^{3}H-LABELLING OF SUBSTANCE P BY TRITIUM EXCHANGE AND DESHALOGENATION

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1. Introduction

In 1931 Von Euler and Gaddum discovered substance P (SP) in crude acid alcohol extracts of equine brain and intestine [1]. It was then isolated from bovine hypothalamus [2,3]. SP was determined as an undecapeptide as two others peptides, eledoisin and physalaemin which exhibit similar pharmacological properties [4]. The proposed structure:

Arg–Pro–Lys–Pro–Gln–Gln–Phe–
Phe–Gly–Leu–Met–NH_2

was confirmed by numerous syntheses [4–7]. With the development of immunohistochemistry [8] and the use of biochemical approaches [9,10], SP was found to be localized in numerous peripheral and central neurons. SP could act as a neurotransmitter or a neuromodulator [11–15]. However, little is known concerning its biosynthesis and its enzymatic inactivation. Moreover only a few binding studies have been made to characterize the SP receptor(s). This prompted us to synthetize a tritiated SP retaining all its chemical and biological potencies. Since SP does not contain residues such as tyrosine or histidine which can easily be labelled [16,17] and as the analogue Tyr–8-SP is less potent, direct catalytic tritium exchange was investigated [18]. In addition, this report describes labelling of SP and Nle SP (Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Nle) (NH_2) by catalytic deshalogenation of their Phe (p-Cl)^{7,8} derivatives. Nle-SP has recently been shown to possess a high biological activity [19] and in contrast to methionine SP, is not as sensitive to oxidizing reagents. The ^{3}H-labelling by exchange or by deshalogenation yielded products with specific radioactivities of 15–50 Ci/mmole, respectively.

2. Materials and methods

2.1. *Materials*

Substance P and Phe (p-Cl)^{7,8} substance P were purchased from Penninsula Labs (Mallet) and Phe-(p-Cl)^{7,8} Nle-SP was a generous gift from Dr M. Bienert and P. Oehme (Institut für Wirkstofforschung, Akademie der Wissenschaften der DDR, Berlin). The final ^{3}H-derivatives were purified by thin-layer chromatography (TLC) on silicagel (Merck 11845) or cellulose plates (Merck 5716) and by HPLC (Waters: pump 6000, injector U6K and UV detector 440). The catalysts Pd/Al_2O_3 (10%) and Pd/C were supplied by Engelhard (Rome). Tritium gas was made by CEA, Saclay. ^{3}H-scanning of TLC plates was performed with a Berthold Scanner II (FRG).

Albino male guinea pigs (~300 g), starved for 24 h were used for bioassays. Immunochemical reactivities of [^{3}H]SP and ^{3}H-analogues were measured with SP antibodies prepared by P. Pradelles [20] using a modification of the method in [21].

2.2. *Tritiation methods*

SP or analogues (0.5–1 μmol) were dissolved in 1 ml Na-phosphate buffer (0.1 M, pH 7) and solutions frozen. The catalyst (10–20 mg Pd/Al_2O_3) was then added and the reacting vial was connected to the tritium supplying automatic apparatus [22]. At 10^{-4} Torr, pure tritium gas (30–40 C) was introduced and compressed until 700–750 Torr. After thawing the reaction mixture was kept at 20°C and magnetically stirred for 20 min. The absorption of tritium gas produced a reduction of pressure of ~80–100 Torr. The catalyst was separated from the reacting solution by filtration over Millipore (Mitex, LSWP 01300) and labile tritium was eliminated by successive flash evaporations in dilute acetic acid (1%, v/v).

2.3. *^{3}H-Labelling by catalytic deshalogenation*

The procedure selected was similar to that above except for the nature and amount of the catalyst used. We have observed that the Pd/Charcoal catalyst used at concentrations slightly higher (40–50 mg) than for the tritiation method by exchange was less poisoned by halogen ions formed during the reaction.

3. Results

3.1. *Analytical identification of [^{3}H]SP and of [^{3}H]Nle–SP*

The first attempts to purify the [^{3}H]SP reaction mixtures were performed using TLC on cellulose, with *n*-butanol:acetic acid:water, 75:10:25 (v/v/v) as the eluent (4°C, elution time 8 h). The radiogram (autoradiography or scanning) revealed a main peak (R_F 0.42) comigrating with authentic SP and corresponding to the spot detectable with ninhydrin. In addition, 3 minor peaks (R_F 0.21, 0.59, 0.77) were seen. Although this procedure was analytically satisfactory the yield of peptide extraction by organic solvents from the cellulose layer was quite low and oxidation of the methionine residue could not be avoided. Amino acid analysis reflected the expected formula except from the methionine residue destroyed during hydrolysis. These drawbacks were overcome using high pressure liquid chromatography (HPLC) (μ C18 Bondapak column, 1 ml/min, methanol:water: N ammonium acetate, 1850:1350:30 (v/v/v) retention volume of authentic SP, 8 ml). Under these conditions SP oxidation was prevented. The labelled SP could be stored for 3 months in liquid nitrogen without appreciable degradation.

3.2. *Biological tests: Contraction of the isolated guinea pig ileum*

The isolated organ was suspended in an oxygenated tyrode solution (30 ml) at 37°C containing per ml: 0.1 μg atropine, 1.0 μg mepyramine and 0.1 μg methysergide.

Synthetic SP or Nle-SP were used as standards in a cumulative-dose assay [23,24]. The tritiated compounds behave exactly as authentic SP and Nle-SP (fig.1).

3.3. *Radioimmunoassays (RIA)*

RIA were performed as in [20] with a rabbit antiserum produced by coupling synthetic SP and ovalbumine with benzoquinone. The tracer used was ^{125}I-labelled Tyr–Gly–SP. After incubation (48 h at

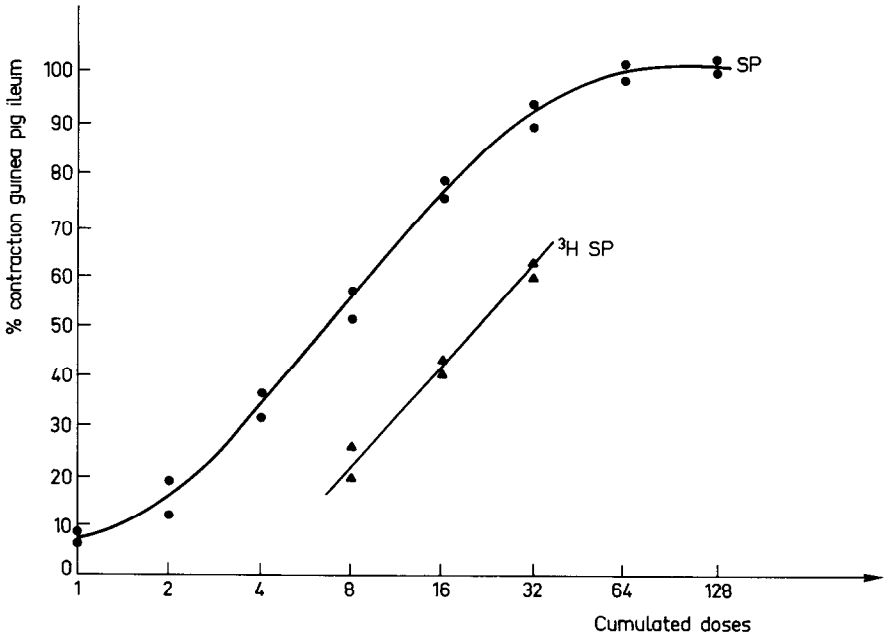

Fig.1. Concentration–response curves for SP and [^{3}H]SP tyrode bath 30 ml. SP added in cumulative doses of 15.6 × 10^{-9} g each. [^{3}H]SP solution, 15.4 × 10^4 dpm/dose.

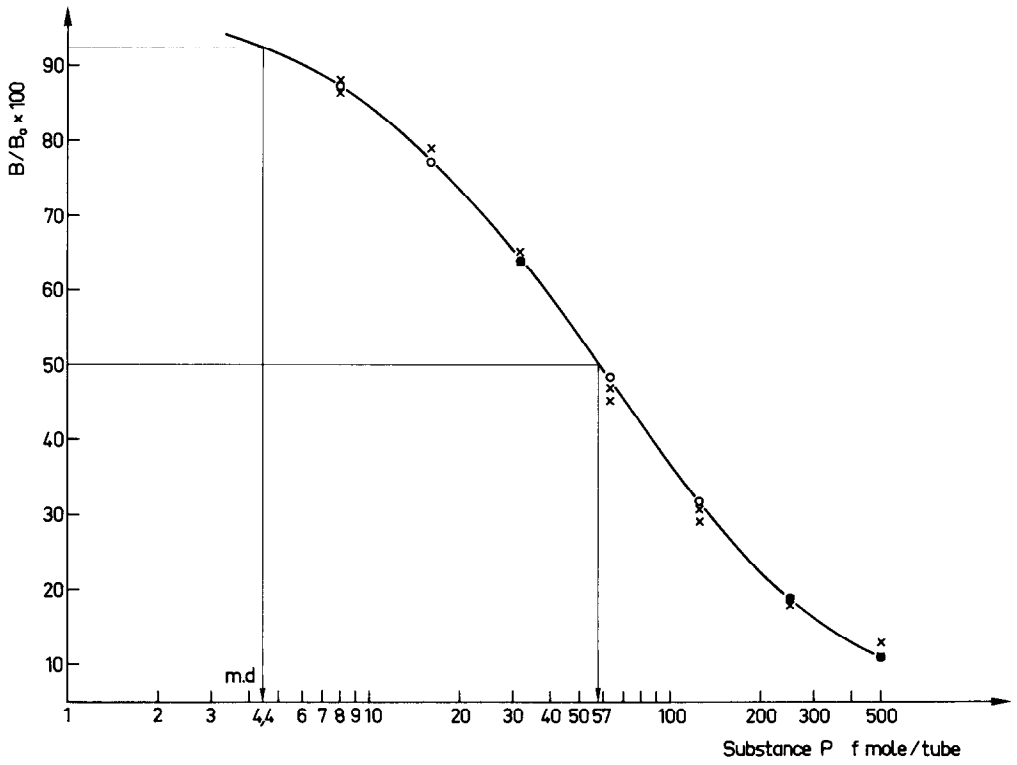

Fig.2. Radioimmunoassay curve for [^{3}H]SP (o——o) and for synthetic SP as standard (x——x); (m.d. minimum detectable amount).

4°C) of sample (0.1 ml) tracer (0.1 ml, 10 000 dpm) and diluted antiserum (0.1 ml, 1:10 000 final in 0.02 M phosphate buffer (pH 7.4), 0.5% BSA), the reacting mixture was precipitated by adding *n*-propanol (1 ml) and the pellets were then processed for γ-counting. The minimum detectable amount of SP was about 5 fmol and maximum binding was 40% with a half displacement observed with 50 fmol synthetic SP (fig.2). In this assay [^{3}H]SP of [^{3}H]Nle-SP behaved exactly as pure synthetic SP or Nle-SP. Nevertheless, it should be pointed out that the antiserum used exhibits an important crossreactivity with the C-terminal sequences of SP [20]. Bioassay and radioimmunoassays were consistent for the calculations of the specific radioactivities of the tritiated compounds ([^{3}H]SP, 15 Ci/mmol and 50 Ci/mmol; [^{3}H]Nle-SP, 25 Ci/mmol).

4. Discussion

In the binding study [25] a [^{3}H]SP of 187 Ci/mmol prepared by exchange was used. This technique usually leads to compounds of much lower specific radioactivities. In fact, in our own conditions SP tritiated by catalytic exchange had spec. radioact. 15 Ci/mmol. Howeve, deshalogenation of the dichloro-SP leads to a compound of 50 Ci/mmol whereas dichloro-derivative of the Nle analogue reached 25 Ci/mmol. A similar specific radioactivity for [^{3}H]Nle-SP has also been obtained [26]. In all cases indicated by bioassays as well as by radioimmunoassays the labelled materials were indistinguishable from the corresponding unlabelled peptides. Catalytic exchange of the aromatic protons, despite the presence of 2 phenylalanine residues consistently leads to lower specific radioactivities when compared to the results obtained after tritium substitution of an heteroatom. This could result from dilution of [^{3}H]SP by cold SP in the exchange procedure or by differences in the rates of exchanges of the aromatic protons and solvent protons at the surface of the catalyst. From our results, catalytic removal of

halogenatom(s) affords a convenient route for tritium labelling providing that the incorporation of *p*-chloro (or *p*-bromo) phenylalanine into the peptide is made during the peptide synthesis. The specific radioactivities of the products prepared should allow studies on SP metabolism but unfortunately according to the data [25], they seem to be too low for the characterization of the SP receptor(s).

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