# TRITIUM LABELLING OF SUBSTANCE P AND THE CORRESPONDING N-TERMINAL 1-7 FRAGMENT IN THE $PRO^2$, $PRO^4$ POSITIONS

by

J.M. Stewart*, C. Sundqvist**, J.-K. Chang*** and L. Terenius**

*Department of Biochemistry, University of Colorado, Denver, Colorado, USA

**Department of Pharmacology, University of Uppsala, Sweden

***Peninsula Laboratories, San Carlos, California, USA

Tritium labelling of substance P has been performed by several groups. Nakata et al (1) used the Wilzbach exchange technique and reported a good yield and excellent specific activity. In our hands this procedure gives almost complete degradation of the molecule; in fact also the simpler [Leu]enkephalin molecule degrades almost completely (unpublished observation). Reductive dehalogenation of para-substituted phenyl groups ($Phe^7$ and $Phe^8$) has also been used (2-4). Hanley and co-workers (4) report good specific activity (23 Ci/mmol) of substance P labelled in the $Phe^8$ position and Berger et al (2) the $Nle^{11}$- substance P analogue, labelled at $Phe^7$ and $Phe^8$ to 27 Ci/mmol.

We have used the alternative approach (5) of introducing tritium by catalytic hydrogenation of the unsaturated precursors with 3,4 dehydro-$Pro^2$, 3,4 dehydro-$Pro^4$ substitution. The tritiated

0362-4803/83/030317-08$01.00

Received August 16, 1982

products had a specific activity of about 15 Ci/mmol and showed good radiochemical stability.

## Experimental

### Synthesis of [3,4-dehydro-Pro$^{2,4}$]-substance P and [3,4-dehydro-Pro$^{2,4}$]-substance P (1-7)

Peptides were synthesized by the Merrifield solid phase method (6) using the Beckman Model 990 automatic synthesizer. Substance P was synthesized on methylbenzhydralamine resin(7) and SP (1-7) was synthesized on the standard Merrifield resin (Lab Systems, Inc.). BOC amino acids were purchased from Bachem or Beckman or were synthesized in this laboratory. Amino acid side chains were protected as follows: Arg(Tos), Lys(2-ClZ), and Gln(Xan). Standard procedures were followed using 2.5-fold excesses of BOC amino acids and dicyclohexylcarbodiimide for each coupling. Deprotection was by 25% trifluoroacetic acid in dichloromethane. The deprotection reagent contained 0.1% indole. Peptides were cleaved from the resins by treatment with anhydrous HF at 0°C for 30 min in the presence of anisole, and were purified by countercurrent distribution. Homogeneity of the peptides was demonstrated by paper electrophoresis and thin layer chromatography. Amino acid analysis was done on a Beckman 120C analyzer after hydrolysis at 110°C for 22 hr in the presence of mercaptoethanol and phenol.

### Tritium-labelling step

The above compounds (2 mg)were dissolved in ethanol and

subjected to catalytic hydrogenation (**Tritium Labelling Service, Amersham International plc**, Amersham, England). After removal of labile tritium, the product was kept in absolute ethanol under nitrogen. The product was analyzed on reversed phase HPLC which showed about 30% to chromatograph as authentic substance P.

Preparative chromatographic isolation of tritium-labelled substance P

Ethanol was removed from the reaction product which was redissolved in dilute pyridine formate buffer (0.1M pyridine, 0.1M formic acid). About 5 millicurie of crude product was applied to a 1 ml SP-Sephadex (H+) ion exchanger equilibrated in the same buffer. The ion exchanger was washed with 10 volumes of the buffer and then eluted with 6 volumes of stronger pyridine formate buffer (0.8M pyridine, 0.8M formic acid). All solvents contained 0.01% of mercapto-ethanol. The elute was lyophilized to dryness, subjected to HPLC chromatography in a water-methanol solution (40% methanol, 0.005% trifluoracetic acid, 0.01% mercaptoethanol) on a Waters C18 reversed phase column. The peak corresponding to substance P was taken to dryness in vacuo. Absolute ethanol with a trace of mercaptoethanol was added to a concentration of about 20 µCi/ml. This ethanol solution was kept at -90°C under nitrogen for about 4 months, with minimal decomposition.

Checks of purity included several HPLC systems on the same column, ion-exchange chromatography on SP-Sephadex and absorption to antibodies raised against substance P -

thyroglobulin conjugates. Finally, an aliquot was tested on the guinea-pig ileum for smooth-muscle activity (8).

## Preparative chromatographic isolation of tritium-labelled substance P (1-7) fragment

Preliminary purification was carried out as described above. The product was isolated by reversed phase HPLC chromatography in a water-methanol gradient (starting buffer 10% MeOH; final buffer 80% MeOH; conditions otherwise identical to those for substance P). Storage conditions were analogous to those of substance P.

Checks of purity included absorption to antibodies raised against conjugates of substance P (1-7) to thyroglobulin.

## Results and Discussion

The HPLC systems separate compounds in the expected order of polarity, for instance substance P from the sulphoxide (Figure 1a). The peak of radioactivity with an elution volume corresponding to substance P was symmetric. Analytical HPLC revealed that about 98% of the activity eluted in the position of SP (1-11) (Figure 1b). Incubation with several antibodies directed against the C-terminus of substance P revealed that over 80% of the total added radioactivity is absorbed. Estimates of specific activity comparing with

the potency of standard substance on the guinea-pig ileum gave about 15 Ci/mmol.

Several unsuccessful runs preceeded the final successful labelling. We learned that conditions of storage are critical and that storage in aqueous solvents may result in loss of radioactive peptide. The radioactive material arising from such storage elutes in the void volume of the HPLC system and may represent tritiated water. The mechanism for this degradation is not known. Storage in absolute ethanol, on the other hand, reduced decomposition, and after 3 months of storage the labelled material is still more that 90% pure. Rechromatography at regular intervals may still be advisable.

The labelled substance P (1-7) has not been characterized as fully as substance P itself. However, it absorbs to more than 80% to six different antisera directed against this fragment and shows no absorption to several substance P antisera which do not cross-react with the (1-7) fragment.

Peptides labelled in the Pro-positions may be of general interest in relation to studies of enzymatic degradation, since post-proline cleaving enzymes are thought to be important for the posttranslational processing of peptides. This may be the case also for substance P. The (1-4) fragment has its own characteristic biologic properties, stimulating neurite extension (9) while the (1-7) fragment appears to have specific neurotropic activity but no spasmogenic activity (10). Confirming the observation of Hanley et al. (4) we also find rapid degradation of substance P if incubated

with synaptosomal membranes. This occurs mainly at the $Pro^4$-$Gln^5$ and $Phe^7$-$Phe^8$ bonds (unpublished). The $Pro^2$-$Pro^4$-labelled fragments which can arise seem to be fairly stable and free Pro is released very slowly (unpublished).

The access to labelled substance P and a choice between labelling in several positions will no doubt provide a tool for neurobiological research with this peptide.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Medical Research Council (Grant No B25X-03766) and by grant NS-09199 from the US-NIH.

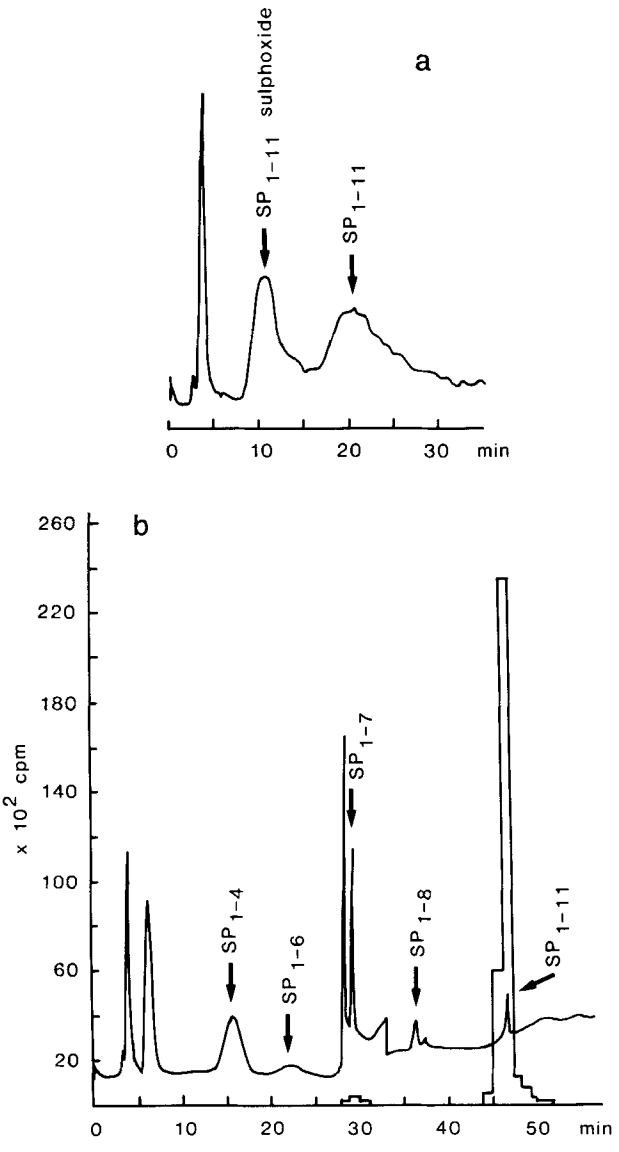


Figure 1.

Separation of substance P with HPLC: a) conditions for preparative separation removing the major contaminant, the sulphoxide (isocratic 40% methanol and 0.005% trifluoroacetic acid) b) analytical separation (non-linear gradient, starting buffer was 0.05% phosphoric acid, pH = 3.0 with NaOH, terminating buffer was 40% acetonitrile in starting buffer)

REFERENCES

1. Nakata, Y., Kusaka, Y., Segawa, T., Yajima, H. and Kitagawa, K. - Life Sci. 22, 259-268 (1978).

2. Berger, H., Fechner, K., Albrecht, E. and Niedrich, H. - Biochem. Pharmacol. 28, 3173-3180 (1979).

3. Sandberg, B.E.B., Hanley, M.R., Watson, S.P., Brundish, D.E., Wade, R. and Eison, A.S. - Febs Letters 137, 236-240 (1982).

4. Hanley, M.R., Sandberg, B.E.B., Lee, C.M., Iversen, L.L., Brundish, D.E. and Wade, R. - Nature 286, 810-812 (1980)

5. Felix, A., Wang, C.T., Liebman, A.A., Delaney, Ch.M., Mowles, T., Burghard, B.A., Charnecki, A.M. and Meienhofer, J. - Int. J. Peptide Protein Res. 10, 299-310 (1977).

6. Stewart, J.M. and Young, J.D. Solid phase peptide synthesis (Freeman, W.H. and Co., San Francisco) (1969).

7. Matsueda, G.R. and Stewart, J.M. - Peptides 2, 45-50 (1981).

8. Cleugh, J., Gaddum, J.H., Mitchell, A.A., Smith, M.W. and Whittaker, V.P. - J. Physiol. 170, 69 (1964).

9. Bar-Shavit, Z., Goldman, R., Stabinsky, Y., Gottlieb, P., Fridkin, M., Teichberg, V.I. and Blumberg, S. - Biochem. Biophys. Res. Comm. 94, 1445-1451 (1980).

10. Stewart, J.M., Hall, M.E., Harkins, J., Frederickson, R., C.A., Terenius, L., Hökfelt, T. and Krivoy, W.A. - Peptides (in press).