 40 per cent) occurred during drying of the skins[52, 53]. Authentic caerulein was later found in extracts of the skin of the South American leptodactylid frog *Leptodactylus pentadactylus labyrinthicus* and of the South African amphibian *Xenopus laevis* (300 to 800 $\mu g\ g^{-1}$ fresh skin). That is of interest because *Xenopus laevis* may be easily bred in an aquarium[54]. It is probable that caerulein is present also in the skin of a number of other Australian hylid frogs (in *Litoria* (*Hyla*)*infrafrenata* and *Litoria* (*Hyla*)*moorei* up to 2500 to 3000 $\mu g\ g^{-1}$ dry tissue), in other South American leptodactylid frogs (in *Leptodactylus laticeps* up to 1300 $\mu g g^{-1}$ fresh tissue) and in other South African pipid frogs (in *Xenopus gilli* up to 1000 to 1500 $\mu g g^{-1}$ fresh tissue)[53, 54].

The skin of the South American hylid frogs of the genus *Phyllomedusa* contained, in its turn, phyllocaerulein, a nonapeptide strictly related to caerulein. In *Phyllomedusa sauvagei*, whence it has been isolated in a pure form, it was present in amounts of 200 to 650 $\mu g\ g^{-1}$ fresh skin. Similar amounts were present in the fresh skin of *Phyllomedusa bicolor*[55].

Systematic screening carried out in the last few years has shown that distribution of caerulein-like peptides in amphibian skin is broader than so far suspected. In fact, conspicuous amounts of caerulein-like peptides have been traced in *Nictimystes disrupta*, an amphibian of New Guinea, in *Hylambates maculatus* of South Africa and finally in some authentic frogs of New Guinea, Borneo and the Philippines, including *Rana erythraea*. The isolation of some of the above caerulein-like peptides is in progress. It is highly probable that the peptide in *Rana erythraea* is different from authentic caerulein[56–58].

The formulae below show the close chemical resemblance existing between the caeruleins, on the one side, and the gastrins and cholecystokinin–pancreozymin on the other side.

Pyr–Gln–Asp–Tyr(SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	Caerulein
Pyr–Glu———Tyr(SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	Phyllocaerulein
–Asp–Tyr(SO_3H)–Met–Gly–Trp–Met–Asp–Phe–NH_2	C-terminal octapeptide of cholecystokinin
–Tyr(SO_3H)———Gly–Trp–Met–Asp–Phe–NH_2	C-terminal hexapeptide of gastrin II

It may be seen that caerulein has in common with gastrin II the C-terminal pentapeptide and the sulphated tyrosyl residue, and with cholecystokinin the entire C-terminal octapeptide, with only the unimportant difference of a methionyl residue substituted for the threonyl residue at the 6-position from the C-terminus. It may be further noted that caerulein differs from phyllocaerulein only by the fact that the N-terminal tripeptide Pyr–Gln–Asp of the former is replaced in the latter by the dipeptide Pyr–Glu.

Caerulein has been subjected to extensive pharmacological investigation which showed that the polypeptide possessed an activity spectrum very similar to that of cholecystokinin–pancreozymin, together with conspicuous gastrin-like effects. The main pharmacological effects of caerulein may be summarized as follows:

(a) Potent spasmogenic action on the *in vivo* and *in vitro* gall bladder

musculature of all the tested animals, including man. A few nanograms per kilogram of weight injected intravenously were sufficient to stimulate the organ *in situ* and less than 1 ng kg^{-1} min^{-1} was effective when infused intravenously. The isolated gall bladder was contracted by caerulein in concentrations as low as 0.01 to 2 ng ml^{-1} of nutrient solution. There was no tachyphylaxis and, generally, a good dose–response relationship. The spasmogenic action of the polypeptide was atropine resistant[59].

In the dog the threshold cholecystokinetic doses by intravenous and subcutaneous routes were 1 and 10 ng kg^{-1}, respectively. The gall bladder contraction began very soon and lasted 3 to 4 hours after intravenous administration and 6 to 7 hours after subcutaneous administration. In some cases a certain degree of contraction was appreciable up to 24 hours. The cholecystokinetic activity was more striking after subcutaneous than after intravenous administration, except for threshold doses. Also the intrahepatic and extrahepatic bile ducts were apparently contracted by caerulein. On a molar basis caerulein was 16 times as potent as cholecystokinin and 170 times as potent as both gastrin I and gastrin II[60, 61].

In man caerulein has been largely used in cholecystography and cholangiography. Doses were 5 to 30 ng kg^{-1} by the intravenous route, 0.25 to 1 μg kg^{-1} by intramuscular or subcutaneous injection, and 0.75 to 1 μg kg^{-1} by nasal insufflation. The threshold intravenous dose was of the order of 1 ng kg^{-1}. The spasmogenic action of the polypeptide began soon after the injection, reached its peak after 10 to 15 minutes, and disappeared after 90 minutes; 2·5 to 5 ng kg^{-1} of intravenous caerulein produced a response very similar to that caused by a fatty meal[62–64].

(b) Relaxing action on the choledocho–duodenal junction, which was particularly evident when the tone of the sphincter was elevated, either spontaneously or following premedication with spasmogenic drugs[65]. As a consequence of the relaxation of the sphincter of Oddi the choledochal resistance was lowered and bile flow increased in conscious dogs. Of the peptides examined the most potent relaxant was caerulein[66].

(c) Powerful stimulant action on the *in situ* musculature of the gut, with the possible exception of the duodenum, at least in man.

In the intact, conscious dog, caerulein caused emesis and evacuation of the bowel. The mean effective dose by rapid intravenous injection was 0.4 to 0.5 μg kg^{-1}, and by subcutaneous administration 3 to 4 μg kg^{-1}. By intravenous infusion caerulein produced retching in most dogs when doses exceeded 10 ng kg^{-1} min^{-1}. Intravenous doses as low as 1 to 5 ng kg^{-1} had a spasmogenic action on jejunal loops of the dog, and slightly larger doses contracted the small intestine of the cat. The stomach and the large intestine were less sensitive to the polypeptide. Caerulein also displayed a considerable spasmogenic action on the rat pylorus. All these effects, with the exception of the latter, could be reduced or abolished by atropine[59].

In human subjects studied by the balloon method caerulein caused inhibition of the duodenal motility and stimulation of the jejunal motility and tone. Threshold doses by intravenous infusion were of the order of 1 ng kg^{-1} min^{-1}. The effect subsided a few minutes after the infusion had been discontinued. By the subcutaneous route the threshold dose was 25 to 30 ng kg^{-1}. With 0.75 μg kg^{-1} the stimulant effect lasted 30 to 40 minutes[67].

In small bowel contrast studies it could be seen, by fluorography and cinematography, that caerulein administration (1 to 2 ng kg^{-1} min^{-1} by infusion or 0.5 to 1 μg kg^{-1} by intramuscular injection) produced a conspicuous reduction in the transit time of the contrast medium. The barium took only 20 to 30 minutes to reach the colon and peristalsis was very lively. Not infrequently the contrast medium was present simultaneously in the colon and the stomach[68].

Some isolated preparations of intestinal smooth muscle were extremely sensitive to caerulein. They were used in the study of the mechanism of action of caerulein, which appeared to be different depending on the animal species and the intestinal section considered. In most cases it was predominantly cholinergic, in other instances it seemed a direct one[69, 70].

In the guinea-pig ileum caerulein has been shown to act on non-nicotinic receptors in the intramural ganglia causing a conspicuous release of acetylcholine. A similar release of acetylcholine occurred also in the rabbit duodenum[71].

Because of its actions on the intestinal smooth muscle, caerulein has been employed, apparently with success, in the treatment of postoperative gastro-intestinal atony and other forms of adynamic ileus[72].

(d) Potent stimulant action on the exocrine pancreas causing the secretion of abundant pancreatic juice rich in enzymes. In the anaesthetized dog with an acutely cannulated Wirsung duct, threshold doses of caerulein were 1 to 5 ng kg^{-1} by rapid intravenous injection, 0.25 to 1 ng kg^{-1} min^{-1} by intravenous infusion, and 50 to 100 ng kg^{-1} by subcutaneous injection. An intense, constant flow of pancreatic juice could be maintained by infusion of caerulein up to 10 to 20 hours[73]. In conscious dogs provided with chronic pancreatic fistulas the dose of caerulein required for 50 per cent of maximal response was 0.5 ng kg^{-1} min^{-1}, volume output, and 0.7 ng kg^{-1} min^{-1}, enzyme output. Depending on experimental conditions, caerulein was 7 to 50 times more potent than gastrin, and 3 to 6 times more potent than cholecystokinin on pancreatic juice flow and enzyme output. Secretin, however, stimulated flow 2.5 to 20 times more than caerulein, on a molar basis[74, 75].

Repeated subcutaneous administration of caerulein caused a remarkable increase (up to 100 per cent) of amylase and chymotrypsin concentration in the rat pancreas[76] and a 80 to 100 per cent increase in the incorporation of ^{14}C–leucine into protein by pancreas tissue slices of fasted guinea-pigs[77]. In chickens the threshold dose of caerulein active on pancreatic secretion was 0.1 to 0.3 ng kg^{-1} min^{-1}. Maximal observed increases were 8-fold for volume output, 23-fold for amylase output, 18-fold for lipase output, and 28-fold for output of total tryptic activity. The concentration of enzymes in caerulein juice was 3- to 6-fold the concentration in control juice[78].

The pancreatic islets, too, were stimulated by doses of caerulein of the same order of magnitude as those active on the exocrine pancreas. In fact, in the dog, doses of the polypeptide as low as 2 ng kg^{-1} min^{-1} produced a 2.5- to 4-fold increase of the immunoreactive insulin levels in pancreatico-duodenal venous blood and a 3.5-fold increase of the immunoreactive glucagon levels. The effect lasted as long as the infusion was continued. Discontinuing the infusion caused a prompt return to basal values; recommencing the infusion resulted in a renewed release of insulin[79, 80].

In normal human subjects 10 ng kg^{-1} of caerulein administered by rapid intravenous injection produced a slight increase in blood glucose levels, while plasma immunoreactive insulin did not change. However, in some patients with insuloma, caerulein provoked a powerful insulin response[81].

Concomitantly with, and probably resulting from, the stimulation of the pancreas there was an increase in the blood flow through the duodenal–pancreatic artery. Intravenous doses of caerulein active on this vascular area (1 to 2 ng kg^{-1}) were at least 10 times lower than those causing systemic hypotension[82].

(e) Conspicuous stimulant action on the Brunner glands. The intravenous infusion of 2.5 ng kg^{-1} min^{-1} of caerulein stimulated the glands of the dog to produce 0.56 ml of secretion per 15 minutes, and the glands of the cat to produce 0.36 ml of secretion per 60 minutes[83].

(f) Potent stimulant action on gastric secretion, with increase in volume, acid and pepsin outputs. Concentration of hydrochloric acid in the dog juice increased up to 50 per cent, and concentration of pepsin up to 250 per cent[84].

In dogs with denervated gastric pouches the dose of caerulein needed to produce one-half of the maximal acid secretion was 8 ng kg^{-1} min^{-1}; in gastric fistula dogs 2.7 ng kg^{-1}. On both a molar and a weight basis caerulein has been found to be more potent than gastrin in stimulating acid secretion. However, it should be pointed out that gastrin was capable of producing much higher observed and calculated maximal responses than was caerulein. Calculated and observed acid outputs to maximal doses of gastrin were usually twice those found for caerulein. Caerulein was then extremely potent in that low doses produced significant amounts of secretion, but it was not effective in producing high rates of acid secretion comparable to those seen with maximal doses of gastrin or histamine[85, 86].

In man the threshold intramuscular dose of caerulein was 50 to 100 ng kg^{-1} and the optimum response was obtained with 250 ng kg^{-1}. At this dosage the effect lasted 60 to 90 minutes and peak gastric secretion (40 ml juice and 3.3 m equiv total HCl output) was reached between 15 and 30 minutes. During the course of the response to 250 ng kg^{-1} of caerulein, 35 m equiv Cl^-, 10 m equiv Na^+, and 5 m equiv K^+ were secreted[86, 87].

Gastrin- and pentagastrin-induced gastric secretion was inhibited by caerulein; the effect of the polypeptide on histamine-induced secretion was, on the contrary, variable. In its turn, the effect of caerulein was completely abolished by atropine in the dog, man and chicken, but was atropine-resistant in the rat and the pigeon[84, 87, 88].

Caerulein increased 'short circuit current' and simultaneously secretion of hydrochloric acid in the isolated gastric mucosa, starting from concentrations as low as 10^{-10} M. The effect was atropine-resistant. On this preparation caerulein was 10000 times more active, on a molar basis, than either pentagastrin and human gastrin I, 30 times as active as cholecystokinin and 1000 times as active as histamine[90].

In the rat, caerulein caused also a remarkable increase in the secretion of the intrinsic factor. With 0.5 μg kg^{-1} of the polypeptide given subcutaneously the increase in intrinsic factor secretion was 100 per cent; with 5 μg kg^{-1}, 350 per cent. By intravenous infusion the threshold dose of caerulein was

10 ng kg^{-1} min^{-1}. On a molar basis caerulein was more than 10 000 times as active as histamine[91].

Finally, caerulein always produced an increase in the histidine decarboxylase activity of the gastric mucosa in the rat. The threshold dose for a three-hour infusion period appeared to be 0.5 μg kg^{-1} h^{-1}. With 5 μg kg^{-1} h^{-1} the enzyme activity was increased by 400 per cent[92].

(g) Variable effects, depending on the animal species considered, on flow and composition of hepatic bile, as well as on transhepatic transport of bile salts, bile pigments and organic anions[93–97].

In the chicken, which was the species most thoroughly studied up to the present, the intravenous infusion of caerulein elicited the following effects: (*i*) increase in the volume of bile flow. The threshold dose of the polypeptide was 0.5 ng kg^{-1} min^{-1} and maximum increase in bile flow was 8-fold; (*ii*) increase in output of bile salts, cholesterol, pigments, and bicarbonate. At an infusion rate of 0.5 ng kg^{-1} min^{-1} the output of bile salts increased by 25 per cent and that of cholesterol by 75 per cent. Except for bicarbonate, the concentration of the bile components was usually higher in the caerulein bile than in control bile; (*iii*) increase in the excretion rate of exogenous bile salts, with removal of the autoinhibition produced by infusion of large amounts of these salts. With 15 ng kg^{-1} min^{-1} increase was 5-fold; (*iv*) acceleration of the transhepatic transport of sulphobromophthalein with simultaneous increase in plasma BSP clearance (threshold 1 ng kg^{-1} min^{-1} caerulein); (*v*) acceleration of the transhepatic transport of rose Bengal and indocyanine, with simultaneous increase in the secretion of endogenous bile salts[93].

Table 4. The effect of caerulein on bile flow and bile salts secretion in different animal species

Animal species	Bile flow	Secretion of bile salts	
		Total output	Concentration
Chicken	+ + +	+ +	+, 0
Goose	+ + +	+ +	0
Pigeon	+	+ + +	+ + +
Rabbit	+	+ +	+ + +
Cat	+ +	+ +	+ +
Dog	+ +	+	(?)
Rat	(+)	0	(−)
Guinea pig	0	0	0

0, no effect; +, increased; −, reduced

In the dog, half of the maximal calculated increase in bile flow was produced by 0.25 ng kg^{-1} min^{-1} of caerulein[94]. At variance with previous findings, not only the output of bicarbonate and chloride but also that of bile salts was increased following the administration of the polypeptide.

A synopsis of the actions produced by caerulein on biliary secretion in different animal species is presented in *Table 4*. Results have been obtained in animals with their gall bladder excluded, with total diversion of bile for collection and with continuous administration of bile salts to support bile flow[97].

It may be seen that the rat and guinea-pig were totally, or nearly, unresponsive to caerulein. In the goose the powerful stimulation of bile flow was not accompanied by any increase in the concentration of bile salts, whereas in the rabbit, cat and pigeon bile secreted under the influence of caerulein showed a high concentration of bile salts.

Of considerable interest are the findings that caerulein (10 to 20 ng kg^{-1} min^{-1}) produced also in the rabbit an increase in the maximal rate of secretion of sulphobromophthalein and bilirubin into the bile and that in the chicken, rabbit and pigeon the choleretic effect of caerulein was generally magnified if the animal was supplied with exogenous bile salts via the duodenum instead of intravenously.

More work is necessary to elucidate the mechanism of the salteretic effect of caerulein. It is certain that the polypeptide enhances the transhepatic transport of bile salts and hence their canalicular excretion; it may also be that it acts on intestinal reabsorption of bile salts and that it affects 7-α-hydroxylation of cholesterol, which is the rate-limiting step in the biosynthesis of bile acids.

(h) Conspicuous villokinetic activity in the cat and in birds. The intravenous infusion of caerulein or cholecystokinin in animals with or without drainage of digestive secretions stimulated the pump-like movements of chicken, cat and pigeon duodenal–jejunal villi. Threshold infusion rates for caerulein were 0.25, 0.5 and 2 ng kg^{-1} min^{-1}, respectively. Dose–response relationship was usually modest, and an inhibitory action could supervene with supramaximal rates in the chicken and cat, but not in the pigeon[98, 99].

(i) Moderate action on the systemic blood pressure. In dogs and rabbits caerulein nearly always produced hypotension with a good dose–response relationship. In the dog the threshold dose was 10 to 100 ng kg^{-1} by intravenous injection, 5 to 15 ng kg^{-1} min^{-1} by intravenous infusion, and 5 to 10 μg kg^{-1} by subcutaneous injection. In other animal species blood pressure response was more erratic and unpredictable[100].

(j) Potent stimulant effect on calcitonin release from porcine thyroid, perfused *in situ*. Approximately 1.3 ng ml^{-1} of caerulein doubled the secretion rate of calcitonin. On a molar basis the polypeptide was about twice as active as cholecystokinin[101].

The acute toxicity of caerulein in animals seems to be very low: in the mouse LD50 by intravenous injection was 1030 mg kg^{-1}.

Very high doses of caerulein (50 and 250 μg kg^{-1}) given daily to rats by subcutaneous injection, for periods of 1, 3 and 6 months, produced in the pancreas severe acinar cell damage ending in parenchymatous atrophy with diffuse fibrosis and increase of ductular and centroacinous cells. The endocrine pancreas, however, was not damaged and the above severe lesions of the exocrine tissue did heal spontaneously after discontinuing the injections of caerulein. Pancreatic lesions were accompanied by an impairment of fat absorption, evidently due to lack of pancreatic lipases, and by reduction of fat deposits. In addition to the pancreatic changes caerulein produced also hypertrophy of the duodenal wall and of the gastric parietal cells, events which could suggest a trophic action of the polypeptide.

Daily doses of 10 μg kg^{-1} caerulein, administered subcutaneously up to 6

months, resulted only in hypertrophy of the pancreas and no pathological changes.

No foetal malformations were observed in rats and rabbits given caerulein during the whole pregnancy at daily subcutaneous doses of 50 and 25 $\mu g\,kg^{-1}$, respectively[102, 103].

Because caerulein is now very often studied and discussed together with cholecystokinin and the gastrins it seems useful to condense in *Table 5* available data on the relative potency of the three peptides on a number of test preparations. The activity of caerulein was considered equal to 100 and that of cholecystokinin and human gastrin I was expressed in terms of this activity.

Table 5. Relative potency, on a molar basis, of caerulein, cholecystokinin and human gastrin I (caerulein = 100)

Test preparation	Cholecystokinin	Gastrin I
Gall bladder *in situ*		
man	6–7	0.6
guinea-pig	30–35	n.t.
dog	10	0.25
Dog small intestine *in vivo*	17	2–2.5
Choledocho-duodenal junction (guinea-pig)	5	n.t.
Intestinal villi (chicken)	50	n.t.
Dog systemic blood pressure	13–25	2–3
Duodenal–pancreatic artery (dog)	10–15	n.t.
Perfused rat stomach preparation	35	5–20
Isolated frog gastric mucosa	3	0.01
Dog exocrine pancreas	15–30	2–15
Dog Brunner glands	10	10

n.t., not tested.

Approximately 75 caerulein-like polypeptides have been synthesized by our research group in order to contribute to the elucidation of the problem of structure–activity relationship for caerulein and cholecystokinin and in an attempt to dissociate the different actions of caerulein[104, 105].

The relative activity of a number of synthetic peptides related to caerulein is shown in *Table 6*, in which the activity of a given weight of caerulein was considered equal to 100 and the activity of the same weight of the other peptides was expressed in per cent.

From the tabulated data and from other published and unpublished results, the following conclusions may be drawn:

(i) The whole activity spectrum of caerulein was present in the C-terminal heptapeptide, and further lengthening of the peptide chain was of limited effect.

(ii) A necessary prerequisite for the activity of the heptapeptide was the presence at its N-terminus of an *O*-sulphated tyrosyl residue or another appropriate negatively charged residue. Thus, desulphation of the tyrosyl residue or its replacement by a *p*-sulphonamido–phenylalanyl residue produced a drastic decay of activity. Substitution of the sulphuric acid by

Table 6. Relative potency of caerulein-like peptides, on a weight basis caerulein = 100)

	Peptide	Test preparation: Denervated gastric pouch of the dog	Dog pancreas	Guinea-pig gall bladder
1	Pyr–Gln–Asp–Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2 Caerulein	100	100	100
2	Pyr–Glu——Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2 Phyllocaerulein	110–150	110–150	120–150
3	Pyr–Gln–Asp–Tyr–Thr–Gly–Trp–Met–Asp–Phe–NH_2	2.5	4	<1
4	Pyr——Asp–Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	40	50	40–60
5	Tyr(S)–Ala–Ala–Gly–Trp–Met–Asp–Phe–NH_2	3	1.4–4	<1
6	Tyr–Thr–Gly–Trp–Met–Asp–Phe–NH_2	<1	1.2–2.5	0.05
7	Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	20–40	60–80	30–50
8	Tyr(P)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	5·5–7	5–10	<1
9	Tyr——Gly–Trp–Met–Asp–Phe–NH_2	0.5–1	1.5–3	<0.1
10	Tyr(S)——Gly–Trp–Met–Asp–Phe–NH_2	8–20	10–30	<0.5
11	Tyr(P)——Gly–Trp–Met–Asp–Phe–NH_2	2	5–7	<1
12	Tyr(S)–Thr——Trp–Met–Asp–Phe–NH_2	2–3	3–5	10–15
13	Tyr(S)————Trp–Met–Asp–Phe–NH_2	2–3	1–2	<1
14	Tyr(P)————Trp–Met–Asp–Phe–NH_2	2–3	2–3	<1
15	Tyr(S)–Gly–Gly–Trp–Met–Asp–Phe–NH_2	3–4	10–15	4–10
16	Tyr(S)–Met–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	65–70	80–150
17	Tyr(P)–Met–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	3.5	<1

Table 6—continued

	Peptide	Test preparation: Denervated gastric pouch of the dog	Test preparation: Dog pancreas	Test preparation: Guinea-pig gall bladder
18	BOC–$\overset{\text{S}}{\text{Tyr}}$–Met–Gly–Trp–Met–Asp–Phe–NH_2	50–75	55–75	50–75
19	BOC–$\overset{\text{S}}{\text{Tyr}}$–Thr–Gly–Trp–Met–Asp–Phe–NH_2	40–50	30–40	60–70
20	BOC–$\overset{\text{S}}{\text{Tyr}}$–Nle–Gly–Trp–Met–Asp–Phe–NH_2	30–60	60–70	110–150
21	BOC–$\overset{\text{S}}{\text{Tyr}}$–Tyr–Gly–Trp–Met–Asp–Phe–NH_2	20	15–50	10–20
22	BOC–$\overset{\text{S}}{\text{Tyr}}$–Trp–Gly–Trp–Met–Asp–Phe–NH_2	10–15	10	20–35
23	BOC–$\overset{\text{S}}{\text{Tyr}}$–Phe–Gly–Trp–Met–Asp–Phe–NH_2	10–20	10–15	10–20
24	BOC–$\overset{\text{S}}{\text{Tyr}}$–$\overset{\text{S}}{\text{Tyr}}$–Gly–Trp–Met–Asp–Phe–$NH_2$	n.t.	1.4	1
25	BOC–$\overset{\text{S}}{\text{Tyr}}$–Thr–Gly–Trp–Nle–Asp–Phe–NH_2	120–140	130–140	130–140
26	BOC–$\overset{\text{S}}{\text{Tyr}}$–Abu–Gly–Trp–Nle–Asp–Phe–NH_2	n.t.	15–20	40–60
27	$\overset{\text{S}}{\text{Hcy}}$–Abu–Gly–Trp–Nle–Asp–Phe–NH_2	n.t.	3–5	2–6
28	$\overset{\text{S}}{\text{Hpp}}$–Abu–Gly–Trp–Nle–Asp–Phe–NH_2	30	30	30–60
29	BOC–(D)$\overset{\text{S}}{\text{Tyr}}$–Val–Gly–Trp–Met–Asp–Phe–NH_2	20–22	6–15	5–7
30	BOC–*m*.$\overset{\text{S}}{\text{Tyr}}$–Val–Gly–Trp–Met–Asp–Phe–NH_2	2	2–2.5	<1
31	BOC–3Cl.$\overset{\text{S}}{\text{Tyr}}$–Val–Gly–Trp–Met–Asp–Phe–NH_2	7–8	20–25	10–15
32	BOC–3,5Br.$\overset{\text{S}}{\text{Tyr}}$–Val–Gly–Trp–Met–Asp–Phe–NH_2	2	7–8	<1–1.5
33	$\overset{\text{S}}{\text{Phe}}$–Thr–Gly–Trp–Met–Asp–Phe–NH_2	10–20	10–20	10
34	$\overset{SO_2NH_2}{\text{Phe}}$–Thr–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	0.5–1	<1

BOC, *tert*-butyloxycarbonyl; Pyr, pyroglutamic acid; $\overset{\text{S}}{\text{Tyr}}$, tyrosine *O*-sulphate; $\overset{\text{P}}{\text{Tyr}}$, tyrosine *O*-phosphate; *m*.Tyr, *m*.tyrosine; 3Cl.Tyr, 3-chloro-tyrosine; 3,5 Br.Tyr, 3,5-dibromo-tyrosine; Hcy, *p*-hydroxycinnamic acid; Hpp, *p*-hydroxyphenylpropionic acid; $\overset{\text{S}}{\text{Phe}}$, *p*-sulphonylphenylalanine; $\overset{SO_2NH_2}{\text{Phe}}$, *p*-sulphonamidophenylalanine; n.t., not tested.

phosphoric acid yielded considerably less active compounds. Similarly, substitution of *p*-sulphonyl–phenylalanine for tyrosine *O*-sulphate caused a 50 to 80 per cent reduction of activity. Desamination of tyrosine *O*-sulphate to *O*-sulphate of *p*-hydroxyphenylpropionic acid, on the contrary, produced no change in biological activity. However, the *p*-tyrosyl sulphate residue could not be replaced by the *m*-tyrosyl sulphate residue. Finally, substitution of L-tyrosine *O*-sulphate with D-tyrosine *O*-sulphate produced a reduction of activity of different intensity, depending on the test preparation.

(iii) Substitution of the threonyl residue in the C-terminal heptapeptide produced different results, depending on the entering amino acid. Tryptophan, phenylalanine and still more glycine and tyrosine *O*-sulphate gave unfavourable results, whereas methionine produced an increase in biological activity, and tyrosine caused only a minor decrease in activity. It is evident from these results that the negative charge at the N-terminus must not exceed certain limits.

(iv) Omission of the threonyl residue, with consequent shifting of the sulphated tyrosyl residue to position 6, as in the C-terminal hexapeptide of gastrin II, produced a peptide which still retained a considerable activity on gastric and pancreatic secretions, but was deprived of any significant action on the smooth muscle of the intestines and gall bladder as well as on systemic blood pressure.

(v) Omission of the glycyl residue, again with shifting of the sulphated tyrosyl residue to position 6, produced a hexapeptide possessing a moderate activity on gastric secretion (5 to 10 per cent), a poor activity on pancreatic secretion and virtually no activity on vascular and extravascular smooth muscle.

(vi) The tryptophanyl residue could not be replaced by a phenylalanyl residue.

(vii) The easily oxidized methionyl residue could be replaced, with advantage, by the stable norleucyl residue, as in the case of gastrin analogues[106].

(viii) The C-terminal tetrapeptide and the C-terminal pentapeptide of caerulein, which incidentally are also the C-terminal tetrapeptide and pentapeptide of the gastrins I and II, were completely devoid of any cholecystokinetic activity and of any stimulant activity on the intestinal smooth muscle, but still retained some secretagogue activity on the pancreas (< 3 per cent) and the rat stomach (1 to 2 per cent). The same was true for the C-terminal hexapeptide of caerulein.

The above conclusions have been largely confirmed using other test systems. Even in the isolated mucosa of the frog stomach, using the 'short circuit current' method, a prerequisite for activity of caerulein-like peptides was the occurrence of a sulphated tyrosyl residue at position 7. The hexapeptide of gastrin II possessed barely 2 to 3 per cent of the activity of caerulein, and gastrin I, like all other desulphated peptides of this group, less than 0.2 per cent of the caerulein activity[90].

These results point to the possibility that gastrins in lower vertebrates may be related in their amino acid composition and sequence (especially in the position of their probable sulphated tyrosyl residue) more closely to the caeruleins than to the gastrins.

It has been demonstrated, quite recently, that even the C-terminal dipeptide

of caerulein, Asp–Phe–NH_2, displayed some pharmacological actions on certain preparations of intestinal smooth muscle. On the isolated guinea-pig ileum, for example, the dipeptide showed 0.2 to 0.3 per cent of the activity of caerulein, on a molar basis, and as much as 2 per cent of the activity of caerulein on the pylorus of the rat stomach *in situ*. This is another striking example of the pharmacological possibilities of the oligopeptides, in this case of the smallest possible oligopeptide.

Needless to say the results obtained in the study of caerulein-like peptides are fully valid also for cholecystokinin and, subordinately, for the gastrins. The converse is similarly true, because each peptide of one of the three series is similar to the peptides of the two other series.

BOMBESIN-LIKE PEPTIDES

Three polypeptides belonging to this family have been recently isolated in a pure form, reproduced by synthesis, and submitted to a pharmacological study by two groups of research workers, independently[107–110]. From the formulae below it is evident that we have to do with a new class of polypeptides.

(I) Pyr–Gln–Arg–Leu–Gly–Asn–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
(II) Pyr–Gly–Arg–Leu–Gly–Thr–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
(III) Pyr————————Val–Pro–Gln–Trp–Ala–Val–Gly–His–Phe–Met–NH_2

Bombesin (I) is a tetradecapeptide obtained from methanol extracts of the skin of the two European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* where it is contained in amounts ranging from 200 to 700 $\mu g\ g^{-1}$ fresh tissue. It is highly probable that authentic bombesin is present also in the skin of *Bombina variegata pachypus*.

Alytesin (II) is again a tetradecapeptide, strictly related to bombesin, which is found in extracts of the skin of another European discoglossid frog, *Alytes obstetricans*, in amounts usually ranging between 600 and 1300 $\mu g\ g^{-1}$ wet skin, but sometimes as low as 50 $\mu g\ g^{-1}$, when expressed in terms of bombesin[108].

Discoglossus pictus, from both Sardinia and Sicily, although belonging to the same family as *Bombina* and *Alytes*, did not contain detectable amounts of bombesin-like peptides.

Finally, ranatensin (III) is an endecapeptide prepared from extracts of the skin of the American frog *Rana pipiens*. No quantitative data on ranatensin contents in the skin were presented by Nakajima *et al.*[107]. In our methanol extracts of dried skins of *Rana pipiens*, the content of ranatensin, assayed on rat uterus preparation and expressed in terms of bombesin, ranged between 0.5 and 120 $\mu g\ g^{-1}$ dry tissue[111].

From the formulae above it may be seen that the three peptides have in common the pyroglutamyl residue at the N-terminus and the C-terminal octapeptide with the sole exception, for ranatensin, of a phenylalanyl residue replacing the leucyl residue, in position 2 from the C-terminus.

Bombesin and alytesin could be easily demonstrated on paper chromatograms and electropherograms of crude or, much better, semi-purified

extracts by means of colour reactions: the Pauly reaction (histidine), the coupling reaction with the NNCD reagent or *p*-dimethylaminobenzaldehyde reaction (tryptophan) and the Sachaguki reaction (arginine). Bombesin and alytesin were accompanied by other compounds giving similar colour reactions.

The first stage in the purification of bombesin-like peptides was chromatography on an alkaline alumina column, followed by elution with descending concentrations of ethanol. Bombesin and alytesin emerged in 85 to 80 per cent ethanol eluates, other bombesin-like peptides in 95 to 90 per cent ethanol eluates.

In fact, bombesin, alytesin and ranatensin are by no means the only representatives of this peptide family and the occurrence of bombesin-like peptides is not confined to discoglossid frogs and to *Rana pipiens*. At least three other bombesin-like peptides have been traced, in our screening, in amphibians of Australia and New Guinea, demonstrating that peptides of the bombesin group also have a fairly broad distribution. The elucidation of the structure of the new bombesins is in progress[112].

The spectrum of biological activity of alytesin and bombesin is characteristic, and the distinction of these polypeptides from the other polypeptides of the amphibian skin is easily accomplished by means of parallel bioassay. All the bombesin-like peptides so far tested have been found to display similar pharmacological actions, with only quantitative differences; thus, bombesin will be used as a prototype of this polypeptide group. The pharmacological study of bombesin is still in progress in several directions and here only the effects which may be considered as well established will be reported in some detail.

(i) Bombesin displayed a stimulant action on numerous preparations of intestinal, uterine and urinary tract smooth muscle. Sometimes the effect was easily repeatable and showed a fair proportionality to the dose, but at other times a prompt and intense tachyphylaxis was observed[111,113,114].

On the oestrous uterus of the rat, bombesin was approximately as active as bradykinin and oxytocin, and 3 to 5 times more potent than Val^5–angiotensin II. The threshold dose was of the order of 0.005 to 0.05 ng ml^{-1} nutrient liquid and there was a good dose–response relationship. With large doses of bombesin the increase in tone often persisted for hours, in spite of repeated washing of the organ with fresh nutrient liquid.

Although the *in vivo* pregnant uterus contracted in response to ranatensin or bombesin, the foetuses did not abort. In fact, the normal gestation periods of rats in the 18th, 20th and 21st day of pregnancy was not altered either by intravenous infusion of 1 μg kg^{-1} min^{-1} of ranatensin for 2 to 3 hours or by single intravenous doses of 10 μg administered every 10 minutes for the same period[114].

On the kitten small-intestine bombesin was again approximately as active as bradykinin (threshold 0.1 to 0.5 ng ml^{-1}), but far more potent than most biogenic substances known to stimulate smooth muscle, including Val^5–angiotensin II. For this reason and because of the frequent lack of tachyphylaxis the kitten small intestine must be included among the most suitable preparations for the quantitative assay of bombesin-like peptides.

Two additional preparations can be recommended: the guinea-pig colon

(threshold 0.03 to 0.5 ng ml^{-1}) and the rat urinary bladder, both isolated (threshold 0.2 to 1 ng ml^{-1}) and *in situ*. The last preparation responded to bombesin with a long-lasting increase in tone, often accompanied by reinforcement of movements. The threshold dose for rapid intravenous injection was 50 to 100 ng kg^{-1}, for intravenous infusion 30 to 50 ng kg^{-1} min^{-1}, and for subcutaneous injection 3 to 10 μg kg^{-1}. With 100 μg kg^{-1} a spasm of the urinary bladder could be observed, lasting longer than 3 to 4 hours.

Other smooth-muscle preparations were sensitive to bombesin but owing to prompt and intense tachyphylaxis they were unsuitable for the bioassay of the polypeptide (guinea-pig ileum, rat large intestine, rabbit duodenum and colon, guinea-pig urinary bladder and ureter); still other preparations were poorly sensitive or insensitive (rabbit, cat, guinea-pig and hamster uteri; hamster, chicken and frog intestinal loops, rat ureter, dog urinary bladder and ureter, monkey urinary bladder). Like bradykinin, bombesin caused a prevalent relaxation of the rat duodenum.

Bombesin seems to act on extravascular smooth muscle mainly by a direct effect. However, in some cases cholinergic and adrenergic mechanisms cannot be excluded.

(ii) In most species (dog, cat, rabbit, rat and chicken) bombesin elicited moderate hypertension, rarely exceeding 40 to 50 mmHg, which was usually gradual in onset and slow to disappear. Tachyphylaxis was frequent. During an intravenous infusion of bombesin in the dog (threshold 1 to 3 ng kg^{-1} min^{-1}) the rise in blood pressure could sometimes be maintained at a steady level as long as the infusion was continued, but at other times the rise of pressure slowly subsided with continued administration of the polypeptide. In the rat and the chicken hypertension elicited by high doses of bombesin was often followed by secondary hypotension. Bombesin-induced hypertension was apparently not affected by pretreatment with either α- or β-adrenergic blocking agents. Similarly, secondary hypotension was not abolished by atropine. Thus, the effect of bombesin on vascular smooth muscle seems to be predominantly a direct one. Angiotensin was usually more potent than bombesin, and its effect on blood pressure was more rapid and of shorter duration.

In sharp contrast to other species, the monkey responded to bombesin with frank hypotension (threshold 2 to 10 ng kg^{-1}, by rapid intravenous injection), which was usually proportional to the dose, and which was equal to, or greater than, that caused by eledoisin or physalaemin, and of longer duration. Tachyphylaxis was moderate for low and adequately spaced doses of the polypeptide, but prompt and intense for high doses. Long-lasting hypotension was obtained by intravenous infusion of bombesin (threshold 1 to 2 ng kg^{-1} min^{-1}), but repeated infusions caused tachyphylaxis[115].

(iii) Bombesin caused in the anaesthetized dog a potent antidiuretic effect, up to complete arrest of urine flow. Threshold doses, by intravenous infusion, were of the order of 0.5 to 1 ng kg^{-1} min^{-1}. Antidiuresis was the result of a reduction in glomerular filtration rate caused by a fall in intraglomerular hydrostatic pressure. This, in its turn, was provoked by afferent vasoconstriction. The spasmogenic effect of bombesin on the smooth muscle of the afferent arterioles was directly demonstrated by the radioactive microspheres technique and indirectly by the ^{85}Kr washout method and the ^{3}H-*p*-amino-

hippurate clearance. The vascular compartment most sensitive to bombesin was that of the outer cortical zone, especially in its external half. Filtration fraction decreased under the influence of bombesin, indicating that the effect of the polypeptide on postglomerular arterioles was, if present, only of minor importance.

At an infusion rate of 12 ng kg^{-1} min^{-1} glomerular filtration rate was virtually abolished whereas blood flow in the outer cortex and in the juxta-medullary cortex, as measured by the radioactive microspheres method, was reduced by 70 and 57 per cent, respectively.

Radioautographs of the kidney after injection of ^{85}Kr confirmed that washout of the gas was strikingly retarded following infusion of bombesin. For example, a similar pattern of distribution of radioactivity in external cortex was seen after 15 seconds in the kidney of control dogs, and after 4 minutes in the kidney of bombesin-treated animals.

At high infusion rates (above 6 ng kg^{-1} min^{-1}), bombesin produced a decrease in ^{3}H-*p*-aminohippurate extraction. The effect of the polypeptide on fractional distal delivery of sodium varied depending on the dose: at low infusion rates it decreased, at high infusion rates it increased. The total glucose appearing in urine following a glucose load was sharply reduced by bombesin. However, the ratio GRF:TmG, i.e. the concentration of glucose in urine, did not show any appreciable change. It is highly probable that the above events largely depend on the vascular effects of the polypeptide, but the possibility that they are in part due to a direct action of bombesin on the tubules cannot be excluded. Research is in progress to elucidate this crucial point[116, 117].

(iv) Afferent vasoconstriction produced by bombesin was accompanied by a conspicuous activation of the renin–angiotensin system and, if sufficiently prolonged and kept within certain limits, by a considerable release of erythropoietin[116–118].

Activation of the renin–angiotensin system was observed at threshold infusion rates less than 3 ng kg^{-1} min^{-1}. There was an increase in renin secretion, followed by increases of renin activity and angiotensin II concentrations in systemic arterial blood. At an infusion rate of 6 ng kg^{-1} min^{-1} renin secretion rose by 3 to 20 times, renin activity in arterial blood by 2 to 4 times, and finally angiotensin II concentration in arterial blood by 2 to 6 times. When bombesin was infused into one renal artery only the infused kidney presented afferent vasoconstriction and increased renin secretion.

The pattern of renin secretion under the influence of bombesin was different depending on the infusion rates of the polypeptide. At low infusion rates an increased renin secretion was seen throughout the infusion period, at high infusion rates two peaks of renin secretion could be observed, one at the beginning of the infusion, the other soon after the infusion had been discontinued. Arrest or reduction of renin discharge from the juxtaglomerular apparatus during bombesin infusion may be interpreted as a direct consequence of afferent vasoconstriction which slows down, owing to lack of *vis a tergo*, the flow of fluid through the interstitial space of the juxtaglomerular apparatus into which renin is released from the granular cells.

As already stated, a prolonged infusion of bombesin caused in the dog the liberation of erythropoietin from renal tissue[118]. 3 ng kg^{-1} min^{-1} infused

over a 6-hour period was virtually ineffective; 6 ng kg^{-1} min^{-1} produced a maximum erythropoietin release, up to 4- to 6-times the basal values; with 12 ng kg^{-1} min^{-1} erythropoietin release was considerably less pronounced. Erythropoietin in the dog blood began to increase 2 hours after starting the bombesin infusion, but increase outlasted interruption of infusion by more than 6 hours.

The causal agent for erythropoietin release is considered to be hypoxia. When this was mild (low rates of bombesin infusion) no evident erythropoietin release occurred; when it was exceedingly severe (excessive afferent vasoconstriction following high rates of bombesin infusion) erythropoietin release decreased.

From preliminary experiments it seems that liberation of erythropoietin and liberation of renin are independent events. In fact, at the time of maximum erythropoietin concentration no excess of renin activity was found in blood.

This would indicate that the site of renin production in the kidney is different from the site of erythropoietin production.

During the infusion of bombesin in the dog (6 ng kg^{-1} min^{-1} for 6 hours), oxygen consumption by the renal tissue decreased in parallel to the renal blood flow. However, oxygen extraction (i.e. arterio-venous oxygen difference) was critically dependent on the magnitude of the reduction in renal blood flow. When passage of blood through the kidney remained above 1 ml g^{-1} min^{-1} no appreciable changes in oxygen extraction occurred, in spite of a 60 to 80 per cent decrease in oxygen consumption; when renal blood flow fell below 1 ml g^{-1} min^{-1}, oxygen extraction clearly increased, to return again to basal values as soon as a renal blood flow exceeding 1 ml g^{-1} min^{-1} was restored[118].

(v) Although the actions of bombesin on the kidney are presently those most thoroughly studied, another important target organ for bombesin has emerged, quite unexpectedly, from the pharmacological screening. It is the stomach, especially that of the dog[119].

Bombesin may be considered a formidable stimulant of acid secretion in the denervated fundic pouch of the conscious dog. By subcutaneous injection the threshold dose of bombesin was of the order of 5 to 10 ng kg^{-1}, which is ten times less than the threshold dose of caerulein. A clear dose–response relationship could be seen up to an optimum of 2 µg kg^{-1}, where both volume and acid outputs attained peak values which were 6- to 20-times the basal values. For large doses of bombesin the effect lasted more than 4 to 5 hours. The concentration of hydrochloric acid in the gastric juice was 3 to 5 times above the basal values throughout the period of secretory response, whereas concentration of pepsin was always below the basal values. The threshold dose of bombesin capable of producing an appreciable increase in acid gastric secretion by intravenous infusion was 0.05 µg kg^{-1} h^{-1} and the effect was proportional to the dose up to 1 µg kg^{-1} h^{-1}.

In contrast to gastrin and caerulein, bombesin stimulated acid secretion by the dog stomach even when given by rapid intravenous injection. However, by this route of administration the polypeptide was less active than by either subcutaneous injection or intravenous infusion.

Atropine nearly completely inhibited the gastric secretory response to bombesin.

The polypeptide was a poor and irregular stimulant of the acid secretion in the perfused stomach preparation of the rat. Preliminary experiments in the chicken seem, on the contrary, to indicate that in this species bombesin is a good gastric secretagogue.

Finally, bombesin produced an increase in the 'short circuit current' of the isolated gastric mucosa of *Rana esculenta*, which was only 0.1 per cent of that produced by caerulein[120].

Research is in progress to elucidate the mechanism by which bombesin stimulates gastric acid secretion. There is strong experimental evidence that bombesin is a potent releaser of gastrin from the dog and man antral mucosa. In fact, intravenous infusions of bombesin produced in both species a conspicuous increase of immunoreactive plasma gastrin, which was particularly intense when acidification of the antrum was hindered and was lacking or strongly reduced in dogs and human patients subjected to antrectomy.

The panorama of the pharmacological actions of bombesin, although already broad, is far from being complete. Apart from the fact that the study of the effects of the polypeptide in the stomach and especially in the kidney may be considered little more than commenced, new actions of bombesin are emerging as our screening procedures are improving and expanding.

Available pharmacological data concerning ranatensin are limited to the effects of the polypeptide on some isolated smooth muscle preparations and on blood pressure of the common laboratory animals. On the whole ranatensin showed, as expected, a spectrum of biological activity very similar to that of bombesin. In fact, the peptide displayed a stimulant action on the guinea-pig ileum and the rat uterus and a relaxant effect on the rat duodenum. In addition it produced a relatively weak sustained contraction of the rabbit aortic strip, but not of the rat aortic strip[114].

Ranatensin raised blood pressure in the dog and rabbit, where it showed 10 per cent of the potency of angiotensin, but did not alter blood pressure in cats and had a variable action in the guinea-pig and rat. The peptide lowered blood pressure in the monkey, being as potent as eledoisin (threshold by intravenous injection, 2 to 9 ng kg^{-1}). In the dog the threshold intravenous doses were similar (10 ng kg^{-1}) for angiotensin and ranatensin, but the dose–response curve for ranatensin was more horizontal and the duration of action substantially longer.

Both hypertensive and hypotensive responses elicited by ranatensin are believed to be the result of a direct effect of the peptide on vascular smooth muscle[121, 122].

So far, only a relatively small number of bombesin-like peptides has been prepared by synthesis, essentially with the aim of determining the minimum length of the amino acid chain required for the first appearance of the bombesin effects on different smooth muscle preparations, and the minimum length necessary for maximal effects on the same preparations. Results are in part shown in *Table 7*. The activity of bombesin was set equal to 100, that of the bombesin-like peptides was expressed as a percentage.

From the comparative bioassay of bombesin-like peptides the following conclusions may be drawn:

(a) bombesin-like effects began to appear in the C-terminal heptapeptide.

Table 7. Relative potency of bombesin-like peptides, on a weight basis (bombesin = 100)

Peptides	Test preparations Guinea-pig large intestine	Cat small intestine	Rat uterus	Rat urinary bladder
Pyr–Gln–Arg–Leu–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2 Bombesin	100	100	100	100
Pyr–Gly–Arg–Leu–Gly–Thr–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2 Alytesin	50–150	70–300	70–100	n.t.
Ala–Val–Gly–His–Leu–Met–NH_2	<0.1	<0.1	<0.1	<0.1
BOC–Ala–Val–Gly–His–Leu–Met–NH_2	<0.1	<0.1	<0.1	<0.1
Trp–Ala–Val–Gly–His–Leu–Met–NH_2	0.1–0.5	<0.1–0.3	2.5–10	0.2–0.5
BOC–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	1–2	0.5–1	40–50	6–14
Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	2–8	1–4	20–30	2–10
BOC–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	25–60	40–100	40–75	30–60
Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	100–120	100–150	150–300	150–300
Thr–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	100–140	100–150	150–400	150–350
BOC–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	110–220	110–160	160–500	300–500
Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	110–200	90–200	120–200	100–150
BOC–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	30–60	30–110	30–60	25–40
Leu–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	100–160	80–150	50–200	100–170
Leu–Gly–Thr–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	80–130	80–150	45–180	60–100
BOC–Leu–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	120–210	60–120	35–130	70–120

Pyr, pyroglutamic acid; BOC, ter-butyloxycarbonyl.

The hexapeptide was fully inactive (<0.1 per cent). The preparation most sensitive to the heptapeptide was the rat uterus.

(b) addition of the glutamine residue to the N-terminus of the heptapeptide produced a 5- to 10-fold increase of activity on all tested preparations. Addition of the glycine residue to the N-terminus of the octapeptide likewise produced a striking increase of activity on all tested preparations. The C-terminal nonapeptide of bombesin was as active as, or even more active than, bombesin itself.

(c) protection of the N-terminal alanine residue of the hexapeptide with a *tert*-butyloxycarbonyl group (BOC-hexapeptide) did not cause the appearance of any activity. However, protection with BOC of the N-terminal tryptophan residue of the heptapeptide (BOC–heptapeptide) produced 5- to 20-fold increases of activity, yielding a compound which was as active as the octapeptide itself; similarly the BOC–octapeptide was several times more potent than the octapeptide, approaching the activity of the nonapeptide. The BOC–nonapeptide, on the contrary, was approximately as active as the nonapeptide, and the BOC–decapeptide consistently less active then the decapeptide.

Thus, the presence of the tryptophan residue seemed necessary for the appearance of the bombesin-like activity. Tryptophan could not be substituted by the BOC group. However, further lengthening of the peptide chain from the hepta- to the nona-peptide, which caused striking increase in activity up to an optimum in the nonapeptide, could be obtained not only by addition of amino acid residues, but also by the addition of the BOC group.

(d) no appreciable differences in activity could be seen between the nona- and the deca-peptide, and between the deca- and the endeca-peptide. These peptides were also hardly distinguishable from each other from a qualitative point of view, for example in the appearance of tachyphylaxis and in the shape of the response.

(e) substitution in the C-terminal nonapeptide of bombesin of threonine for asparagine, as in alytesin, did not change biological activity.

(f) replacement in the C-terminal octapeptide of bombesin of glutamic acid by asparagine or valine produced no changes in activity, except perhaps in the stimulant action on the rat uterus, which appeared to be increased. Replacement of glutamic acid by phenylalanine, on the contrary, increased the activity on all tested preparations.

As previously stated, the isolation of new natural bombesin-like peptides is in progress. It may be anticipated that they will differ consistently from bombesin in the intensity and duration of their effects on different test preparations. It is possible that they may represent models for the synthesis of bombesin-like peptides possessing peculiar characteristics.

Bombesin and bombesin-like peptides occupy in some respects a unique position among the active peptides of the amphibian skin. In fact, whereas all other hitherto described polypeptide groups of the amphibian skin have their counterpart in peptides occurring in mammalian tissues, this does not occur for bombesin-like peptides. The bradykinins of the amphibian skin have their duplicate in the mammalian plasma kinins, the physalaemin-like peptides of the amphibian skin are strictly related to substance(s) P of the mammalian brain and gastro-intestinal tract, and finally the caeruleins bear the strictest chemical and biological resemblance to cholecystokinin and, subordinately, to the gastrins.

So far a counterpart of the bombesin-like peptides is lacking in the mammalian organism. The question is whether this lack is a real one or whether it simply depends on the fact that bombesin-like peptides have never been sought with suitable methods. Our research group is presently trying to solve this problem.

MISCELLANEOUS POLYPEPTIDES

In addition to the previously described polypeptides which must be considered as firmly established chemical and pharmacological entities, several other active peptides have been traced in the skin of different amphibian species.

For example, two peptides mimicking, on the whole, physalaemin in their pharmacological effects, but certainly different from the tachykinins, have been traced in the skin of African and Australian amphibians, respectively.

Another peptide producing prolonged hypotension and possibly acting also as a releasing factor on endocrine glands has been found in a South American frog.

A major obstacle for the isolation and study of these peptides is the scarcity of material. However, sometimes there are also serious methodological difficulties, due to the considerable length of the amino acid chain.

The polypeptides found by Michl and coworkers[124–126] in the cutaneous venom of *Bombina variegata variegata* deserve a particular mention. So far the following peptides have been isolated in a pure form:

(I) Ala–Glu–His–Phe–Ala–Asp–NH_2
(II) Ser–Ala–Lys–Gly–Leu–Ala–Glu–His–Phe
(III) Gly–Ala–Lys–Gly–Leu–Ala–Glu–His–Phe
(IV) Gly–Ile–Gly–Ala–Leu–Ser–Ala–Lys–Gly–Ala–Leu–Lys–Gly–Leu–Ala–Lys–Gly–Leu–Ala–Glu–His–Phe–Ala–Asp–NH_2 Bombinin

It may be seen that peptide (I) is nothing but the C-terminal hexapeptide of bombinin and, similarly, that peptides (II) and (III) are strictly related to each other and to bombinin. These peptides, on the other side, have nothing to do with bombesin-like peptides.

Very little is known about the pharmacological properties of bombinin, apart from the indication that it is endowed with a potent haemolytic action.

DISCUSSION

It has been repeatedly pointed out that polypeptides herein described represent only a part of the active peptides occurring in amphibian skin. Not only do several already identified peptides still await isolation and thorough pharmacological study, but it is highly probable that other peptides are escaping our attention because their activity lies beyond the limits of our screening methods, although these are covering a progressively increasing number of pharmacological effects.

As previously noted, amino acid sequences found in amphibian skin are the repetition of identical or similar sequences present in mammalian tissues. There are sound reasons to believe that elucidation of the structure of other amphibian peptides will confirm this statement.

Why does the genetic code cause the allineation in the same sequence of the same amino acid residues in amphibian skin, in the posterior salivary glands of octopods and in different, sometimes highly differentiated tissues of mammals? What is the origin, the significance and the function of active peptides in amphibian skin?

These questions must be left open until the analytic, descriptive phase of research is more advanced and until sufficient information is available about the biosynthesis and fate of the polypeptide molecules.

For example we have at present no idea whether amphibian peptides are the result of a progressive aggregation of smaller peptides, single amino acid residues or both, or of a splitting or hydrolysis of larger peptides. Both peptide fragments and larger precursors have been occasionally traced in the skin. Research on the occurrence in the cutaneous tissue of enzyme systems

catalyzing on the one side the formation of active peptides and on the other side their breakdown and inactivation is completely lacking.

Anyhow, it seems probable that peptides present in methanol extracts pre-exist in the living skin in a free form or very loosely bound. In fact, by means of an injection of adrenaline into the lymphatic dorsal sac of a *Xenopus* it was possible to produce the prompt appearance of large amounts of caerulein in the water in which the frogs were immersed. This experiment shows two things: first that caerulein is ready for secretion in the skin, and secondly that it may be secreted externally together with the other components of the so called 'cutaneous venom'. The fact that polypeptides in the skin are mainly localized in the cutaneous glands, the secretion of which is generally held to be only external, and that peptides actually appear in the cutaneous secretion, does complicate the understanding of the possible significance and function of these extremely active molecules in the amphibian skin. A true endocrine function, following their discharge into the blood stream, lacks at present any experimental evidence. The intervention of the skin polypeptides in local regulation seems more conceivable. It has been tentatively suggested that they may interfere in some basic functions of the skin, for example in the regulation of the secretion of the skin or in the control of water and electrolyte exchanges through the skin.

It is obvious that the same function may be displayed in the different amphibian species by different polypeptides, and it is possible that polypeptides inactive in our screening systems are active in amphibian skin.

ACKNOWLEDGEMENTS

Results reported in this paper are the results of the common effort of several groups of research workers active in Italy and abroad. This research was supported throughout by grants from the Consiglio Nazionale delle Ricerche, Rome.

REFERENCES

[1] V. Erspamer, *Ann. Rev. Pharmacol.* **11**, 327 (1971).
[2] A. Anastasi, V. Erspamer and J. M. Cei, *Arch. Biochem. Biophys.* **108**, 341 (1964).
[3] G. Bertaccini, J. M. Cei and V. Erspamer, *Br. J. Pharmac.* **25**, 363 (1963).
[4] A. Anastasi and G. Falconieri Erspamer, *Experientia* **26**, 866 (1970).
[5] V. Erspamer, G. De Caro and R. Endean, *Experientia* **22**, 738 (1966).
[6] A. Anastasi and V. Erspamer, *Arch. Biochem. Biophys.* **101**, 56 (1963).
[7] M. M. Chang and S. E. Leeman, *J. Biol. Chem.* **245**, 4784 (1970).
[8] M. M. Chang, S. E. Leeman and H. D. Niall, *Nature (New Biology)* **232**, 86 (1971).
[9] G. Bertaccini, J. M. Cei and V. Erspamer, *Br. J. Pharmac.* **25**, 380 (1963).
[10] G. B. Fregnan and A. Glaesser, *Arch. Int. Pharmacodyn.* **171**, 435 (1968).
[11] M. Bergamaschi and A. H. Glaesser, *Circulation Res.* **15**, 391 (1964).
[12] W. Lochner and J. R. Parratt, *Br. J. Pharmac.* **26**, 17 (1966).
[13] J. Nakano, B. A. Darrow and J. R. McCurdy, *Arch. Int. Pharmacodyn.* **172**, 429 (1968).
[14] H. A. Kontos, W. Shapiro, H. P. Mauck and J. L. Patterson jr., *J. Appl. Physiol.* **19**, 113 (1964).
[15] G. Bertaccini and G. De Caro, *J. Physiol. (London)* **181**, 68 (1965).
[16] F. Lembeck, F. Geipert and K. Starke, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **261**, 422 (1968).
[17] G. De Caro and M. Cordella, *Ann. Oftalmol. Clin. ocul.* **91**, 933 (1965).

[18] N. Emmelin, P. Ohlin and A. Thulin, *Br. J. Pharmac.* **37**, 666 (1969).
[19] Ch. A. Schneyer and H. D. Hall, *Proc. Soc. Exp. Biol. Med.* **127**, 1245 (1968).
[20] G. Bertaccini, G. De Caro and M. Impicciatore, *J. Physiol. (London)* **193**, 487 (1967).
[21] G. Zetler, D. Mönkemeier and H. Wiechell, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **262**, 97 (1969).
[22] G. Falconieri Erspamer, L. Negri and D. Piccinelli, *Naunyn-Schmiedeberg's Arch. Pharmak. exp.* in press.
[23] P. Mantovani, G. L. Piccinin and G. Bertaccini, *Pharmacol. Res. Commun.* **1**, 172 (1969).
[24] G. De Caro, *Arch. Int. Pharmacodyn.* **146**, 27 (1963).
[25] E. Stürmer and B. Berde, *J. Pharmacol. Exp. Ther.* **140**, 349 (1963).
[26] T. G. Kantor, M. E. Jarvik and B. B. Wolff, *Proc. Soc. Exp. Biol. Med.* **126**, 505 (1967).
[27] S. H. Ferreira and J. R. Vane, *Br. J. Pharmac.* **30**, 417 (1967).
[28] H. Broghammer, *Klin. Wschr.* **41**, 1097 (1963).
[29] E. F. Gersmeyer, A. Castenholz and M. Nicolay, *Klin. Wschr.* **43**, 309 (1965).
[30] F. Pratesi, A. Nuti, S. Brunetti, R. P. Dabizzi and L. Caramelli, *Folia angiologica* **3**, 266 (1966).
[31] I. Szám, D. Kusztos and G. Csapó, *Arzneimittelforschg.* **16**, 1671 (1966).
[32] G. De Caro, M. Cordella and P. Miani, *Ophthalmologica* **158**, 284 (1969).
[33] G. Falconieri Erspamer, A. Anastasi and J. M. Cei, *J. Pharm. Pharmac.* **22**, 466 (1970).
[34] B. Camerino, G. De Caro, R. A. Boissonnas, Ed. Sandrin and E. Stürmer, *Experientia* **19**, 339 (1963).
[35] E. Stürmer, Ed. Sandrin and R. A. Boissonnas, *Experientia* **20**, 303 (1964).
[36] L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer, A. Glaesser and O. Goffredo, *Experientia*, **20**, 306 (1964).
[37] L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer, A. Glaesser and O. Goffredo, *Experientia*, **21**, 695 (1965).
[38] L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer and O. Goffredo, *Experientia* **22**, 29 (1966).
[39] E. Schröder and K. Lübke, *Experientia* **20**, 19 (1964).
[40] K. Lübke, R. Hempel and E. Schröder, *Experientia* **21**, 84 (1965).
[41] E. Schröder, K. Lübke and R. Hempel, *Experientia* **21**, 70 (1965).
[42] F. Lembeck, A. Oberdorf, K. Starke and R. Hettich, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **261**, 338 (1968).
[43] R. S. Dunn and A. M. Perks, *Experientia* **26**, 1220 (1970).
[44] J. J. Pisano, *Fed. Proc.* **27**, 58 (1968).
[45] A. Anastasi, V. Erspamer and G. Bertaccini, *Comp. Biochem. Physiol.* **14**, 43 (1964).
[46] A. Anastasi, V. Erspamer, G. Bertaccini and J. M. Cei, in *Hypotensive Peptides*, p. 76. (Ed. E. G. Erdös, N. Back and F. Sicuteri). Springer Verlag, New York (1966).
[47] A. Anastasi, V. Erspamer and G. Bertaccini, *Brit. J. Pharmacol.* **27**, 479 (1966).
[48] T. Nakajima, *Chem. Pharm. Bull.* **16**, 769 (1968).
[49] M. L. Roseghini and J. M. Cei, *J. Gen. Comp. Pharmacol.* **3**, 195 (1972).
[50] M. R. F. Furtado, *Biochem. Pharmacol.* **21**, 118 (1972).
[51] G. Fischer and W. Albert, *Z. Naturforschg.* **26b**, 1021 (1971).
[52] A. Anastasi, V. Erspamer and R. Endean, *Arch. Biochem. Biophys.* **125**, 57 (1968).
[53] G. De Caro, R. Endean, V. Erspamer and M. Roseghini, *Br. J. Pharmac.* **33**, 48 (1968).
[54] A. Anastasi, G. Bertaccini, J. M. Cei, G. De Caro, V. Erspamer, M. Impicciatore and M. Roseghini, *Br. J. Pharmac.* **38**, 221 (1970).
[55] A. Anastasi, G. Bertaccini, J. M. Cei, G. De Caro, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **37**, 198 (1969).
[56] B. Endean, V. Erspamer, G. Falconieri Erspamer and L. Negri, To be published.
[57] V. Erspamer, G. Falconieri Erspamer, L. Negri and J. Visser, To be published.
[58] A. C. Alcala, V. Erspamer, G. Falconieri Erspamer and L. Negri, To be published.
[59] G. Bertaccini, G. De Caro, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **34**, 291 (1968).
[60] G. Bertaccini, G. Ballarini, A. Agosti and G. Zannetti, *Arch. Int. Pharmacodyn.* **183**, 261 (1970).
[61] M. Vagne and M. I. Grossman, *Amer. J. Physiol.* **215**, 881 (1968).
[62] G. Bertaccini, T. Braibanti and F. Uva, *Gastroenterology* **56**, 862 (1969).
[63] I. Orlandini and A. Agosti, *Radiol. Medica, Torino* **55**, 1061 (1969).

[64] R. Carratù, G. Arcangeli and F. Pallone, *Rendic. Rom. Gastroenterol.* **3**, 28 (1971).
[65] A. Agosti, P. Mantovani and L. Mori, *Naunyn-Schiedebergs Arch. Pharmakol.* **268**, 114 (1971).
[66] T. M. Lin and G. F. Spray, *Gastroenterology* **56**, 1178 (1969).
[67] G. Bertaccini and A. Agosti, *Gastroenterology* **60**, 55 (1971).
[68] M. L. Ramorino, M. V. Ammaturo and F. Anzini, *Rend. Rom. Gastroenterol.* **2**, 172 (1970).
[69] M. Del Tacca, G. Soldani and A. Crema, *Agents and Actions* **1**, 176 (1970).
[70] M. Del Tacca, S. Pacini, G. Amato, C. Falaschi and A. Crema, *Europ. J. Pharmacol.* **17**, 171 (1972).
[71] A. Vizi, G. Bertaccini, M. Impicciatore and J. Knoll, *Gastroenterology* **64**, 268 (1973).
[72] A. Agosti, G. Bertaccini, R. Paulucci and E. Zanella, *Lancet* **1**, 395 (1971).
[73] G. Bertaccini, G. De Caro, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **37**, 185 (1969).
[74] M. I. Grossman, in *Proc. Symp. Exocrine Pancreas*. Queen's University, Kingston, Ontario, Canada, June 5–7, 1969.
[75] G. F. Stening and I. M. Grossman, *Amer. J. Physiol.* **217**, 262 (1969).
[76] G. De Caro, I. Ronconi and N. Sopranzi, in *Prostaglandins, Peptides and Amines* (Symposium Florence 1968), p. 167. (Ed. P. Mantegazza and E. W. Horton), Academic Press, New York and London (1969).
[77] J. Meldolesi, *Brit. J. Pharmacol.* **40**, 731 (1970).
[78] L. Angelucci and G. Linari, *Eur. J. Pharmacol.* **11**, 204 (1970).
[79] G. Bertaccini, G. De Caro and P. Melchiorri, *Brit. J. Pharmacol.* **40**, 78 (1970).
[80] G. De Caro, G. Improta and P. Melchiorri, *Experientia* **26**, 1145 (1970).
[81] F. Fallucca, R. Carratù, G. Tamburrano, M. Javicoli, G. Menzinger and D. Andreani, *Horm. Metab. Res.* **4**, 55 (1972).
[82] L. Dorigotti and A. H. Glaesser, *Experientia* **24**, 806 (1968).
[83] G. F. Stening and I. M. Grossman, *Gastroenterology* **56**, 1047 (1969).
[84] G. Bertaccini, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **34**, 311 (1968).
[85] L. R. Johnson, G. F. Stening and M. I. Grossman, *Gastroenterology* **56**, 1255 (1969).
[86] A. Agosti, S. Biasioli and G. Naranjo, *Boll. Soc. Ital. Biol. Sper.* **45**, 778 (1969).
[87] A. Agosti, S. Biasioli and G. Bertaccini, *Gastroenterology* **59**, 727 (1970).
[88] G. F. Stening, L. R. Johnson and M. I. Grossman, *Gastroenterology* **57**, 44 (1969).
[89] A. M. Brooks, A. Agosti, G. Bertaccini and I. M. Grossman, *New England J. Med.* **282**, 535 (1970).
[90] L. Negri and V. Erspamer, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **277**, 401 (1973).
[91] P. Melchiorri and N. Sopranzi, *Pharmacol. Res. Commun.* **2**, 135 (1970).
[92] P. Melchiorri and N. Sopranzi, *Agents and Actions* **2**, 58 (1971).
[93] L. Angelucci, M. Baldieri and G. Linari, *Eur. J. Pharmacol.* **11**, 217 (1970).
[94] R. S. Jones and M. I. Grossman, *Amer. J. Physiol.* **219**, 1013 (1970).
[95] L. Angelucci, L. Micossi, F. Cantalamessa and G. Linari, *Arch. Int. Pharmacodyn.* **196** Suppl., 92 (1972).
[96] L. Angelucci, G. Linari, L. Micossi and F. Cantalamessa, *Abstr. 5th Internat. Congress Pharmacology*, p. 132. SanFrancisco, 23–28 July 1972.
[97] L. Angelucci, G. Linari, L. Micossi and F. Cantalamessa, to be published.
[98] L. Angelucci, L. Micossi and F. Cantalamessa, *Arch. Int. Pharmacodyn.* **196**, Suppl., 89 (1972).
[99] L. Angelucci, L. Micossi and M. Parri, *Abstr. 5th Internat. Congress Pharmacology*, San Francisco p. 133, 23–28 July 1972.
[100] G. Bertaccini, G. De Caro, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **33**, 59 (1968).
[101] A. D. Care, J. B. Bruce, J. Boelkins, A. D. Kenny, H. Conaway and G. S. Anast, *Endocrinology* **89**, 262 (1971).
[102] E. Solcia, C. Capella, T. Chieli, O. Bellini and C. Bertazzoli, in *Gastrointestinal Hormones* p. 103. (Ed. L. Demling). G. Thieme Verlag, Stuttgart (1972).
[103] T. Chieli, C. Bertazzoli, G. Ferni, I. Dell'Oro, C. Capella and E. Solcia, *Toxicol. Appl. Pharmacol.* **23**, 480 (1972).
[104] A. Anastasi, L. Bernardi, G. Bertaccini, G. Bosisio, R. De Castiglione, V. Erspamer, O. Goffredo and M. Impicciatore, *Experientia* **24**, 771 (1968).

[105] L. Bernardi, G. Bertaccini, G. Bosisio, R. Bucci, R. De Castiglione, V. Erspamer, O. Goffredo and M. Impicciatore, *Experientia* **28**, 7 (1972).
[106] J. S. Morley, *Proc. 8th Europ. Peptide Symposium*, p. 226. Noordwijk-on-Sea, The Netherlands, Sept. 1966. North Holland Publishing Co., Amsterdam (1967).
[107] T. Nakajima, T. Tanimura and J. J. Pisano, *Feder. Proc.* **29**, 284 Abstr. (1970).
[108] A. Anastasi, V. Erspamer and M. Bucci, *Arch. Biochem. Biophys.* **148**, 433 (1972).
[109] A. Anastasi, *Naunyn-Schmiedebergs Arch. Pharmakol*, **269**, 135 (1971).
[110] L. Bernardi, R. De Castiglione, O. Goffredo and F. Angelucci, *Experientia*, **27**, 873 (1971).
[111] V. Erspamer, G. Falconieri Erspamer, M. Inselvini and L. Negri, *Brit. J. Pharmacol.* **45**, 333 (1972).
[112] A. Anastasi, R. Endean, V. Erspamer, G. Falconieri Erspamer and L. Negri, to be published.
[113] V. Erspamer, G. Falconieri Erspamer and M. Inselvini, *J. Pharm. Pharmacol.* **22**, 875 (1970).
[114] R. G. Geller, W. C. Govier, J. J. Pisano, T. Tanimura and V. Van Clineschmidt, *Brit. J. Pharmacol.* **40**, 605 (1970).
[115] V. Erspamer, P. Melchiorri and N. Sopranzi, *Brit. J. Pharmacol.* **45**, 442 (1972).
[116] P. Melchiorri, N. Sopranzi and V. Erspamer, *J. Pharm. Pharmacol.* **23**, 981 (1971).
[117] V. Erspamer, P. Melchiorri and N. Sopranzi, *Brit. J. Pharmacol.*, in press.
[118] P. Melchiorri and N. Sopranzi, *J. Pharmacol. Exp. Ther.* in press
[119] G. Bertaccini, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* in press.
[120] L. Negri, personal communication.
[121] B. V. Clineschmidt, R. G. Geller, W. C. Govier, J. J. Pisano and T. Tanimura, *Brit. J. Pharmacol.* **41**, 622 (1971).
[122] W. J. Louis, T. Tanimura and J. J. Pisano, *Eur. J. Pharmacol.* **14**, 340 (1971).
[123] R. De Castiglione, F. Angelucci, V. Erspamer, G. Falconieri Erspamer and L. Negri, to be published.
[124] G. Kiss and H. Michl, *Toxicon* **1**, 33 (1962).
[125] A. Csordás and H. Michl, *Toxicon* **7**, 103 (1969).
[126] A. Csordás and H. Michl, *Mschr. Chem.* **101**, 182 (1970).