0270-6474/85/0504-0851$02.00/0

The Journal of Neuroscience
Vol. 5, No. 4, pp. 851–856
April 1985

Opioid Pathways in an Avian Retina

I. The Content, Biosynthesis, and Release of Met^5-Enkephalin[1]

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Abstract

By means of an enzyme-linked immunosorbent assay, the concentration of enkephalin-immunoreactive substances was estimated to be about 25 nM in the chicken retina. The biosynthesis of ^{3}H-Met^5-enkephalin in this retina was studied by a pulse-chase incubation technique. Isolated retinas were incubated with 0.2 ml of oxygenated Ringer's solution containing 40 μCi of [^{3}H]methionine and trasylol, a peptidase inhibitor, for 30 min at room temperature. The tissue was then rinsed three times in large volumes of Ringer's solution and incubated in the same solution containing unlabeled methionine (100 μg/ml) and trasylol for at least another hour. The products synthesized were extracted in acetic acid and assayed by high performance liquid chromatography (HPLC) and immunoassay. A peak of radioactivity that comigrated with Met^5-enkephalin on HPLC and cross-reacted with antibodies against enkephalins was detected. The level of ^{3}H-Met^5-enkephalin radioactivity increased approximately 10-fold as the chase-incubation period increased from 0 to 120 min, suggesting that, as in other tissues, Met^5-enkephalin may be synthesized as part of a larger precursor. The newly synthesized Met^5-enkephalin could be released by depolarization of the retina with high extracellular K$^+$ concentration. Furthermore, this K$^+$-stimulated release was greatly suppressed by 5 mM Co^{2+} in the medium, suggesting that this release is Ca^{2+}-dependent and may be synaptically mediated.

One of the most exciting developments in neuroscience during recent years has been the discovery that, in addition to the handful of classical neurotransmitters, such as γ-aminobutyric acid, glycine, acetylcholine, and the catecholamines, a large number of peptides and hormones have been localized to and found to be physiologically active in many regions of the nervous system (Hokfelt et al., 1980). However, the mechanisms by which these neuroactive peptides operate at the cellular and molecular level are, to a large extent, unknown, although there is increasing evidence that they serve a role as transmitter and/or modulatory agents in the processing of neural information (Burnstock, 1976; Iversen et al., 1978; Burnstock et al., 1979; Hokfelt et al., 1980; Iversen, 1983). Utilizing the vertebrate retina as a model system, we have initiated studies to examine the functional roles played by a class of opioid peptides, Leu^5- and Met^5-enkephalins, in the processing of neural information. We chose the enkephalins for our studies because these important neuroactive peptides not only are well characterized both biochemically and pharmacologically (Loh and Ross, 1979), but have been localized to and shown to be physiologically active in the vertebrate retina (see Watt et al., 1985, for references).

Two of the most important criteria in the establishment of any substance as a neurotransmitter or neuromodulator involve the demonstrations that the substance is present in an identified neuron at significant concentrations and that it is released from the cell upon its depolarization. Using the vertebrate retina as a model system, we have attempted to determine the functional roles, if any, for a class of opioid peptides, Leu^5-enkephalin and Met^5-enkephalin, in neurotransmission. In the chicken retina, our immunocytochemical and immunochemical analyses utilizing both polyclonal and monoclonal antibodies against enkephalins (Watt et al., 1983, 1985; Tavella et al., 1985), together with studies by other investigators (Brecha et al., 1979; Humbert et al., 1979), point to the existence of enkephalins or enkephalin-like peptides in a small subpopulation of amacrine cells. As a logical extension of previous studies on retinal enkephalinergic systems, this paper and the following one (Watt et al., 1985) address: (*1*) the content, biosynthesis, and Ca^{2+}-dependent release of Met^5-enkephalin and (*2*) the synaptic organization of enkephalin-immunoreactive amacrine cells in the chicken retina. Specifically, in the present paper, we report the use of combined immunochemistry and high performance liquid chromatography (HPLC) to demonstrate that authentic Met^5-enkephalin, or a peptide very similar to enkephalin (Hoffman, 1983), is synthesized by the chick retina. Furthermore, we show that this peptide can be released from the retina in response to its depolarization and that the release mechanism is dependent on the presence of extracellular Ca^{2+}.

Materials and Methods

Measurement of enkephalin content. Enzyme-linked immunosorbent assay (ELISA) was used to determine the enkephalin content in the chicken retina. The basic principles of this method are the same as those of the radioimmunoassay and are based on the competition binding of the antibody with the antigen in the sample and that conjugated onto the polystyrene microtitration plate (from Dynatech Laboratories). The number of antigen-antibody complexes on the plate decreased as the concentration of antigen in the sample increased. The antigen-antibody complexes of the plate were incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) and then visualized with HRP substrates. The detailed procedures for this assay method are summarized as follows. One microgram of the antigen (Met^5-enkephalin conjugated to bovine serum albumin) in 100 μl of 0.1 M sodium carbonate (pH 9.6) was added to each well. The plate which has 96 wells was incubated overnight at 4°C and then washed three times with

Received January 17, 1984; Revised August 27, 1984;
Accepted October 3, 1984

[1] This work was a collaborative project with the Clayton Foundation for Research and was also supported in part by United States National Eye Institute Grants EY03701 (to Y. Y. T. S.), EY07001 and EY05690 (to C. B. W.), and EY02423 (to D. M. -K. L.), and by the Retina Research Foundation.

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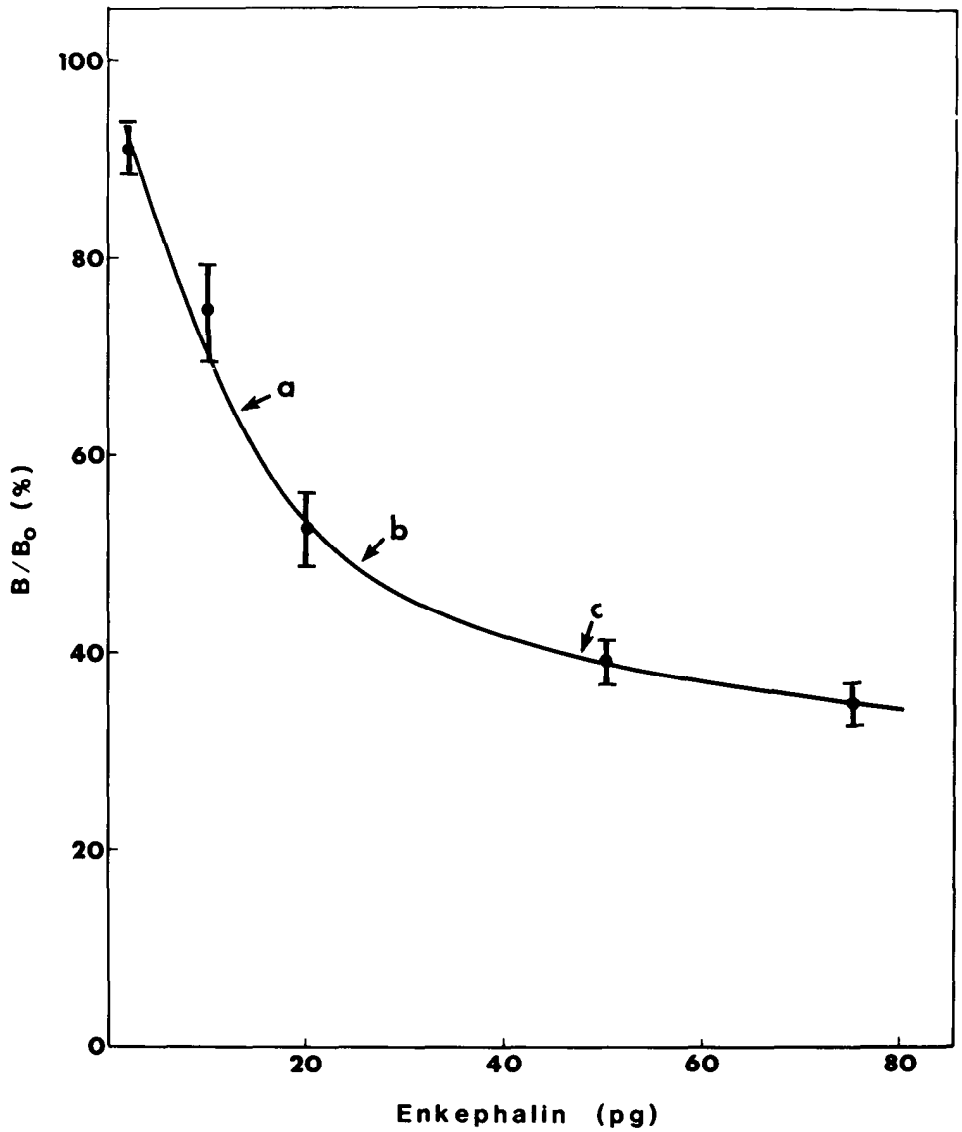

Figure 1. A standard curve for the ELISA test. Each *point* represents at least two measurements that are assayed in triplicate. The enkephalin content in the chick retina was estimated. Points a, b, and c represent different volumes of tissue extract with their protein concentrations corresponding to 0.1, 0.2, and 0.4 mg. The enkephalin concentrations for these points are 13, 26, and 47.5 pg, respectively. The average concentration of enkephalin in the retina is 126 pg/mg of protein or approximately 2.5×10^{-8} M.

phosphate-buffered saline, pH 9) (PBS-9). Fifty microliters of the standard or the unknown sample in PBS-9 were added to each well. Fifty microliters of the diluted antiserum (either polyclonal antibody A206 from Dr. K. -J. Chang, 1:250 in PBS; or monoclonal antibody AD4, 1:50 in PBS) were added to the plate and incubated for 90 min at room temperature or overnight at 4°C. After the plate was washed 10 times with 0.05% Triton X-100 in water, 50 μl of the HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (1:3000 dilution in 0.5 M NaCl, 0.5% Triton X-100, and 0.01 M Na_2HPO_4) were added to each well and incubated for 10 min at room temperature. After washing the plate 10 times with 0.05% Triton X-100 in water, 100 μl of the substrates (0.4 mM 2,2′-azion-di-(3-ethyl-benzthiazoline sulfonic acid) diammonium salt (ABTS) and 0.3% H_2O_2 in 0.1 M sodium citrate (pH 4) were added to each well. After 15 min, the optical densities at 400 nm were measured for each well.

The optical density of these wells without any free antigen was designated *Bo* and for wells containing a known amount of antigen, *B*. For each concentration of free antigen the ratio of optical density (*B*/*Bo*) was calculated. This ratio (*B*/*Bo*) versus the concentrations of free antigen in the solution was plotted to generate a standard curve.

Biosynthesis of Met⁵-enkephalin. Isolated chicken retinas were incubated in 0.2 ml of oxygenated (95% O_2 and 5% CO_2) Ringer's solution containing 40 μCi of [^{3}H]methionine (specific activity, 50 Ci/mmol; New England Nuclear Corp.) and the peptidase inhibitor, trasylol (250 Kallikrein units/ml), for 30 min. The tissue was washed three times in large volumes of Ringer's solution containing trasylol and then incubated in the same Ringer's solution containing 100 μg/ml of methionine for another hour (chase incubation). The retina was then homogenized in 1 ml of ice-cold 2 N acetic acid and boiled in a water bath for 3 min. An aliquot of the homogenate was used to measure the protein content as well as the total radioactivity in the homogenate. The remaining homogenate was centrifuged at 40,000 × *g* for 45 min. The supernatant was lyophilized and resuspended in 0.1 ml of sodium phosphate buffer (0.01 M, pH 7.4). The radioactive products synthesized by the tissue were separated and measured by HPLC.

The effects of different chase incubation periods on the biosynthesis of ^{3}H-Met⁵-enkephalin in the labeled tissue were also studied. In these experiments, the isolated retina was cut radially into four pieces and incubated in 40 μCi of [^{3}H]Met for 30 min. After the retina was washed three times in large volumes of Ringer's solution, one piece of tissue was removed for extraction and assayed for ^{3}H-Met⁵-enkephalin. The remaining three pieces of retina were each incubated in the same unlabeled solution for an additional 30, 60, and 120 min, respectively. Similar biosynthetic studies were also made using [^{3}H]glycine (44 Ci/mmol) as the labeled precursor.

HPLC. The HPLC system consisted of Waters models 6000A and M45 pumps that were controlled by a model 660 solvent programmer, a model 86K injector, a 0.4 × 30 cm μBondapak C18 column, and a Waters model 450 UV detector. Twenty-five microliters of each acid extract were applied to the system. The column was eluted with a convex gradient of 0.0125 M sodium phosphate buffer, pH 6.4 (primary solvent), and acetonitrile (secondary solvent) from 12 to 40% in 50 min with a flow rate of 1 ml/min. Fractions of 1 ml were collected. An aliquot of each fraction (0.1 ml) was used to determine the radioactivity by liquid scintillation counting. The remaining 0.9 ml from each fraction was lyophilized and resuspended in 0.09 ml of 0.9% NaCl solution for immunoassay.

Immunoassay. Twenty microliters of each resuspended fraction were mixed with 0.05 ml of an antiserum against enkephalins (batch A206 from

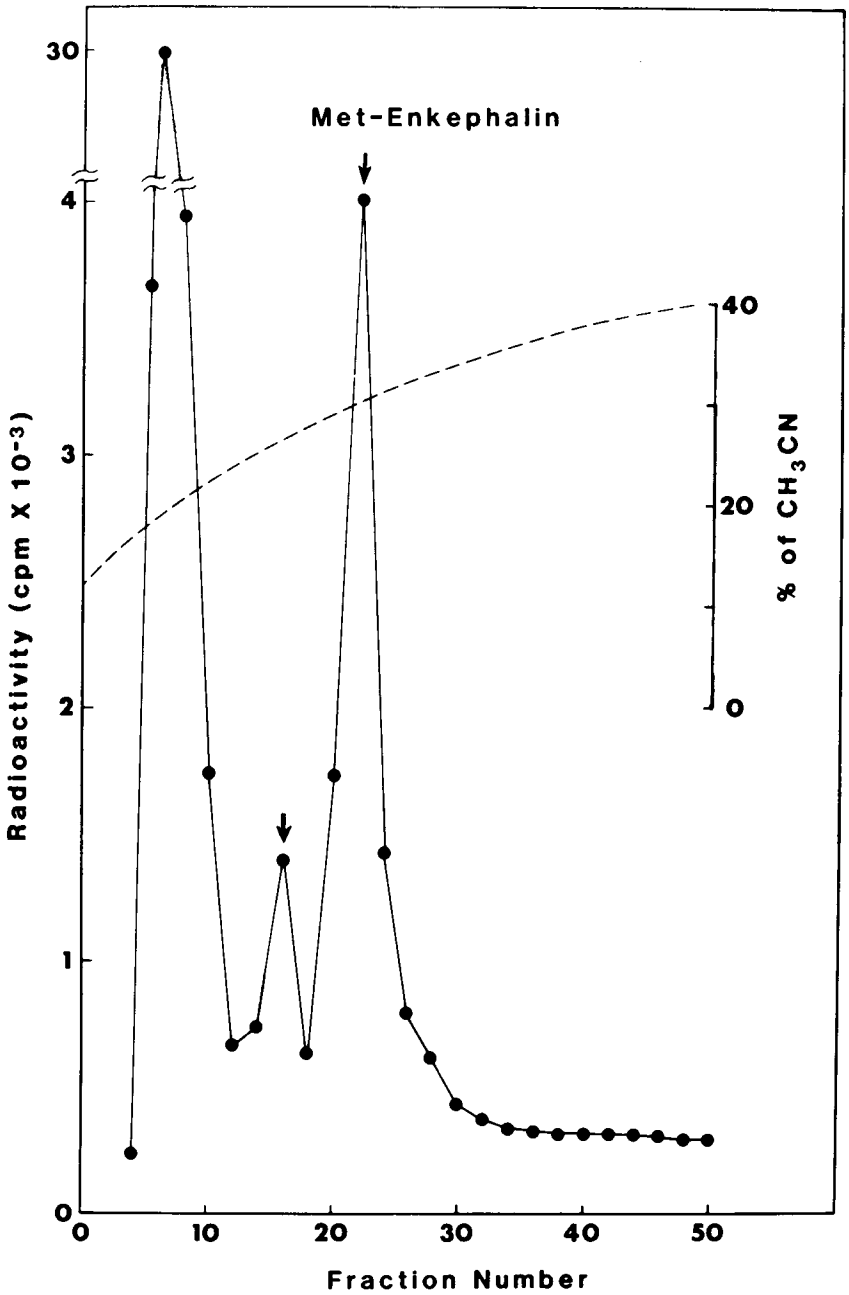

Figure 2. Elution profile of the tissue extract on a μBondapak C18 column. The column was eluted with a convex gradient of 12.5 mM phosphate, pH 6.4 (primary solvent), and acetonitrile (secondary solvent), with acetonitrile from 12 to 40% in 50 min. The flow rate was set at 1 ml/min and fractions with 1 ml were collected. Radioactivity of each fraction was measured in a liquid scintillation counter. The retention time corresponding to Met⁵-enkephalin was indicated. Immunoassays of the fractions demonstrated that two peaks, indicated by *arrows*, showed enkephalin-like immunoreactivity.

Dr. K. -J. Chang; 1:200 dilution in PBS) or a monoclonal antibody AD4, produced in our laboratory (Tavella et al., 1985) at dilution in PBS and incubated at 4°C overnight. Antigen-antibody complexes were precipitated with a standard polyethylene glycol technique. After centrifugation at 12,000 × *g* for 3 min, the precipitate was resuspended in 0.2 ml of water, mixed with 5 ml of Scintiverse, and counted in a liquid scintillation counter.

Release experiments. A piece of isolated retina was incubated in Ringer's solution containing [^{3}H]Met, using a procedure identical to that for the biosynthesis experiments. After 1 hr of chase incubation, the retina was washed extensively in Ringer's solution and placed for 3 min each at room temperature in 1.5 ml of either normal Ringer's solution or isotonic K^+-rich Ringer's solution (in which 60 mM Na^+ in the normal solution had been replaced by K^+). All of the Ringer's solutions used in the release experiments contained trasylol. The K^+-stimulated release of enkephalin in the presence of 5 mM Co^{2+} and in the absence of Ca^{2+} was also measured. Each eluate was collected and lyophilized. For desalting, each residue was extracted with 4 ml of 5% 6 N HCl in acetone, and the mixture was centrifuged. The supernatant was taken to dryness with N_2 and the dry sample was reconstituted in 0.15 ml of primary solvent (12.5 mM phosphate buffer, pH 6.4). The solution was applied to the HPLC to identify the compounds released into the eluate. For each sample, at least two measurements were made.

Results

Enkephalin content in the chicken retina. The concentration of enkephalin-immunoreactive substances in the chicken retina was determined by the ELISA test described under "Materials and Methods." As shown in Figure 1, each of the positions *a*, *b*, and *c* correspond to the ratio of optical density (*B*/*Bo*) in the presence of a different protein content (0.1, 0.2, and 0.4 mg, respectively) from a retinal homogenate. From the *curve* in Figure 1, the average concentration of enkephalin-immunoreactive substances in the chicken retina was estimated to be 126 pg/mg of protein or 0.25 pmol/mg of protein. Assuming that the protein content in the retina is 10% of its wet weight, the concentration of the enkephalin-immunoreactive substances is then on the order of 25 nM.

Biosynthesis of ^{3}H-Met⁵-enkephalin. Three major peaks of radioactivity were observed on HPLC after the retina was incubated in the unlabeled medium for 1 hr. A typical elution profile is shown in Figure 2. The peak that eluted at 28% acetonitrile, with a retention time of 22 min (fraction 22), co-migrated with authentic Met⁵-enkephalin. The peak (fraction 17) which eluted immediately before

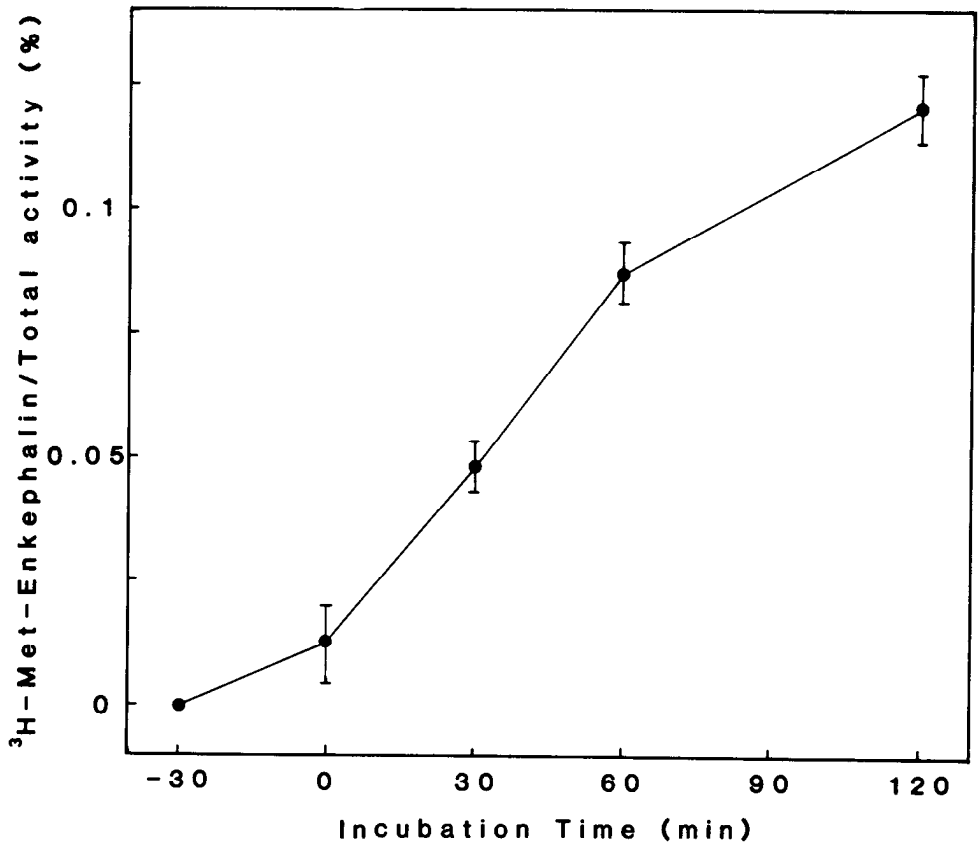

Figure 3. The effect of the chase incubation period on the ^{3}H-Met-enkephalin synthesized in the retinal tissue. The isolated chicken retina was cut radially into four pieces. The tissues were labeled with 40 μCi of ^{3}H-Met for 30 min (from −30 to 0 min). After rinsing three times in a large volume of Ringer's solution (containing the peptidase inhibitor), one piece of retina was removed for extraction (time *0*). The other three pieces were incubated in the same Ringer's solution containing 100 μg/ml of unlabeled Met for an additional 30, 60, and 120 min, respectively. The amount of ^{3}H-Met^5-enkephalin synthesized and accumulated in the retina at each time point was measured as described under "Materials and Methods." The ratio of ^{3}H-Met-enkephalin to total radioactivity in each extract was calculated and plotted against time of incubation.

Met^5-enkephalin co-migrated with the synthetic peptide Gly-Gly-Phe-Met and may correspond to a degradative product of Met^5-enkephalin. The identities of the products in the peak with the highest amount of radioactivity (fraction 8, eluted at 8 min) are not known. Under our experimental conditions, no other [^{3}H]Met-labeled enkephalin-related peptides were identified.

Immunoassays of the fractions from HPLC showed that the peak which co-migrated with Met^5-enkephalin (fraction 22) cross-reacted with both the polyclonal antibody against enkephalins (A206) and the monoclonal antibody AD4 (Tavella et al., 1985). Additionally, the peak at fraction 17 also possessed enkephalin immunoreactivity. Greater than 95% of the radioactivity presented in the sample solutions from these two peaks was precipitated by anti-enkephalin serum. In contrast, the peak with the highest radioactivity (fraction 8) did not cross-react with the enkephalin antibodies. These results indicate that Met^5-enkephalin, or a peptide very similar to Met^5-enkephalin, is synthesized and accumulated by the chicken retina.

The percentages of [^{3}H]Met that were incorporated into Met^5-enkephalin after a 30-min incubation in a medium containing [^{3}H]Met and followed by different times of postincubation (chase in an unlabeled medium) are shown in Figure 3. Without any chase period (time 0), very little ^{3}H-Met^5-enkephalin was detected in the retina. The amount of ^{3}H-Met^5-enkephalin in the retina increased linearly during the first hour of chase incubation before gradually approaching the maximal level.

Biosynthesis of (^{3}H-Gly)-enkephalin. Using [^{3}H]Gly as the precursor, we were able to separate the (^{3}H-Gly)-Met^5-enkephalin from (^{3}H-Gly)-Leu^5-enkephalin by our HPLC procedure. Our results showed that the concentration of newly synthesized (^{3}H-Gly)-Met^5-enkephalin was between 2 and 6 times that of (^{3}H-Gly)-Leu^5-enkephalin.

Release of ^{3}H-Met^5-enkephalin. In addition to possessing the mechanisms for the biosynthesis and accumulation of Met^5-enkephalin, we also examined whether the newly synthesized enkephalin could be released from the chicken retina upon depolarization. Using elevated K^+ in the medium to depolarize the retina, we showed that the newly synthesized ^{3}H-Met^5-enkephalin could indeed be released in response to this depolarizing stimulus (Fig. 4). The identities of the radioactive substances in each eluate were determined by HPLC and were shown to consist of approximately 95% Met^5-enkephalin. Additionally, the K^+-induced release of ^{3}H-Met^5-enkephalin was greatly reduced by the presence of 5 mM Co^{2+} in the medium, suggesting that the release mechanism is dependent on the presence of extracellular Ca^{2+}.

Discussion

Enkephalin content. The content and biosynthesis of enkephalins have been demonstrated in a variety of neural tissues (Yang et al., 1978; Wilson et al., 1980; Lindberg et al., 1982; Su, 1982; Su and Lam, 1982; Su et al., 1983). Using the ELISA test, we have compared the concentrations of enkephalin immunoreactivity in teleost, avian, and mammalian retinas. Our results show that, among these retinas, the enkephalin concentration in the chicken retina (about 25 nM) was almost an order of magnitude higher than those for rabbit (about 4 nM) and goldfish (about 2.5 nM) retinas (Y. Y. T. Su, unpublished data). This result in part led us to select the chicken retina for our analyses of retinal enkephalinergic systems. As a confirmation of the ELISA test, we compared the concentrations of enkephalin-immunoreactive substances in retinas and in rat brain as measured by ELISA and the more commonly employed technique of radioimmunoassay (Miller et al., 1978) and found that these two methods yield similar results. Additionally, the concentration of enkephalin immunoreactivity in the chicken retina is in the same range as those reported for the rat cortex and whole brain using radioimmunoassays (about 5 nM; Miller et al., 1978). However, it must be emphasized that in ELISA, as in radioimmunoassays, the values determined represent not only the content of enkephalins alone but also of all of the substances in the extract that are immunoreactive against the particular antibody used. Thus, the values that we obtained using

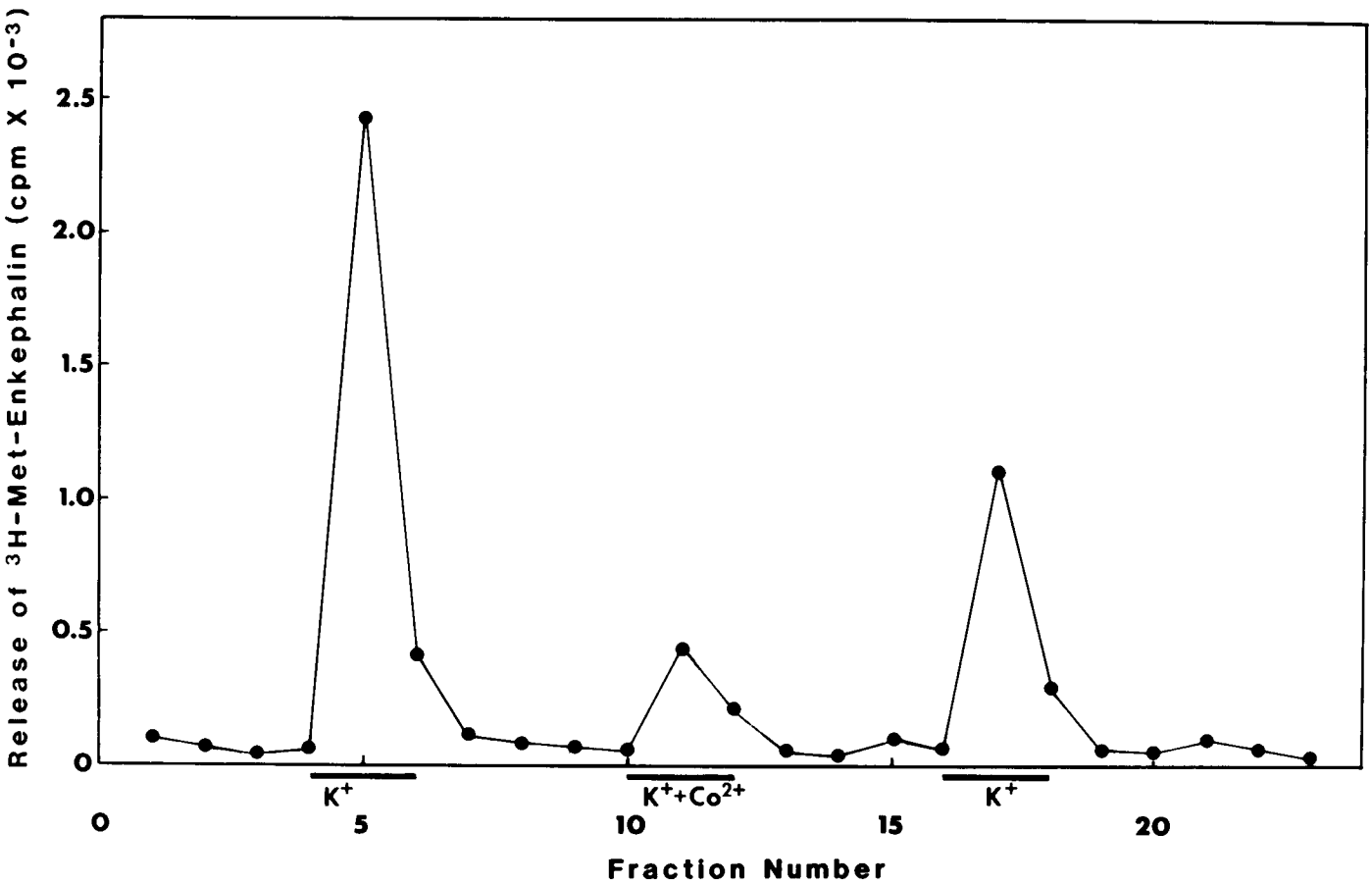

Figure 4. The release of ^{3}H-Met^5-enkephalin from the chicken retina. Each fraction represents a retina incubated for 3 min with 1.5 ml of either normal or K^+-rich Ringer's solution. The eluates were individually collected, lyophilized, desalted, and assayed for Met^5-enkephalin by HPLC. This figure shows that ^{3}H-Met^5-enkephalin is released in response to high extracellular K^+ concentration, and that this release is greatly reduced by the presence of 5 mM Co^{2+} in the medium.

these techniques should only be taken as an upper limit of the true enkephalin concentration.

Finally, although an enkephalin concentration of 25 nM in the chicken retina may appear to be very low, this perhaps reflects the small number of enkephalin-containing retinal neurons rather than the low enkephalin content in these cells. Indeed, if one estimates from our imumocytochemical study (Watt et al., 1985) that fewer than 1% of the retinal cells contain enkephalins, then the actual concentration of these peptides in enkephalinergic neurons of the chicken retina may be on the order of 2.5 μM, a value that is comparable to that found in adrenomedullary chromaffin cells which are known to contain enkephalins (about 5 μM, Wilson et al., 1980).

Enkephalin biosynthesis. Uptake and/or biosynthesis are two common mechanisms by which a neuron accumulates a neurotransmitter or peptide. In an earlier study, we found by autoradiography that the chicken retina does not possess a mechanism for taking up exogenous enkephalins (D. M. -K. Lam, unpublished data). In contrast, using HPLC and immunoassays with both polyclonal and monoclonal antibodies, the present study demonstrates that the chicken retina is capable of synthesizing and accumulating Met^5-enkephalin or a substance very similar to Met^5-enkephalin. Studies are currently in progress to elucidate the cDNA and amino acid sequence of this substance. Additionally, our preliminary study with chase incubations (Fig. 3) suggests that, similar to reports of enkephalin biosynthesis in other tissues (Yang et al., 1978; Wilson et al., 1980; Lindberg et al., 1982), Met^5-enkephalin in the chicken retina is probably also synthesized as part of a larger precursor. Once again, the identity of this precursor and the mechanism of enkephalin biosynthesis in the retina must await further biochemical and genetic studies. In addition, our study using [^{3}H]Gly as the precursor suggests that, for each Leu^5-enkephalin molecule synthesized, between two and six molecules of Met^5-enkephalin are made.

Enkephalin release. A crucial criterion in the identification of any substance as a neurotransmitter or modulator is the demonstration that the substance is released from the presynaptic neuron in response to a depolarizing stimulus. This synaptically mediated release is also generally dependent upon the extracellular Ca^{2+} concentration and is inhibited by Co^{2+} in the medium. With respect to the retinal enkephalinergic system, we have shown here that Met^5-enkephalin is synthesized by the chicken retina. Additionally, the newly synthesized Met^5-enkephalin is released upon depolarization of the retina by high K^+ in the medium and the release is greatly reduced by external Co^{2+}. Since our immunocytochemical studies using both monoclonal and polyclonal antibodies against enkephalins have revealed that the enkephalin immunoreactivity is localized only to a subpopulation of amacrine cells (Tavella et al., 1985; Watt et al., 1985), it is most likely that these neurons are responsible for the biosynthesis and accumulation of enkephalins in this retina. It therefore follows that depolarization of these amacrine cells probably also leads to the release of Met^5-enkephalin in a Ca^{2+}-dependent manner. Although the effects of enkephalin release on the processing of visual information are still unknown, we have recently demonstrated that enkephalin receptors exist in the chicken retina and that exogenously applied enkephalins inhibit transmitter release from GABAergic and dopaminergic amacrine cells (Su et al., 1984). Furthermore, as shown in the following paper (Watt et al., 1985), synaptic contacts between enkephalin-immunoreactive and other amacrine cells have been observed by electron microscopic immunocytochemistry. Taken together, our studies show that the chicken

retina possesses the necessary pre- and postsynaptic mechanisms for an enkephalinergic system.

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