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Peptides – From Research Tools to 'Soft' Drugs? [1]

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Abstract. Over the last 30 years, regulatory peptides have played an ever increasing role as research tools but as therapeutics they have been applied in only a very limited number of indications. The recent call by the public for 'soft' pharmaceuticals and 'soft' chemistry, the increasing knowledge on peptide receptors and intracellular signalling, the design of more potent and specific peptide ligands as well as the availability of sufficient quantities of large peptides by genetic engineering may be the starting point for a new era of peptides as 'natural' therapeutic agents in human and animal medicine. In the first part, this review focusses on new achievements in peptide chemistry as well as on the role of peptides as research tools, *i.e.* their pleiotropic nature and their mechanisms of action. The second part summarizes new concepts for the application of peptides in medical diagnosis and therapy.

1. Introduction

The peptides comprise a great variety of biologically active linear and cyclic biopolymers with diverse functions. They can be divided into different classes, such as signalling or regulatory factors (*i.e.* hormones and growth regulators), antibiotics, alkaloids, toxins, and sweeteners (*Table 1*). While antibiotics became very important therapeutics as soon as they could be produced in large enough quantities after world war II, there has been a continued argument on the potential for peptide hormones in medical therapy, except perhaps for insulin. The high biological potency of peptides and their complete metabolism by the organism into the natural subunits, the amino acids, would suggest that they are ideal therapeutic agents. However, the susceptibility of peptides to enzymatic attack in the gut and the circulation with a resulting short half-life as well as the need to administer them by the route of injection were the principal arguments against their application as drugs.

Since the 1950's, when chemists began to develop methods for efficient peptide synthesis, signalling peptides became more and more important as research tools, resulting in an ever increasing demand for these substances by biologists and pharmacologists. The great interest in peptides is also reflected by the continuously growing number of participants at peptide symposia as well as of specialized companies that produce and sell peptides. However, the number of peptide drugs on the market has remained comparatively small although a few of them have become very important therapeutics. The availability of large peptide and protein factors by genetic engineering together with new therapeutic concepts will considerably widen the list of peptide medicals in the future.

The scope of this review is to give a short account of the present role of peptides as research tools and pharmaceuticals, the emphasis being put on some of the major chemical and biological topics of interest. A short historical retrospective will be followed by a few remarks on modern methods for the preparation of peptides and by a summary on the mechanisms of action of regulatory peptides and their interaction with membrane receptors. The second part of the review will concentrate on the use of peptides as diagnostics and 'soft' therapeutics.

Table 1. *The Different Classes of Peptides and Some Selected Examples*

Antibiotics	Actinomycin D, bacitracin, penicillin, gramicidin
Enzyme inhibitors and substrates	ACE inhibitors, renin inhibitors, HIV protease inhibitors; synthetic oligopeptides for enzyme diagnostics
Hormones and regulatory factors	Pituitary hormones, neuropeptides, insulin, growth factors, cytokines
Peptide alkaloids	Ergotamine
Toxins	Amanitine, phalloidine, α-bungarotoxin, abrin, ricin
Sweeteners	Aspartam

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Alex N. Eberle: Born 1945 in St.Gallen, Switzerland, studied chemistry, biochemistry, and molecular biology at the Swiss Federal Institute of Technology (ETH) in Zürich. There he also wrote his Ph.D. thesis on peptide synthesis and biology. He then worked as a research fellow at the MRC Laboratory of Molecular Biology in Cambridge U.K. together with *F. Sanger, J.E. Walker, C. Milstein*, and *R.C. Sheppard*. In 1982 he joined the Department of Research of the University Hospitals in Basel where he became its deputy chairman in 1990. His research focusses on the mechanism of action of peptides, *i.e.* peptide receptors and signal transduction pathways, as well as on the application of peptides to medical diagnosis and therapy. His particular interest at present are the effects of the melanotropins and various growth factors on melanoma. In 1988 he received the *Leonidas Zervas Award for Peptide Chemistry* and in 1991 the *Robert-Wenner-Prize of the Swiss Cancer League*.

Only a few original publications and reviews will be cited. The reader will find more detailed information in the proceedings of the last three peptide symposia [2] and in the relevant journals [3].

2. A Short Historical Retrospective

The beginning of peptide chemistry is usually traced back to the early 20th century when *Emil Fischer* in Berlin coined the term 'peptide' after he and *E. Fourneau* had synthesized glycylglycin, the simplest dipeptide. *Fischer*, the nobel laureate of chemistry of 1902, attracted many scientists to his Institute, among them famous names such as *Max Bergmann*, and with his team made important contributions to the field of peptides during its first epoch until world war I. The real roots of peptide chemistry, however, are to be found much earlier in Munich where *Theodor Curtius* in 1881 accidentally obtained benzoyldiglycine by condensing hippuric acid with glycine. The second epoch of peptide chemistry between the two world wars was characterized by a number of important discoveries of peptidic factors, such as insulin by *Banting* and *Best*, penicillin by *Fleming*, *Chain*, and *Florey*, glutathione by *Harrington* and *Mead* and the peptide alkaloid ergotamin by *Stoll*. In this period, the first useful protecting group still used in peptide chemistry today, the benzyloxycarbonyl or Z group, was introduced by *Bergmann* and *Zervas*. The third epoch after world war II was very productive and brought

Table 2. *Regulatory Peptides Arranged According to the Year of Structural Analysis*

1951	Oxytocin
1953	Insulin
1954	Adrenocorticotropin, vasopressin
1956	β-Melanotropin, angiotensin
1957	α-Melanotropin, glucagon
1960	Bradykinin
1962	Eledoisin
1964	Gastrin, physalaemin
1965	β-Lipotropin
1966	Secretin, somatotropin (growth hormone)
1968	Calcitonin, cholecystokinin, C-peptide
1969	Thyrotropin-releasing hormone, nerve growth factor, prolactin
1971	Choriongonadotropin, vasointestinal peptide, gastric inhibitory peptide, lutropin, lutropin releasing hormone, substance P, bombesin, neurophysin
1972	Follitropin, epidermal growth factor
1973	Motilin, somatostatin, tuftsin
1975	Enkephalin, pancreatic polypeptide, thymopoietin
1976	Delta-sleep inducing peptide, β-endorphin, insulin-like growth factor
1977	Relaxin, granuloliberin
1978	Fibroblast growth factor
1979	Gastrin releasing peptide, glicentin, α-/β-neoendorphin,kyotorphin, thymosin
1980	Interferons-α/β
1981	Corticotropin-releasing factor, dynorphin, oxyntomodulin, peptide histidine isoleucine (PHI), calcitonin gene-related peptide, erythropoietin, sauvagine
1982	Growth hormone releasing factor, neuropeptide Y, peptide YY, rimorphin, katacalcin, urotensin, interferon-γ
1983	Neuromedin B, neuromedin K, galanin, melanin-concentrating hormone, atriopeptin, platelet-derived growth factor, transforming growth factor α/β, interleukin-2
1984	Tumour necrosis factor α and β, cerebellin, interleukin-3
1985	Valosin, granulocyte-macrophage colony stimulating factor, interleukin-1α and β, neuropeptide K, neutrophil peptide
1986	Cyclolinopeptide A, leucopyrokinin, leucokinin, leukomyosuppressin, pancreastatin, galanin-associated peptide, interleukin-6
1987	Follicular gonadotropin-releasing peptide, cardioactive peptide, interleukin-4, interleukin-5, interleukin-8
1988	Corticostatin, interleukin-7, neuropeptide-γ, endothelin

The list covers the first 100 regulatory peptides discovered between 1951 and mid 1988, *i.e.* from oxytocin to endothelin.

the field of peptide chemistry much attention [4]: after the structure of oxytocin had been published by *DuVigneau* in 1951, more than fifty new peptide hormones and regulatory factors were structurally analyzed and chemically synthesized up to 1980 (*Table 2*). Important contributions were made by the peptide groups in Basel which synthesized, amongst others, adrenocorticotropin, calcitonin, parathormone, and insulin. Among the new synthetic methods introduced during this period, the solid-phase peptide chemistry technique invented by *B. Merrifield* (the *Nobel* laureate of chemistry of 1984) in the early sixties made it possible to produce thousands of peptide analogues at reasonable costs and in a short time. During the fourth epoch of the last ten years the number of known peptides has doubled (*Table 2*) and the field of peptide and protein chemistry has been opened up to molecular biology and genetic engineering. The latter has resulted in the structural analysis of an increasing number of prohormones and receptors as well as the preparation of large peptides which are not accessible through conventional chemical synthesis.

3. Modern Synthetic Methods

Peptides for research purposes are usually required in only mg-to-g amounts. This is the optimal scale for the time-saving solid-phase peptide synthesis which is the method of choice in any modern peptide research laboratory [5]. Originally, the *Merrifield* technique was based on the attachment of the first N^{α}-protected amino-acid residue to gelatinous chloromethylated polystyrene beads, obtained by copolymerization of styrene with 1% divinylbenzene. The N^{α}-protecting group was usually a *t*-butoxycarbonyl (Boc) group which is cleaved under moderately acidic conditions, leaving the benzyl-type side-chain protecting groups as well as the Ts groups (→ protection of arginine) unaffected. As the polystyrene resin requires apolar solvents for swelling, each synthesis cycle consisted of a complex wash scheme with various solvents in order to meet the criteria for optimal solvation of both the gel resin and the growing peptide chain. The whole procedure offered itself to automation, which resulted in a number of commercially available instruments [6]. The cleavage of the peptide from the resin and the side-chain deprotection, however, still remains a manual step and is carried out in strong acid, such as liquid HF. As these conditions frequently caused a number of side-reactions, new resins were developed with which novel strategies of synthesis could be applied, in particular simplified coupling reaction cycles and relatively mild cleavage conditions. The most noteworthy method was developed by *Sheppard* and *Atherton* [7] who introduced a physically supported polyamide gel resin consisting of

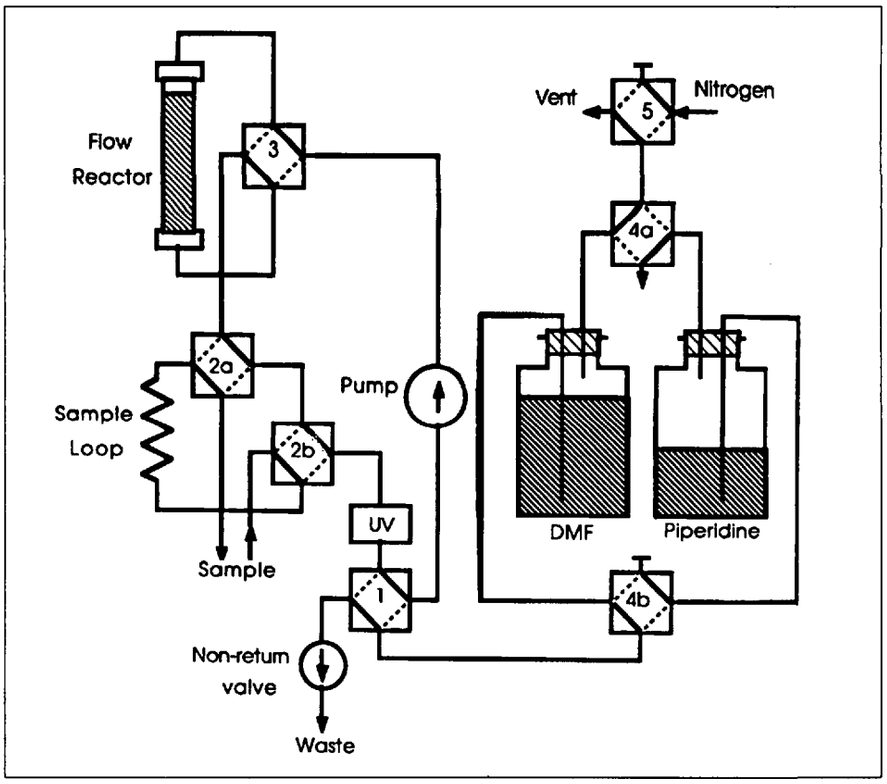

Fig. 1. *Scheme of a (semi-)automatic equipment for continuous-flow peptide synthesis designed by* Atherton *and* Sheppard [7]. The polymer is packed into a flow reactor which is connected to a pump, UV monitor and sample loop. *Valve 1* switches the system from recirculation (coupling reaction) to flow-through into the waste (washing steps; deprotection). *Valve 2a/b* introduces loaded sample from the sample loop into the system. *Valve 3* reverses the flow direction in the reactor. *Valve 4* selects the solvent (DMF or piperidine). *Valve 5* is activated for flushing the bottles with nitrogen. Fully automated instruments contain a sample pipetting system with which not only the amino acids but also additional reagents for the coupling step can be introduced into the recirculation system. An increased number of valves allows the selection between more than two different solvents.

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a macroporous *Kieselgur* carrier filled with a copolymer of *N,N*-dimethylacrylamide, ethylene bisacrylamide, and acryloylsarcosine methyl ester. The latter is converted to a primary amine by reaction with ethylene diamine, thus forming the C-terminal attachment point. This pressure-resistant polymer is characterized by excellent swelling properties in *N,N*-dimethylformamide (DMF) and its suitability for the continuous flow technique: the polymer is packed into a column and the reactions are carried out by either recirculation of the activated amino acid residues (→ coupling reactions) or flow-through of solvents (→ cleavage of the N^{α}-protecting group and washing steps). The orthogonal protection strategy is based on the base-labile fluorenylmethoxycarbonyl (Fmoc) group for N^{α}-protection and *t*-Bu and other acid-labile groups for ω-protection. This results in a simple reaction cycle where the solvent is either DMF (for coupling reactions and washing steps) or 20% piperidine in DMF (for the cleavage of Fmoc). Automated instruments based on this concept have been developed by *R.C. Sheppard* and *E. Atherton* (*Fig. 1*) and have also become commercially available [8].

The current methodology used by many investigators, including the author, is summarized in *Fig. 2*. The resin is either the macroporous *Kieselgur* (→ Pepsyn K) or a macroporous polystyrene containing a polydimethylacrylamide gel (→ Polyhipe) or polyoxyethylene grafted onto polystyrene (→ TentaGel). All these resins have excellent performance in DMF as the sole solvent. They are usually derivatized with an internal amino-acid residue (→ reference) and an acid-labile linker which, after cleavage, either produces a C-terminal free acid or carboxamide. The protection by Fmoc of N^{α}-groups is combined with *t*-Bu-type protecting groups for side chain $-NH_2$, $-OH$ and $-COOH$. Arginine is frequently protected by N^{G}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl) (Pmc) and histidine, asparagine and glutamine by trityl (Trt) whereas the protection of cysteine may consist of acetamidomethyl (Acm), trityl or combinations thereof (*Fig. 2*). In addition, a number of other valuable protecting groups have been introduced in the past few years [5][7]. The coupling reaction is carried out *a*) by the active ester method using pentafluoromethyl (Pfp) or dihydrobenzotriazine (DHBt) esters of Fmoc-amino acids, *b*) by applying a condensation reagent forming an intermediary uronium ester with the Fmoc-amino acid (*e.g.* TBTU; see *Fig. 2*), *c*) by the 'classical' carbodiimide method using the reagent pair diisopropylcarbodiimide and hydroxybenzotriazole (DIC/HOBt), or *d*) by symmetrical anhydrides of Fmoc-amino acids. Cleavage from the resin with trifluoroacetic acid (TFA) requires the presence of scavengers such as 20% dithioethane, thioanisol, or others, particularly when the peptide contains tryptophan, in order to suppress side-reactions of the cations originating from cleaved protecting groups. The purification scheme usually combines ion exchange and gel chromatography with reversed-phase chromatography on C_{18} columns and, less frequently, partition chromatography, counter-current distribution, and ion pair chromatography. Modern analysis no longer relies solely on HPLC runs, TLC and amino-acid analysis and sequencing, but includes high performance capillary electrophoresis, fast atom bombardment (FAB) mass spectroscopy, HPLC combined with ion spray mass spectroscopy or laser-desorption ionization (LDI) mass spectroscopy and other specialized analytical methods (CD, NMR spectroscopy, IR spectroscopy, polarimetry *etc.*).

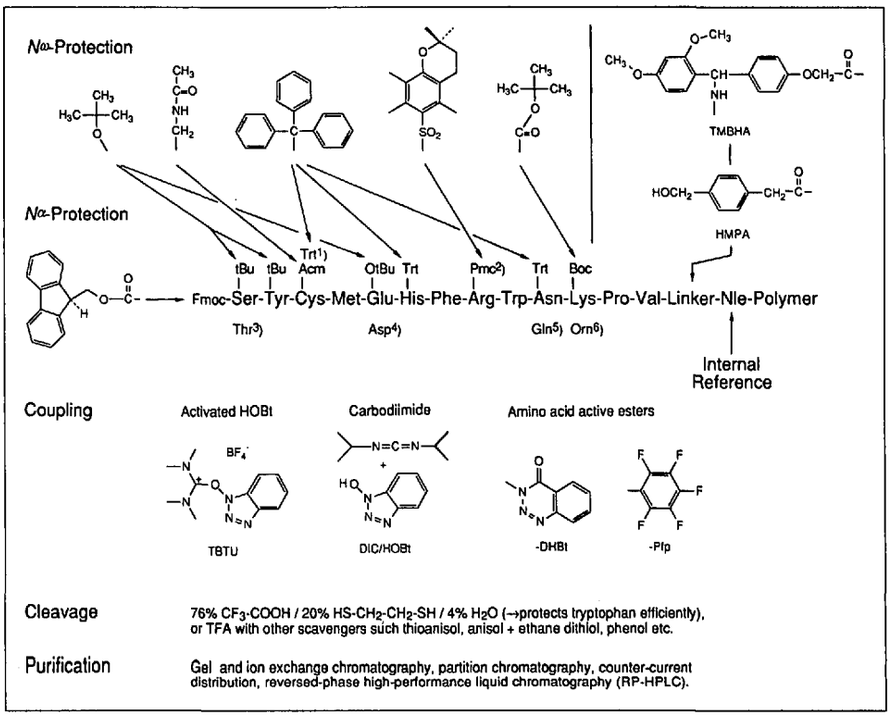

Fig. 2. *Elements of the continuous-flow solid-phase peptide synthesis using continuous-flow methodology*. The example shows an analogue of α-melanotropin with 13 different amino acids containing all those residues for which specific side-chain protection is required. The orthogonal protection strategy uses Fmoc for N^{α}-protection and acid-labile groups for side-chain protection. The linkers are also labile to diluted TFA (TMBHA) or more concentrated TFA (HMPA). Both types of linker exist in several variants with different acid-lability, forming either C-terminal free acid functions upon cleavage (→ HMPA) or carboxamide groups (→ TMBHA). The physically supported polymers are either Kieselgur/polyamide, Polyhipe or TentaGel. The coupling reaction is carried out by using coupling reagents such as TBTU and related compounds, carbodiimides with HOBt, active esters of the Fmoc-amino acids or preformed symmetrical anhydrides. The cleavage and deprotection is performed in TFA using scavengers to protect residues such as tryptophan, histidine, tyrosine, methionine and others. The purification and analysis employs established methodology.

Abbreviations: Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; DHBt, dihydrobenzotriazinyl; DIC, diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazol; HMPA, 4-hydromethylphenylacetic acid; Pfp, pentafluorophenyl; Pmc, N^{G}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl); TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate; TFA, trifluoroacetic acid; TMBHA, trimethoxybenzhydrylamine; Trt, trityl. [1]) Trityl is cleaved by acid treatment; if disulfide formation is required, this must be done before cleavage from the resin. [2]) Arginine may alternatively be protected by Mtr (methoxytrimethylphenylsulfonyl) or be applied in unprotected, protonated form. [3]) Thr is protected as Ser. [4]) Gln is protected as Asn. [5]) Orn is protected as Lys.

Synthesis with the continuous-flow technology is very rapid, particularly with the TentaGel resin where a synthesis cycle is complete in less than 30 min. For more rapid syntheses of a large number of peptides, multiple synthesis methods have been developed, such as the so-called tea-bag method by *Houghton* [9], the pin-synthesis by *Geysen et al.* [10] and a robotic work-station with a 48-column reactor by *Gausepohl et al.* [11]. While peptides synthesized in this way may be useful in the search for antigenic determinants *etc.*, the quality of the products are often insufficient for pharmacological studies, as the peptides are frequently used without further purification.

The classical peptide synthesis by fragment condensation still plays an important role for those sequences which cannot be obtained in good yield or quality by the solid-phase technology. The synthesis in homogeneous solution is also the most frequently used method for the production of large quantities of peptides (although an increasing number of peptide products is prepared by solid-phase technology). The special features of peptide production were reviewed by *Feurer* [12] many years ago and are still valid. Like all chemical processes, both solid-phase and classical peptide chemistry require energy and considerable amounts of solvents, particularly when medium-sized or long peptides are produced, such as the 32-residue peptide calcitonin or the 39-residue peptide corticotropin which require almost a hundred steps or more. However, as practically all reactions take

Table 3. *Peptide Hormones and Cell Growth Factors Produced by Genetic Engineering which Are Either Commercially Available or Being Developed for Medical Application*

Anti-AIDS therapeutics	
CD-4	Soluble receptor acts as a decoy for the AIDS virus
AIDS vaccine	Viral proteins or peptide fragments are being tested as vaccine candidates
Cardiovascular and clotting factors	
Atrial natriuretic peptide	Vasodilator/diuretic peptide; potential use in the prophylaxis and treatment of acute renal failure
Applaggin	Peptide from snake venom, inhibits platelet aggregation, potential use in prevention and treatment of arterial clots
Hirudin	Thrombin inhibitor; potential use in prevention and treatment of venous blood clots
Hirugen	Peptide derivative of hirudin; same application
Plasminogen activator	Helps dissolve blood clots in heart attack and cardiovascular disorders
Factor VIII	Blood clotting factor protein deficient in the major forms of hemophilia
Colony stimulating factors (CSF) and erythropoietin	
Erythropoietin	Regulates production of red blood cells; used to treat anemia in kidney dialysis patients, AIDS and cancer
G-CSF	Granulocyte-CSF stimulates production of white blood cells; used to treat cancer patients following chemotherapy and infectious diseases
GM-CSF	Granulocyte-macrophage-CSF stimulates production and functional activities of white blood cells; use similar as for G-CSF
M-CSF	Macrophage-CSF stimulates production and functional activities of monocytes and macrophages; potential use in cancer therapy
Growth factors (GFs) and metabolic regulators	
BDNF	Brain-derived neurotrophic factor affects survival and maintenance of central nervous system
EGF	Epidermal growth factor stimulates growth of skin cells; potential use in skin grafting, eye surgery, burns
FGF	Fibroblast growth factor promotes growth of fibroblasts, keratinocytes and formation of new blood vessels
Growth hormone	Growth regulator acting *via* IGF; promotes growth in children
IGF	Insulin-like growth factor is a metabolic regulator; potential use in wasting syndromes, severe burns and wound healing
Insulin	Regulates blood sucrose levels; important therapeutic for the treatment of type I diabetes
Insulinotropin	Peptide hormone that modulates release of insulin by the pancreas; potential use in type II diabetes
NGF	Nerve growth factor affects growth and differentiation Alzheimer's disease
PDGF	Platelet-derived growth factor has a potential similar to FGF
TGF-β	Transforming growth factor-β stimulates growth of numerous cell types; potential application in wound healing and burns
Interferons (INFs)	
α-Interferon	Immune stimulant for cancer therapy
β-Interferon	Immune stimulant for treatment of viral diseases and multiple sclerosis
γ-Interferon	Immune stimulant for treatment of infectious diseases, cancer and rheumatoid arthritis
Interleukins (ILs)	
Interleukin-1α/β	Multiple direct and indirect effects on blood cells and various tissues; potential use in cancer therapy and inflammation
IL-1-RA	Interleukin-1 receptor antagonist; potential use as antiinflammatory agent
Interleukin-2	Stimulates production and function of T cells; application in cancer therapy and infectious diseases
Interleukin-3	Stimulates differentiation of white blood cells. May be used in combination with GM-CSF
Interleukin-4	Promotes growth of activated T and B cells to kill tumours; treatment of immune deficiency
Interleukin-5	Acts on specific white blood cells (eosinophils, B cells)
Interleukin-6	Stimulates T and B cells, early blood cells and platelet production; potential application in cancer therapy
Interleukin-7	Stimulates T and B cells; potential use to speed up recovery following chemotherapy
Interleukin-8	Chemotactic factor for neutrophils
Interleukin-9	Regulator of the hematopoietic and immune systems; stimulates growth of T cells
Interleukin-10	Inhibits cytokine synthesis by T helper cells
Interleukin-11	Synergistic action with IL-3 in stimulating white blood cells. May be used in combination with IL-3 and GM-CSF
IL receptors	Soluble versions of natural cytokine receptors; potential use to regulate autoimmune and inflammatory reactions
Other peptide factors	
Collagenase inhibitor	Protein inhibitor of collagenase; potential application in rheumatoid arthritis and other inflammatory conditions
Elastase inhibitor	Protein inhibitor of elastase; potential use in cystic fibrosis and emphysema
Relaxin	Hormone that softens birth canal
TNF-α	Tumour necrosis factor-α is toxic for many cells and displays a wide range of immunoregulatory activities, similar to IL-1. A potential use in tumour killing is by transplantation of its gene into TILs (see text)
TNF-β	Tumour necrosis factor-β (see TNF-α)
TNF inhibitor	Tumour necrosis factor inhibitors may limit the systemic damage in certain immune-mediated conditions, *e.g.* septic shock, cachexia
Trypsin inhibitor	Protein inhibitor of trypsin for treatment of pancreatitis
TSH	Thyroid stimulating hormone; for use in thyroid imaging procedures
Vaccines, antibodies, immunotoxins	
Antibodies	Monoclonal antibodies are used for diagnostic and imaging purposes of various diseases
Immunotoxins	Monoclonal antibodies linked to toxin molecules for potential use against cancer cells
Vaccines	Recombinant viral and bacterial proteins used to prepare subunit vaccines (safer and more effective than killed whole organisms)

place at ambient temperature and since the amounts produced are very much lower than those of other chemicals (because of the high biological potency of most regulatory peptides), the risks involved with peptide production are very much smaller. Therefore, it is not unreasonable to regard peptides as substances that are produced by so-called 'low-risk' or 'soft' chemistry.

The weight share of peptides of the world-wide annual chemical production is only about 0.002–0.003%, and most of this can be accounted for by peptide antibiotics, the immunoregulator ciclosporin (cyclosporin A) and the sweetener aspartam. The hormones and regulatory factors play only a minor role. However, in terms of value added, they are very much more important. Some of these peptides, *e.g.* insulin, reach a turnover of more than one billion dollars per year. Slightly lower is the share for calcitonin, a peptide hormone which in the past few years has become an important drug for the treatment of hypercalcemia, *Paget*'s disease, osteoporosis and pain (mainly of patients with bone cancer). The 'softness' of these peptides is illustrated by the fact that *e.g.* 1 kg of salmon calcitonin yields *ca.* 30 million single therapeutic doses.

4. Recombinant Peptide Medicals

An alternative and even 'softer' method for the preparation of medium-sized peptides such as calcitonin and corticotropin is

the biotechnological production in bacteria, yeast, or cultured mammalian cells. This method is also the only economic way to produce peptides of more than 50 amino acids in length and peptides with complicated glycosyl or other functional groups. Attempts are even being made to apply this method to the production of short peptides such as aspartam, *e.g.* by using DNA clones containing up to 150 repeats of the dipeptide [13].

The principle of the expression of a 'foreign' gene in host cells is relatively simple: in order to be expressed and controlled through the normal biosynthetic and genetic machinery of the host cell, the cloned gene has to be linked to the genetic control elements that are characteristic of the host species. This is accomplished through the use of specialized extra-chromosomal elements called expression vectors (*e.g.* plasmids) that are specifically designed for overexpression of the relevant genes in the host cells. The different expression vectors available to date are specific for the different types of host cell (prokaryotic and eukaryotic cells) and there are a number of different host cell systems available for the production of polypeptides [14]. The relevant problems with which the biotechnologists are confronted include *1*) the selection of the appropriate expression strategy, *2*) the selection of the host cell system, *3*) the selection of a plasmid that displays a high enough copy number and good stability (→ stability of DNA), *4*) the control of transcript stability (→ stability of mRNA), *5*) the control of stability of the translated polypeptide, and *6*) the isolation and purification of the protein product.

The expression strategy is either based on direct expression or on secretion. In the first, promoter sequences are linked directly to the ATG translation initiation codon followed by the coding region of the 'foreign' gene. The protein product is usually highly expressed but accumulates in the cytoplasm of the cells and has to be isolated from cell lysates, purified and (if it contains disulfide bonds) refolded and cyclized. In addition, the N-terminal extra methionine is generally not part of the desired sequence and has to be cleaved. Secretion vectors contain as additional element the gene of a signal peptide which directs the translated protein into the secretion pathway of the host cell. The signal peptide is removed during secretion by a signal peptidase, generating the desired amino terminus. Disulfide bond formation, and when eukaryotic cells are being used as hosts, glycosylation or other modifications also occur during the secretory process. Thus, the secretion strategy is the method of choice if posttranslational modifications of the product are required.

There are at present more than 50 regulatory peptide factors produced by genetic engineering that are being developed for medical application (*Table 3*). Some of these recombinant products have already been introduced as therapeutics, including human insulin, human growth hormone, erythropoietin, interferons, interleukin-2, colony stimulating factors and several growth and other regulatory factors [15]. For research purposes an increasing number of mutants thereof are prepared *via* site-directed mutagenesis of the gene in order to analyze the contribution of a specific structure of a protein to its function. This is an important part of modern protein engineering: potentially important side chains are identified in the structural model of a protein and the corresponding residues are then removed or changed by mutagenesis. This technique is not only applied to the structure-function analysis of peptide and protein ligands but also to the study of structure-function relationships in receptors [16].

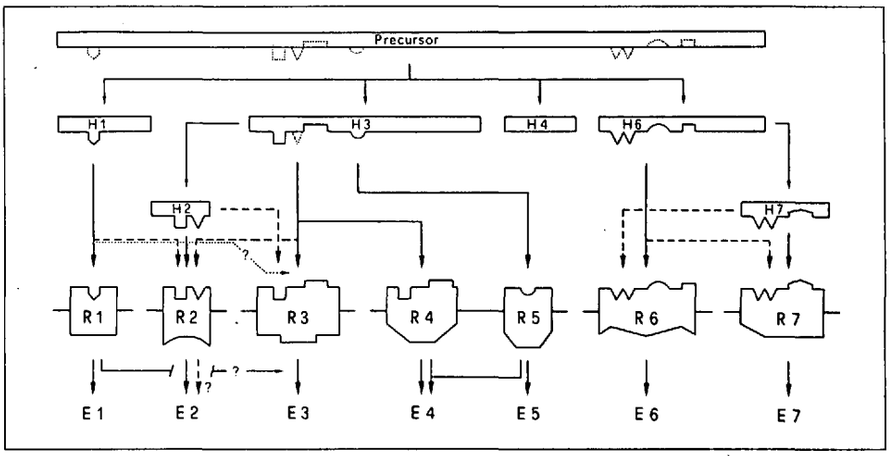

Fig. 3. *Pleiotropic action of peptide hormones* [17]. A biologically inactive precursor is processed into active peptides which themselves may be further cleaved into smaller fragments with a different function. Certain parts of the information within these peptides – although contained in the larger sequence – are scarcely transferred at all when the respective message sequences are obscured. Hormone H stimulates receptor R which transduces the signal, eventually leading to the effect E. (⊔) = Message (stimulating portion of the hormone); (⊔) = buried (obscured) message; (⊓) = address (binding portion of the hormone). H1 interacts well with R1 (—) and weakly with R2 (·····). H2 contains two message sequences, both of which are necessary for full stimulation of R2 (it is not known whether R2 transduces the signal *via* one or two pathways). H3 stimulates with one of its messages R3 and R4 (receptors of different tissues having the same topography) and with another message R5 (a different kind of receptor); the full effect E4 may depend on simultaneous stimulation of R4 and R5. H6 and its fragment H7 interact well only with their own respective receptors R6 and R7, despite an identical message; truncation at the C-terminus of H6 changes the topography of the address region.

5. Peptides Are Pleiotropic Molecules

Peptides are known to elicit a variety of effects and to interact with different tissues and cells. There are two reasons for this multiple or 'pleiotropic' action of most peptide hormones and regulatory factors: *i*) receptors for almost all peptides are expressed by different target cells, linking the hormonal signal to slightly differing biological effects; *ii*) many peptides contain a multiplicity of signalling information, resulting in the interaction with different types of receptors, thus again linking the hormonal signal to several biological responses (*Fig. 3*). Another important aspect is that peptide hormones are produced *via* large prohormones which frequently serve as precursor molecules for several biologically active peptides with different functions. The various forms of pleiotropy of peptides is best illustrated by the group of opiomelanocortins, comprising adrenocorticotropin (ACTH), the lipotropins, the melanotropins (MSHs), and the endorphins, each of which has a major target (ACTH: adrenal cortex; MSH: melanocytes and melanoma cells; endorphins: certain neurons) but also interact with a number of additional tissues. The precursor of these peptides, proopiomelanocortin (POMC), occurs in many tissues, mainly the pituitary gland and the brain, and is processed differently according to the gland cells or neurons in which it is produced [17]. *Fig. 3* is a schematic representation of a hypothetical prohormone similar to POMC showing the different possible forms of interrelations.

The pleiotropy of peptides is a disadvantage when they are used in therapy because they may induce side effects owing to the redundancy of signalling information. A well-known example is the problem of ACTH treatment of young children affected by the *West* syndrome, one of the most frequent forms of epilepsy in the very young. Although the severe side effects induced by prolonged administration of ACTH (mainly metabolic and psychic disorders) disappear after termination of therapy, they could perhaps be avoided if a derivative was at hand that contained precisely the essential signalling information but had a reduced adrenocortical activity. An ACTH/MSH compound with a very much reduced pleiotropic character has been developed by *D. DeWied* and *W.H. Gispen* and their colleagues in Holland for the treatment of nerve regeneration and the protection from nerve damage by cisplatin which is used to treat cancer patients. This compound, *Org 2766*, was derived from the $ACTH_{4-10}$ fragment which still contains very weak adrenocortical activity; oxidation of Met, replacement of Arg by D-Lys and of Trp-Gly by Phe yielded a compound

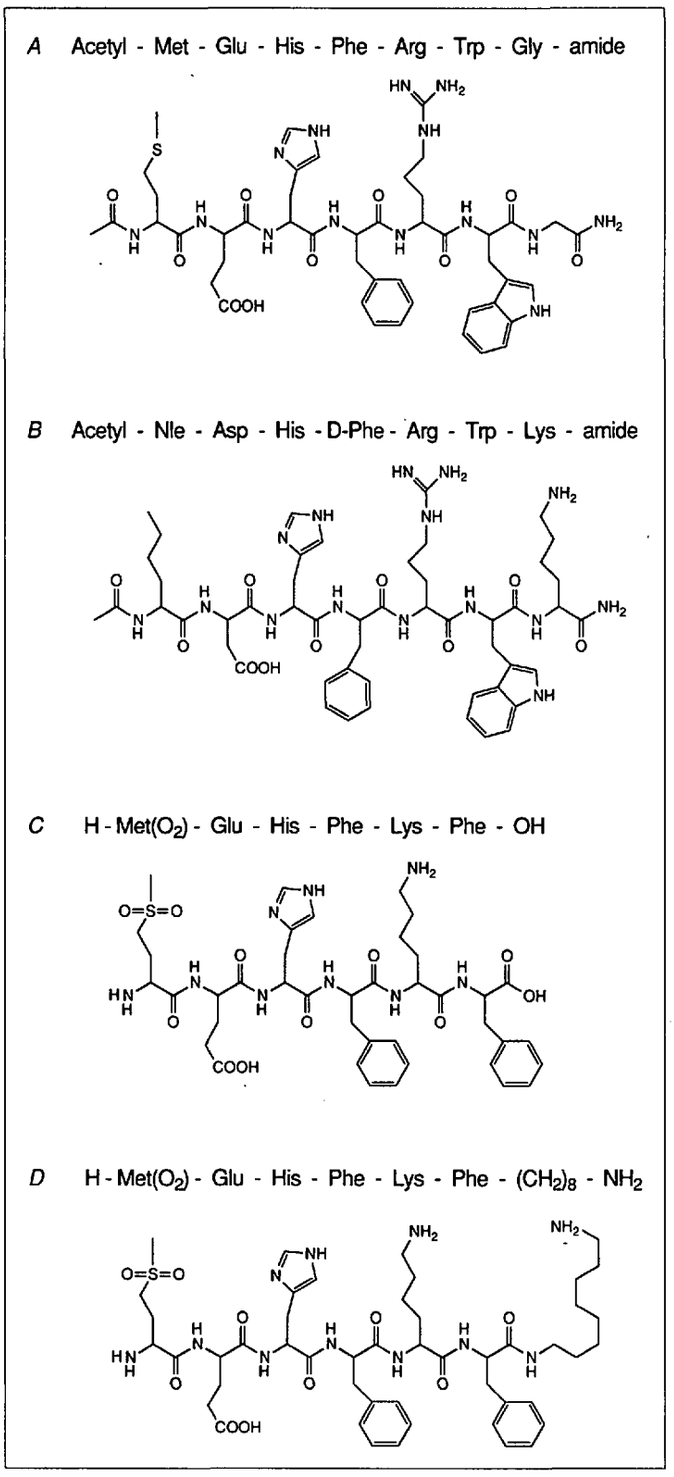

Fig. 4. *Modified structures of the MSH/ACTH$_{4-10}$ fragment with selective acitivity on the pigmentary or central nervous systems. A*) Structure of the MSH$_{4-10}$ fragment. *B*) Structure of an analogue with 1–2000fold higher pigmentary activity than *A*. *C*) Structure of the *Org 2766* analogue with a 1000fold higher CNS activity than *A* and undetectable pigmentary activity. *D*) Structure of the *Hoe 427* analogue with a 10fold higher CNS activity than *C*.

which was 1000fold more potent in certain neuronal assays. Additional modifications to this molecule by *R. Geiger* and collaborators in Frankfurt yielded a compound (*Hoe 427*) suitable for treating mild mental disorders without side effects (*Fig. 4*). It is interesting to note that the original ACTH$_{4-10}$ fragment is also a weak agonist for melanocytes and melanoma cells (with an acetylated N-terminus and amidated C-terminus *ca.* 1–2000 times less potent than α-MSH). If Met is exchanged by Nle, Glu by Asp, Phe by D-Phe and Gly by Lys (*Fig. 4*), the biological activity is increased over 1000fold. Thus, the potency difference between structure B and C of *Fig. 4* in the pigment cell system is more than 10^7 which demonstrates that it is possible to produce very selective agonists.

Structure-activity studies during which a large number of peptide analogues and fragments are synthesized and tested in different biological assays have become the most popular tool for the development of new peptide drugs during the past 20 years. The principal aim of such studies is the design of hormone analogues with features of practical utility for medical application, such as increased potency, greater metabolic stability, increased selectivity, prolonged time course of action or ability to inhibit the natural hormone (antagonistic properties). Such studies have also proved to be very useful for the elucidation of both the mechanism of hormone-receptor interactions and the organization of information within the peptide. It was found, *e.g.*, that in many linear (short) peptides the hormonal messages are formed by discrete sequences of adjacent amino-acid residues and that a peptide may contain several different messages which may even partly overlap. Typical examples are the POMC peptides ACTH, MSH, and endorphin [17].

6. Peptides for Receptor Analysis

When *Paul Ehrlich*, the father of chemotherapy and *Nobel* laureate in medicine of 1908, performed his studies on toxins and antitoxins, he discovered the principle of the receptor: '*corpora non agunt nisi fixata*'. The dual function of a receptor as discriminator for the ligand on the cell surface and as transducer of the hormonal signal into the cell was established in the nineteen fifties and sixties but our knowledge on the structural features of peptide hormone receptors is very young. To know more about the discrimination function of a receptor and in particular to develop more specific ligands (agonists and antagonists), a complete three-dimensional structure of a receptor is of greathelp. One of the major problems, however, is the low number of receptor molecules that are expressed per cell. In most cases the identification of receptor molecules requires elaborate protein chemical techniques, such as photoaffinity labelling. There are only a few fortunate examples where a partial protein structure could be obtained. When the modern DNA cloning strategies were applied to receptor cDNA and genes, it became possible to obtain complete primary sequences of receptors. Such sequences are now known for about two dozen peptide receptors but there is still only one crystallographic diffraction analysis, namely that of the G protein-coupled type of receptor molecule bacteriorhodopsin.

Most analyses of receptors would be impossible without a set of labelled peptide ligands containing tritium, iodine-125, a fluorescence marker, biotin or another affinity or photoaffinity group (*Fig. 5*). As for structure-activity studies (see above), it is important to apply different types of peptide ligands that are labelled with different types of marker groups suitable for, *e.g.*, hormone-receptor binding studies, chemical or photochemical cross-linking of the hormone with its receptor or quantification of receptors in tissues by receptor autoradiography. Labelled peptides are also indispensable for their measurement in biological fluids (see below).

The study of peptide-receptor interactions and signal transduction as well as the structural analysis by cloning of receptor

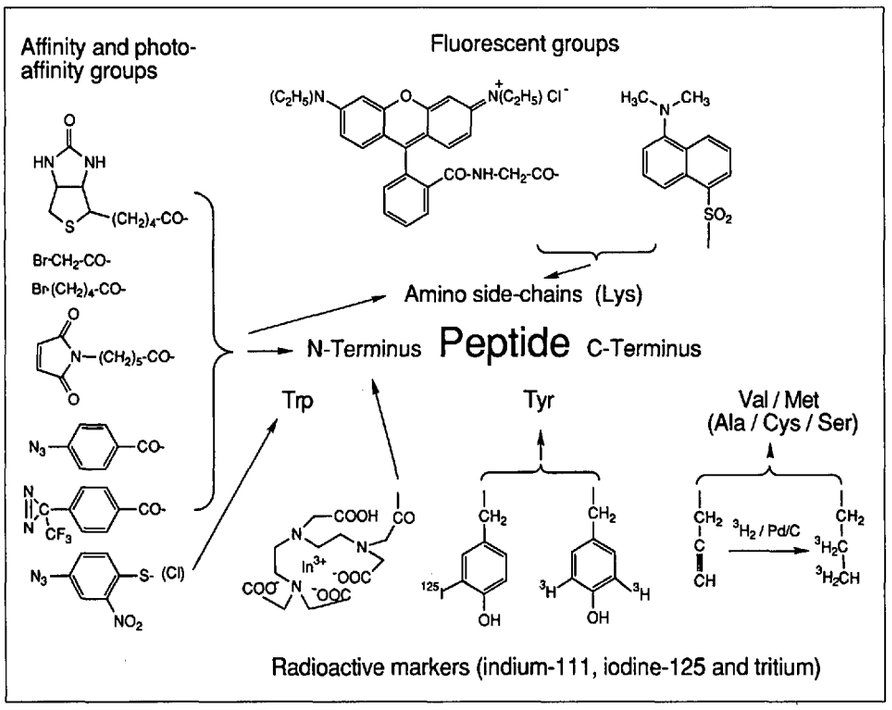

Fig. 5. *Radioactive, fluorescent, and chemical marker groups for peptides.* The fluorescent groups (*e.g.* rhodamine-glycyl; dansyl) and the affinity and photoaffinity groups (*e.g.* biotinyl; bromoacetyl; 5-bromovaleryl; N^{ε}-maleimidocaproyl; 4-azidobenzoyl; 4-(3-trifluoromethyldiazirino)benzoyl) are introduced into the amino terminus or a side-chain amino group at a late stage during synthesis. The same applies to the 2-nitro-4-azidophenylsulfenyl chloride reagent (bottom, left) which can be attached specifically to the 2'-position of tryptophan. Photoreactive amino acids (*e.g. p*-azidophenylalanine, not displayed) can be introduced at any time during the synthesis. For radioactive labelling, a suitable precursor is prepared, *e.g.* a peptide containing L-propargylglycine, which incorporates 4 tritium atoms (→ $^3H_4$-L-norvaline), or L-3',5'-diiodotyrosine which incorporates 2 tritium atoms (→ $^3H_2$-L-tyrosine). Radioiodination with $Na^{125}I$ requires a tyrosine (or a histidine) residue in the peptide chain. The DTPA chelator for ^{111}In (see text) is usually attached to the N-terminus of the peptide.

molecules has led to a fairly precise idea how receptors are arranged in a cell membrane and coupled to signal transducing elements. An example for the arrangement of two G protein-coupled receptors linked to each other is given in *Fig. 6*: the MSH receptor, which induces dispersion of melanin granules in melanophores and melanin (pigment) production in melanocytes and melanoma cells, and the α_2-adrenergic receptor, which reverses the action of the MSH receptor. The MSH receptor stimulates adenylate cyclase by activation of the G_s regulatory protein whereas the α_2-adrenergic receptor inhibits adenylate cyclase by activation of the G_i regulatory protein. Peptide receptors frequently interact with G_p regulatory proteins that activate phospholipase C, resulting in inositol-triphosphate as intracellular signal (→ releases calcium ions from intracellular calcium stores) and diacylglycerol which stimulates protein kinase C. Another group of receptors, those for growth factors, contain tyrosine kinase activity and regulate cellular processes directly by phosphorylation of the relevant proteins. Yet another group of receptors are ligand-induced ion channels transporting either calcium, sodium or potassium ions. It is interesting that one and the same peptide may interact with more than one receptor. Thus, the understanding of intracellular signalling is crucial for the development of new and specific therapeutics.

7. Peptides in Medical Diagnosis

Endocrinological diagnosis has long been based on the measurement of peptides in biological samples. The radioimmunological techniques required have become very important in the clinic since *S. Berson* and *R. Yalow* introduced the method for insulin about thirty years ago. Numerous radioimmunoassays have been developed for research purposes as well as for clinical application, and there is an increasing number of immunometric assays available that do no longer require radioisotopes as markers (bioluminescence, chemiluminescence, fluorescence *etc.*). An important aspect is the use of peptides as antigens for the generation of specific antibodies: a partial peptide sequence is selected as optimal antigenic determinant and attached specifically and uniformly to the carrier protein. This usually results in more specific antibodies than random attachment to the carrier. Alternatively, the peptides may be linked in small sets to carrier templates thus stabilizing secondary structures in these peptides. An example for this kind of concept are the template-assembled synthetic proteins (TASP) developed by *Mutter* and *Vuilleumier* [18], which can be applied to the generation of specific antibodies or to the study of peptide-protein interactions. *Jung* and coworkers [19] introduced tripalmitoyl-*S*-glycerylcysteinyl peptides as immunoadjuvants in combination with or covalently linked to the antigens. These lipopeptides are potent stimulators of B lymphocytes, similar to the lipoproteins from the outer cell membrane of bacteria, and thus appear to be very promising as synthetic vaccines.

Another important application of synthetic peptides is their use as substrates in enzymology. At present hundreds of enzyme substrates containing suitable chromophores are on the market for the quantification of the activity of the various enzymes.

More recently tumour-specific synthetic peptides carrying a suitable radioactive marker have proved to be valuable tools for tumour diagnosis. They are either applied *in vitro* on tumour sections for the determination of the 'receptor status' of the cells (*Fig. 7*) or injected into the body for tumour localization. For the *in vitro* application, ^{125}I is the most frequently used radioisotope whereas for *in vivo* administration, the peptides are labelled with the short-lived ^{123}I or with ^{111}In or ^{99}Tc both of which require peptides carrying a suitable chelator. Diethylenetriaminepentaacetic acid (DTPA) as chelator for ^{111}In has been studied extensively for labelling tumour-specific antibodies [20] and several peptides, *e.g.* the somatostatin-octapeptide

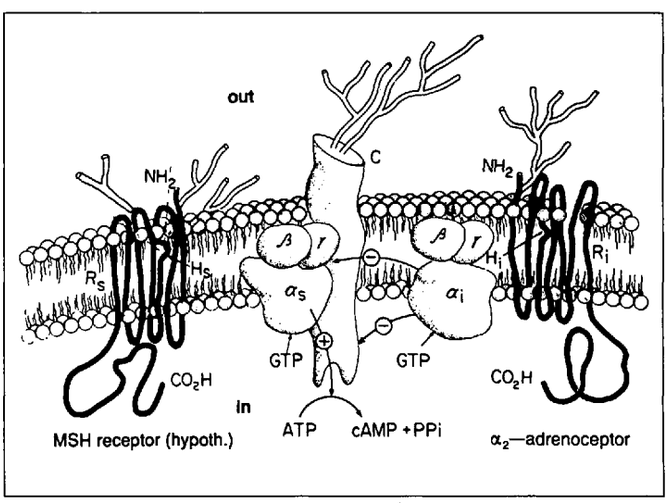

Fig. 6. *Schematic representation of the structure of two associated G protein-coupled receptors.* Both the MSH receptor (hypothetical) and the α_2-adrenoceptor are present on pigment cells and both are linked *via* stimulatory G proteins (MSH) and inhibitory G proteins (α_2) to the same adenylate cyclase (C). The G proteins are composed of three different subunits (α, β, γ) and require GTP. The intracellular signal of MSH is cAMP formed by C from ATP.

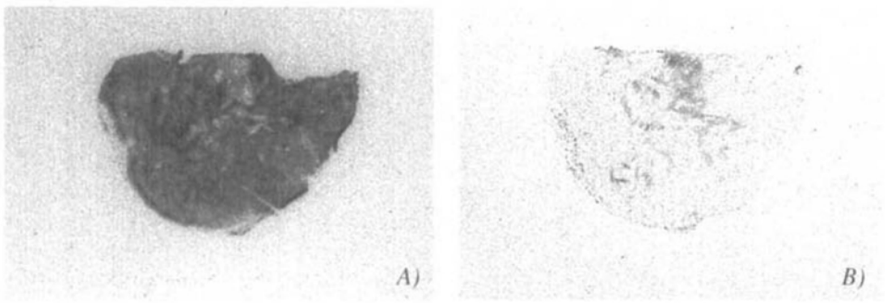

Fig. 7. *Study of the MSH receptor 'status' of melanoma tissue by quantitative receptor autoradiography.* Cryosections of melanoma were incubated with MSH radioligand in the absence *A*) and presence *B*) of excess unlabelled hormone. Quantitative anlaysis of a series of such autoradiograms reveals the receptor number per cell.

(*Sandostatin*) and a fragment of α-melanotropin. Such peptides appear to be very promising not only for tumour diagnosis but also for therapeutic application.

8. Peptides in Therapy

One of the principal problems in the general use of peptides as 'natural' therapeutic agents is less the availability of suitable compounds but rather their route of application, which should circumvent the syringe. Several alternatives have already been developed and are being used in the clinic, such as the nasal sprays for calcitonin, luteinizing hormone-releasing hormone (LHRH; *Buserelin*), oxytocin, and vasopressin. Rectal suppositories for calcitonin will soon be on the market, and ointments are being tested for application of peptides through the skin. While the oral administration of the vasopressin analogue desmopressin is well established, a first series of tests has been carried out on orally administered insulin: a microemulsion of peptide, fatty acids, lecithin, and cholesterol was formed and absorbed on cellulose and then embedded in gelatine capsules. In this way, it was possible to treat diabetic patients for a short period of time almost as effectively as with injections [21]. However, long-term administration of horm-ones such as insulin requires a well controlled dosage adapted to each individual patient and, therefore, will probably prove unsuitable for oral application.

Although a number of excellent peptide compounds have become available for therapeutic application over the last ten years, there is always a need for more specific substances (see *e.g.* the problem with the ACTH therapy of the *West* syndrome mentioned above). Theoretical modelling of bioactive conformations and synthesis of stabilized molecular forms of peptides are getting more and more important in the search of new therapeutics. The aim is to find peptides with reduced structures exhibiting a higher potency and longer duration of action than the natural compounds. An increased stability of the peptides to biodegradation frequently turns out to be the most decisive factor. *E.g.*, it was possible to synthesize forms of somatostatin with reduced structures that retain the pharmacological properties of the parent hormone but exhibit a very much prolonged duration of the effect (*Fig. 8*). Similarly, truncated or conformationally restricted analogues of α-MSH were found to be over tenfold more stable in the circulation than the natural hormone and to bind at least as well to the receptor or even better (see *Fig. 4* and [17]). Such compounds are potential drugs for cancer-diagnosis and therapy, *e.g.* as carriers of radioisotopes or cytotoxins.

9. Pseudopeptides and Peptidomimetics

Increasingly important are peptidomimetics, compounds that are derived from peptide structures but are no longer true peptides. In particular, protease inhibitors are currently developed for the treatment of cardiovascular diseases, such as renin inhibitors and angiotensin-converting enzyme inhibitors, as well as for the control of infections (*e.g.* inhibitors of HIV protease). The aim of these efforts is to find molecules that can be administered orally and that exhibit an equally high specificity as the corresponding peptides. *Fig. 9* displays the structure of two transition state inhibitors of aspartyl proteases developed by *Roche*: *A*) an orally active inhibitor of renin that is specific for the primate enzyme and does hardly affect the activity of other aspartyl proteases (*e.g.* pepsin, cathepsin D or angiotensin-converting enzmye), and *B*) a HIV protease inhibitor which contains a pseudo-peptide group and displays a high specificity and potency [22]. The latter is currently being tested in AIDS patients.

Another important area are non-peptide receptor antagonists which block the effect of excessively produced endogenous peptide [23]. Well known examples are opioid peptide antagonists such as naloxone or norbinaltorphimine, and CCK and gastrin antagonists such as lorglumide and loxiglumide. Naloxone is an important pharmacological agent for experimental studies but is also used to treat opiate intoxications. Loxiglumide is a very specific blocker of the CCK receptor in the gut and may be used to treat pancreatitis.

10. New Therapeutic Concepts

An area of great interest at present are the cytokines, a group of large polypeptides that regulate a variety of cells, mainly of the hematopoietic and immune systems (see also *Table 3*). As the cytokines communicate between the neural, endocrine, and immune systems, they affect a number of tissues and participate in the regulation of various processes, including tumour growth and suppression, activation of T cells and control of inflammation. Genetic engineering has made it possible to clone and express the genes of most of these regulatory factors and to produce them in quantities large enough for medical application (see above). The cytokines are regarded as potentially important therapeutics for the treatment of

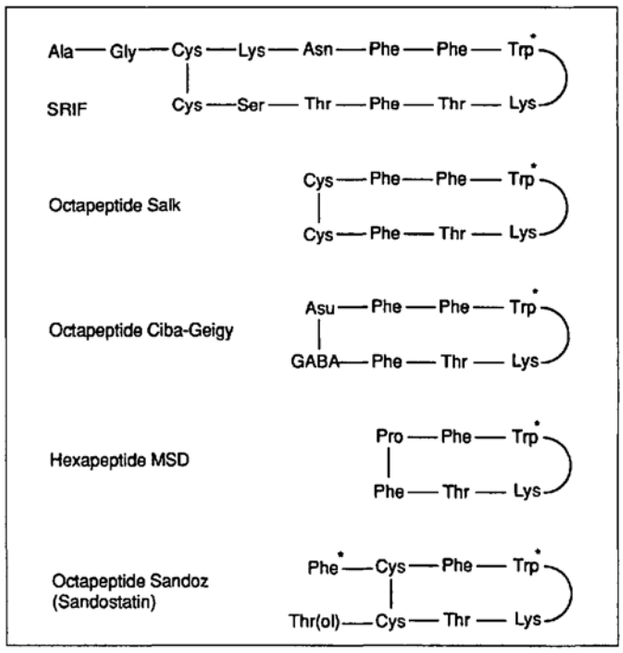

Fig 8. *Structure of miniforms of somatostatin (SRIF).* These structurally reduced cyclic peptides retain pharmacological properties similar to the parent hormone and display a higher potency and/or a prolonged duration of action. *E.g.*, the octapeptide *Sandoz* (Sandostatin) exhibits a 3fold higher potency *in vitro* and a 70fold increased activity *in vivo* as compared to SRIF.

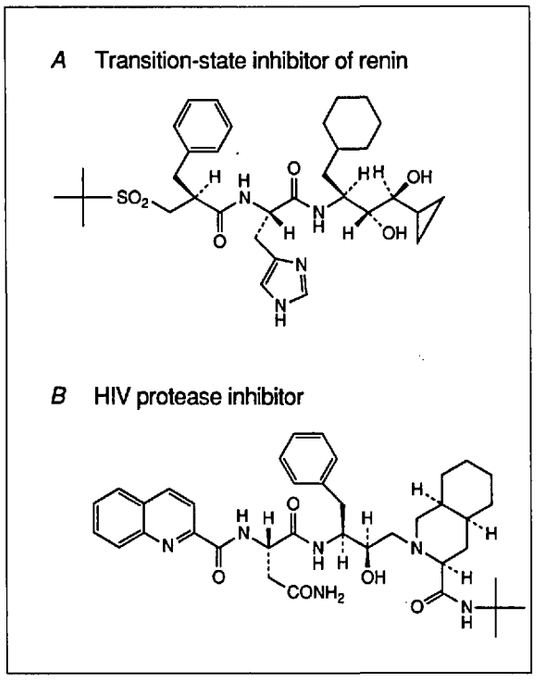

Fig. 9. *Structure of two orally active peptidomimetic inhibitors of aspartyl proteases. A*) A new transition state renin inhibitor with high specificity for renin of primates (*Ro 42-5892; F. Hoffmann-La Roche AG*, Basel). *B*) A transition state inhibitor of HIV protease with excellent selectivity and potency; the *in vitro* antiviral activity is in the nanomolar range (*Roche* U.K. [22]).

hematopoietic deficiencies (*e.g.* by erythropoietin, colony stimulating factors), viral infections (by interferons), cancer (by interleukins, tumour necrosis factor), inflammatory processes (by interleukins) and in wound healing (by tissue growth factors such as TGF-β). Some of them have already been administered successfully to large groups of patients, *e.g.* erythropoietin which is used to treat anemia of hemodialyzed patients with kidney disease.

The general application of certain cytokines *in vivo* may however produce severe side effects owing to their multiple biological activity (→ pleiotropic character). There are two possibilities to transform such molecules into 'soft' medicals, *i*) by designing analogues with a much reduced pleiotropy thus displaying only the desired effect, and *ii*) by delivery of the cytokine directly into the site of action, *e.g.* into a tumour. This could be achieved either by coupling the cytokine to a tumour-specific antibody or by local production at the site of action. This latter approach is currently being investigated by research teams in the United States where, *e.g.*, the tumour necrosis factor (TNF-α) gene is cloned into tumour-infiltrating lymphocytes (TILs) isolated from a patient's tumour. These genetically altered TILs are reinjected into the patient and should find their way back into the tumour where TNF-α is locally produced and released, thus killing the tumour [24].

Other concepts for the treatment of tumours include the construction of peptide-toxin (or antibody-toxin) conjugates or the application of boron-containing peptides through which radioactivity is generated *in situ* by activation with thermal neutrons. The first approach targets peptide receptors on tumour cells whereby the peptide acts as carrier for a partially inactivated toxin and directs it to the site of action. The partially inactivated toxin is, *e.g.*, the abrin or ricin A chain which is toxic to cells only in the presence of the B chain (the B chain of the native toxin binds to the cell surface and induces uptake of the toxic A chain) [25]. Thus, in the peptide-toxin conjugates the toxic part becomes only activated upon binding of the hormone to its receptor. The second concept, the boron-activation, is based on the ability of the ^{10}B isotope to capture slow neutrons, thus producing α and ^{7}Li particles. This process is very efficient in cell killing [26] and seems to be less damaging for the surrounding tissue than γ-irradiation. The application of boron-containing peptides to target tumours may also have the advantage that side effects can be minimized as compared to peptide-toxin conjugates. The principal problem is, however, the specific accumulation of large enough quantities of boron in the tumour.

11. Conclusion

Peptides have become very widely used tools for research and are part of almost any modern project in the field of immunology, oncology, infectiology, endocrinology, cardiovascular diseases, or neurobiology. In medical therapy too, peptides play an increasingly important role: apart from the established treatment of certain infectious diseases with peptide antibiotics and of hormone deficiencies with natural or synthetic peptide hormones, new peptide factors such as cyclosporin and several cytokines are being used in modern therapeutic concepts. Most of the larger peptide factors are produced by biotechnological methods and, thus, from the point of view of production, can be regarded as 'soft' medicals. However, many of them still induce severe side effects during treatment. In order to become soft medicals also for the patient, new peptide analogues and possiby orally active and metabolically more stable peptidomimetics will have to be designed which display a higher specificity and less side reactions than the natural compounds. Another challenge will be to develop 'softer' routes of application for the new peptide medicals.

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