ENZYMATIC MODIFICATION OF PEPTIDES IN THE BRAIN

By

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ENZYMATIC MODIFICATION OF PEPTIDES IN THE BRAIN

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ABSTRACT

Certain post-translational modifications of peptides in the brain were studied in order to answer three questions. (1) Does an enzyme exist in the brain with properties similar to glutamine cyclotransferase of papaya latex? (2) Does formation of carboxyamide residues only require the presence of a carboxyl terminal glycine in the respective precursor? (3) Does a nonspecific ligase exist in the brain? Experiments are described that attempted to answer these questions. Amino terminal glutaminyl peptides were used to assay for glutamine cyclotransferase in brain homogenates. Cyclization of the amino terminal glutamine would enable these peptides to pass through a cation exchange column. Glutamic acid would be released from the product after acid hydrolysis of the eluate and determined by an enzyme assay. Isotopically labelled carboxyl terminal glycyl peptides were synthesized and used to assay for an enzyme that exchanges glycine for ammonia. The product was separated from the substrates with an anion exchange column. A random mixture of peptides was used to assay for a nonspecific ligase. One group was [^{3}H]-acetylated to block the amino terminals. A second group was coupled to glycinamide to block the carboxyl terminals. Coupling would alter the ion-exchange properties of these substrates.

No evidence was obtained for the existence of the postulated enzymes. These results are discussed in relation to a novel hypothesis implicating peptides with memory.

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Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
CTP	cytosine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GTP	guanidine 5'-triphosphate
Lys	lysine
MSM	mercaptoethanol
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
Phe	phenylalanine
Ser	serine
TCA	trichloroacetic acid
UTP	uridine 5'-triphosphate
Val	valine

INTRODUCTION

I. Peptides and Memory

More than 20 peptides have been identified in the neurons of the brain, spinal cord and periphery (Hokfelt et al. 1980). A partial list includes substance P, neurotensin, angiotensin II, oxytocin, vasopressin, members of the gastrin/cholecystokinin family, somatostatin, enkephalins, thyrotropin releasing hormone and luteinizing hormone releasing hormone (Bloom, 1977; Hughes and Iversen, 1978). Even some of the larger pituitary hormones, growth hormone, prolactin, thyroid stimulating hormone and adrenocorticotropin and the pancreatic hormones insulin and glucagon, may be present in the brain (Krieger and Liotta, 1979).

Besides the hormonal peptides, a large variety of brain peptides have been identified (Sano, 1970). These are mostly small peptides: lysyl peptides (Gatfield and Taller, 1971), tryptophanyl peptides (Edvinsson et al. 1973), and glutamyl peptides (Reichelt, 1970). There are no estimates of the total peptide content of the brain, except for the hog hypothalamus which may contain several grams per kilogram (Shome and Saffran, 1966). There is no reason to believe that neurobiologists have identified all the peptides in the brain. A whole potpourri of as yet undiscovered peptides may reside in the brain.

The role of these peptides in the brain is open to speculation. Various suggestions have been put forward (for review see Guillemin, 1978). There is experimental evidence

that at least some of these peptides act as neurotransmitters. Much work has focused on substance P (Iversen et al. 1976; Marx, 1979; Nicoll et al. 1980), luteinizing hormone releasing hormone. (Jan et al. 1979) and the enkephalins (Frederickson, 1977). Neuromodulation of synaptic transmission has also been demonstrated experimentally (Mudge, 1979; Krivoy, 1979).

But the most fascinating aspect in the study of brain peptides is their effect on behaviour. Luteinizing hormone releasing hormone induces mating behaviour (Moss, 1973, 1977), cholecystokinin octapeptide induces satiety (Straus and Yalow, 1979), angiotensin II stimulates drinking (Epstein et al. 1970), a nonapeptide induces sleep (Schoenenberger and Monnier, 1977), drinking can be inhibited by vasopressin (Ungar, 1975), prolactin is implicated in bird migration (Farner et al. 1967), the enkephalins may be involved in narcotic tolerance (Snyder, 1977), scotophobin induces dark avoidance behaviour (Ungar et al. 1972) and the posterior pituitary hormones may be involved in memory processes (De Weid and Veersteeg, 1979). The effects mentioned above represent a narrow selection of the work going on in the field of neuropeptide physiology. The field has been reviewed extensively (Donovan, 1978; Nemeroff and Prange, 1978). The next logical question is to ask whether of not peptides play a role in the acquisition and storage of all learned behaviour.

There are several reasons why peptides are good candidates as memory molecules. 1) They have a high information content in that the number of different permutations is large even for a fairly short peptide. 2) Immunoglobulins can distinguish

between peptides of different sequences. Thus it is quite conceivable that receptors on neurons can recognize specific peptides. 3) Various biologically active peptides are found in the brain. 4) Peptides have been implicated in behaviour and memory processes.

One approach to the study of memory is to consider the theoretical requirements of the information storage system. These considerations then lead to a prediction of various components and we then go and look for these components. A similar example was the prediction of tRNA by Crick simply on the theoretical grounds that nucleic acids cannot interact directly with amino acids.

The crucial theoretical consideration lies in the nature of the information in memory. It operates by linking in some way two neurons (or group of neurons) which have not been connected previously. Therefore the key component in the memory storage system must be an enzyme which joins molecules. If the molecules are peptides then this component is an oligopeptide ligase (an enzyme which joins peptides).

The working hypothesis is that each neuron (or group of neurons) is specified by receptors on its surface which recognize a particular oligopeptide. When the neuron fires (electrically excited) the receptors release the peptides into the extracellular fluid. If two groups of neurons fire at the same time in response to two sensory inputs (e.g. the sound of a bell plus the sight and smell of food), then two different peptides are released. It is postulated that these

peptides are then joined together by the action of the oligopeptide ligase. In order to prevent the formation of extended polymers we must postulate that each peptide is blocked at one end so that only a single bond is made. The joined peptide can now bind at either of the two neurons. To complete the mechanism we need to postulate that the binding of a peptide to the specific receptor causes the neuron to fire again. The joined peptide molecules are now divided between the two neurons, each of which can bind one end of it. The memory is recalled when one of the two neurons becomes excited as a result of sensory input (e.g. the sound of a bell). The firing of this neuron now releases the joined peptide which can diffuse and bind to the other neuron (i.e. governing the sight and smell of food). This binding now causes the latter to fire. Thus the sound of the bell leads to the sense of food even in the absence of food.

The hypothesis can be tested by assaying for three enzymes. The most important is the ligase discussed above. The other enzymes are involved in blocking the amino and carboxyl terminals of these peptides.

If these enzymes are detected then the next task is to make sure that they are involved in the process of learning. To do this, the enzymes would be studied <u>in vitro</u> in order to develop specific inhibitors. These inhibitors could then be tested to see if they are inhibitors of learning.

The first goal of the project is to test the aforementioned hypothesis. A second goal is to shed some light on the metabolism of

brain peptides.

II Metabolism of Brain Peptides

Because the study of neuropeptides is still in the early stages, little is known about their metabolism. Many questions remain unanswered. Are inactive precursors converted to active products during axonal transport or extracellularly? How are they synthesized? What is the rate of biosynthesis? What is the rate of proteolysis? Is the action of peptides terminated by specific enzymes, reabsorption, or by simple diffusion? What are the mechanisms of posttranslational modification? Do peptides undergo novel forms of metabolism? The answers to these questions will shed light on the function of these peptides in the brain. Therefore the search for ligase activity in the brain and for enzymes involved in the blocking of amino and carboxyl terminals may lead to a better understanding of the metabolism of brain peptides. This in turn will lead to a better understanding of their functions. Using metabolic studies to gain insight into function is not a new approach. An example is glutathione. Glutathione was for many years a compound looking for a function. Only by studying amino acid metabolism did Meister (1974) realize that glutathione plays an integral role in amino acid transport.

A) Oligopeptide Ligase

The formation of peptide bonds by a nonribosomal mechanism is well documented. Some examples include the

synthesis of the tripeptide glutathione (Mooz and Meister, 1967); opthalamic acid (Cliffe and Waley, 1958); carnosine (Kalyankar and Meister, 1959); homocarnosine (Winnick and Winnick, 1959); and the polypeptide antibiotics gramicidin (Gevers et al. 1968) and tyrocidine (Roskoski et al. 1970). The biosynthesis of thyrotropin releasing hormone occurs in the presence of puromycin and RNase, thus indicating that the tripeptide is not formed by ribosomal action as such or as part of a prohormone (Mitnick and Reichlin, 1971; Grimm-Jorgensen and McKelvy, 1974). Many proteolytic enzymes have been observed to catalyze peptidyl transferase reactions (Mycek, 1970). The best studied of these, termed "dipeptidyl transferase" or cathepsin C occurs in the brain (Metrione et al. 1966).

The faithfulness of production of a given sequence of amino acids does not appear to be as great as in the nucleic acid template mechanism since the amino acid composition of tyrocidine can vary with the culture medium used (Roskoski, 1970), and the amino acid and peptide specificities of the synthetases which have been studied are not stringent (Kalyankar and Meister, 1959; Winnick and Winnick, 1959). The presence of nonspecific enzymes capable of synthesizing compounds from amino acids and peptides by nonribosomal mechanisms gives credibility to the hypothesis that a nonspecific oligopeptide ligase could occur in the brain.

Since the substrate specificity for the ligase activity was not known, a complex mixture of peptides was used. Random peptides were prepared by digestion of proteins by a nonspecific

proteolytic enzyme. In order to follow the reaction, the peptides were isotopically labelled. One group of peptides was acetylated using [^{3}H]-acetic anhydride, blocking the amino terminals. The carboxyl terminals of another group of peptides were blocked by amidation. An assay for ligase activity was carried out by adding the amino and carboxyl terminal blocked peptides along with appropriate cofactors to a brain homogenate. A ligase would join the amino and carboxyl terminal blocked peptides, giving a peptide with altered ion exchange properties. The labelled acetylated peptide would 'stick' to an anion exchange column due to the free carboxyl group, whereas a peptide formed by the joining of an acetylated and an amidated peptide would pass through an anion exchange column. Hence, any product could be detected by liquid scintillation counting of the eluate.

B) Modification of the Carboxyl Terminal of Peptides

Many peptides do not contain a free α-carboxyl group but instead terminate with an amino acid amide (see Table 1). Many of these peptides have been found in the mammalian brain. Preliminary studies indicate that there are other unknown peptide amides in the brain tissue (Tatemoto and Mutt, 1978). So far, at least 14 different amino acids have been found to occur at such amidated terminals. This modification has hitherto only been detected in peptides containing fewer than 80 residues.

The function of the amide residue is open to speculation. For many peptides such as bradykinin, eledoisin, (Stewart and Woolley, 1965), oxytocin (Ferrier and Du Vigneaud, 1966), and

TABLE 1

Blocked Peptides

Peptide/Protein	N-terminal	C-terminal
Fibrinogen β chain	pGlu-Gly-	
Eledoisin	pGlu-Pro-	-Leu-Met-NH_2
Thyrotropin releasing hormone	pGlu-His-	-His-Pro-NH_2
α-Melanotropin		-Pro-Val-NH_2
Calcitonin		-Ala-Pro-NH_2
Secretin		-Leu-Val-NH_2
Cholecystokinin		-Asp-Phe-NH_2
Substance P		-Leu-Met-NH_2
Luteinizing hormone releasing hormone	pGlu-His-	-Pro-Gly-NH_2
Vasopressin		-Arg-Gly-NH_2
Oxytocin		-Leu-Gly-NH_2
Phospholipase A2	pGlu-Glu-	
Bombesin	pGlu-Gln-	-Leu-Met-NH_2
Melittin		-Gln-Gln-NH_2
Phyllocaerulein	pGlu-Glu-	-Asp-Phe-NH_2
Lipoprotein	pGlu-Ala-	
Bombesin	pGlu-Glu-	-Leu-Met-NH_2
Alytesin	pGlu-Gly-	-Leu-Met-NH_2
Pancreatic hormone		-Arg-Tyr-NH_2

(Dayhoff, 1972, 1976)

... continued

Table 1 continued ...

Peptide/Protein	N-terminal	C-terminal
Phsalaemin (Erspamer et al. 1977)	pGlu-Ala-	-Leu-Met-NH_2
Gastrin (Gregory & Tracy, 1975)	pGlu-Gly-	-Asp-Phe-NH_2
Kinin (Haberman, 1972)	pGlu-Thr-	
Cardioexcitatory peptide (Price and Greenberg, 1977)		-Arg-Phe-NH_2
Xenopsin (Stewart & Channalbasavaiah, 1979)	pGlu-Gly-	
Cholecystokinin-octapeptide (Dockray et al. 1978)		-Asp-Phe-NH_2
C-Reactive protein (Oliveira et al. 1977)	pGlu-Thr-	
Neurotensin (Carraway et al. 1978)	pGlu-Leu-	
Vasoactive intestinal peptide (Bodansky et al. 1976)		-Leu-Thr-NH_2
Melittin F (Gauldie et al. 1978)		-Lys-Asn-NH_2
Apamine (Habermann, 1972)		-Gln-His-NH_2
Caerulein (Anastasi et al. 1968)		-Leu-Met-NH_2
Blanching hormone (Fernlund and Josefsson, 1972)	pGlu-Gln-	-Asp-Phe-NH_2
Adipokinetic hormone (Stone et al. 1976)	pGlu-Leu-	-Gly-Thr-NH_2
Neurotoxin (Maeda & Tamiya, 1978)		-Gly-Arg-NH_2
Phyllomedusin (Vale, 1978)	pGlu-Asn-	-Leu-Met-NH_2
Ranatensin (Vale, 1978)	pGlu-Val-	-Phe-Met-NH_2

gastrin (Tracy and Gregory, 1964), the amide group is essential for activity. The amide residue is not required for activity of other peptides such as α-melanotropin (Stewart and Woolley, 1965). It has been suggested that the process of amidation may represent a control point in the formation and release of the peptide (McKelvy and Epelbaum, 1978). Others have postulated that the carboxyl terminal amide is important for resistance to the action of carboxypeptidases (Stewart and Channabasavaiah, 1979).

The mechanism of this amidation, which is undoubtedly a post-translational event, is at present unknown. The modification is not universal, and even closely related peptides may or may not be amidated. For example, in the case of the two hormones secretin and glucagon, which exhibit similar chain length and considerable sequence homology, only the former has a blocked carboxyl end (Suchaneck and Kreil, 1977).

Various mechanisms have been postulated. From the variety of carboxyl terminal residues that may in one case be amidated, in others not, it appears unlikely that a free α-carboxyl group and side chain of the terminal amino acid could be a specific recognition site for this type of modification.

One plausible mechanism was suggested by Smyth (1975). He postulated that the peptides are produced in the form of precursors and that activation may take place not by hydrolysis but by aminolysis of the peptide chain. This transamidation reaction would account for the carboxyl terminal amide.

Another mechanism that is linked to the release of the peptide from a precursor was proposed by Walter and Hoffman (1977).

The proposal is as follows: The active peptide-to-be must be elongated at the carboxyl terminal and the residue which is to bear the carboxyamide function must be followed by a residue which can be enzymatically converted to a dehydroalanine residue. Cysteine or serine residues are excellent candidates. Hydrolysis of the dehydroalanyl precursor would result in the release of the peptide amide, thus completing the formal conversion of the amino moiety of the cysteine or serine residue to the carboxyl terminal carboxyamide. However, there is little experimental evidence to support either of the hypotheses.

A recent conjecture has been put forward by Suchanek and Kreil (1977). They envisage that a carboxyl terminal glycine represents the recognition site for an enzyme that exchanges glycine for ammonia. Support for the hypothesis comes from three areas.

The first piece of evidence comes from work done on the biosynthesis of a secretory peptide in honey bee venom glands - melittin (Suchanek et al. 1978). This peptide is 26 amino acids long with a blocked carboxyl terminal; its carboxyl end is amidated. Melittin mRNA has been translated in a cell-free system and yields a product termed prepromelittin which has the carboxyl terminal sequence -Gln-Gln-Gly-OH (Suchanek and Kreil, 1977). Melittin and promelittin terminate with -Gln-Gln-NH_2. This carboxyl terminal glycine may represent a recognition site for a venom gland enzyme that exchanges glycine for ammonia.

The second system which suggests that glycine is the recognition site comes from studies on the biosynthesis of α-melanotropin. α-Melanotropin is considered to be derived from a

corticotropin-β-lipotropin precursor (Nakanishi et al. 1979). If the release of α-melanotropin from its precursor proceeds via the action of trypsin- and carboxypeptidase B-like activities, as has been postulated for proinsulin and other prohormones (Steiner, 1976), an α-melanotropin precursor would first be generated with the carboxyl terminal structure -Val-Gly-OH (Suchaneck and Kreil, 1977). An enzyme similar to the one postulated in bee venom glands would convert this structure to valinamide.

The third piece of evidence comes from an examination of the sequences of two snake toxins. Although both are almost homologous for the first 74 residues, one terminates -Gly-Ser-NH_2, the other terminates -Gly-Ser-Gly-Arg-NH_2. We can speculate that the arginine in the latter is cleaved off, leaving carboxyl terminal glycine to participate in the amidation reaction.

Several mechanisms for such a reaction could be envisaged, of which transamidation with, for example, glutamine appears to be the most likely possibility. The proposed mechanism would, of course, be independent of the nature of the carboxyl terminal residue of the final product and would only require the presence of a terminal glycine in the respective precursor polypeptide.

This hypothesis was tested by preparing N-[^{3}H]-acetyl peptides terminating with glycine. These peptides were incubated with brain homogenates, followed by elution through an anion exchange column. Any neutral blocked peptides would pass through the column to be counted, whereas the unmodified peptides 'stick' to the column.

C) Modification of the Amino Terminal

Pyroglutamic acid has been identified as the amino terminal residue in a number of naturally occurring peptides and proteins. Although the chemistry of pyroglutamic acid-containing peptides has been studied in some detail, little is known about the biosynthesis or function of this unusual amino terminal residue.

A number of authors have suggested that pyroglutamic acid may be involved in the initiation of protein synthesis in eukaryotic cells, as pyroglutamic acid has a blocked α-amino group similar to that of N-formyl-methionine which is the initiator residue for protein synthesis in many systems (Moav and Harris, 1967; Bernfield and Nestor, 1968; Rush and Starr, 1970; Baglioni, 1970). Although a specific tRNA could not be identified for pyroglutamic acid, Bernfield and Nestor (1968) showed that the formation of pyroglutamic acid-tRNA from glutamine-tRNA is readily catalyzed by glutamine cyclotransferase. However, recent studies have shown that methionine is indeed the initiator for synthesis of proteins containing amino terminal pyroglutamic acid (Burnstein and Schechter, 1977) and there is no evidence that pyroglutamic acid coupled to tRNA is donated to the growing polypeptide chain.

Some insight into the mechanism for the formation of amino terminal pyroglutamic acid may come from an examination of the formation of free pyroglutamic acid which has been extensively studied. High levels of pyroglutamic acid are found in the brain and other tissues (Lam et al. 1978). Wilk and Orlowski (1976) reported a concentration of 58.7 nmoles/g in the mouse brain whereas

Wolfersberger (1973) reported a concentration of 4.06 moles/g in the guinea pig brain.

Glutamic acid can be converted to pyroglutamic acid by a number of enzymes. D-Glutamate cyclotransferase catalyzes the reversible conversion of D-glutamic acid to D-pyroglutamic acid (Meister and Buckenberger, 1962). D-Glutamic acid may exert toxic effects in the mammalian organism and perhaps this enzyme serves the function of removing D-glutamic acid from the system (Van Der Werf and Meister, 1971). Purified preparations of glutamine synthetase (Meister, 1968) and γ-glutamylcysteine synthetase (Orlowski and Meister, 1971) can also catalyze the formation of pyroglutamic acid from glutamic acid but it is unlikely that the reactions are of physiological significance.

One pathway of potential physiological significance is the conversion of glutamic acid to pyroglutamic acid in rat tissues (Niwaguchi et al., 1965). The activity is stimulated by intermediates of the citric acid cycle.

Probably the most significant pathway for the formation of pyroglutamic acid is through the action of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase of the γ-glutamyl cycle (Orlowski and Meister, 1970). γ-Glutamyl transpeptidase transfers the γ-glutamyl moiety of glutathione to an amino acid acceptor: (Tate and Meister, 1974).

Glutathione + amino acid $\rightleftharpoons$ γ-glutamyl-amino acid + cysteinylglycine

This is followed by conversion of γ-glutamyl amino acid to pyroglutamic acid and the amino acid by γ-glutamyl cyclotransferase

(Tananguchi and Meister, 1978).

$$\gamma\text{-glutamyl-amino acid} \rightleftharpoons \text{pyroglutamic acid} + \text{amino acid}$$

An enzyme which directly converts glutamine to pyroglutamic acid may also be of physiological importance. Wolfersberger and Tabachnick (1974) have found this enzyme termed glutamine cyclotransferase, in kidney, liver and brain homogenates of the guinea pig.

Although free pyroglutamic acid does exist in tissues, it is not incorporated into protein (Moav and Harris, 1967). Early reports indicated that glutamic acid might be the precursor of amino terminal pyroglutamic acid (Twardzik and Peterkofsky, 1972) but these results are probably erroneous in light of recent data.

Most investigators feel that amino terminal pyroglutamic acid is derived by cyclization of glutamine (Gregory and Tracy, 1975; Burnstein et al. 1976; Burnstein and Schechter, 1977; Jilka and Pestka, 1977). This general consensus has been reached through a study of precursors to proteins and peptides. The cyclization of glutamine at the amino terminal of polypeptides may be universal. To the best of my knowledge none of the reported sequences of any polypeptides found in multicellular organisms have been shown conclusively to contain amino terminal glutamine.

The crux of the problem is whether the cyclization occurs after, or concomitantly with cleavage of an extra piece from the precursors. Two lines of evidence point to the former. First, two investigators studying the synthesis of immuno-

globulins have tentatively demonstrated that the immunoglobulins contain amino terminal glutamine within the cell after synthesis, but the secreted products terminate with pyroglutamic acid (Baglioni, 1970; Stott and Munro, 1972). Secondly, an enzyme has been identified and purified from papaya latex which catalyzes the conversion of N-glutaminyl peptides to pyroglutamyl peptides and ammonia (Messer and Ottesen, 1965). Does an enzyme with similar capabilities exist in the brain?

The question can be answered by assaying brain homogenates with N-glutaminyl peptides. Cyclization of glutamine to pyroglutamic acid would allow these peptides to pass through a cation exchange column. The amount of product would be proportional to the amount of glutamic acid released after digestion of the eluate with strong acid. The amount of glutamic acid may be determined by an enzymatic assay.

Therefore, the purpose of this project was to answer three questions: (1) Does a nonspecific oligopeptide ligase exist in the brain? (2) Does the formation of carboxyamide residues require only the presence of a carboxyl terminal glycine in the respective precursor? (3) Does an enzyme exist in the brain with properties similar to glutamine cyclotransferase of papaya latex?

METHOD

I Preparation of Random Peptides from Rabbit Muscle

A. Preparation of Rabbit Muscle

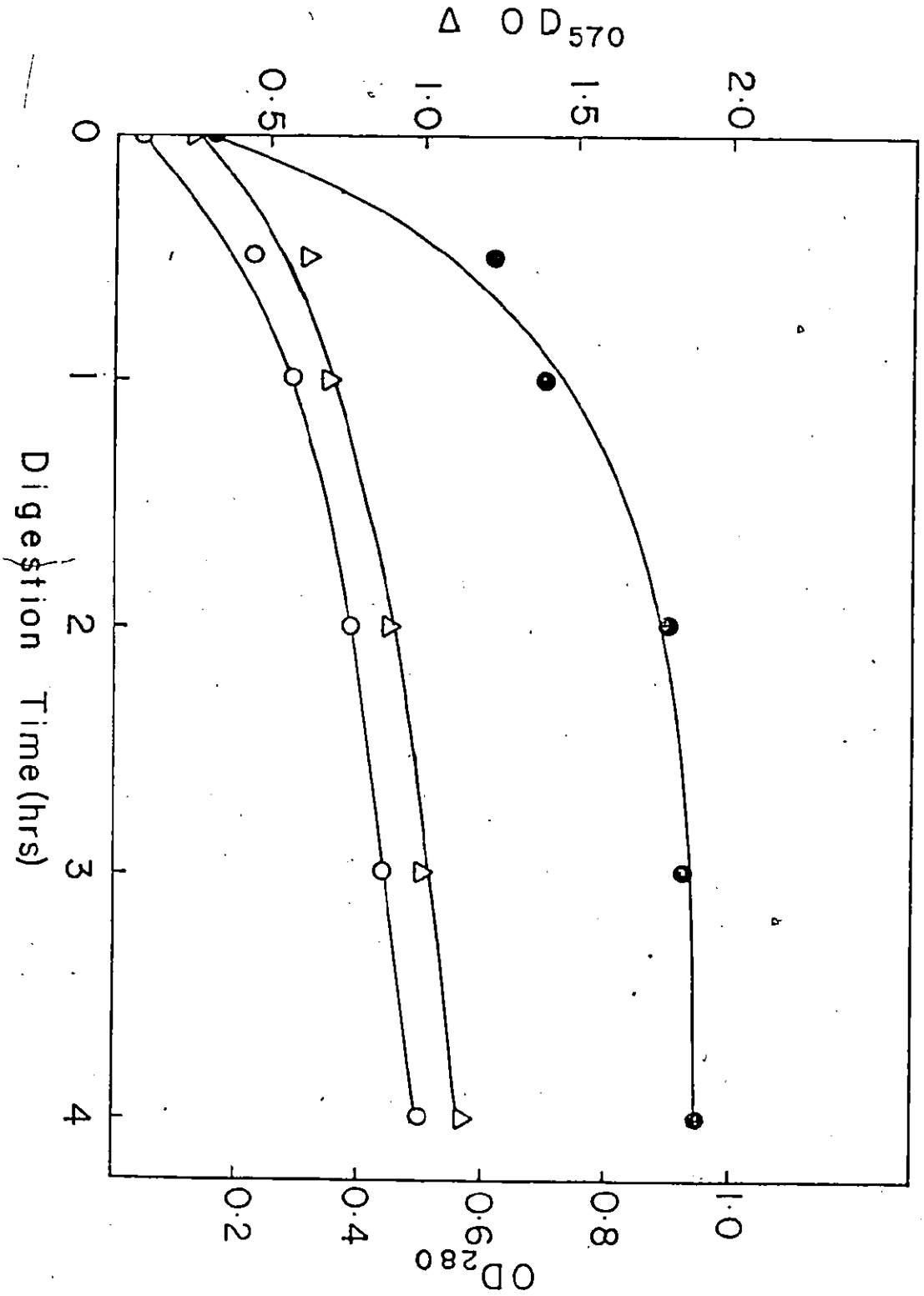
Rabbits were freshly killed by breaking the neck and exsanguinated. After removal of the skin, muscle tissue was dissected from the back and either used immediately or frozen at -20°C.

Ten g of muscle tissue was suspended in 40 ml of buffer consisting of 0.1 M $NaPO_4$ pH 7.5, 1 mM EDTA and 5 mM MSH. The mixture was homogenized in a Waring blender for 40 seconds at 70 volts at room temperature. The homogenized muscle was kept frozen at -20°C until needed.

B. Determination of Optimal Time of Digestion with Papain

Ten ml of homogenized rabbit muscle was digested with 12.5 mg of papain (containing 20% by weight buffer salt) in a 25°C water bath with occasional mixing. At times 0.5, 1, 2, 3 and 4 hours a 1 ml sample was removed and 0.111 ml of a solution of TCA(1g/ml). Zero time was done without papain added. Samples were centrifuged at 12,000 g at 4°C for 15 min in a Sorvall RC2-B centrifuge. Supernatants were decanted off and the absorbance read at 280 nm. Ninhydrin analysis before and after hydrolysis (see Analytical Methods) was also performed using 20 μl of a sample diluted 10-fold (see Fig. I).

Figure 1. Digestion of Rabbit Muscle with Papain. (Δ-Δ) OD_{280}; (o-o) ninhydrin analysis before hydrolysis; (•-•) ninhydrin analysis after hydrolysis.


Δ OD570
0·5
1·0
1·5
2·0
0
1
2
3
4
Digestion Time (hrs)
OD280
0·2
0·4
0·6
0·8
1·0

C. Papain Digestion of Rabbit Muscle

To a 20 ml sample of rabbit muscle homogenate was added 25 mg of papain (2 x crystallized from Papaya Latex containing 20% NaCl and NaOAc). The muscle tissue was digested for 2 hours at 25°C. The reaction was stopped by the addition of 2.22 ml of 100% (w/v) TCA. The resulting precipitate was pelleted by centrifugation at 12,000 g for 15 min at 4°C in the Sorvall RC2-B. The supernatant was decanted off and analyzed by a ninhydrin assay before and after hydrolysis (see Analytical Methods).

Buffer salts and TCA were removed by elution on a cation exchange column. Dowex 50 x 8 200-400 mesh cation exchange resin was washed successively with water, 1N NaOH, water, 3N HCl, and water until neutral. A 1.6 x 16 cm column was packed and several volumes of water were passed through the column. The TCA supernatant was layered on the column and eluted with 3 volumes of water (100 ml) and 2 ml fractions were collected. Peptides were then eluted off the column with redistilled pyridine. Fractions obtained after the change to pyridine were analyzed by the ninhydrin assay (see Analytical Methods). Those fractions giving a positive reaction were pooled, evaporated to dryness under reduced pressure at 40°C and washed twice with small volumes of water followed by evaporation. The residue was suspended in 1 ml of water and analyzed by ninhydrin assay before and after hydrolysis (see Analytical Methods). The peptides were stored at -20°C.

II Preparation of Acetylated Peptides

A. Acetylation of Rabbit Muscle Peptides

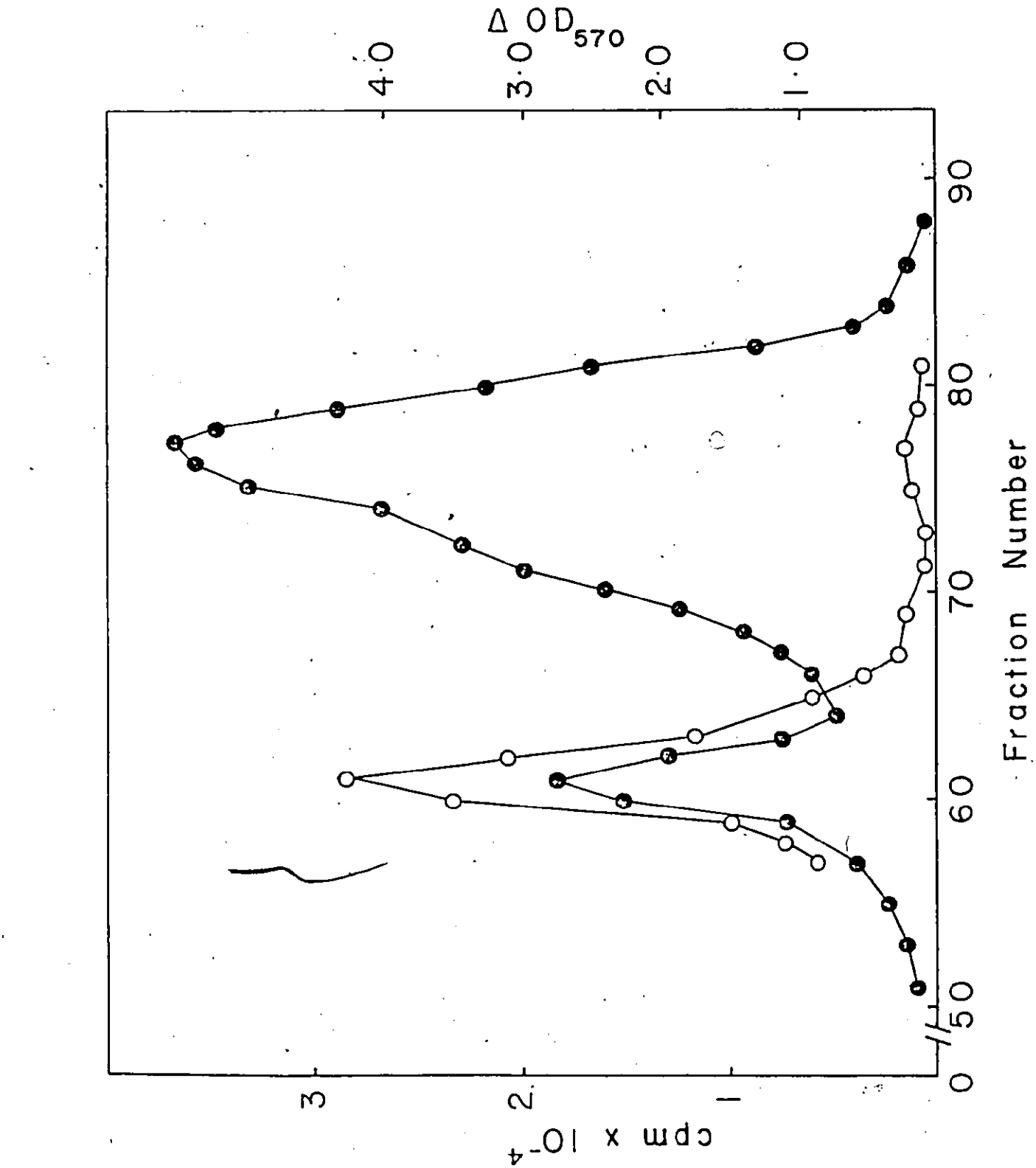
Rabbit muscle peptides (96.4 μmoles/ml) were suspended in redistilled pyridine and water (2:1) to give 50 μmoles in 0.6 ml. The acetylation was started with the addition of 200 μmoles of [^{3}H]-acetic anhydride with a specific activity of 50 μCi/μmole. After 0.5 hours at room temperature 25 μl of 10.6 M 'cold' acetic anhydride was added and the reaction was allowed to proceed for an additional 2 hours. After 2 hours, one drop of a solution of MSH was added and the pH was adjusted to 2.5 with concentrated formic acid. The MSH was added in order to determine the included volume of the gel filteration column.

B. Purification of Acetylated Peptides

Bio-Gel P-2 200-400 mesh was swollen overnight in 0.5 M pyridine formate buffer pH 2.5. The gel was de-aerated at reduced pressure. A 1.6 x 50 cm column was packed and equilibrated with two volumes of 0.5 M pyridine formate pH 2.5.

The sample was applied to the column and eluted with 0.5 M pyridine formate pH 2.5 at a flow rate of 10 ml/hr and 1 ml fractions were collected. Fractions were analyzed three ways: a 20 μl aliquot was assayed with ninhydrin after hydrolysis (see Analytical Methods); a 10 μl aliquot was analyzed using 5,5-dithiobis-(2-nitrobenzoic acid) (see Analytical Methods) in order to detect MSH; and 10-50 μl was dissolved in Triton X-114 scintillation fluid and analyzed in a liquid scintillation counter. A typical elution pattern is shown in Figure 2.

Figure 2. Bio-Gel P-2 Gel Filtration of Acetylated Peptides (●-●) cpm; (o-o) OD_{570}.

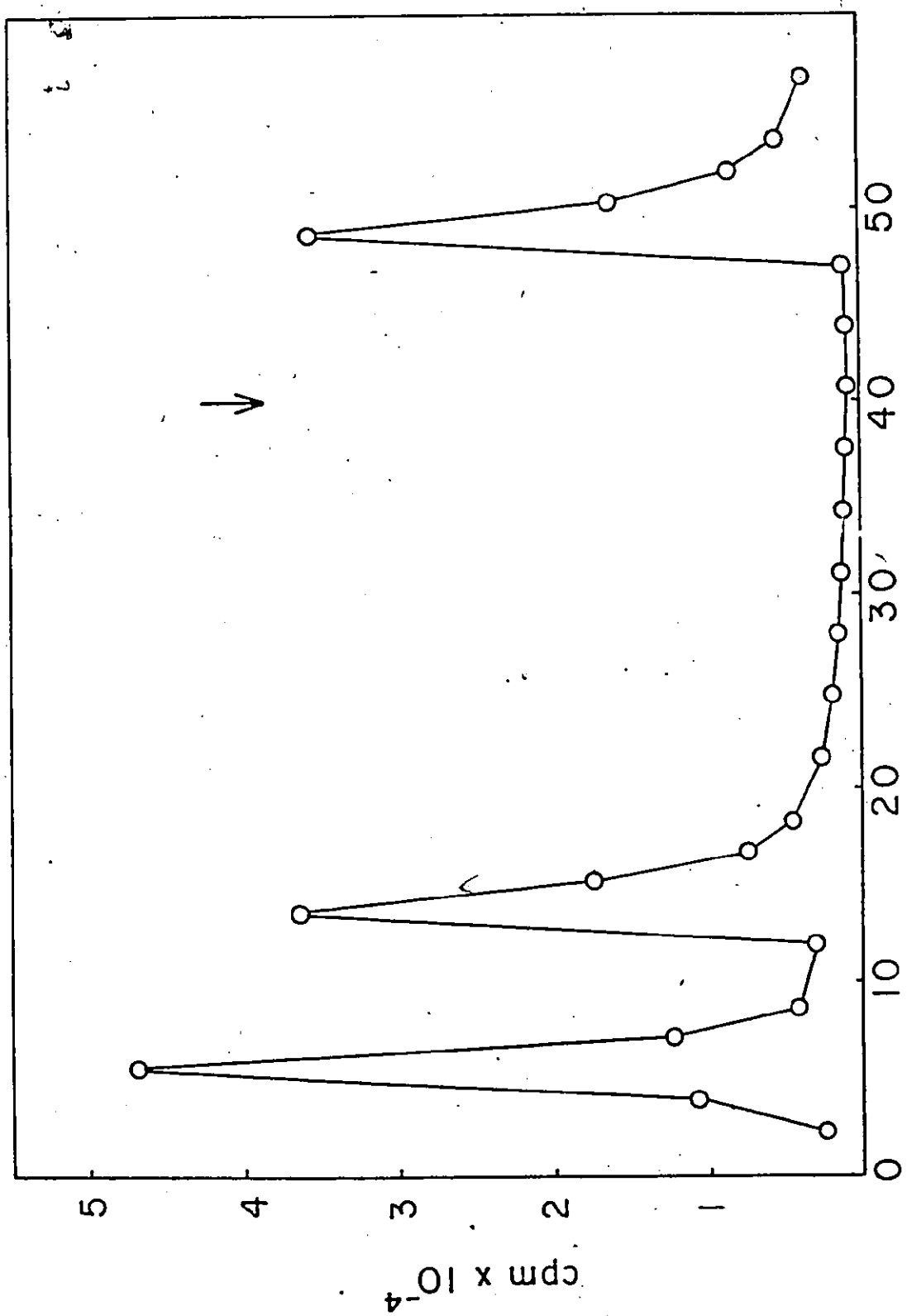

Δ OD570
4·0
3·0
2·0
1·0
90
80
70
60
50
0
Fraction Number
cpm x 10-4
3
2
1

Those fractions which contained the labelled peptides were pooled and evaporated to dryness under reduced pressure. The peptides were resuspended in a small volume of water and evaporated under reduced pressure. The peptides were then dissolved in 2 ml of water. The concentration was determined by reacting with ninhydrin after hydrolysis (see Analytical Methods). The concentration of the product was determined as the number of amino acid equivalents since the acetylated peptides do not react with ninhydrin. The specific activity was determined by dissolving a small aliquot in 10 ml of Triton X-114 scintillation fluid and counting in a liquid scintillation counter.

The peptides were further purified by ion exchange chromatography. Dowex 50 x 8 200-400 mesh was washed successively with water, 1N NaOH, water, 3N HCl, and water until neutral. The resin was then suspended in 0.1 M $NaPO_4$ pH 7.0. A 0.8 x 20 cm column was packed and equilibrated with the same buffer. A sample of acetylated peptides containing about 100 μmoles (after hydrolysis) was added to $NaPO_4$ buffer pH 7.0 to give a final buffer concentration of 0.1 M $NaPO_4$. The sample was loaded on the column and eluted with 0.1 M $NaPO_4$ pH 7.0, and 1.5 ml fractions were collected at a rate of 6 ml/hr. All operations were carried out at 4°C.

After 40 ml was collected, which represented four column volumes, the eluant was changed to 2 M formic acid. Aliquots (20 μl) from each sample were added to 10 ml Triton X-114 scintillation fluid and counted in a scintillation counter. A typical elution curve is shown in Fig. 3. The acetylated peptides emerging with

Figure 3. Dowex 50 x 8 Ion Exchange Column of Acetylated Peptides.
Initial elution was with 0.1 M $NaPO_4$ pH 7.0 which was changed to 2 M formic acid at the fraction indicated by the arrow.


cpm x 10^{-4}
5
4
3
2
1
0
10
20
30
40
50
Elution Volume (ml)

2 M formic acid were pooled, evaporated to dryness under reduced pressure and washed twice with small volumes of water followed by evaporation. The final product was suspended in 0.5 ml of water and analyzed by ninhydrin assay (see Analytical Methods) and liquid scintillation counting.

III Preparation of Amidated Peptides

A. Amidation with Glycinamide

Glycinamide-HCl and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were dissolved in 0.2 M pyridine HCl pH 6.0 buffer to give a final concentration of 100 μmoles/ml of glycinamide and 200 μmoles/ml of carbodiimide in a final volume of 3.75 ml. The reaction was started by the addition of 150 μmoles of rabbit muscle peptides to give a final concentration of 40 μmoles/ml. The reaction was done in a 25°C water bath. After 30 min, the pH of the solution was adjusted to pH 9.5 with 1 N NaOH. The sample volume was reduced to about 1 ml by evaporation under reduced pressure.

B. Purification of Amidated Peptides

Bio-Gel P-2 200-400 mesh was swollen overnight in 1 M α-picoline acetate pH 9.5. After de-aeration a 1 x 50 cm column was packed and equilibrated with two bed volumes of buffer. A drop of a solution of MSH was added to the sample which was then layered on the column and eluted with 1 M α-picoline acetate pH 9.5. Fractions (2 ml) were collected at a rate of 6 ml/hr. Fractions were analyzed by ninhydrin assays before

and after hydrolysis and with 5,5'-dithiobis-(2-nitrobenzoic acid) (see Analytical Methods). The peptides emerging in the void volume were pooled, evaporated to dryness under reduced pressure and washed twice with small volumes of water followed by evaporation. The final product was suspended in 1 ml of water and analyzed by ninhydrin assay before and after hydrolysis (see Analytical Methods).

IV Assay for Oligopeptide Ligase

A. Preparation of Brain Homogenates

Either rabbit or rat brains were used. The procedure for removal of rat brains was as follows:

A male Sprague-Dawley rat, 200-300 g, was stunned by a blow to the neck and exsanguinated by cutting the blood vessels in the neck. The skull was exposed by cutting the skin sagittally from the back of the neck to the nose. A cut was made just posterior to the occipital bone to just below the spinal cord. Scissors were inserted into the foramen and cuts were made around the occipital bone which was removed. The parietal and frontal bones were removed by cutting along the outer edges of the cranium and removing the bone cap. The membrane was cut and the brain removed with a curved spatula. The pituitary was covered by a thin dura membrane which was pulled away and the pituitary removed with a narrow spatula. The brain was washed in cold buffer. The procedure was similar for removal of the rabbit brain except that bone forceps were used instead of scissors and the bone must be cracked to remove the pituitary gland.

The brain was weighed and suspended in cold buffer (usually 0.1 M $NaPO_4$ pH 7.0) to give a 20% w/v suspension. Homogenization was carried out in a Waring blender for one min at 70 volts on the rheostat or by 12 strokes of a Potter-Elvehjem (teflon on glass) homogenizer at 1000 rpm. The homogenates were used immediately.

B. Assay of Brain Homogenates

The specific conditions used in each assay are given in the Results. Typically 100 μl of 20% w/v homogenate was added to an assay mixture containing ATP, $MgCl_2$, acetylated peptides, amidated peptides and buffer to give a final volume of 200 μl. After equilibration the reactions were started by the addition of homogenate to the assay tubes. Incubations were for 15 or 30 min at 37°C. Heated homogenate was prepared by placing the homogenate in a boiling water bath for 10 min and cooling to 37°C before use. Those control tubes which did not contain homogenate were brought up to the required volume with 100 μl of the homogenization medium. Reactions were stopped by placing the assay tubes in a boiling water bath for 5 min.

Dowex 1 x 8 100-200 mesh resin was prepared by washing successively in 1N NaOH, 3N HCl, and water until neutral and then suspended in 0.025 M $NaPO_4$ pH 7.0. Assay mixtures were layered on the 0.5 x 5 cm columns and eluted with 2.4 ml of 0.025 M $NaPO_4$ pH 7.0. Triton X-114 scintillation fluid (18 ml) was added to the eluates and the vials were counted

in a liquid scintillation counter for 20 min. Standards containing the assay mixture but no homogenate were treated in the same manner but not eluted through the columns. Instead they were added to 18 ml of scintillation fluid along with 2.4 ml of 0.025 M $NaPO_4$ pH 7.0.

V Preparation of Aldolase Peptides

A crystalline suspension of aldolase (16.5 mg protein/ml, 14 units/ mg protein) in 2.5 M $(NH_4)_2$ SO_4 - 0.01 M Tris - 0.001 M EDTA, pH 6.5 was used to prepare a random mixture of peptides. A solution of aldolase (60.7 ml), representing 1 g of protein, was centrifuged for 30 min at 12,000 g in a Sorvall RC2-B centrifuge at 4°C. The supernatant was poured off and the pellet resuspended in 70 ml of H_2O. This preparation was dialyzed against four changes of 6 litres of H_2O over 3 days at 4°C. After dialysis the final volume was 100 ml. Protein determination was done by the method of Lowry et al. (1951).

The aldolase was suspended in 5 mM MSH and 0.1 M $NaPO_4$ pH 7.0 and placed in a 90°C water bath for 1 min in order to denature the protein for papain digestion. The procedures used for digestion with papain, acetylation and amidation were similar to those used to prepare the rabbit muscle peptides described earlier.

VI Acetylation of Glycyl Peptides

A. Preparation of [^{3}H]-acetyl-Phe-Gly-Gly

A total of 376 μmoles of Phe-Gly-Gly was dissolved

in 2.4 ml of a 1:1 solution of redistilled pyridine and water. The reaction was started by the addition of 300 μmoles of [^{3}H]-acetic anhydride with a specific activity of 50 μCi/μmole. The reaction mixture was stirred at room temperature. After 30 min, 176 μl of 10.6 M 'cold' acetic anhydride was added which represented a 5 molar excess. The pH of the solution was kept above 7.0 with the addition of redistilled pyridine. The solution was stirred for 2 hours at room temperature. After 2 hours, concentrated formic acid was used to adjust the pH of the solution to 2.5. The solvent and [^{3}H]-acetic anhydride were removed by evaporation under reduced pressure. Further purification was done by gel filtration.

Bio-Gel P-2 200-400 mesh was swollen for several hours in 0.5 M pyridine formate pH 2.5. A 1.5 x 70 cm column was poured and equilibrated with 2 volumes of the same buffer. Acetylated Phe-Gly-Gly was dissolved in 3 ml of buffer, layered on the column and eluted with 0.5 M pyridine formate pH 2.5. Fractions (1.5 ml) were collected at a rate of 5 ml/hr. A drop of MSH was included in the sample in order to determine the included volume of the column. Fractions were assayed by scintillation counting, 5,5'-dithiobis-(2-nitrobenzoic acid) analysis, and ninhydrin analysis, before and after hydrolysis (see Analytical Methods). Those fractions containing the peptide were pooled, evaporated to dryness under reduced pressure, washed with water followed by evaporation, and suspended in 3 ml of water. Ninhydrin analysis after hydrolysis and liquid scintillation counting revealed a concentration

of 166 μmoles (after hydrolysis)/ml with a specific activity of 2.27 x 10^6 cpm/μmole(after hydrolysis).

B. Preparation of [^{3}H]-acetyl-Phe-Gly

The acetylated peptide was prepared by the same procedures described for the preparation of [^{3}H]-acetyl-Phe-Gly-Gly. A total of 562 μmoles(after hydrolysis) was obtained with a specific activity of 2.28 x 10^6 cpm/μmole(after hydrolysis).

C. Preparation of [^{3}H]-acetyl-Phe-Gly-(Ser-Gly)

The peptide was prepared by coupling with carbodiimide. Forty μmoles of [^{3}H]-acetyl-Phe-Gly and 100 μmoles of Ser-Gly were suspended in 1 ml of 0.2 M pyridine HCl pH 6.0. The reaction was started by the addition of 200 μmoles of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. After 30 min at 25°C the reaction was stopped by adjusting the pH to 9.0 with 2 N NaOH.

The peptide was purified by a combination of anion and cation exchange chromatography. A Bio-Rad AG 1 x 8 200-400 mesh column was prepared and equilibrated with 0.2 M pyridine. The sample was layered on the column and eluted with 3 column volumes of 0.2 M pyridine. The eluant was then changed to 2 M formic acid. The peptide was detected by liquid scintillation counting. After evaporation under reduced pressure the product was suspended in 1.5 ml of H_2O and pH adjusted to 3.0 with concentrated formic acid. The sample was then layered on a 0.7 x 10 cm Bio-Rad AG 1 x 4

100-200 mesh column and eluted with 5 volumes of water. The eluate was evaporated to dryness under reduced pressure, washed with water and evaporated to dryness. The residue was suspended in 1.5 ml of water. The sample was analyzed by ninhydrin analysis after hydrolysis, and liquid scintillation counting (see Analytical Methods). A total of 151 μmoles (after hydrolysis) of product was obtained with a specific activity of 4.4×10^5 cpm/μmole(after hydrolysis).

D. Preparation of [^{3}H]-acetyl-Phe-Gly-(Ala-Gly)

The procedures used for the preparation of [^{3}H]-acetyl-Phe-Gly-(Ala-Gly) were essentially the same as those described above. A total of 150 μmoles(after hydrolysis) of product was obtained with a specific activity of 4.5×10^5 cpm/μmole(after hydrolysis).

E. Preparation of Brain Homogenates

Sprague-Dawley 200-400 g male rats were used for most experiments. The removal and preparation of whole brain homogenate was described earlier.

Hypothalamic homogenates included both the hypothalamus and pituitary gland. A 0.4-0.5 mm cube, bounded anteriorly by the front of the optic chiasma and posteriorly by the pons was removed. The pituitary was found in the sella turcica at the base of the brain, covered by a thin membrane. The membrane was cut and the pituitary excised. Homogenates were then prepared as described for the whole brain.

F. Assay Conditions

Usually a 20% w/v homogenate, prepared in 0.1 M $NaPO_4$ pH 7.0, was used. A 100 µl portion of brain homogenate was assayed in the presence of 5 mM $MgCl_2$, 5 mM ATP, 2.5 mM glutamine, 2.5 mM asparagine and 0.1 M $NaPO_4$ buffer pH 7.0 in a total volume of 200 µl. Incubation was for [illegible] min at 37°C with the reaction terminated by placing the assay tubes in a boiling water bath for 5 min. Controls contained either homogenate which was heated previously in a boiling water bath for 10 mins, or just buffer alone.

The assay mixtures were then eluted with 2.4 ml of 0.02 M $NaPO_4$ pH 7.0 through 0.5 x 5 cm Dowex 1 x 8 200-400 mesh columns. Triton X-114 scintillation fluid (18 ml) was added to the eluates and the samples counted on a Beckman liquid scintillation counter for 20 mins each.

VII Amino Terminal Pyroglutamic Acid

A. Assay Methods

A 20% w/v brain homogenate was prepared in 0.25 M sucrose-0.01 M Tris-HCl pH 7.5. A 100 µl portion of this homogenate was incubated in the presence of 5 mM ATP, 10 mM $MgCl_2$, 0.5 mg/ml bacitracin, 1 µmole of either Gln-Gln, Gln-Gly or Gln-Gln-Gln, and 0.1 M Tris-HCl pH 8.0 in a total assay volume of 200 µl. Incubation was for 30 min at 37°C. The control contained brain homogenate which had been heated for 5 min in a boiling water bath. The reaction was stopped by the addition of 10 µl of 100% w/v TCA. The precipitate

was removed by centrifugation in an International clinical centrifuge at maximum setting for 10 min. The supernatant was removed, eluted through a 0.5 x 4 cm Dowex 1 x 8 100-200 mesh column with 2.5 ml of H_2O. The eluate was evaporated to dryness under reduced pressure, and heated for 12 hours at 110 C in a sealed tube. The HCl was either neutralized or removed by evaporation.

Glutamic acid was then determined by the method of Bergmeyer (1965) and modified according to Engel and Jones (1978). A 100 μl aliquot of the acid digest was pipetted into a 1 cm cuvette along with 1.5 ml of glycine-hydrazine buffer (0.5 M glycine; 0.4 M hydrazine; pH 9), 100 μl of nicotinamide adenine dinucleotide (3 x 10^{-2}M NAD^+) and 100 μl of 0.25 M EDTA. The assay was started by the addition of 5 μl of glutamic dehydrogenase (23.7 mg protein/ml, 43 units/mg protein in 50% glycerol. The optical density was read at 340 nm on the Cary spectrophotometer.

VIII Analytical Methods

A. Protein Estimation

Protein measurements were made using the methods of Lowry et al. (1951) using bovine serum albumin as a standard.

B. Radioactivity Estimation

Radioactivity was determined by liquid scintillation counting at room temperature in 10 ml of Triton X-114 and xylene (1:3 v/v) described by Anderson and McClure (1973)

containing 3 g of 2,5-diphenyloxazole and 0.2 g of p-bis [2-(5-phenyloxazalyl)]-benzene per liter. The maximum aqueous sample used was 2.7 ml. Counting efficiency was approximately 36% for tritium. In each series of assays a quenched standard was always included. Samples were counted for 20 min in a liquid scintillation counter.

C. Ninhydrin Assay

The assay was done using methods of Hirs et al. (1956) and Rosen et al. (1962). Samples of 100 µl were hydrolyzed in 1.67 N NaOH for 2-3 hours at 110°C in nalgene tubes and neutralized with 0.2 ml of 20% acetic acid. Both 0.2 ml of acetate buffer (3.8 N; pH 5.5; 1.5×10^{-4} M NaCN) and 0.2 ml of 3% ninhydrin in methoxyethanol were added to the sample. Samples were developed in a boiling water bath for 15 min. After the addition of 1 ml of 50% isopropanol the tubes were read at 570 nm on a Cary spectrophotometer.

D. Assay for MSH

One ml of 0.05 M Na Borate pH 10 and 0.1 ml of 10^{-2}M 5,5'-dithiobis-(2-nitrobenzoic acid) were added to a tube containing 10 µl of the sample. The samples were mixed. A colour change was due to the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with MSH.

RESULTS

I Oligopeptide Ligase

The search for ligase activity was started by incubating a complex mixture of peptides derived from muscle tissue by digestion with papain. One group of these peptides was acetylated using [^{3}H]-acetic anhydride, blocking the amino terminals. The carboxyl terminals of another group of peptides were blocked by amidation. Any ligase activity will result in the coupling of such peptides, which will result in the coupled peptide being eluted through an anion exchange column.

Significant ligase activity was not detected in either rat or rabbit brain homogenates (see Table 2). The lower limit of significant activity was chosen to be 5% of the counts added or 5 times the background. A number of experimental problems surfaced but were subsequently ruled out.

The use of a large volume of brain homogenate in each assay ensured that any required cofactors would be present in sufficient quantities.

Since the initial experiments failed to yield any detectable activity, the peptides used were tested for their behaviour on the anion exchange columns. Elution of 0.3 μmoles of amidated peptides which were treated under assay conditions showed that more than 90% of the peptides emerged in the eluate as determined by ninhydrin analysis. This confirmed that the amidation reaction did yield blocked peptides.

TABLE 2

Assay of Brain Homogenates

Components	Rat Brain Homogenate cpm	Rabbit Brain Homogenate cpm
Control with heated extract	129	60
Control without homogenate	118	58
- Amidated Peptides	173	86
- ATP	143	82
- $MgCl_2$	137	94
- ATP, $MgCl_2$	145	77
Complete system	128	74
cpm added	10,183	9,362

The incubation mixture contained 100 μl of 20% w/v brain homogenate (1-2 mg protein), 5 mM ATP, 10 mM $MgCl_2$, 0.15 μmoles of acetylated rabbit muscle peptides (specific activity 67,000 cpm/μmole), 0.3 μmoles of amidated peptides and 0.1 M $NaPO_4$ pH 7.0 in a total assay volume of 200 μl. The assay tubes were placed in a 37°C water bath for 30 min. The results are the average of duplicate assays.

The second check of the assay was to see if the acetylated and amidated peptides would couple and emerge through the column. This was done by reacting the acetylated peptides with excess carbodiimide and amidated peptides in 0.2 M pyridine HCl pH 6.0 under conditions previously described for the amidation of rabbit muscle peptides. After evaporation of the solvent under reduced pressure, the resulting product was eluted through a 0.5 x 5 cm Dowex 1 x 8 200-400 mesh column under conditions of the assay. Over 75% of the product emerged from the column. These experiments showed that the amidated peptides do emerge from an anion exchange column under the conditions employed and that the peptides could be coupled, at least synthetically.

One problem with the use of a complex mixture of peptide derived from muscle tissue was that other elements present in the initial digest may contaminate the peptides and inhibit the enzyme. For this reason a complex array of peptides was prepared from a pure protein. The enzyme aldolase, available commercially as a crystalline suspension was used to prepare peptides by the same procedures used for the preparation of muscle peptides. These peptides were acetylated and amidated as before. The results of the assay can be seen in Table 3. Significant activity was not obtained. If 5% of the initial counts was arbitrarily taken to represent a significant activity, this would represent 65 pmoles of product formed in 30 min.

There existed the possibility that the high level of

TABLE 3

Assay of Brain Homogenate with Acetylated Aldolase Peptides

Components	Product Formed (cpm)
Control with heated extract	56
Control without homogenate	48
- ATP	44
- $MgCl_2$	61
- ATP, $MgCl_2$	47
- Amidated peptides	32
Complete system	49
cpm added	10,578

The incubation mixture contained 100 μl of 20% w/v rabbit brain homogenate, 5 mM ATP, 5 mM $MgCl_2$, .04 μmoles of acetylated aldolase peptides, 0.1 μmoles of amidated aldolase peptides and 0.1 M $NaPO_4$ pH 7.0 in a total volume of 200 μl. The assay tubes were placed in a 37°C water bath for 30 min. Reactions were stopped by placing the tubes in a boiling water bath for 5 min. The results are the average of duplicate assays.

proteolytic activity which exists in the brain may be degrading the peptides. Therefore phenylmethylsulfonyl fluoride was included to inhibit serine proteases, iodoacetamide to inhibit sulfhydryl proteases, or EDTA to inhibit metal requiring proteases. The addition of any of these compounds did not result in any increase of labelled product being eluted from the anion exchange columns. Inclusion of bacitracin at 0.5 mg/ml did not affect results.

There existed the possibility that other nucleotides besides ATP may be required; however, the inclusion of CTP, UTP or GTP did not enhance the reaction.

The use of alternative buffers to phosphate was also explored as phosphate buffers are notorious for chelating metal ions. Assays were carried out with Tris and 2-(N morpholino) ethane sulfate buffers at pH 7.5 and 7.0 respectively. Neither caused any enhancement of activity above background. The same results were obtained using a range of pH values shown in Table 4.

The information of peptide bonds may require ATP. However, ATP is rapidly hydrolyzed in brain homogenates due to ATPases participating in the maintenance of Na^+/K^+ gradients. To compensate for this activity, both ouabain, a potent inhibitor of Na^+/K^+ ATPases, and an ATP regenerating system of phosphoenolpyruvate and pyruvate were included. Their inclusion did not change the results obtained previously (Table 5).

The presence of large amounts of peptides in the brain

TABLE 4

Cofactors and Conditions Used During Assay

Component	Final Concentration
Iodoacetamide	5×10^{-2} M
Phenylmethylsulfonyl fluoride	2×10^{-3} M
EDTA	2.5×10^{-3} M
Bacitracin	0.5 mg/ml
CTP	5×10^{-2} M
UTP	5×10^{-2} M
GTP	5×10^{-2} M
Tris-HCl pH 7.5	5×10^{-2} M
2-(N morpholino) ethane sulfonate buffer pH 7.0	5×10^{-2} M
Citrate buffer pH 5.5	0.1 M
Phosphate buffer pH 6.0, 6.5, 7.0, 7.5	0.1 M
Tris-HCl pH 8.0, 8.5, 9.0	0.1 M

The above conditions were included in the standard assay mixtures of 5 mM ATP, 5 mM $MgCl_2$, 0.15 μmoles of acetylated peptides, 0.3 μmoles of amidated peptide, 0.1 M $NaPO_4$ pH 7.0 unless stated, and 100 μl of 20% w/v brain homogenate in a total assay volume of 200 μl. Incubations were for 15 or 30 min at 37°C. The reactions were stopped by placing the tubes in a boiling water bath for 5 min.

TABLE 5

Assay of Brain Homogenate with an ATP Regenerating System

Components	Product Formed (cpm)
ATP regenerating system	123
Ouabain	118
ATP regenerating system and ouabain	101
- ATP regenerating system and ouabain	112
Complete system with heated extract	104
cpm added	8,654

The incubation mixture contained 100 μl of 20% w/v rat brain homogenate, 5 mM ATP, 5 mM $MgCl_2$, 5 x 10^{-4}M ouabain, 25 mM phosphoenolypyruvate, 5 units of pyruvate kinase, 0.15 μmoles of acetylated rabbit muscle peptides, 0.3 μmoles of amidated rabbit muscle peptides and 0.1 M $NaPO_4$ pH 7.0 in a total volume of 200 μl. The assay tubes were placed in a 37°C water bath for 30 min. The reactions were stopped by placing the tubes in a boiling water bath for 10 min. The results are the average of duplicate assays.

homogenates could compete with the amidated peptides. However, this possibility was unlikely due to the fact that the addition of a ten molar excess of amidated peptide did not result in any detectable product formation.

II Studies on the Formation of Carboxyamide Residues

Four [^{3}H]-acetylated peptides were prepared. If the synthetic peptides were processed as proposed, the resulting products would terminate with glycinamide, alaninamide, phenylalaninamide, or serinamide. All four are known to exist at the carboxyl terminal of peptides occurring in vivo.

Assaying for the activity using the synthetic peptides in whole brain or hypothalamic homogenates did not reveal any amidation of the substrate (Table 6). Significant results would have been obtained if 0.2 nmoles of the smaller peptides were modified or 2 nmoles of the larger peptides were modified.

The conditions used for subsequent assays are outlined in Table 7. Bacitracin was used to inhibit the degradation of the peptides (Cuello et al. 1978). The activity of ATPases was countered by the use of ouabain and an ATP regenerating system. Possible substrates participating in the reaction such as NH_3 and NADPH were also included.

One question needed to be answered. Would the amidation of the acetylated peptides allow them to pass through the anion exchange columns? [^{3}H]-acetyl-Phe-Gly-Gly and [^{3}H]-acetyl-Phe-Gly-Gly were coupled to glycinamide with carbodiimide. The blocked

TABLE 6

Assay for Carboxyamide Formation with Peptides Terminating with Glycine

Subtrate Added			Product Formed (cpm)		
Substrate	μmoles	cpm	Control with heated extract	Brain Homogenate	Hypothalamic Homogenate
[^{3}H]-acetyl-Phe-Gly	0.01	19,483	58	52	
[^{3}H]-acetyl-Phe-Gly-Gly	0.01	19,476	63	61	62
[^{3}H-acetyl-Phe-Gly-(Ala-Gly)	0.05	18,185	169	239	144
[^{3}H-acetyl-Phe-Gly-(Ser-Gly)	0.05	18,321	143	161	156

Incubation mixtures consisted of 100 μl of 25% w/v homogenate, 5 mM ATP, 5 mM $MgCl_2$, 5 mM Gln, Asn, Asp, Glu and 0.1 M $NaPO_4$ pH 7.0 in a total assay volume of 200 μl. The assay tubes were placed in a 37°C water bath for 30 min. The reactions were stopped by placing the assay tubes in a boiling water bath for 5 min. The results are the average of duplicate assays.

TABLE 7

Conditions for Assay of Carboxyamide Formation

	Product Formed Using the Following Substances (cpm)							
	[^{3}H]-acetyl-Phe-Gly .01 μmoles,		[^{3}H]-acetyl-Phe-Gly-Gly .01 μmoles,		[^{3}H]-acetyl-Phe-Gly-(Ala-Gly) .05 μmoles,		[^{3}H]-acetyl-Phe-Gly-(Ser-Gly) .05 μmoles	
Component	Control		Control		Control		Control	
Bacitracin 0.5 mg/ml	66	79	67	74	220	240	204	259
PEP (10 mM) and pyruvate kinase (2U)	84	77	72	89	108	133	138	193
Tris-HCl pH 7.5 as buffer	78	84	71	67	153	176	147	167
NH_4Cl 10 mM	91	66	90	77	130	118	193	204
Ouabain 1 mM	79	68	84	81	183	185	235	247
NADPH 5mM	-	-	81	71	166	194	335	279
Standard (cpm added)	19,500		19,500		16,500		14,000	

These results summarize a number of experiments. The conditions used are detailed in the methods. These results were taken from incubations with whole brain homogenates.

peptides were subjected to the conditions used for the enzyme assay and eluted through the 0.5 x 5 cm anion exchange columns. The eluates were analyzed by liquid scintillation counting. A recovery of 60 to 70% was obtained for both peptides.

III Amino Terminal Pyroglutamic Acid

Three peptides, Gln-Gln, Gln-Gly and Gln-Gln-Gln, were used to assay for glutamine cyclotransferase. Cyclization of the amino terminal glutamine of the peptides would allow them to pass through the cation exchange columns. The eluates were subjected to acid hydrolysis. The quantity of glutamic acid released was determined by an enzymatic assay using glutamic dehydrogenase. The existence of an enzyme similar in properties to glutamine cyclotransferase was not detected in rat brain homogenates (Table 8). The accuracy of the assay method was tested using a known amount of authentic pyroglutamic acid. More than 90% of pyroglutamic acid was recovered after simulating conditions of the assay.

A number of conditions were changed in the assay in order to detect activity. Due to the high level of ATPase activity an ATP regenerating system of phosphoenolpyruvate and pyruvate kinase was included. Ouabain, a potent inhibitor of $(Na^+ + K^+)$-stimulated ATPases in the brain (Sweadner, 1979; Wallick et al. 1979) was also included.

TABLE 8

Formation of Amino Terminal Pyroglutamic Acid

Subtrate	Product Formed (μmoles)
Gln-Gln	<15
Gln-Gly	<7.5
Gln-Gln-Gln	<5

Assay conditions are described under Methods. Controls were done with heated homogenates. Limits of detection were 15 μmoles for Gln-Gly, 7.5 μmoles for Gln-Gln and 5 μmoles for Gln-Gln-Gln.

DISCUSSION

The experimental results obtained during the last year and a half failed to give any evidence for the existence of the three postulated enzymes: an oligopeptide ligase; an enzyme which amidates the carboxyl terminal through a mechanism involving glycine; or an enzyme with properties similiar to glutamine cyclotransferase of papaya latex. These results must be interpreted in light of their implications to the two original objectives of the project; namely, to test Chan's hypothesis concerning the storage of information in the brain, and to obtain a better understanding of the metabolism of brain peptides.

The most important enzyme of the memory hypothesis was the oligopeptide ligase. A variety of conditions and co-factors were used during the experiments, but evidence for the postulated enzyme was not obtained. The failure to detect activity could be due to a number of reasons: (1) a nonspecific ligase simply does not exist in the brain tissue of either rabbits or rats; (2) activity was very low; (3) the substrate peptides were unsuitable; (4) the necessary co-factors were not available; (5) inhibitors were present in the assay systems; (6) peptidases degraded the peptides.

The last four difficulties can be ruled out for the following reasons. First, a random mixture of peptides was used in the experiments. The postulated enzyme must be nonspecific for the hypothesis to be confirmed. Demonstration

of coupling between amino and carboxyl blocked peptides was shown with the use of carbodiimide. Secondly, the necessary cofactors should be present as a high concentration of homogenate was used in each assay. Thirdly, the presence of low molecular weight inhibitors was studied by passing the homogenate through a short Sephadex G-25 column prior to the assay. The protein fractions, identified by reading the absorbance at 280 nm, were assayed for ligase activity. Enzyme activity was not observed. One drawback of the procedure was that small molecular weight cofactors may also be removed.

The presence of high levels of proteolytic activity in brain homogenates requires careful consideration as all work with peptides and proteins in the brain will be subject to this problem. Peptides are rapidly degraded in the brain (Benuck and Marks, 1975; Marks, 1976, 1978; Huang and Lajtha, 1978; Martins et al. 1977). This problem can be overcome by the use of bacitracin which has been found to inhibit peptide degradation (McKelvy et al. 1976; Miller et al. 1977). This antibiotic was included in the assays along with other protease inhibitors. However, there was no evidence of ligase activity.

The possibility that the level of ligase activity was below the limit of detection of the assay cannot be ignored. If the enzyme was always present in the extracellular space then the amount of enzyme activity could be quite high as the cells must continually produce the enzyme to compensate

for breakdown. However, only low concentrations would be needed if the enzyme was only released during synaptic transmission. The possibility of enzymes being released at synapses in conjunction with the release of transmitter has also been postulated by Guillemin (1978).

The inability to detect a nonspecific oligopeptide ligase calls the original hypothesis into question. Upon reflecting back to the hypothesis certain problems come to mind. First, the presence of a nonspecific ligase would not only join peptides involved in memory, but also any hormones, peptide transmitters or protein fragments. A second problem is that there is a high level of proteolytic activity in the brain which would degrade the newly formed peptides. Since the joined peptide is not synthesized continually by the neurons, the peptides, and hence the information would be lost. A third problem is also the result of the inability of a single neuron to sythesize the joined peptide. A repetitive firing of one neuron would rapidly release all of the joined peptide with which it was associated. The neuron cannot synthesize the released peptides. Therefore, the neuron cannot transmit the impulse and the memory is lost.

The question as to whether or not peptides play a role in the acquisition of all learned behavior could be better answered if we knew the quantity and range of peptides in the brain. In the past this task has been difficult because of the small quantities of peptides involved and difficulties

in separation of similiar peptides. Progress in the field has been made by the use of reverse phase high pressure liquid chromatography in conjunction with micro ion-exchange chromatography. These techniques allow the use of crude extracts, give good recoveries of peptide (85% of 2-4 pmols), and is applicable to picomolar quantities of peptides (Morris et al. 1980).

In conjunction with work on the peptide pool of the brain, more information must be learned about how the neurons communicate with each other. Neurons have been seen as a one way information-transmitting cellular system with a sometimes vast but passive receptive dendritic surface with integrative capabilities focussed on the axon hillock and with an axonal self-regenerative mechanism for rapid transmission of the message to axonal terminals (Schmitt et al. 1976). The new view of the neuron is that the dendrite is no longer seen as a 'passive receptor surface', but rather as a locus for transmitting as well as receiving information in traffic with dendrites of other neurons by low voltage depolarization (Guillemin, 1978). This local circuitry actually represents the structure of the greatest mass or volume of the central nervous system, with the projection neurons probably a minority in number as well as space (Guillemin, 1978). Therefore, the very characteristics of neuronal activity most pertinent to information processing may lie, not in the action potentials and conventional synaptic activity of long-axon neurons, but in the

decremental activity of often axonless local neuronal circuits (Irwin, 1978). Little can be studied about learning and memory at the neuronal level until we understand how neurons 'talk'.

The inability to detect nonspecific ligase activity has certain implications in the metabolism of brain peptides. Although a nonspecific ligase may not exist, the possibility of enzymes present which catalyze the formation of specific peptide bonds cannot be ruled out. Many workers have presented evidence that small neuropeptides may be formed by non-ribosomal mechanisms. In addition to the synthetases and peptidyl transferase enzymes which act to form new peptide bonds, a group of enzymes has been identified that catalyze the transfer of the aminoacyl moiety of certain aminoacyl tRNA's to the amino terminal of acceptor peptides and proteins (McKelvy, 1977). While this process is dependent on amino acid activation by adenylation and aminoacyl tRNA formation, as is the nucleic acid template mechanism, peptide bond formation is independent of mRNA, ribosomes, GTP, or other components of ribosomal protein synthesis (Brownstein, 1978). Not all amino acids have been shown to participate in the reaction. Whether or not any of these mechanisms are of physiological significance remains to be demonstrated. Most work has focused on thyrotropin releasing hormone but actual demonstration of synthesis is difficult to reproduce (Bauer and Lipman, 1976).

The other two reactions studied, namely, amino and

carboxyl terminal blockage, involve derivatization of existing amino acid residues. This process is quite common. While only 20 primary amino acids are specified in the genetic code and are involved as monomer building blocks in the assembly of the polypeptide chain, over 140 amino acids and amino acid derivatives have been identified as constituents of different proteins in different organisms (Uy and Wold, 1977). As suggested by Uy and Wold (1977) three questions need to be answered. (1) Where in the cell and at what stage of protein synthesis does the derivatization take place? (2) What determines the specificity of the process in which only one or a few residues of a large number of like residues are selectively derivatized? (3) What is the relationship of any given modification to a specific biological function? This last question will have to be pursued for each individual derivatization reaction.

The first modification to be studied was the formation of a carboxyamide residue. The results of the experiments suggests that glycine does not alone constitute the specific recognition site in the rat brain. It is possible that glycine and one or more amino acids constitute the recognition site just as thrombin only recognizes -Gly-Arg- in the conversion of fibrinogen to fibrin (Doolittle, 1973). Secondary and tertiary structure could also be important. Such a situation exists in the processing of hormone precursors in which adjacent basic residues are cleaved in unstructured or β-turn arrangements but not when in an α-helix arrangement

(Geisow, 1978). Predictions as to whether or not primary and secondary structure, or both are involved as the recognition site must wait until the sequences of precursors to carboxyamide containing peptides are known.

Several systems lend themselves to further investigation. The release of α-melanotropin from adrenocorticotrophin or the initial corticotrophin-β-lipotropin precursor could be studied. The second system is the processing of the precursor to melittin. The techniques for the isolation of mRNA and subsequent translation have been developed (Kreil et al. 1977). Translation of the mRNA yields a product termed prepromelittin which is subsequently processed to promelittin, terminating with a carboxyamide residue. The processing of prepromelittin could be studied in brain homogenates. It is already known that enzymes are present in the rat liver which cleave prepromelittin to give a product with the same amino terminal sequence as promelittin (Kaschnitz and Kreil, 1978). Unfortunately these workers did not study the carboxyl terminal of the product to see if a carboxyamide residue was also formed. The removal of the prepeptide is not species specific (Suchaneck et al. 1978). Does the same hold true for the formation of the carboxyl terminal amide?

The second amino acid modification to be studied was the conversion of amino terminal glutamine to pyroglutamic acid. There was no evidence of an enzyme with properties similiar to glutamine cyclotransferase of papaya latex in the rat brain. An earlier report that glutamine cyclotransferase

exists in guinea-pig homogenates may be incorrect (Wolfersberger and Tabachnick 1974). They failed to properly characterize the product or rule out alternate pathways such as glutamine/glutamic acid interconversion or the γ-glutamyl transferase/γ-glutamyl cyclotransferase pathway.

The failure to identify a glutamine cyclotransferase in the brain homogenates leaves the door open to a mechanism involving cyclization concomitant with cleavage of an extra piece from a precursor. All secretory proteins may be synthesized as precursors (Blobel and Dobberstein, 1975). The extra piece on the amino end is referred to as a signal peptide. The signal peptide has been postulated to bind to the membranes of the endoplasmic reticulum. When the newly synthesized protein enters the matrix of the endoplasmic reticulum the signal peptide is cleaved off. The formation of pyroglutamic acid may take place concomitantly with cleavage of the signal peptide for a number of secretory peptides such as the immunoglobulins (Jilka and Pestka, 1977; Burstein and Schecter, 1977). The isolation of the 'signalase' may yield some answers.

Many hormones and peptides are derived from precursors; growth hormone and prolactin (Lingappa et al. 1977); calcitonin (Jacobs et al. 1979); epidermal growth factor (Frey et al. 1979); somatostatin (Noe et al. 1979); vasopressin and neurophysin (Russel et al. 1979); gastrin (Dockray et al. 1978); parathyroid hormone (Habener et al. 1978); and oxytocin (Brownstein

et al. 1980). This list is by no means exhaustive. An important recognition signal in the processing of peptide precursors are two adjacent basic amino acids (Hales, 1978; Steiner, 1979). Cyclization of glutamine may be catalyzed by the trypsin-like enzyme as it cleaves the pair of basic amino acids from the amino group of glutamine. However, not all proteases share this property. Trypsin will cleave -Lys-Lys-Gln- of the gastrin precursor with the release of free amino terminal glutamine (Gregory and Tracy, 1978). However the trypsin-like enzyme found in the tissues is also sensitive to secondary structure, cleaving only in regions of unstructured or β-turn arrangements but not the adjacent basic residues in an α-helix arrangement (Geisow, 1978).

The function of pyroglutamyl and carboxyamide residues remains unknown. Both may represent control points in the processing of biosynthetic precursors. They may also serve as specific deactivation/activation sites analogous to the acetyl group of acetylcholine. Both deamidase and pyroglutamate aminopeptidases are known to exist (Prasad, 1976; Taylor and Dixon, 1978; Matsin et al. 1979).

APPENDIX: MATERIALS

1. Chemicals

All chemicals, where possible, were reagent grade. Triton X-114 was a product of Rohm and Haas.

Bacitracin, NAD, NADH, phosphoenolpyruvate, ATP, CTP, UTP, GTP, α-ketoglutaric acid, Phe-Gly, Ala-Gly, Ser-Gly, Phe-Gly-Gly, glycinamide hydrochloride, 5;5-dithio-bis (2-nitrobenzoic acid) and bovine serum albumin were purchased from Sigma Chemical Co.

Gln-Gln, Gln-Gly, Gln-Gln-Gln were obtained from Chemical Dynamics Corp.

L-[3,4-^{3}H(N)]-Glutamine (45 Ci/mmol) and [2-^{14}C] acetic anhydride were products of New England Nuclear.

1-Ethyl-3-(3-dimethyaminopropyl) carbodiimide hydrochloride was a product of Pierce Chemical Co.

2. Enzymes

Rabbit muscle pyruvate kinase, bovine L-glutamic dehydrogenase, rabbit muscle aldolase, papain, rabbit muscle lactate dehydrogenase and porcine γ-glutamyl transpeptidase were purchased from Sigma Chemical Co.

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