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COMMENTARY

THE *IN VIVO* FATE OF BRAIN OLIGOPEPTIDES

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In this commentary brain oligopeptides are defined as peptides containing 3–15 amino acid residues for which the central nervous system, including the pituitary, is the (assumed) target tissue. They include releasing hormones [1]*, neuropeptides influencing behavior [2], peptides involved in memory transfer [3], and putative neurotransmitter peptides [4].

DETERMINATION AND IDENTIFICATION OF OLIGOPEPTIDES AND THEIR METABOLITES

In studies concerned with the *in vivo* fate of biologically active compounds, the detectability of the parent compound and its metabolites is crucial. The high biological potencies of brain oligopeptides necessitate detection at a level of less than 1 ng, implicating very sensitive analytical procedures.

1. *Bioassay*

The bioassay offers a straightforward approach to follow the appearance and disappearance of biological activity in various tissues with time. However, bioassay, by its nature, detects only rather large changes in biological activity. The specificity and sensitivity of the particular bioassay have to be considered. In behavioral assays a routine measurement of biological activity is hardly feasible.

2. *Radioimmunoassay*

Oligopeptide concentrations obtained by radioimmunoassay ideally reflect those of the parent molecule. However, the specificity of the assay does not always allow differentiation between the original oligopeptide and closely resembling metabolites, precursor molecules or structurally analogous peptides. Bioassay and radioimmunoassay provide little or no information on metabolic pathways.

3. *Labelled oligopeptides*

The use of suitable labelled oligopeptides offers a general applicable approach in studying *in vivo* fate. The labelled peptides should possess full biological activity and have physiochemical properties, including (radio)chemical stability, that are identical with those of the natural peptides. Operational specific activities should be in the order of 1 nCi/0·1 ng or 10 Ci/m-mole. A number of approaches for labelling oligopeptides are available.

(a) *Extrinsic labelling.* All extrinsic labelling procedures suffer from the disadvantage that peptide derivatives rather than the natural peptides are used. The derivatives might deviate in biological activity and physiochemical properties.

(a.1) Extrinsic labelling with ^{125}I or ^{131}I—This procedure is widely used, because radioactive iodine is easily introduced and high specific activities can be obtained. However, a number of serious drawbacks are inherent to this procedure. The presence of a tyrosine or histidine residue is essential in the classical procedure. This can be overcome by application of a recently developed method, in which radioactive iodine is introduced by reaction of free amino groups of the peptide with ^{125}I iodinated *N*-succinimidyl 3-(4-hydroxyphenyl) propionate [5]. This avoids exposure of the peptide to the oxydative iodination reaction, thereby protecting the peptide against damage by excessive radiation and oxydative side reactions [6]. This procedure might also, at least in some cases, overcome the frequently observed interference of iodination with biological activity. Experience with this procedure is awaited with interest. Finally, iodinated peptides are frequently deiodinated enzymatically *in vivo*. This makes results based on measurement of radioactivity alone questionable.

(a.2) Extrinsic labelling with ^{3}H—Various extrinsic labelling procedures using ^{3}H have been described. They usually involve the reaction of ^{3}H-labelled reagents with free amino groups of the peptide [7, 8]. The maximum specific activity obtainable with ^{14}C will usually be too low for the purpose.

(b) *Intrinsic labelling.* These labelling procedures yield labelled peptides that are identical, except for the radioactive atoms, to the natural peptide. Biological activity and physiochemical properties will be virtually unchanged. Intrinsic labelling is almost exclusively limited to labelling with ^{3}H. The stability of tritiated molecules at high specific activities is usually less than that of the natural peptides.

(b.1) Intrinsic labelling by random tritiation—This is usually carried out with tritium-gas by classical or modified Wilzbach procedures. During labelling modifications of the parent peptide take place owing to radiolysis and partial racemisation. Extensive purification is essential as well as removal of labile tritium atoms.

* For the releasing hormones the conventional abbreviations will be used: thyrotropin-releasing hormone = TRH; luteinizing hormone-releasing hormone = LH-RH; follicle-stimulating hormone-releasing hormone = FSH-RH; melanocyte-stimulating hormone-release inhibiting factor = MIF; melanocyte-stimulating hormone-releasing factor = MSH-RF; growth hormone-releasing hormone = GH-RH.

(b.2) Intrinsic labelling by direct synthesis—It is technically feasible to prepare labelled oligopeptides by direct synthesis from the constitutive radioactive amino acids, although purification problems will probably be high. Moreover, the expense of synthesis, starting with individual radioactive labelled amino acids, would be excessive.

(*b*.3) Intrinsic labelling by catalytic tritiation—In these approaches halogenated, usually iodinated, peptides are catalytically converted to their ^{3}H-labelled, natural counterparts [9]. The presence of the catalyst greatly reduces exposure times of the peptide to tritium-gas, thereby circumventing most of the disadvantages of the Wilzbach procedure. Peptides containing tyrosine or histidine can be iodinated in one of the usual ways or, preferably, iodinated peptides can be prepared by direct synthesis, using one or more iodinated amino acids as starting material [10]. This alternative route avoids possible damage of the peptide during iodination and is not restricted to tyrosine or histidine containing peptides [11]. For example, tritiated phenylalanine-peptides were obtained by catalytic tritiation of the corresponding synthetic *p*-iodophenylalanine-peptides [12]. Catalytic tritiation of halogenated peptides, prepared by direct synthesis with halogenated amino acids, offers one of the most promising approaches in obtaining radioactive labelled oligopeptides for investigations on *in vivo* fate.

4. *Identification of metabolites*

The *in vivo* fate of peptides is dominated by peptide metabolizing enzymes. As a consequence, metabolites play an important role in studies concerned with the fate of peptides. The number of possible metabolites is high and increases rapidly with chain length. The advantages of shorter peptide sequences are obvious. Nevertheless, the problems encountered even with oligopeptides are considerable. A linear peptide with no repeating amino acids can give rise to $n + (n-1) + (n-2) + \ldots$. metabolites, including the parent molecule, n being the number of amino acid residues. This number increases if *in vivo* modifications to one or more of the constituting amino acids take place [13]. Detection and identification of untagged metabolites by chemical means pose a formidable task. The physiological or even pharmacological concentrations of the peptide and its metabolites are extremely low, both in terms relative to other tissue constituents and in absolute terms. Serious technical problems are involved in detection, purification and separation of the parent molecule and its metabolites. The introduction of a radioactive label enables easy and specific detection. Addition of non-radioactive carrier molecules prior to extraction and separation facilitates purification and separation. Detection, and eventually identification, of all metabolites by measuring radioactivity is only possible if the radioactive label is present in each amino acid residue. However, in most cases only one amino acid residue contains the label. This results in a decrease in the number of radioactive metabolites, the decrease being dependent on the position of the label. This reduction of detectable metabolites simplifies identification, but also limits the amount of information on enzymatic cleavage sites. Knowledge of the biological activity characteristics of the metabolites is essential in translating the metabolic mechanisms into biological consequences.

THE *IN VIVO* FATE OF BRAIN OLIGOPEPTIDES

1. *Route of administration and absorption*

Extensive gastrointestinal breakdown and slow transference through the epithelial linings result in poor absorption of peptides after enteral administration. This is well illustrated in the metabolic fate of adrenocorticotrophin analogues in the intestine of the rat [14]. However, intestinal absorption of oligopeptides seems to be relatively effective because it was found to represent a major mode of protein absorption [15]. Effective oral administration has been reported for some releasing hormones in microgram or sub-microgram quantities [16]. Oral administration and prolonged duration increase the potential therapeutic value of peptide drugs. Oligopeptides can readily be synthetized and the opportunities for synthesis of analogues are excellent. This enables not only the synthesis of analogues with altered biological activity profiles, but also of analogues with increased proteolytic stability and improved absorption characteristics. These goals can be achieved by the introduction of D-amino acid residues, modified *N*- and *C*- terminal residues, amino acid substitutions, side chain alterations and possibly by synthesis of retro-D peptides. The impact of these structural changes on biological activity must of course be determined. An approach to such a design of peptide drugs is well demonstrated in the case of oxytocin [17]. The possible clinical usefulness of synthetic LH-RH analogues has been reported [18, 19].

Intravenous injection is the most frequently used parenteral route of administration in studies concerned with *in vivo* fate of brain oligopeptides. The less complex subcutaneous administration might be regarded as a suitable alternative [20]. Special formulations can prolong the duration of action by sustained release mechanisms [21], thereby increasing the utilization of the administered peptide in a given dose. Depot effects can also be expected after intramuscular injection [22]. Intranasal administration might be an effective alternative for long-term therapy [23, 24].

2. *Distribution studies*

Reliable information on the distribution profile of a peptide can only be obtained if the parent molecule itself is determined. This can be done by specific bio- or radio-immuno-assay or by measuring radioactivity after separation of the labelled parent peptide from its radioactive metabolites. However, in many studies data are based on total radioactivity, representing radioactivity in parent peptide and radioactive metabolites. The distribution patterns of the metabolites might differ considerably from those of the natural peptides. Therefore, results based on total radioactivity measurements should be interpreted with care.

(a) *Apparent volume of distribution.* This can be estimated by extrapolating the plasma disappearance

curve of intravascularly injected peptide back to the time of injection. Assuming first order kinetics for the various processes involved in the disappearance of the peptide from plasma, a semilogarithmic plot of plasma concentration vs time is used. In view of the uncertainties inherent in this approach and the limited reliability in measurements based on total radioactivities, the value of recorded initial volumes of distribution seems restricted. This is illustrated by the divergencies recorded for the initial volumes of distribution of LH-RH [23–26]. In general, oligopeptides and their metabolites cannot be expected to pass readily out of blood vessels with a 'continuous' epithelial lining. This would implicate initial volumes of distribution close to estimated plasma volumes, i.e. 4–5% of bodyweight. However, for TRH [27, 28] and MIF [29] very high values have been found, implicating rapid extravascular distribution. This can possibly be explained by disappearance through interendothelial spaces (kidney, liver), although initial distribution values of 50% [28] are difficult to evaluate.

(b) *Half-life*. Plasma disappearance curves of drugs also allow an estimation of drug half-lives. Brain oligopeptides, like other peptides, have half-lives that range from 1 to 10 min, as based on total radioactivity and from 1 to 5 min, as based on intact peptide concentration. With iodinated peptides increased half-lives have been found [24]. In some cases multi-exponential plasma disappearance curves have been described [24, 28].

(c) *Uptake in various tissues and organs*. Preferential uptake is usually concluded from tissue/plasma ratios greater than one. Again, the restrictions in determining these ratios from measurements of total radioactivity, are apparent. Uptake studies in non-target organs have been confined mainly to liver and kidney. Almost invariably both organs, especially the kidneys, show tissue/plasma ratios considerably greater than one.

Of particular interest is uptake in the brain, target tissue for the title peptides. Preferential accumulation in brain tissues has hardly been recorded, a marked accumulation of some releasing hormones in the pineal gland [26] being a notable exception. Tissue/plasma ratios for brain tissue usually range from 0·2–0·8 [10, 24, 30, 31], corresponding to 0·05–0·5% of injected dose/g brain tissue in periods ranging from some minutes to a few hours [10, 30, 31]. The low uptake in brain is not surprising since penetration of oligopeptides through the blood–brain barrier is not likely. Admittance to the brain might result from local deficiency of the blood–brain barrier, as seems likely for the central action of angiotensin on blood pressure in the area postrema [32]. Alternative to capillary circulation, oligopeptides might gain access to tissues of the central nervous system by passage through cerebrospinal fluid. Recently attention has been focussed on a possible physiological role of CSF in distributing peptides through the brain. More specifically it has been postulated for brain peptides that passage through CSF might be involved in delivering these peptides from their sites of production to their sites of action [33–37]. Also in pharmacological processes, the CSF might be involved in distributing systemically administered oligopeptides over tissues of the central nervous system. Because available evidence demonstrates that the blood–CSF barrier is relatively impermeable to peptides [33, 34], it might be speculated that the proposed transport system for peptide hormones from CSF to hypophyseal portal blood [36] could act reversibly. Delivery to CSF might be accomplished by retrograde transport through the stalk and basal hypothalamus to the third ventricle or by direct leakage from the pituitary surface into the adjacent basilar cisterns [33]. Brain oligopeptides could accumulate in the pituitary, be delivered to CSF and hence be distributed from CSF to effector cells in the brain. The pituitary, residing outside the brain, might function as a gateway for brain oligopeptides to enter the brain. Peptides could reach the pituitary from hypophyseal–hypothalamic sites of production or from systemic sites of administration. In fact, accumulation of systemically administered oligopeptides into the pituitary, as judged from tissue/plasma ratios > 1, has been found for most releasing hormones [10, 24, 26, 30]. This preferential hypophyseal uptake is usually interpreted as evidence for the site of action of the releasing hormones, but such interpretations have to be considered with care. Long lasting accumulation in the pituitary might reflect uptake of metabolites rather than of intact peptide [10] and be of a general rather than of a specific nature [38, 39].

As pointed out, uptake of systemically administered oligopeptides into the brain is very low. Uptake of intact peptide, rather than overall radioactivity, has been demonstrated by bioassay [40] and chemical methods [3, 41]. The physiological concentration of TRH [42–44] and LH-RH [45–47] in regional brain areas has recently been determined. These studies allow a crude estimate of biological effective brain oligopeptide concentrations of $1–10^5$ ng/g fresh brain tissue. The lower values possibly reflect overall brain concentrations rather than concentrations at sites of action which may be considerably higher [45].

Another approach in determining uptake of radioactively labelled brain oligopeptides in various tissues and organs is the use of (whole body)-autoradiography. This powerful technique has only found limited application. Inherent to this method is that total radioactivity is registered, because discrimination between parent peptide and metabolites is not possible. This results in an uncertainty in interpretation of autoradiograms. However, this uncertainty can be reduced by using stabilized peptide analogues and short time intervals between the moment of administration and the moment of preparing sections for autoradiography. High specific activity of the labelled peptides is essential, in reducing both the necessary dose and the period of exposure. Good agreement between autoradiographic data and direct measurements of radioactivity were found in studies of distribution profiles of two releasing hormones [26, 31]. High uptake of radioactivity in the kidney and pituitary and to a lesser extent in the liver, subcutaneous tissue, intestinal wall, pineal and submaxillary glands was found. Accumulation of radioactivity in the brain was negligible. To obtain sufficient uptake of radioactive labelled oligopeptides for localisation studies in the brain, more radioactivity, longer periods of exposure or a more effective route of administration, e.g. intraventricularly, seem necessary.

Combined with micro-autoradiography of different brain areas, interesting results might emerge on central distribution. These results might have direct implications for localizing possible sites of action of brain oligopeptides. Another approach in locating selective sites of action involves implantation and lesion experiments [48].

(d) *Elimination.* Oligopeptides are rapidly eliminated, as is apparent from their low half-lives. The rates of elimination can be determined by measuring the plasma disappearance of intact peptide after i.v. administration.

(d.1) Excretion—The observed high levels of radioactivity in the kidney indicate an important role of this organ in the elimination of brain oligopeptides. This is substantiated by measurements of the amounts actually excreted in the urine. The available data for a number of releasing hormones demonstrate relative large individual variations [13, 29]. As a crude estimate, 10–50% of an intravenously injected dose seems to be excreted in the urine during the first hr after administration. This estimate includes intact peptide and metabolites. The contribution of intact peptides to excretion in the urine is also variable [25, 29, 49–51]. Excretion data based on radioactivity measurements are usually (much) higher than those based on bioassay or radio-immunoassay, because the former include the urinary excretion of radioactive metabolites. Besides, data based on urinary excretion of radioactivity can produce equivocal estimates, because they depend on the position of the label in the peptide and the fate of the various metabolites. It appears that even active tubular secretion might be involved in urinary excretion [49].

(d.2) Metabolism—The fast and abundant appearance of radioactive metabolites after administration of radioactive labelled peptides, demonstrates the importance of biotransformation as another main route of elimination. The products of metabolism have been demonstrated in a variety of tissues and the underlying metabolic processes have been investigated for a number of peptides [52]. Proteolytic breakdown can occur in a variety of tissues, with differing specificities and potencies for the same or different peptides. The moderate preferential uptake of radioactivity in the liver after administration of radioactive labelled oligopeptides indicates a possible role of this organ in metabolism. The metabolic capacities of various tissues have been tested in *in vitro* incubation studies [52]. For brain oligopeptides the number of such studies is limited and usually restricted to plasma. Individual peptides are metabolized at quite different rates by plasma enzymes [51, 53]. Not only peripheral tissues, but also brain tissue is capable of metabolizing brain oligopeptides [54, 55]. The available evidence seems to warrant a dominating role of peptide metabolizing enzymes on the *in vivo* fate of oligopeptides.

(d.3) Storage—This represents a third mechanism of elimination, but has hardly been investigated. The distribution patterns obtained in whole body autoradiography might indicate that this mechanism is operative, because a modest concentration of radioactivity in subcutaneous tissue and the bones has been found in these studies [26, 31]. There is, at present, little evidence of the binding of oligopeptides to plasma proteins [51].

BIOSYNTHESIS AND MECHANISM OF ACTION

1. *Biosynthesis*

A number of biosynthetic routes are probably functional in the formation of brain oligopeptides. In biosynthesis, as in elimination, enzymes seem to play a dominant role. The physiological significance of enzymes on the *in vivo* fate of brain oligopeptides is not only to terminate the action of these peptides by inactivation, but also to initiate the action of these peptides by catalyzing their release from prohormones or possibly by catalyzing *de novo* peptide synthesis [52, 56].

(a) *Enzymatic release from prohormones.* The release of brain oligopeptides from precursor peptides has been found operative in the formation of MSH-RF and MIF from oxytocin [52]. A similar situation might exist with ACTH as a precursor peptide. A number of oligopeptides, possibly proteolytic derivatives of ACTH, have been shown to possess behavioral activity comparable to that of the precursor peptide. The endocrine activities of these ACTH-oligopeptides are markedly decreased as compared to those of the parent molecule [57]. This shift in biological activity profile between precursor and product molecule might have physiological significance.

(b) *Synthesis* de novo. The possibility of synthesis *de novo* of brain oligopeptides is apparent from findings that precursor amino acids, incubated with hypothalamic tissue *in vitro*, can be incorporated in various releasing hormones [58]. Because the radioactive precursor amino acid is liable to be incorporated in a great number of peptides, extensive purification of the incubation mixture is essential to prove identity with the oligopeptide under investigation [59]. There is evidence that these syntheses *de novo* do not represent ribosomal synthesis, but are being effected enzymatically [60].

2. *Mechanism of action*

Specific binding to membrane preparations, suggestive of receptor interaction, has been reported for a number of brain oligopeptides [40]. Receptor mediated stimulation of the adenylate cyclase–cyclic AMP–protein kinase system has been demonstrated in a number of cases [61–63]. Changes in intracellular protein kinase activity might be reflected in observed changes in biogenic amine metabolism, which in turn influence synaptic transmission. The effects of brain oligopeptides on central neurotransmitter metabolism have usually been described as being possibly connected with behavioral effects of these peptides [64–72]. Alternatively, oligopeptides as such might have a possible functional role as neurotransmitters [4]. Data obtained from electrophysiological studies indicate increased excitability of midbrain limbic structures by neuropeptides of the ACTH 4–10 type [73], suggestive of facilitated transmission. Other possible mechanisms of action, for example on membrane permeability via protein kinase-mediated phosphorylation of functional membrane proteins presently remain open.

In summary, the excellent achievements in isolation, sequence determination and synthesis of the releasing hormones have evoked intense investigations on the *in vivo* fate of these peptides. The results

of these studies ultimately will lead to a complete picture of the fate of these peptides and will be beneficial in elucidating their physiological functioning and clinical applicability.

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