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Synthesis and Some Biological Activities of Substance P†

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The undecapeptide, substance P, having the structure H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, has been synthesized by a solid-phase technique on a Beckman automatic peptide synthesizer, appropriately purified, and biologically characterized. In nanogram dosage, synthetic substance P stimulated contraction of the isolated guinea pig ileum, decreased systemic arterial blood pressure, and increased local blood flow in the dog's gracilis muscle. The biological activities of this synthetic undecapeptide are in reasonable agreement with the known activities of preparations of substance P from tissue. Substance P released, *in vitro*, the luteinizing and follicle-stimulating hormones but only at high dosage. Substance P did not release growth hormone, prolactin, or thyrotropin.

In 1931 von Euler and Gaddum[1] described some properties of "an unidentified depressor substance in certain tissue extracts," particularly from intestinal plain muscles and brains of horses. This substance stimulated smooth muscle contraction in some organs and lowered the arterial blood pressure of atropinized rabbits by peripheral vasodilation. A standard preparation of this substance was called "P;" samples also became known as "preparation P" and "powder P." In the following years the expression "substance P" was extensively used.

The active compound, together with other peptides, could be effectively salted out from an aqueous solution by ammonium sulfate[2] and was inactivated by proteolytic enzymes,[3,4] indicating its polypeptide nature. By applying chromatographic separation, Pernow[5] obtained a 1000-fold increase in activity from the starting material of peptides and also showed the presence of substance P in high concentrations in the hypothalamus. It was also found in extracts and subcellular particles of peripheral nerves,[6] pointing to a general localization in nervous tissue.

A number of effects on the central and peripheral nervous system have also been noted.[7,8] Further studies on biological activities and chemical properties of preparations of substance P were reported in the 1960's by various workers.[9,10]

Leeman and Hammerschlag[11] reported in 1967 that extracts of bovine and rat hypothalami contained a peptide that stimulated salivary secretion when such extracts were injected into anesthetized rats. Lembeck and Starke[12] then reported sialogogic activity of their preparations of substance P and suggested that substance P and the sialogogic peptide might be identical.

Chang and Leeman[13] isolated in 1970 the sialogogic peptide from bovine hypothalami and found it to be an undecapeptide with biological properties indistinguishable from those described for substance P. The amino acid sequence was found by Chang, *et al.*,[14] to be H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 and was confirmed by a solid-phase synthesis;[15] the behavior of the synthetic and natural products was identical. In 1973, Studer, *et al.*,[16] isolated substance P from horse intestine and found an amino acid sequence identical with that re-

†Hypothalamic Hormones. 54.

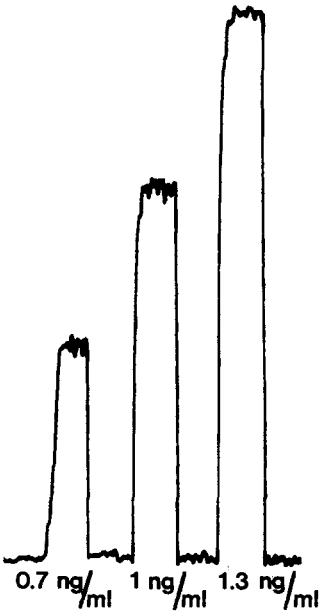

Figure 1. Effect of 2-4 ng of substance P on the isolated guinea pig ileum suspended in a 3-ml bath of oxygenated Tyrode's solution at 38°.

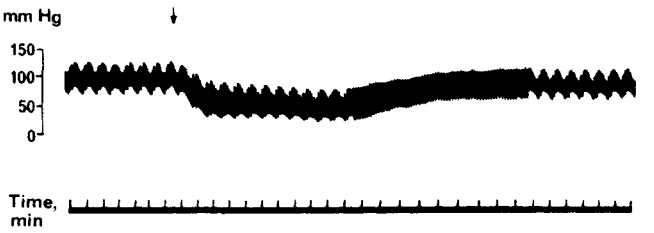

Figure 2. Effect of intravenous injection of 5 ng/kg of body weight of substance P in the dog on arterial blood pressure (mm). The arrow indicates injection time.

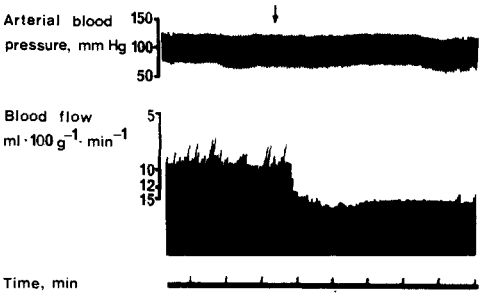

Figure 3. Effect of close arterial infusion of substance P (70 ng/100 g of muscle tissue/mm) on arterial blood pressure and blood flow through the gracilis muscle in the dog. The initial blood flow of 7.4 ml/100 g/min increased during the infusion to a mean value of 17.5 ml/100 g/min. Infusion was started at the arrow and continued over the whole period shown in the figure.

ported for substance P from bovine hypothalamus,[14] thus confirming the identity between Leeman's sialogogic peptide and substance P.

We have also synthesized this undecapeptide and have made additional comparisons of the biological activities of the synthetic product and the known activities of our previous samples of natural substance P. The greater availability of the synthetic peptide supports expanded biological studies and opens up evaluations of the potential clinical importance of substance P.

Tissue Origin of Substance P. von Euler and Gaddum[1] emphasized the presence of their substance P in intestinal muscle and brain. They also observed similar activities from extracts of tissues of many organs but recognized that some of these activities could be due to other substances.

Leeman and Hammerschlag[11] isolated their sialogogic peptide from approximately 100,000 bovine hypothalamic fragments. Subsequently, a radioimmunoassay for this peptide revealed about 500 ng/g of substance P in substantia nigra tissue of the human brain stem and smaller concentrations in other brain tissues.[17]

Synthesis. The benzhydrylamine-type resin,[18] originally described by Pietta and Marshall,[19] was used for the synthesis by the Beckman Model 990 solid phase peptide synthesizer. This type of resin avoids difficulties with alkylation[20] encountered in esterifying methionine to conventional chloromethylated resins.

Dried benzhydrylamine resin hydrochloride was neutralized with Et_3N and stirred overnight with an excess of *tert*-butyloxycarbonylmethionine and DCI in CH_2Cl_2. The remaining unreacted amino groups of the resin were acetylated with a mixture of Ac_2O and Et_3N in DMF overnight. Amino acid analysis of the resin, after hydrolysis in 6 *N* HCl–propionic acid (1:1), gave a value of 0.22 m*M*/g for Met.

N^{α}-Boc protection was used throughout the synthesis. Side-chain protecting groups were Z for Lys and Tos for Arg. A 2.5-fold excess of each *tert*-butyloxycarbonylamino acid was used with a coupling time of 6 hr except for Boc-Gln which was incorporated as a fivefold excess of its ONP active ester with an 11-hr coupling time. Dried (P_2O_5), redistilled CH_2Cl_2 was used as the coupling solvent except for Arg and Gln which required purified DMF. Deprotection was accomplished by 50% TFA in CH_2Cl_2 with neutralization by 10% Et_3N in CH_2Cl_2. Completeness of coupling was monitored by the ninhydrin color test procedure of Kaiser, *et al.*,[21] and when necessary the coupling reaction was repeated (particularly in the case of Pro). From 1.5 g of Boc-Met-BHA resin a 1.98-g yield of the protected undecapeptide-BHA resin was obtained.

Cleavage of the peptide from the resin, with simultaneous removal of the protecting groups and formation of the carboxyl terminal amide, was effected with 20 ml of dried (CoF_3), liquid HF in the presence of 2 ml of anisole for 1 hr at 0°.[22] After removal of the excess HF *in vacuo*, the resin was washed with EtOAc to remove anisole, followed by 0.5 *N* HOAc to extract the peptide.

The crude, lyophilized product (424 mg) was purified by gel filtration on a 102 × 2.5 cm column of Bio-Gel P2 eluted with 1.3% HOAc, with detection of the peptide peaks by uv at 256 nm (Phe). The main fraction (253 mg) was partitioned on a 100 × 1.5 cm column of Sephadex G-25 eluted with the system 0.1% HOAc–*n*-BuOH–Pyr (11:5:3) with detection of the peptide peaks by the Folin–Lowry[23] procedure at 700 nm, giving 90 mg (16.7%) of pure substance P.

Amino acid analysis of the product was done on a Beckman Model 119 amino acid analyzer set up for single-column methodology after hydrolysis of the peptide with 6 *N* HCl containing 4% thioglycolic acid. Amino acid analysis of the pure substance P gave the following ratios: Arg 0.97, 2 Pro 1.92, Lys 1.03, 2 Glu 2.08, 2 Phe 1.94, Gly 1.03, Leu 1.11, Met 0.91, 3 NH_3 2.83. Standard tlc methods on silica gel showed one spot with the following R_f's in the given systems when detected by iodine, ninhydrin, or chlorotolidine: R_f 0.17, *n*-BuOH–HOAc–EtOAc–H_2O (1:1:1:1); R_f 0.00, EtOH–H_2O (7:3); R_f 0.09, $CHCl_3$–MeOH–NH_4OH (60:45:20); R_f 0.44, EtOAc–Pyr–HOAc–H_2O (5:5:1:3). The pure product showed a specific optical rotation of $[\alpha]^{24}D$ −76.5° (*c* 0.666, 1% HOAc).

Table I. Effect of Substance P on LH and FSH Release

No.	Dose of substance P, I_3, I_4, ng/ml of medium	LH			FSH		
		Δ ng/ml of medium[a]	S.E.M.	*p* value *vs.* 1	Δ ng/ml of medium[a]	S.E.M.	*p* value *vs.* 1
1		71	±15		398	±258	
2	1,000	61	±26	ns	299	±227	ns
3	10,000	236	±33	0.001	3246	±660	0.01
4	100,000	455	±138	0.02	5864	±1203	ns

[a] Δ = mean of 12.

Our synthesis of substance P was similar to that reported by Tregear, *et al.*,[15] for substance P except that we used the Beckman automatic peptide synthesizer with deprotection by 50% TFA-CH_2Cl_2 instead of classical solid-phase techniques with deprotection by 4 *N* HCl–dioxane. Our purification also differed from that previously reported. We used partition chromatography over Sephadex G-25 using entirely physical methods (uv) to detect the synthetic product. Tregear, *et al.*,[15] used column chromatography on Sephadex G-15 in 0.5 *M* HOAc with detection of their synthetic substance P by comparison of its biological activity with that of natural preparations.

Biological Activities of Synthetic Substance P. Activity on Isolated Intestinal Tissue. A section of guinea pig ileum was suspended in a 3-ml bath of the standard oxygenated Tyrode's solution at 38°. The synthetic substance P stimulated contraction of the isolated guinea pig ileum in concentrations of tenths of a nanogram per milliliter of bath fluid (Figure 1). The activity of the synthetic substance P was about 500,000 Euler units per milligram. This effect was not influenced by atropin, antihistaminics, or LSD.

Activity on Blood Pressure. Analyses of the circulatory effect of substance P were performed on dogs anesthetized with pentobarbital. The arterial blood pressure was recorded through a catheter inserted in a carotid artery connected to a Statham pressure transducer.

Intravenous injections of synthetic substance P in doses higher than 2 ng/kg of body weight elicited a decrease in the systemic arterial blood pressure lasting from 2 to 20 min depending on the dose (Figure 2).

Activity on Skeletal Muscle Blood Flow. The blood flow through the dog's gracilis muscle was recorded according to the procedure of Renkin and Rosell.[24] Pressure and flow were recorded on a Grass polygraph.

Close arterial infusion of substance P to the dog's gracilis muscle increased the local blood flow when doses not affecting the systemic arterial blood pressure were used, *i.e.*, less than 100 ng/100 g of tissue/min (Figure 3). When higher doses were given, the muscle blood flow decreased concomitant with an arterial hypotension.

Activities to Release Pituitary Hormones. All hormonal activities were obtained from *in vitro* studies using pituitaries of 20-day-old female Sprague–Dawley rats (Charles River Laboratory). To determine agonist, antagonist, synergistic, or additive activities of substance P, in the absence and in the presence of LHRH and TRH, two pituitaries were incubated at 37° in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10-ml Teflon beakers in a Dubnoff shaker. The pituitaries were incubated for a total of 6 hr. Medium was removed each hour for radioimmunoassay[25] of LH, FSH, GH, PRL, and TSH, and then fresh medium was added. After two preincubation periods (P_1, P_2), substance P was added to the incubation medium (I_3, I_4, I_5, I_6) and LHRH or TRH was added at I_5 and I_6. Agonist activity was determined from the hormonal release at I_3 and I_4, and antagonist or synergistic activity from I_5 and I_6. When both peptides were added together, substance P was always added to the incubation medium 5 min before LHRH or TRH. The RIA reagents for FSH, PRL, GH, and TSH were distributed by NIAMDD, NIH. Dr. G. Niswender supplied the antiovine LH serum No. 15 for the rat LH assay, and Dr. L. E. Reichert supplied an ovine LH preparatin for labeling and the LH rat reference preparation. The values for these assays are calculated in terms of nanograms of the following standards: LH-LER-1240-2 (0.60 NIH-LH-SI unit/mg), FSH (2.1 NIH-FSH-SI units/mg), GH (0.61 unit/mg), PRL (111 units/mg), and TSH [0.22 USP (bovine) unit/mg].

As shown in Table I, synthetic substance P definitely releases LH and FSH. It has about 0.0003% of the LH-releasing activity of LHRH and about 0.00015% of the FSH-releasing activity of LHRH. Substance P has no LHRH antagonist activity on LH or FSH release. Substance P and LHRH were slightly additive but not synergistic in their effects on LH release. The effect of substance P on the LHRH-induced release of FSH was neither additive nor synergistic. Large doses of substance P (100 μg/ml) have no GH, PRL, or TSH releasing activities.

Discussion of Biological Activities and Mechanism of Action. Synthetic substance P has a powerful vasodilating action which is probably due to a direct effect on the cells of smooth muscle in the vessel wall; the effective nanogram dosage in dogs is impressive. Picogram levels of the synthetic substance P were sufficient to stimulate contraction of the isolated ileum of the guinea pig. Comparable potent activity was observed by the increase in local blood flow when synthetic substance P was infused into the dog's gracilis muscle. All these pharmacological activities are in reasonable agreement with the previously observed activities of impure preparations of substance P from tissue.[1-13]

The fact that substance P shows even low activities to release LH and FSH is surprising. However, this undecapeptide has an Arg moiety and adjacent Phe-Phe moieties and possibly some conformational aspects for slight acceptability at the receptor site for the decapeptide, LHRH, when LH is released.

When the organic structure of substance P is appraised on the basis of current interpretations of sequence–conformation–activity relationships[26-28] of the two hypothalamic releasing hormones, TRH and LHRH, the following considerations can serve as guidelines to studies on the mechanism of action. Of the eight different amino acids in the undecapeptide sequence of substance P, Arg^1, Lys^3, Phe^7, and Phe^8 appear to be the four crucial amino acids. the Arg^1 moiety, in protonated form, could be very important for activity and potency because it contributes an ionic nature to the molecule. The Lys^3 moiety could make a similar contribution, but perhaps secondary to that of Arg^1. The Phe^7-Phe^8 moiety could contribute unique structural specificity to the entire molecule, because of possible π–π bond interaction with a receptor site. Doubtless, the conformation of the entire molecule of

substance P also significantly contributes to its function at a site.

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Synthesis and Hypocholesterolemic Activities of Eritadenine Derivatives

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More than 100 derivatives of eritadenine were synthesized and their hypocholesterolemic activities were evaluated. The most active derivatives were carboxylic acid esters with short-chain monohydroxy alcohols, which were 50 times more active than eritadenine and effective in lowering serum cholesterol of rats at the dose of 0.0001% in the diet. The carboxyl function and at least one hydroxy group appear to be essential for activity. The intact adenine structure seems to be required for activity except for the N^1 position where an oxygen or an alkoxy group could be attached without loss of activity.

Eritadenine is a hypolipidemic substance isolated from the Japanese mushroom shiitake (*Lentinus edodes*) and its chemical structure has been identified as 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)butyric acid by two independent groups of workers.[1,2] Eritadenine lowers all the lipid components of plasma lipoproteins (cholesterol, triglyceride, and phospholipid) and is active in several animal species including man.[3] Its hypocholesterolemic property has been studied in detail in the rat.[4-6]

In order to study the structure-activity relationship of this naturally occurring hypolipidemic agent, and also in the hope of finding a derivative with the best possible therapeutic index, numerous derivatives and analogs of eritadenine were synthesized in these laboratories and their hypocholesterolemic activities were evaluated in normal rats.

Chemical Procedures. Preparation of the eritadenine esters and amides (compounds **1–50**) was carried out by the common methods, which are described in the Experimental Section.

Syntheses of the stereoisomers of eritadenine (compounds **51–56**), noreritadenine (compound **70**), homoeritadenine (compounds **73–77**), α-*O*-alkyleritadenine (compound **68b**) and the reversed nucleosides (compounds **78–82**) have been reported by Kawazu, *et al.*[7,8]

Compounds **57** and **58** were synthesized by direct alkylation of adenine with an O-protected ethyl 4-bromo-2,3-dioxybutyrate. The latter was obtained by warming D-erythronolactone in ethanol saturated with hydrogen bromide and, after removal of the ethanol, by treating the residue with a protecting agent such as ethyl orthoformate, acetone, or acetaldehyde. Compounds **59**, **60**, and **61** were prepared by condensation of the eritadenine ester with the cyclic ketones in the presence of an acid.

Compound **63** was prepared by reaction of the sodium salt of adenine with pantolactone.

Compounds **64** and **65** were synthesized from the pyrimidine derivatives employing the usual synthetic method for the purine skeleton as reported for the synthesis of eritadenine.[1,9]

The amino acids (compounds **66–68**) were synthesized from the reversed nucleoside of adenine, 5-(adenine-9*H*-9-yl)-3-acylamino-D-ribose, by oxidation with oxygen under conditions similar to those for synthesis of homoerit-