zation by the finding that *Ds* in m1, m3, and m9 had inserted closely distal (*12*) to the site that gives (Sc) its distinctive tissue-specific effect? One possibility is that the controlling element receptor may act by insertion within the affected gene (*13*). If so, *Ds* acts by inserting within an essential *R* component or between components whose function depends on a contiguous cis relationship (Fig. 2A). Crossover restitution of (Sc) function would involve replacing *Ds* by a segment from an *R* element in the homologous chromosome. This interpretation implies the presence of a segment of equivalent effect in different *R* genic elements. Components common to different *R* elements have not been disclosed by other means.

Alternatively, *Ds* may exert a regional effect in chromosome 10, inhibiting (Sc) action when inserted beyond the bounds of the *R* element (Fig. 2B). In this case, the crossover event that exchanges *Ds* between homologs need not involve components common to different *R* elements. This alternative would be ruled out for instances of *Ds* insertion that map between the sites of recessive *r* mutations induced by conventional means. Such data are not available for *R*. Comparable data have been obtained, however, through fine structure analysis of the *waxy* gene (*8*); three controlling element mutants mapped within the region delimited by recessive mutation that had occurred spontaneously or had been induced by gamma rays. A highly localized effect of controlling elements, such as is indicated by the data for the *waxy* gene, is clearly necessary if they are to resolve components within an *R* element. In inter se combinations, the three *waxy* variants recombined with one another. Again, the property of insertion at various sites on a local scale is essential if the present approach is to yield a representative picture of the *R* gene.

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References and Notes

1. Reviewed in J. R. S. Fincham and G. R. K. Sastry [*Annu. Rev. Genet.* **8**, 15 (1974)] and P. A. Peterson [in *Maize Breeding and Genetics*, D. B. Walden, Ed. (Wiley, New York, 1978), p. 601].
2. S. R. Jaskunas and M. Nomura, in *DNA Insertion Elements, Plasmids and Episomes*, A. I. Bukhari *et al.*, Eds. (Cold Spring Harbor Laboratory, Cold Water Harbor, N.Y., 1977), p. 487.
3. L. J. Stadler, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 49 (1951); E. D. Styles, O. Ceska, K. T. Seah, *Can. J. Genet. Cytol.* **15**, 59 (1973).
4. L. J. Stadler, *Science* **120**, 811 (1954).
5. ——— and M. G. Neuffer, *ibid.* **117**, 471 (1953); L. J. Stadler and M. H. Emmerling, *Genetics* **41**, 124 (1956); J. L. Kermicle, *ibid.* **64**, 247 (1970); H. K. Dooner and J. L. Kermicle, *ibid.* **67**, 427 (1971); *ibid.* **82**, 309 (1976).
6. K. S. McWhirter and R. A. Brink, *Genetics* **47**, 1053 (1962).
7. The *R* alleles used in the present analysis and their probable genic element compositions are as follows: *R-sc:124* = (Sc), *R-r:standard* = (P)(S), *r-r:n35* & *r-r:n101* = (P)(s) and *r-g* = (p) or (s).
8. O. E. Nelson, *Genetics* **60**, 509 (1968).
9. B. McClintock, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13 (1951); R. A. Brink and R. A. Wilson, *Genetics* **37**, 519 (1952); P. C. Barclay and R. A. Brink, *Proc. Natl. Acad. Sci. U.S.A.* **40**, 1118 (1954).
10. M. G. Neuffer, *Genetics* **52**, 521 (1965).
11. H. K. Dooner and J. L. Kermicle, *ibid.* **78**, 691 (1974).
12. The percentage of recombination between (Sc) and *Ds* in m1, for example, may be calculated from the data in Table 1 as (45/87,100) × 100 × 2 = 0.10. The factor 2 enters in because only one of the two products of recombination is considered.
13. P. Nevers and H. Saedler, *Nature (London)* **268**, 109 (1977).
14. Paper 2396 from the Laboratory of Genetics. Conducted at the Wisconsin Agricultural Experiment Station with funds from DOE contract EY-76-S-02-1300.

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An About 50,000-Dalton Protein in Adrenal Medulla: A Common Precursor of [Met]- and [Leu]Enkephalin

Abstract. *A protein that may be an enkephalin precursor has been identified in extracts of bovine adrenal medulla. This protein (about 50,000 daltons) appears to contain seven copies of [Met]enkephalin and one copy of [Leu]enkephalin. Digestion with trypsin and carboxypeptidase B yields [Met]enkephalin and [Leu]enkephalin in a ratio of almost 7 to 1. The enkephalins were identified by chromatography and by their binding to opiate receptors. Some characteristics of several other adrenal peptides that may serve as intermediates in the biosynthesis of the enkephalins are presented.*

Demonstration of the [Met]enkephalin sequence (Try-Gly-Gly-Phe-Met) (*1*) at the amino terminus of β-endorphin (about 3000 daltons) (*2*) was originally interpreted as being indicative of a precursor-product relationship. However, attempts to demonstrate the presence of β-endorphin in tissues rich in enkephalins other than the pituitary gland, such as striatum (*3, 4*) and adrenal medulla (*5*), have been unsuccessful. In contrast, from extracts of adrenal medulla we were able to isolate a number of small (*6*) and large (*7*) enkephalin-containing peptides ranging from 500 to 22,000 daltons (*8*). In those studies, the enkephalin-like activities in the isolated peptides and proteins were revealed after tryptic digestion by their ability to compete with tritiated [Leu]enkephalin for binding to neuroblastoma-glioma cells; the released peptides were identified by high-performance liquid chromatography (*9*). The presence of free enkephalin in the tryptic digests indicates that the pentapeptide sequence occurred at the carboxyl terminus; hexapeptides with Arg or Lys in position 6 were found when the enkephalin sequence occurred internally. Since enkephalins extended at the carboxyl terminus with Arg or Lys possess very weak receptor binding activity (*10*), their detection and quantification in tryptic digests of peptides

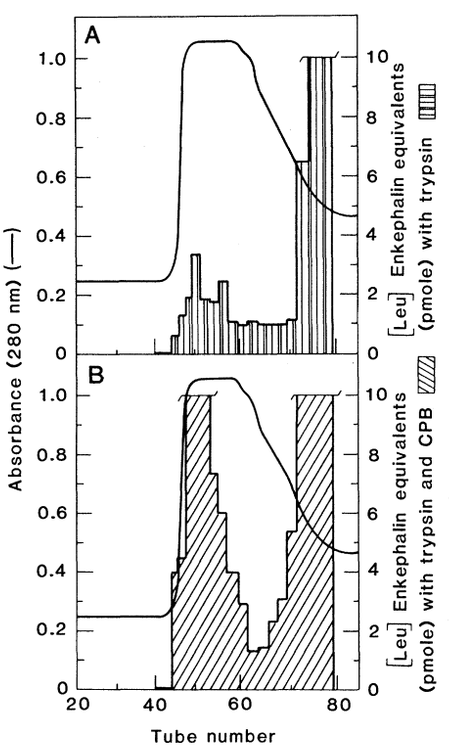

Fig. 1. Chromatography of extracts of adrenal medulla on Sephadex G-75. Bovine adrenal medulla (26 g) were homogenized in 100 ml of 1*M* acetic acid, 20 m*M* HCl, 0.1 percent β-mercaptoethanol containing phenyl methane sulfonyl fluoride (PMSF) (1 μg/ml) and pepstatin (1 μg/ml). The extract was centrifuged at 140,000*g* for 60 minutes. The supernatant was chromatographed on Sephadex G-75 (5 by 100 cm) with the above buffer and without PMSF and pepstatin. Portions (200 μl) from each fraction were lyophilized, redissolved in 400 μl of 50 m*M* tris-HCl (*p*H 8.5), and digested with 1 μg of TPCK-treated trypsin for 16 hours at 37°C. Tryptic digests (200 μl) were treated with 0.1 μg of carboxypeptidase B (*CPB*) for 2 hours at 37°C and then for 20 minutes at 90°C to inactivate the enzyme. Each portion was assayed with the neuroblastoma-glioma cell binding assay (*9*), with trypsin (A), and with trypsin and carboxypeptidase B (B). The activity in tubes 48 to 54 represents the 50,000-dalton protein and the activity after tube 66 represents the beginning of the 22,000-dalton protein.

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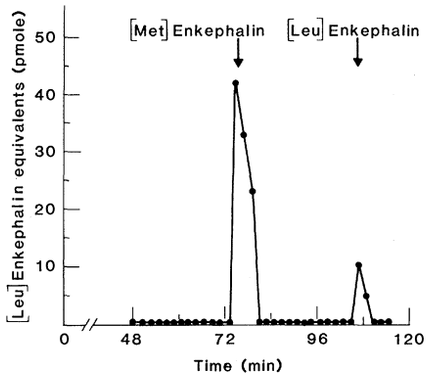

Fig. 2. High-performance liquid chromatography (HPLC) of trypsin and carboxypeptidase B digests of the 50,000-dalton region. Portions (2.0 ml) from fractions 50 to 54 of the Sephadex G-75 chromatography (Fig. 1) were digested with trypsin and carboxypeptidase B as described in the legend to Fig. 1, with 0.5 mg of trypsin and 20 μg of carboxypeptidase B. The resulting peptides were chromatographed on a Lichrosorb RP-18 column (10 μm, 4.6 by 250 mm) and eluted at 30 ml/hour with a mixture of formic acid (0.5*M*) and pyridine (0.4*M*), *p*H 4.0, and a gradient of *n*-propanol: 0 percent (12 minutes), followed by a 2-hour linear gradient of 5 to 9 percent. Fractions (1.0 ml) were collected, lyophilized, and assayed for opioid activity as described in the legend to Fig. 1. The column had been standardized with synthetic [Met]enkephalin and [Leu]enkephalin (arrows).

presented a problem. This was solved by treating enkephalin-containing peptides first with trypsin and then with carboxypeptidase B, thus liberating free enkephalins and improving the characterization of the large enkephalin-containing peptides (up to 22,000 daltons) that had already been isolated (*8*). This procedure has now enabled us to detect an even larger enkephalin-containing polypeptide (about 50,000 daltons), which may be closely related to the primary product of translation in enkephalin biosynthesis.

Bovine adrenal glands were obtained from the slaughterhouse within 30 minutes of animal death, and chromaffin granules were isolated from the medullas (*11*). The granules were homogenized in acid to prevent proteolysis (*2*). The procedures for extraction, elution from Sephadex columns, and digestion with trypsin have been described (*2*).

After extracts from bovine adrenal medullas were subjected to gel filtration on Sephadex G-100, no opiate receptor binding activity was observed in the region containing peptides larger than 22,000 daltons. Treatment of the same fractions with trypsin revealed a small amount of activity (Fig. 1A). This activity was greatly enhanced when the trypsin-digested fractions were further treated with carboxypeptidase B (Fig. 1B). Digestion of the proteins eluting in the region corresponding to 50,000 daltons was found to yield both [Met]enkephalin and [Leu]enkephalin in the ratio of approximately 7 to 1 (Fig. 2). Rechromatography of the 50,000-dalton activity indicated that smaller molecular weight peptides were not present, thereby ruling out aggregation of smaller peptides. Purification to homogeneity and sequencing analysis will be required to determine the precise number of enkephalin sequences in this protein and whether one or more such proteins are present in this fraction.

Several smaller and intermediate-sized peptides in the adrenal medulla contain more than one [Met]enkephalin sequence (*7*, *8*), but only one contains a [Leu]enkephalin sequence (*7*). The properties of the peptides and their relative amounts in extracts of adrenal medulla are summarized in Table 1; schematic comparison of their structures is shown in Fig. 3. In addition to those shown, we have detected several other peptides in

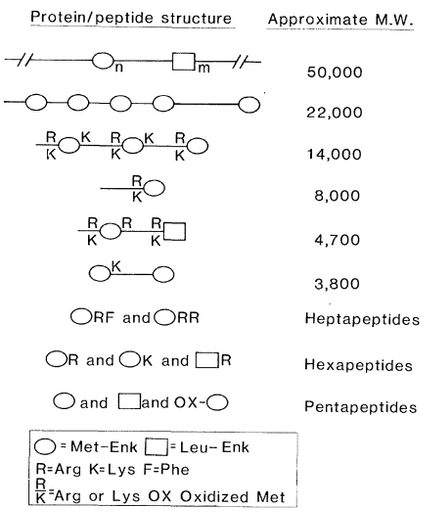

Fig. 3. Structures, as now known, of enkephalin-containing polypeptides; *n* is 6 to 7, *m* is 1, as determined by their ratio.

the range of 2000 to 5000 daltons, and small amounts of others. On the basis of these observations, a possible biosynthetic pathway for the enkephalins begins to emerge. Direct evidence for such a pathway from studies with [^{35}S]methionine indicated that the enkephalin sequences in the 22,000- and 14,000-dalton peptides are labeled before ^{35}S appears in free [Met]enkephalin (*12*).

The primary products of translation of secretory proteins or peptides are generally processed by one or more enzymes to yield the final active substances. Thus, insulin arises by action of a specific insulinase on the precursor, proinsulin (*13*). Pituitary pro-opiocortin (*14*) (pro-opiomelanocortin) (*15*) contains within it three hormone sequences—corticotropin, α-melanocyte-stimulating hormone, and β-endorphin. The individual hormones are presumably released by a trypsin-like protease, with subsequent removal of the carboxyl terminal Arg or Lys residue by carboxypeptidase B activity. In the case of the enkephalins, translation and subsequent processing apparently produce a precursor of about 50,000 daltons containing several copies of the [Met]enkephalin sequence, but only one copy of the [Leu]enkephalin sequence. Here too, there is evidence for the actions of proteases and peptidases giving rise to a series of intermediate-sized enkephalin-containing proteins and peptides, and to enkephalins with or without extensions at the carboxyl terminus.

All studies reported indicate that an enkephalin sequence at the amino terminus is required for interaction with the opiate receptor. Several of the intermediate-sized and small peptides (Fig.

Table 1. Proteins and peptides isolated from adrenal chromaffin granules.

Protein or peptide (daltons)	Amount* (nmole/g)	Active peptides
		From tryptic digests†
~ 50,000	0.1	
22,000	3.3	[Met]enkephalin and [Met]enkephalin-Lys^6‡
14,000	2.9	[Met]enkephalin and [Met]enkephalin-Lys^6
8,000	2.2	[Met]enkephalin
4,700	4.0	[Met]enkephalin-Arg^6 and [Leu]enkephalin
3,300		[Met]enkephalin-Lys^6 and [Met]enkephalin
		Naturally occurring§
<1,000	1.8	[Met]enkephalin; [Met]enkephalin-Arg^6‡; [Met]enkephalin-Lys^6; [Met]enkephalin-Arg^6-Arg^7; [Met]enkephalin-Arg^6-Phe^7; [Leu]enkephalin

*Amounts are based on total enkephalin content as determined by the binding assay after treatment with trypsin and carboxypeptidase B. Values are presented as nanomoles per gram of adrenal medulla. †Peptides produced by trypsin that are active in the opiate receptor binding assay. ‡Other active peptides have been found but not yet identified. §Present in adrenal medulla extracts and active in the binding assay without treatment with trypsin.

3), as well as the free enkephalins, have such amino terminal sequences and interact with the opiate receptor. It remains to be seen, therefore, whether peptides other than the free [Met]- and [Leu]enkephalins are of physiologic importance among all those found in the adrenal gland. On a molar basis the free enkephalins represent no more than 9 percent of the total compounds containing enkephalin sequences (Table 1). On a weight basis, the enkephalins represent less than 0.4 percent of the total. Conceivably, some of the larger peptides and proteins may provide additional specificity for cell recognition, confer additional stability in vivo, or even possess different types of biological activity.

The finding of the sequences of three different hormones in pro-opiocortin was rather surprising at the time (*14*). It is of interest that the putative enkephalin precursors also contain more than one active peptide sequence, [Met]enkephalin and [Leu]enkephalin. What is most unusual about the larger peptides and proteins is that they contain multiple copies of a single sequence, [Met]enkephalin. The full significance of this multiplicity of the [Met]enkephalin sequence in the large peptides is not clear. It does, however, explain the ratio of [Met]enkephalin to [Leu]enkephalin of about 5 to 1 up to 7 to 1 reported by others (*16*), and suggests that this ratio is already encoded in nuclear DNA. Another important conclusion is that a common precursor to [Met]enkephalin and [Leu]enkephalin makes unlikely the proposed occurrence of separate neurons for the two enkephalins (*17*).

A 50,000-dalton enkephalin-containing protein has also been found in the striatum and intestine (*2*), and the heptapeptide, [Met]enkephalin-Arg^6-Phe^7, and other extended enkephalins found in adrenal medulla are also present in striatum (*6*). Thus, the biosynthetic pathway for enkephalins suggested by these studies on the adrenal medulla may be universal for all enkephalin-containing tissues.

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References and Notes

1. Abbreviations: Met, methionine; Tyr, tyrosine; Gly, glycine; Phe, phenylalanine; Leu, leucine; Arg, arginine.
2. J. Hughes, L. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, H. R. Morris, *Nature (London)* **258**, 577 (1975); B. M. Cox, A. Goldstein, C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1821 (1976).
3. R. V. Lewis, S. Stein, L. D. Gerber, M. Rubinstein, S. Udenfriend, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4021 (1978).
4. H.-Y. T. Yang, W. Fratta, J. S. Hong, A. DiGuilio, E. Costa, *Neuropharmacology* **17**, 422 (1978).
5. R. V. Lewis, A. S. Stern, J. Rossier, S. Stein, S. Udenfriend, *Biochem. Biophys. Res. Commun.* **89**, 822 (1979); H.-Y. T. Yang, E. Costa, A. M. DiGiulio, W. Fratta, J. S. Hong, *Fed. Proc. Am. Soc. Exp. Biol.* **38**, 364 (1979).
6. A. S. Stern *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6680 (1979).
7. S. Kimura, R. V. Lewis, A. S. Stern, J. Rossier, S. Stein, S. Udenfriend, *ibid.* **77**, 1681 (1980).
8. R. V. Lewis *et al.*, *Adv. Biochem. Psychopharmacol.*, in press.
9. L. D. Gerber, S. Stein, M. Rubinstein, J. Wideman, S. Udenfriend, *Brain Res.* **151**, 177 (1978).
10. The IC_{50} of these peptides in the binding assay are: [Leu]- and [Met]enkephalin, $3.5 \times 10^{-9} M$; [Met]enkephalin-Arg^6-Phe^7, $6.8 \times 10^{-9} M$; [Met]-enkephalin-Lys^6, $65 \times 10^{-9} M$; and [Met]enkephalin-Arg^6, $95 \times 10^{-9} M$.
11. A. D. Smith and H. Winkler, *Biochem. J.* **103**, 480 (1967).
12. Studies carried out in collaboration with J. P. Rossier, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France, and J. M. Trifaro, McGill University, Montreal, Canada.
13. D. F. Steiner and P. E. Oyer, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 473 (1967); D. F. Steiner, D. Cunningham, L. Spigelman, B. Aten, *Science* **157**, 697 (1967).
14. S. Kimura *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1756 (1979); R. E. Mains, B. A. Eiper, N. Ling, *ibid.* **74**, 3014 (1977).
15. P. Crine, F. Gossard, N. G. Seidak, L. Blanchette, M. Lis, M. Chretien, *ibid.* **76**, 5085 (1979).
16. C. Gros *et al.*, *J. Neurochem.* **31**, 29 (1978); G. Henderson, J. Hughes, H. W. Kosterlitz, *Nature (London)* **271**, 677 (1978).
17. L-I. Larsson, S. Childers, S. H. Snyder, *Nature (London)* **282**, 407 (1979).
18. We thank L. D. Gerber and L. Brink for technical assistance.
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Sodium-Calcium Exchange Activity Generates a Current in Cardiac Membrane Vesicles

Abstract. *Sarcolemmal membrane vesicles isolated from canine ventricular tissue accumulate calcium through the sodium-calcium exchange system when an outwardly directed sodium gradient is generated across the vesicle membrane. Moreover, calcium uptake under these conditions is accompanied by the transient accumulation of the lipophilic cation tetraphenylphosphonium. Since the distribution of tetraphenylphosphonium across biological membranes reflects the magnitude and direction of transmembrane potential differences and the characteristics of the transient accumulation of this cation closely resemble those of sodium-calcium exchange activity, it is concluded that a membrane potential, interior negative, is produced during calcium accumulation through the exchange system. Thus, the operation of the sodium-calcium exchange system generates a current in cardiac membrane vesicles, suggesting that three or more sodium ions exchange for each calcium ion.*

Calcium ions traverse the myocardial cell membrane in both directions during each cycle of contraction and relaxation in the heart. These Ca^{2+} movements appear to play a crucial role in excitation-contraction coupling in cardiac muscle since the removal of extracellular Ca^{2+} leads to an abrupt loss of contractile activity (*1*, *2*). An important mechanism for the transfer of Ca^{2+} across cell membranes in excitable tissues is Na^+-Ca^{2+} exchange, a carrier-mediated transport process that couples the movement of Ca^{2+} in one direction to the movement of Na^+ in the other (*3*-*5*). The precise role of Na^+-Ca^{2+} exchange in cardiac physiology is uncertain. Reuter and co-workers (*3*, *6*) proposed that its primary function is removal of Ca^{2+} from the cell, utilizing the energy of the inwardly directed Na^+ gradient for this purpose. In contrast, Langer *et al.* (*7*) suggested that it brings Ca^{2+} into the cell with each contraction in response to a depolarization-induced elevation in intracellular Na^+. It has also been suggested that Na^+-Ca^{2+} exchange mediates the inotropic effects of cardiac glycoside administration and changes in stimulation frequency (*4*, *8*).

The stoichiometry of the cardiac Na^+-

Table 1. Uptake of TPP^+ and calcium by cardiac membrane vesicles treated with valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone.

Treatment*	TPP^+ uptake† (nmole per mg of protein)		Ca^{2+} uptake† (nmole per mg of protein)
	EGTA	Ca^{2+}	
Control	0.58 ± 0.06	1.64 ± 0.07	35.2 ± 2.4
Valinomycin	0.37 ± 0.04	0.40 ± 0.05	61.8 ± 1.1
Carbonyl cyanide *m*-chlorophenylhydrazone	0.42 ± 0.10	0.60 ± 0.04	51.6 ± 4.4

*Vesicles were treated with $8.3 \times 10^{-6} M$ valinomycin (4 nmole per milligram of protein) or $3.3 \times 10^{-6} M$ carbonyl cyanide *m*-chlorophenylhydrazone (2 nmole per milligram of protein) and then assayed for TPP^+ and Ca^{2+} uptake as described in the legend of Fig. 1. †Values are the means ± standard errors of triplicate determinations. For both the TPP^+ and Ca^{2+} uptake studies, Ca^{2+} was present at a concentration of 50 μM. Uptake was measured over a 10-second interval in each case.

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