Basch, R. S., & Goldstein, G. (1975) *Ann. N.Y. Acad. Sci. 249*, 290–299.
Buchan, A. M. J., Polak, J. M., Solcia, E., Capella, C., Hudson, D., & Pearse, A. G. E. (1978) *Gut 19*, 403–407.
Edman, P. (1970) in *Protein Sequence Determination. A Source Book of Methods and Techniques* (Needleman, S. B., Ed.) pp 211–255, Springer-Verlag, New York.
Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.
Gershwin, M. E., Kruse, W., & Goldstein, G. (1979) *J. Rheumatol. 6*, 610–620.
Goldstein, G. (1966) *Lancet 2*, 1164–1167.
Goldstein, G. (1968) *Lancet 2*, 119–122.
Goldstein, G. (1974) *Nature (London) 247*, 11–14.
Goldstein, G. (1976) *J. Immunol. 117*, 690–692.
Goldstein, G., & Whittingham, S. (1966) *Lancet 2*, 315–318.
Goldstein, G., & Hofmann, W. W. (1968) *J. Neurol. Neurosurg. Psychiatry 31*, 455–459.
Goldstein, G., & Hofmann, W. W. (1969) *Clin. Exp. Immunol. 4*, 181–189.
Goldstein, G., & Lau, C. Y. (1979) *Miles Int. Symp. Ser. No. 12*, 459–465.
Goldstein, G., Scheid, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H., & Niall, H. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 11–15.
Goldstein, G., Scheid, M. P., Boyse, E. A., Schlesinger, D. H., & Van Wauwe, J. (1979) *Science (Washington, D.C.) 204*, 1309–1310.
Horwitz, D., Glynn, M. J., Barada, F. A., Andrews, B. S., O'Brien, W., Kay, H. D., Schindler, J., & Goldstein, G. (1980) in *Immunoregulation and Autoimmunity. Proceedings of Immunodynamics III* (Krakauer, R. S., Ed.) Elsevier/North-Holland, New York (in press).
Kalden, J. R., Williamson, W. G., Johnston, R. J., & Irving, W. J. (1969) *Clin. Exp. Immunol. 5*, 319–340.
Komuro, K., Goldstein, G., & Boyse, E. A. (1975) *J. Immunol. 115*, 195–198.
Lau, C. Y., & Goldstein, G. (1980) *J. Immunol. 124*, 1861–1865.
Lau, C. Y., Freestone, J., & Goldstein, G. (1980) *J. Immunol. 125*, 1634–1638.
Pisano, J. J., & Bronzert, T. J. (1969) *J. Biol. Chem. 244*, 5597–5607.
Polak, J. M., & Bloom, S. R. (1980) *Miles Int. Symp. Ser. No. 12*, 371–394.
Radola, B. J. (1969) *Biochim. Biophys. Acta 194*, 335–345.
Scheid, M. P., Goldstein, G., & Boyse, E. A. (1975) *Science (Washington, D.C.) 190*, 1211–1213.
Scheid, M. P., Goldstein, G., & Boyse, E. A. (1978) *J. Exp. Med. 147*, 1727–1743.
Schlesinger, D. H., & Goldstein, G. (1975) *Cell (Cambridge, Mass.) 5*, 361–365.
Schlesinger, D. H., Goldstein, G., Scheid, M. P., & Boyse, E. A. (1975) *Cell (Cambridge, Mass.) 5*, 367–370.
Simmons, J., & Schlesinger, D. H. (1980) *Anal. Biochem. 104*, 254–258.
Smith, L. (1966) *Am. J. Med. 40*, 662.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Sunshine, G. H., Basch, R. S., Coffey, R. G., Cohen, K. W., Goldstein, G., & Hadden, J. W. (1978) *J. Immunol. 120*, 1594–1599.
Verhaegen, H., DeCock, W., DeCree, J., & Goldstein, G. (1980) *Thymus 1*, 195–204.
Weksler, M. E., Innes, J. B., & Goldstein, G. (1978) *J. Exp. Med. 148*, 996–1006.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem. 77*, 569–573.

Proteolytic Processing in the Biogenesis of the Neurosecretory Egg-Laying Hormone in *Aplysia*. 1. Precursors, Intermediates, and Products†

Robert W. Berry

ABSTRACT: The neurosecretory bag cells of the mollusk, *Aplysia*, produce a peptide egg-laying hormone, ELH, via a multistep proteolytic processing sequence analagous to those which have been demonstrated for secretory peptides in other systems. The goals of the present study were to identify the major members of this processing sequence by sequential sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing of bag cell proteins synthesized in the presence of labeled precursors and to elucidate the precursor–product relationships between these proteins in pulse–chase experiments. Eight major members of the processing sequence were identified. The ultimate precursor is a 29 000-dalton, pI = 7.7, protein which gives rise to a pI = 7.2 protein with an apparent M_r of 6000 as well as heterogeneous species of M_r 16 000–20 000. The latter protein or proteins is/are processed to apparent end products of 13 000–14 500 daltons, while the pI = 7.2 species yields precursors to the final secretory products. These include a pI = 7.5 peptide which is cleaved to ELH (M_r 4385, pI > 9) and a M_r 4500, pI = 4.1 species which yields the other secretory product, AP (M_r 4500, pI = 4.9). Therefore, it appears that a single precursor is processed to yield three products, two of which are known to be secreted, and that each product is generated via at least one intermediate form.

The egg-laying hormone, ELH,[1] of the marine mollusk, *Aplysia californica*, is a 4385-dalton peptide which induces oviposition when injected into recipient animals. ELH is produced and secreted by the bag cells, bilateral clusters of apparently homogeneous neurosecretory cells located at the junction of the pleuroabdominal connective nerves and the

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[1] Abbreviations used: AP, acidic peptide; ELH, egg-laying hormone; M_r, relative molecular mass; MSH, melanocyte stimulating hormone; $NaDodSO_4$, sodium dodecyl sulfate.

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abdominal ganglion (Coggeshall, 1967). Brief synaptic input sends the clusters into a bout of repetitive activity (Kupfermann & Kandel, 1970), resulting in ELH secretion (Stuart et al., 1980), and such a burst of bag cell activity always precedes egg laying in vivo (Pinsker & Dudek, 1977). Since the isolated bag cell organs will continue to synthesize and secrete ELH for extended periods in vitro (Arch, 1972a,b) and since ELH has been isolated and sequenced (Chiu et al., 1979), this preparation is a particularly favorable one with which to study the routes and mechanisms of biogenesis of a neurosecretory peptide of known structure and defined function.

Previous studies have shown that the final secreted bag cell products include both ELH, which is extremely basic (p*I* = 9.3–9.7), and an acidic peptide, AP (Arch, 1981), of similar size, but with a p*I* of about 4.9 (Arch et al., 1976a; Stuart et al., 1980; Yates & Berry, 1981). As with other secretory peptides, these products appear to be generated from a larger protein precursor via a multistep proteolytic processing sequence. Kinetic studies of the passage of label through low molecular weight bag cell proteins suggest that an initial 25 000–29 000-dalton precursor gives rise to 12 000 and ~6000-dalton products, of which the latter group contains the acidic and basic secretory peptide products (Arch, 1972b; Arch et al., 1976b; Loh et al., 1975, 1977).

While the acidic and basic products have been well characterized, much less is known about the precursor(s) and any intermediates in the processing sequence. Owing to their limited solubility (Arch et al., 1976b), these have been identified previously solely by their electrophoretic migration rates. Since the peaks observed on $NaDodSO_4$–polyacrylamide electrophoresis have not been shown to be monodisperse, it is not known how many discrete members of the sequence actually exist, nor can their processing relationships be specified in detail. In view of the fact that the bag cells produce at least two final products (ELH and AP), it is particularly important to know if the initial precursor is a single molecular species. An adequate characterization of the members of the ELH processing sequence and a detailed examination of their processing relations are prerequisites for further studies on the cellular and molecular mechanisms of ELH biogenesis. Accordingly, the series of experiments in this and the following report were undertaken. First, as reported here, members of the sequence were identified by a two-step separation using $NaDodSO_4$–polyacrylamide electrophoresis, followed by isoelectric focusing, and the kinetic relationships between these species were investigated in pulse–chase studies. Next, as reported in the following paper (Berry et al., 1981), the processing relations inferred from the kinetic data were confirmed by peptide mapping techniques.

Materials and Methods

Animals and Incubation Procedures. *Aplysia californica*, weighing 200–400 g, were obtained from Pacific Bio-Marine Laboratories (Venice, CA) and kept at 15 °C in Instant Ocean (Aquarium Systems, Wickliffe, OH). In all of the studies reported here, members of the ELH processing sequence were identified on the basis of biosynthetically incorporated radioactivity. Abdominal ganglia were removed from the animal, and the paired bag cell organs, together with 1–2 cm of pleuroabdominal connective nerve, were isolated from the ganglion. These were combined and preincubated at 15 °C for 2–4 h in artificial seawater (Berry, 1975) and then transferred to 1 mL of artificial seawater containing 1% glucose and 100 μCi of radioactive amino acid. The labeled precursors included L-[4,5-^{3}H]leucine (~50 Ci/mmol, New England Nuclear), L-[^{35}S]methionine (~600 Ci/mmol, New England Nuclear), or a mixture of 15 tritiated amino acids (TRK.440, Amersham). Labeling with the various isotopic precursors produced no major differences in any of the parameters discussed in this paper. For pulse–chase studies, exposure to labeled precursor was terminated after 2 h, and the organs were transferred to 5 mL of supplemented artificial seawater (Berry, 1976) containing 1 mM unlabeled amino acids and 0.2 mg/mL vinblastine (Sigma) to block axonal transport. All incubations were terminated by dissection of the bag cell somata from the organs and homogenization in a buffer containing 1% $NaDodSO_4$ (Berry, 1976).

Analytical Procedures. Members of the ELH processing sequence were identified by electrophoretic mobility on 5 5 × 75 mm cylindrical polyacrylamide gels (8% T, 5% C) containing 0.1% $NaDodSO_4$ (Berry, 1976). Molecular weight estimates were made by reference to the mobility of standard proteins [bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), myoglobin (M_r 18 500), cytochrome *c* (M_r 12 500), corticotropin (M_r 4500), and bacitracin (M_r 1500)] on parallel gels and to internal standards of dansylated myoglobin and corticotropin. The gels were sliced into 1-mm segments and counted in the fluid of Ward et al. (1970).

In some cases, members of the sequence from $NaDodSO_4$–polyacrylamide gels were subjected to further analysis by isoelectric focusing. The procedure of Yates & Berry (1981) was used. Briefly, this involves slicing the $NaDodSO_4$ gel longitudinally into two unequal segments, locating the peaks of interest by scintillation counting of 1-mm slices of the smaller segment, removal of $NaDodSO_4$ by overnight immersion in methanol/acetic acid/water (50:10:60), and elution of the protein from the gel with 1% Triton X-100/6 M urea. The eluates, containing an average of 84% of the radioactivity in the larger segments, were then subjected to isoelectric focusing in polyacrylamide gels in the presence of Triton and urea as described previously (Yates & Berry, 1981).

Terminology. In this and the following report (Berry et al., 1981), members of the ELH processing sequence are designated by a Roman numeral–capital letter combination, the numerals being assigned in order of decreasing apparent molecular weight to prominent peaks on the electrophoretic map following long-duration labeling, with letters to identify species that differ in isoelectric point or labeling kinetics. The prior designations of AP and ELH have been retained for the well-defined product peptides (Arch, 1972a,b, 1981). Though cumbersome, this designation system has the virtues of being based on operational parameters and avoiding a reliance on molecular mass, estimates of which may be in error (Yates & Berry, 1981). The more common "pre" and "pro" notation [see Steiner et al. (1980)] seems inappropriate in this instance, as it would involve a priori assumptions about precursor–product relations and does not make provisions for multiple intermediate forms.

Results

Molecular Weights of Members of the ELH Sequence. Electrophoresis of extracts of bag cells exposed to labeled precursor for long periods (6–18 h) reveals four major peaks of labeled protein (Figure 1). These correspond to apparent molecular weights of 27 000–32 000, 13 000–20 000, 4500–7000, and ~2000, referred to here as peaks I–IV in order of decreasing molecular weight (see above). The only other prominent labeled protein, M_r ~70 000, turns over much more slowly than peaks I–IV and is apparently unrelated to the ELH processing sequence. Peaks I and II clearly correpond to the 25 000–29 000- and 11 300–12 000-dalton material described by Arch (1972b) and by Loh et al. (1975). Both

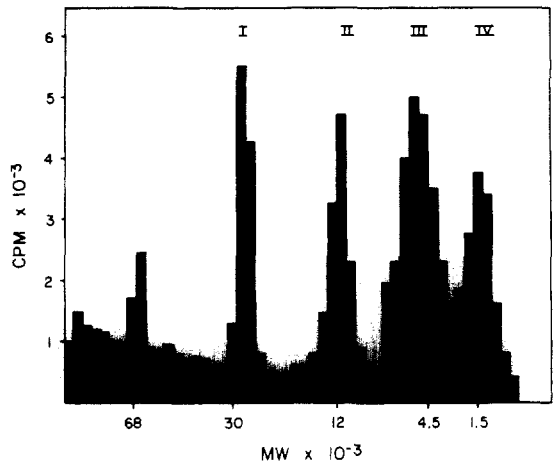

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis separation of proteins synthesized by a single bag cell cluster during 6 h of exposure to tritiated leucine. Identical patterns were obtained with [^{35}S]methionine or a mixture of tritiated amino acids as precursors and with exposure to label lasting as long as 18 h.

groups have also described 6000-dalton material, and Loh et al. (1975) identified a ≤3000-dalton protein which corresponds to peak IV and which has been shown to be ELH, migrating anomalously on $NaDodSO_4$–polyacrylamide gel electrophoresis (Yates & Berry, 1981).

Pulse–chase experiments reveal a clear precursor–product relationship between these peaks (Figure 2). After a 2-h pulse of labeled precursor, only peaks I–III are evident (Figure 2a). Furthermore, while peak I migrates identically after 2 h or 6–18 h of labeling, there is evidence of heterogeneity in peaks II and III; both peaks migrate more slowly after 2 h of labeling than at longer labeling times. The early peak II (IIA) has an apparent M_r of 16 000–20 000, while peak IIIA appears to have a M_r of 6000–7000.

This result was confirmed by interposing chase periods of varying duration between exposure to labeled precursor and extraction for electrophoresis. After a 30-min chase period (Figure 2b), peak II appears as an incompletely resolved doublet, consisting of IIA and a M_r 13 000–14 500 peak (IIB). After 4 h of chase, only peak IIB is evident (Figure 2c). The most parsimonious interpretation of this result is that peak IIA serves as a precursor for peak IIB.

Peak III undergoes a similar, though slower, reduction in apparent M_r, from 6000–7000 (IIIA) at 30 min (Figure 2b) to 4500 (IIIB) at 4 h (Figure 2c). Again, this is suggestive of a precursor–product relationship between IIIA and IIIB. Peak IV arises concomitantly with IIIB (Figure 2c), which suggests that IIIA may serve as the precursor to both these products. As shown in Figure 2d, peaks IIIB and IV are maintained for at least 16 h without additional reduction in molecular weight.

Kinetic Relations between Members of the ELH Sequence. The kinetics of movement of label through the members of the sequence is presented in more detail in Figure 3. Although peaks I, II, and IIIA are all present after 2 h of labeling, shorter labeling periods were not investigated, because Arch (1972b) has presented convincing evidence that peaks II and IIIA arise from peak I and do so in a manner that is inconsistent with IIIA's being derived from II. Peak I decays rapidly, as would be expected if it were the precursor to all other members of the sequence. No radioactivity is lost from peak II during the first 4 h, and it is lost only slowly thereafter. This is consistent with an early conversion of IIA to IIB, followed by slow decay of the latter. This processing step cannot be directly quantitated because of the incomplete

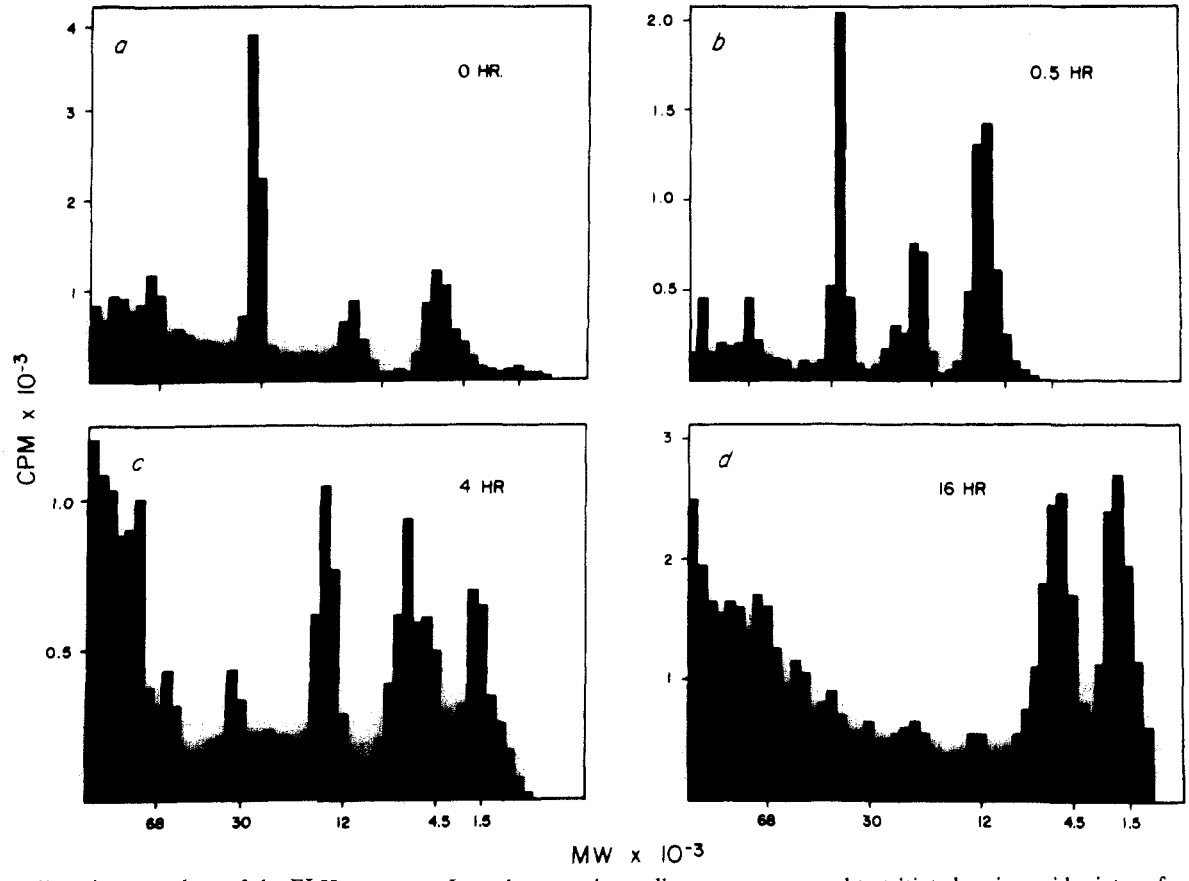

FIGURE 2: Transient members of the ELH sequence. In each case, a bag cell organ was exposed to tritiated amino acid mixture for 2 h and then chased in nonradioactive medium for the times indicated.

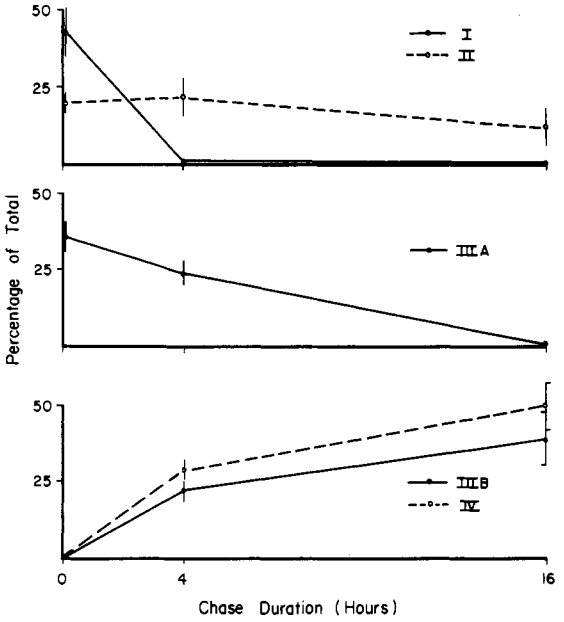

FIGURE 3: Kinetics of passage of label through members of the ELH sequence. Data from pulse–chase experiments such as those illustrated in Figure 2 are presented as the percentage of the total radioactivity in all ELH sequence members that is found in a given member at various times after a 2-h labeling period. Bars represent standard error of the mean; n = 21 at 0 h, 15 at 4 h, and 6 at 16 h.

separation of IIA from IIB at times when both are present.

In contrast, IIIA decays at a constant rate, indicating kinetic homogeneity. This rate is slower than that of peak I and is equivalent to the rate of rise of peaks IIIB and IV during the period from 4 to 16 h, when all of the peak I label has been expended. This suggests that IIIA serves as an intermediate in the production of IIIB and IV. The fact that the amount of label lost from IIIA during this period (4–16 h) is sufficient to account for the amount of label appearing in IIIB and IV over this period strengthens this notion. It should be noted that the quantity of label lost from peak II would not be sufficient to account for the accumulation in IIIB and IV.

These data were obtained with a mixture of 15 tritiated amino acids as precursors, so the radioactivity in each protein species should provide a more accurate estimate of the mass of that protein that is present than would the use of a single amino acid precursor. Thus, the close correspondence in both the quantity of IIIB and IV and the rate at which they are produced provide strong evidence that they arise in equal amounts via a single cleavage step from the same precursor.

Isoelectric Points of the Members of the ELH Sequence. So that the members of the processing sequence could be further characterized and the degree of homogeneity of each of the peaks identified above assessed, the members were eluted from the $NaDodSO_4$ gels and subjected to isoelectric focusing. As shown in Figure 4a, peak I is essentially homogeneous, having a pI of 7.7 ± 0.1 (n = 5). There was no indication of a secondary peak that might correspond to the protein with its "pre" sequence intact. If such a signal sequence (Blobel, 1980; Steiner et al., 1980) exists for this protein, cleavage must be too rapid for the "pre" form to accumulate to any appreciable extent. Neither was there any perceptible difference between material taken from cells labeled for 2 or 8 h.

In contrast, peak II proved to be distinctly heterogeneous, comprising two major peaks at pI = 6.9 and 7.6 (both ±0.1, n = 6) as well as several minor peaks, both more acidic and more basic (Figure 4b). It might be supposed that the major peaks correspond to peaks IIA and IIB seen on $NaDodSO_4$ gels, but the only major difference between the isolelectric

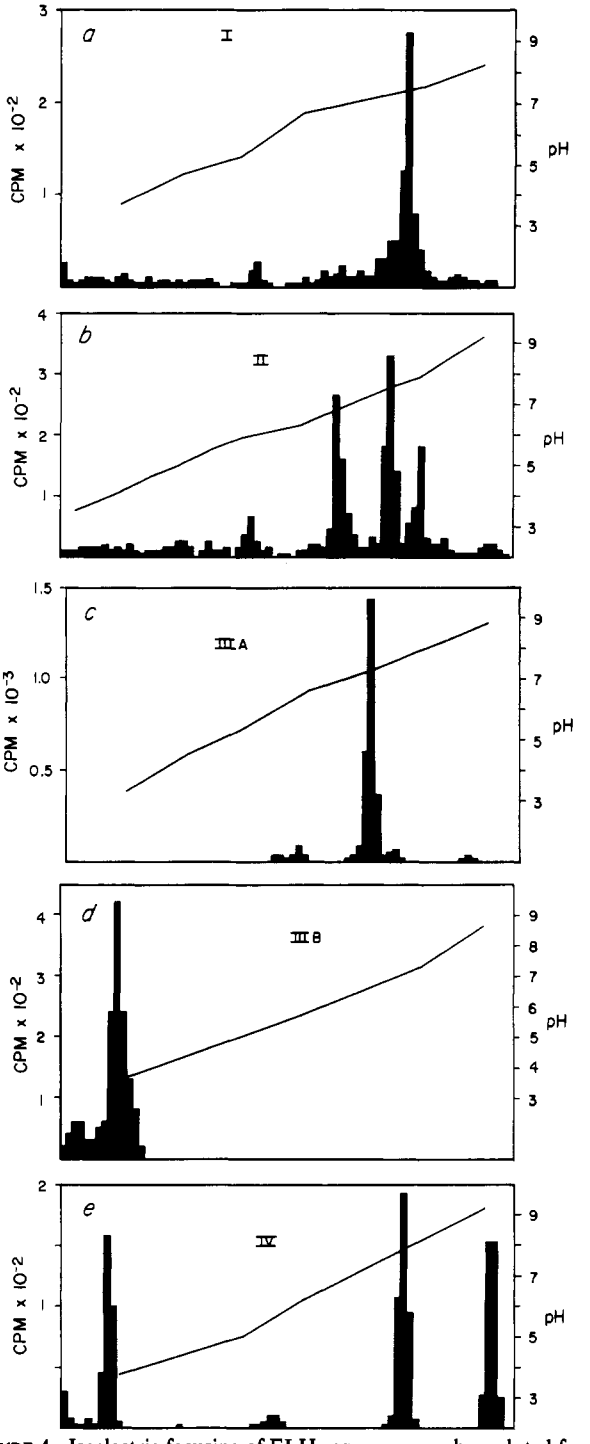

FIGURE 4: Isoelectric focusing of ELH sequence members eluted from $NaDodSO_4$ gels. (a) Peak I, 8-h labeling; (b) peak II, 2-h labeling; (c) peak IIIA, 2-h labeling; (d) peak IIIB, 2-h labeling, 4-h chase; (e) peak IV, 8-h labeling. All examples are from leucine-labeled material. Identical results were obtained with [^{35}S]methionine.

focusing profiles of these two peaks is that the minor species at pI = 8.0 is absent in IIB. Again, no consistent differences were observed between material labeled for 2 h or for 8 h. The possible sources of this heterogeneity will be discussed below.

As expected, peak III was bidisperse on isoelectric focusing, with material labeled for 2 h (i.e., peak IIIA) comprising a single species of pI = 7.2 ± 0.1 (n = 6) (Figure 4c), whereas after an additional 4-h chase period, the pI had shifted to <4.0 (Figure 4d). Since Yates & Berry (1981) observed a significantly higher isoelectric point (4.9) for material of this molecular weight after 16 h of chase, protein IIIB must undergo an additional processing step.

Table I: Members of the ELH Processing Sequence

peak	$M_r \times 10^{-3}$	p*I*	time of appearance (h)	role
I	27-32	7.7	<2	ultimate precursor
IIA	16-20	heterogeneous	<2	precursor to IIB
IIB	13-14.5	heterogeneous	2-6	product?
IIIA	6-7	7.2	<2	precursor to IIIB, IV
IIIB	4.5	4.1	2-6	precursor to AP
		4.9	>6	AP product
IV	2	7.5	2-6	precursor to ELH
		>9	>6	ELH product

Yates & Berry (1981) observed only a single species in late peak IV material: ELH, with a p*I* of 9.6. In contrast, although peak IV material labeled for 8 h or for 2 h with a 4-h chase contains ELH (Figure 4e), it also contains a major peak at p*I* 7.5 ± 0.2 (n = 6) and variable amounts of p*I* < 4.0 material, which is probably due to cross-contamination from IIIB. The more neutral species cannot also be due to cross-contamination from the similar material in IIIA, because the two do not overlap significantly, either in migration rate on electrophoresis or in time. At this low molecular weight, it is conceivable that the p*I* = 7.5 material might be a fragment of the cleavage sequence, but this is not likely. As was noted above, peaks IIIB and IV tend to contain about the same amount of radioactivity throughout the chase period. Peak IIIB is homogeneous, as is late peak IV, whereas early peak IV contains both ELH and the more neutral peptide. Thus, conservation of radioactivity implies that the neutral peptide represents a precursor to ELH.

Discussion

The salient properties of the members of the ELH processing sequence as revealed in this study are summarized in Table I. Eight members of the sequence can be distinguished on the basis of molecular weight, isoelectric point, and turnover kinetics under the conditions used here. It is entirely possible that other, more transient, members of the sequence exist which could be revealed by using shorter pulse or chase intervals. Of the eight species identified here, five have been observed previously [compare Table I with Table I in Arch (1981)]. Protein I corresponds in molecular mass and kinetics to the 29 000-dalton precursor of Arch (1972b) and the 25 000-dalton precursor of Loh et al. (1975, 1977). Both groups also observed peak II but could not resolve IIA from IIB. Both groups also observed a 6000-dalton product, and Loh et al., a ≤3000-dalton product. Yates & Berry (1981) showed that this ≤3000-dalton species is ELH. It should be noted that although the molecular mass of ELH is known from its amino acid sequence to be 4385 (Chiu et al., 1979), it migrates anomalously on $NaDodSO_4$ gels of the type used here and by Loh et al. This is apparently not the case with the gel system used by Arch and co-workers, and their 6000-dalton material contains ELH, as well as a p*I* = 4.8 peptide (AP) and a transient p*I* = 4.6 species, which may correspond to IIIB (Arch et al., 1976b). The 6000-dalton peak of Loh et al. (1975, 1977) has been shown to contain AP (Yates & Berry, 1981).

Taken as a whole, the data are most consistent with the processing sequence shown in Figure 5, whereby a single initial cleavage (step 1) of protein I yields IIA and IIIA. Subsequently, cleavage step 2 yields IIB from IIA and step 3 yields both IIIB and IV from IIIA. Finally, cleavage step 4 converts IIIB to the final acidic product, AP, and cleavage step 5 converts IV to ELH. As discussed below, this scheme is

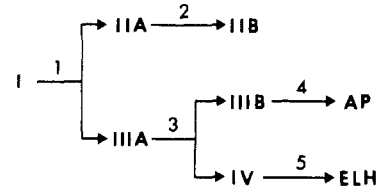

FIGURE 5: Putative ELH processing sequence.

consistent with the available kinetic evidence, and further support comes from peptide maps of the sequence members presented in the following paper (Berry et al., 1981).

The evidence for step 1 comes primarily from the data of Arch (1972b), which shows concomitant production of II and III early in the chase period. Since I is a single species, this would imply that a single cleavage creates both products. The present data also indicate that all other species arise after IIA and IIIA are present, and in particular, that IIIB and IV do not appear in concert with the disappearance of protein I, so there can be no parallel pathway directly to these products from protein I. The evidence for step 2 is based on the facts that IIA precedes IIB in the chase sequence and that the total radioactivity in the sum of these species does not decay until after 4 h of chase. One alternative would be that IIA arises from protein I as before, but turns over rapidly, giving rise to protein IIIA. This, however, would require that IIB arise from protein I independently, via a delayed cleavage, and is therefore a less attractive alternative.

The primary evidence for step 3 is that IIIB and IV must arise from an intermediate, since they are labeled too slowly to be derived from protein I. From the relative rates of decay of II and IIIA, the latter should be the intermediate in question, and only protein IIIA contains enough radioactivity to account for the amount of label subsequently found in the products after depletion of protein I. The concomitant production of peptides IIIB and IV and the fact that protein IIIA is a single species argue that these products arise from a single cleavage of the intermediate. If protein IIIA is in fact the intermediate, however, it would have to be at least as large as the combined molecular mass of its products, or about 9000 daltons. This would mean that its relative molecular mass is underestimated on these $NaDodSO_4$ gels by about the same amount as is that of ELH (3000 daltons; Yates & Berry, 1981). The final products, produced in steps 4 and 5, must arise from IIIB and IV, because they appear so late in the chase period that these are their only possible precursors.

This scheme is consistent with the data of Loh et al. (1975, 1977), who showed a transition between 6000-dalton and ≤3000-dalton (ELH) material late in the chase period. However, this transition did not abolish 6000-dalton material, and although part of this material is AP (Yates & Berry, 1981), the remainder, which turns over rapidly, could well be IIIA. The data of Arch et al. (1976b) are also compatible with this scheme. These workers observed a p*I* = 4.6 to p*I* = 4.8 transition in concert with a reduction in molecular mass from ~6000 daltons to ~5000 daltons and suggested that both reflected the same event, a step analagous to step 4 in the present shceme. However, it may be that the two events are not linked: that the p*I* shift corresponds to step 4, while the molecular mass reduction reflects the IIIA to IIIB and IV transition.

The nature of peak II material is subject to much more uncertainty than that of the other members of the sequence. The data presented here support the observations of Arch (1972b) which suggest that peak II is an end product of peak I rather than an intermediate in the generation of lower mo-

lecular weight peptides or a protein that is unrelated to the ELH sequence, but its function remains a mystery. Its heterogeneity stands in contrast to the monodispersity of the other members of the sequence and deserves comment. At least four sources can be suggested for this heterogeneity: First, some of the peaks observed on isoelectric focusing gels could represent ~12 000-dalton proteins which are synthesized in bag cell organs but which are not members of the ELH sequence. Alternatively, the charge isomers might be artifacts generated during the analytical procedures, which involve extended exposure to urea. However, if this is the case, one might expect similar heterogeneity in peak I material. A third possibility is that a monodisperse peak II protein is subject to variable modification reactions, such as phosphorylation, acetylation, or glycosylation. Finally, the bag cells may generate a number of peak II proteins from the protein I precursor. This could reflect heterogeneity in the cleavage enzyme specificity of individual members of the bag cell cluster, heterogeneous cleavage enzymes within a single bag cell, or multiple cleavage sites on the peak II protein. The possibility that $NaDodSO_4$ polyacrylamide gel electrophoresis yields a seriously distorted estimate of the molecular weight of peak I or peak II and that a single bag cell is capable of generating, for example, two IIA species and a IIIA protein from the protein I precursor must also be considered.

In contrast to peak II, protein I, the precursor to both the acidic and basic product peptides, is a single molecular species. This finding is significant in that it implies that if the two products are generated in different members of the bag cell cluster, those members must differ in their cleavage enzymes (for steps 4 and 5) rather than in their precursor. This result is in agreement with an emerging body of observations on processing of the protein proopiomelanocortin, which is found in the vertebrate pituitary and which contains the nested sequences of a number of peptide hormones. Among these are corticotropin and αMSH, the former being a product of the corticotrophs of the anterior lobe, the latter being produced by cells of the intermediate lobe (Roberts et al., 1978; Mains & Eipper, 1980). The available evidence suggests that the proopiomelanocortin precursor of each lobe is similar, perhaps identical, and that, therefore, specificity of the peptide product might be based on the specificity of the cleavage enzymes in the different cells. It is not yet known if the acidic and basic bag cell products are generated in the same or different cells.

The experiments reported here were designed to identify all of the major members of the proteolytic processing sequence leading to ELH. They have yielded data that unambiguously imply a straightforward processing sequence which is tested in the following paper. Such studies should provide a solid basis for further examinations of the cellular and molecular mechanisms and physiological functions of multistep proteolytic processing in the generation of ELH and, by analogy, of secretory peptides in general.

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References

Arch, S. (1972a) *J. Gen. Physiol. 59*, 47–59.
Arch, S. (1972b) *J. Gen. Physiol. 60*, 102–119.
Arch, S. (1981) in *Eighth International Symposium on Neurosecretion* (Farner, D., Ed.) Plenum, New York.
Arch, S., Earley, P., & Smock, T. (1976a) *J. Gen. Physiol. 68*, 197–210.
Arch S., Smock, T., & Earley, P. (1976b) *J. Gen. Physiol. 68*, 211–225.
Berry, R. W. (1975) *Brain Res. 86*, 323–334.
Berry, R. W. (1976) *J. Neurochem. 26*, 229–231.
Berry, R. W., Trump, M. J., & Baylen, J. T. (1981) *Biochemistry* (following paper in this issue).
Blobel, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1496–1500.
Chiu, A. Y., Hunkapiller, M. W., Heller, E., Stuart, D. K., Hood, L. E., & Strumwasser, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6656–6660.
Coggeshall, R. E. (1967) *J. Neurophysiol. 30*, 1263–1278.
Kupfermann, I., & Kandel, E. R. (1970) *J. Neurophysiol. 33*, 865–876.
Loh, Y. P., Sarne, Y., & Gainer, H. (1975) *J. Comp. Physiol. 100*, 283–295.
Loh, Y. P., Sarne, Y., Daniels, M. P., & Gainer, H. (1977) *J. Neurochem. 29*, 135–139.
Mains, R. E., & Eipper, B. A. (1980) *Ann. N.Y. Acad. Sci. 343*, 94–110.
Pinsker, H. M., & Dudek, F. E. (1977) *Science (Washington, D.C.) 197*, 490–493.
Roberts, J. L., Phillips, M., Rosa, P. A., & Herbert, E. (1978) *Biochemistry 17*, 3609–3168.
Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci. 343*, 1–16.
Stuart, D. K., Chiu, A. Y., & Strumwasser, F. (1980) *J. Neurophysiol. 43*, 488–498.
Ward, S., Wilson, D. L., & Gilliam, J. J. (1970) *Anal. Biochem. 38*, 90–97.
Yates, M. E., & Berry, R. W. (1981) *Gen. Comp. Endocrinol. 43*, 308–314.