1 L.J.D. Zaneveld, B.M. Dragoje and G.F.B. Schumacher, Science *177*, 702 (1972).
2 H. Schiessler, W.D. Schleuning and H. Fritz, in: Adv. Biosciences, Vol. 10, p. 271. Ed. G. Paspé and S. Bernharg. Pergamon Press Vieweg, Oxford 1973.
3 H. Sumi, K. Minakata, Y. Takada and A. Takada, J. physiol. Soc. Jap. *39*, 53 (1977) (in Japanese).
4 H. Sumi, Y. Takada and A. Takada, Thromb. Res. *11*, 747 (1977).
5 H. Sumi, N. Toki, Med. Biol. *96*, 323 (1978) (in Japanese).
6 H. Sumi, Med. Biol. *96*, 307 (1978) (in Japanese).
7 H. Sumi, Y. Takada and A. Takada, Abstracts of XVII Cong. Int. Soc. Hemat. *17*, 631 (1978).
8 H. Sumi, Y. Takada and A. Takada, J. biol. Soc. Jap. *49*, 941 (1977).
9 M. Muramatu and S. Fujii, Biochim. biophys. Acta *242*, 203 (1971).
10 H. Fritz, B. Forg-Brey and H. Umezawa, Hoppe-Seyler's Z. Physiol. Chem. *354*, 1304 (1973).
11 M. Dixon, Biochem. J. *55*, 170 (1955).
12 G.F.B. Schumacher and L.J.D. Zaneveld, in: Proteinase Inhibitors, p. 178. Ed. H. Fritz, H. Tschesche, L.J. Greene and E. Truscheit. Springer-Verlag, New York 1974.
13 O. Wallner, H. Fritz and K. Hochstrasser, in: Proteins and Related Subjects, vol. 23, Protides of the Biological Fluids, p. 177. Ed. H. Peters. Pergamon Press, Oxford 1976.
14 K. Hochstrasser, H. Feuth and K. Hochgesand, in: Proteinase Inhibitors, p. 111. Ed. H. Fritz, H. Tschesche, L.J. Greene and E. Truscheit, Springer-Verlag, New York 1974.

Synthesis and contractile activity of the C-terminal heptapeptide of substance P with N^5-dimethyl glutamine in the 6-position. Active site studies

C.P. Poulos, N. Pinas and D. Theodoropoulos[1]

Laboratory of Organic Chemistry, University of Patras, Patras (Greece), 3 December 1979

Summary. The synthesis and testing of [N^5-dimethyl-Gln^6]-SP_{5-11} showed 37 ± 12% contractile activity relative to SP, and intrinsic efficacy 98 ± 4%. This finding indicates that the carboxamide groups of the dual Gln^5-Gln^6 moiety are not equally related with the contractile response of the C-terminal heptapeptide of SP.

It is well known that the C-terminal tripeptide and tetrapeptide of substance P (SP) are inactive, while the pentapeptide has a weak spasmogenic effect on the guinea-pig ileum[2-5]. However, the hexapeptide and heptapeptide do not differ in their activity (on the guinea-pig ileum) from synthetic SP (SSP)[2-5]. In other words, the N-terminal increase of the C-terminal pentapeptide by glutaminyl residue(s) leads to the enchancement of biological activity. A feature common to the biologically active hexapeptide and heptapeptide is the presence of the hydrophilic side chain of the glutaminyl carboxamide group, at positions 5 and 6. Provided that the carboxamide groups at these positions are exposed and possess maximal structural freedom, they should be more available to the receptor. Recent evidence[6] indicates that a C-terminal heptapeptide analogue of SP, namely [N^5-dimethyl-Gln^5]-SP_{5-11} was found to exhibit only 25% activity relative to SP and about 30% antagonist action, when tested for contractile activity on the guinea-pig ileum; these effects were achieved by the mere change of the carboxamide hydrogens by methyl groups of the glutaminyl residue at position 5 of the hormone.

In the light of this finding and its significance for active-site studies on this hormone, it was decided to investigate whether the same modification of the glutaminyl residue at position 6 would lead to identical or different biological response. Thus, the synthesis of [N^5-dimethyl-Gln^6]-SP_{5-11} (figure) was performed by stepwise solution techniques from H-Phe-Phe-Gly-OBzl[7]. Boc-Glu [$N(CH_3)_2$]-OH[6,8] was preactivated with DCC and 1-hydroxy-benzotriazole[9] and coupled to the tetrapeptide ester to give Boc-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-OBzl, m.p. 128–130 °C; $[\alpha]_D^{20}$ −18.61° (c 0.5, DMF). Thereafter, elongation of the peptide chain, using the same coupling process, afforded Boc-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-OBzl, m.p. 212–214 °C; $[\alpha]_D^{20}$ −23.53° (c 0.5, DMF). Catalytic hydrogenation of the latter produced the acid, Boc-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-OH, m.p. 193–195 °C; $[\alpha]_D^{20}$ −23.2° (c 0.5, DMF), which was coupled with H-Leu-Met-NH_2[10] in the same manner as above. The resulting heptapeptide derivative, Boc-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-Leu-Met-NH_2, m.p. 216–219 °C; $[\alpha]_D^{20}$ −40.89° (c 0.5, DMF), was deprotected with CF_3COOH in the presence of anisole and was permitted to remain in ethanol solution containing thioglycolic acid for 24 h at room temperature. Following the desalting of the product (50 mg) by ion-exchange chromatography (Dowex-1X8) with ethanol as the eluent, purification was attained by gel filtration on Sephadex G-15 (80 × 2.5 cm) with 2 M acetic acid (elution volume 242 ml). At a flow rate of 21 ml/h fractions of 7 ml were collected (32–39) containing the major peak of peptide material and lyophilized; yield 38 mg. A portion (20 mg) was finally purified by partition chromatography on a column (76 × 1.5 cm) of Sephadex G-25 equilibrated with n-BuOH-AcOH-H_2O (4:1:5). The peptide was eluted with the upper phase at a flow rate of 6 ml/h. Fractions of 2 ml were collected (30–38) and lyophilized to give homogenous product (15 mg): m.p. 232–237 °C; $[\alpha]_D^{20}$ −58.5° (c 0.18, DMF); TLC showed a single spot with R_f 0.39 in n-BuOH-AcOH-H_2O (4:1:1), R_f 0.72 in n-BuOH-AcOH-H_2O-pyridine (30:6:24:20) and R_f 0.34 in n-BuOH-AcOH-H_2O (4:1:5, upper phase). Amino acid analysis gave the following molar ratios: Glu, 2.05; Phe, 2.01; Gly, 1.00; Leu, 1.03; Met, 0.89; NH_3, 1.99. Elemental analysis gave the following values: $C_{43}H_{64}N_{10}O_9S$ calculated. C 57.57; H, 7.19; N, 15.61; found C, 57.62; H, 7.24; N, 15.39.

Biological test[1]. Terminal ileum (2 cm) of guinea-pigs (250–300 g) was incubated in a 5-ml bath for 20 min before testing. The bath solution was composed of 9 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.1 g $MgCl_2$, 1 g glucose, 0.12 g tris-(hydroxy-methyl)-aminomethane per 1000 ml distilled water while the temperature was kept at 37 °C and the pH at 7.4. Substances were dissolved in distilled water, diluted

a H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
1 2 3 4 5 6 7 8 9 10 11

b H-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-Leu-Met-NH_2
5 6 7 8 9 10 11

a Amino acid sequence of substance P (SP). *b* [N^5-dimethyl-Gln^6] C-terminal heptapeptide of SP; numbers indicate sequence positions of individual residues. Amino acids, with the exception of glycine, are of the L-configuration.

with physiological solution and injected in the bath solution in a volume of 0.1 ml every 4 min. Isotonic contractions were registered for 30 sec. After this time the bath solution was changed. Dose effect curves of substance P and of the test substance were registrated on each organ.

Results and discussion. In each experiment the ED_{50}, the maximal contraction and the activity relative to SP (set at 100) were estimated. The ED_{50} SP was $1.9 \pm 0.6 \cdot 10^{-8}$ M, against $5.1 \pm 1.5 \cdot 10^{-8}$ M for the analogue. Thus the relative activity of the analogue was found to be $37 \pm 12\%$ and its maximal contraction $98 \pm 4\%$. The identical intrinsic efficacy of [N^5-dimethyl-Gln^6]-SP_{5-11} suggests that alkyl groups on the N^5 of the Gln^6 residue do not interfere with an active element. On the other hand, the carbonyl of the $CONH_2$ portion of the glutaminyl residue in position 5 appears to be an improvable factor for biological response[6], since methylation either causes a detrimental orientation of the active element or inhibits the interaction with the receptor by a steric effect. The observed distinction of the carbonyl groups of the dual Gln^5-Gln^6-moiety in the heptapeptide may allow a further characterization of the particular binding sites and active elements involved in recognition and activation of the specific receptor(s).

1 The authors wish to express their deep appreciation to Professor H. Niedrich and Dr J. Bergmann of the Institute of Drug Research, Academy of Sciences of DDR, Berlin, for their valuable help in providing the biological data.
2 J. Bergmann, M. Bienert, H. Niedrich, B. Mehlis and P. Oehme, Experientia *30*, 401 (1974).
3 R. Bury and M. Mashford, J. med. Chem. *19*, 854 (1976).
4 K. Kitagawa, K. Ujita, Y. Kiso, T. Acita, Y. Nakata, N. Nakamoto, T. Segawa and H. Yajima, Chem. pharm. Bull. *27*, 48 (1979).
5 N. Yanaihara, C. Yanaihara, M. Hirohashi, H. Sato, Y. Iizuka, T. Hashimoto and M. Sakagami, Nobel Symposium 37 (SP) p.27. U.S. von Euler and B. Pernow. Raven Press, New York 1977.
6 N. Pinas, C.P. Poulos and D. Theodoropoulos, FEBS Lett. *108*, 45 (1979).
7 H.-D. Jakubke, Ch. Klessen and K. Neubert, J. prakt. Chem. *319*, 640 (1977).
8 G.L. Stahl, W. Smith, R. Walter, Th. Tsegenidis, G. Stavropoulos and D. Theodoropoulos, J. med. Chem., in press (1980).
9 G. Jager, W. Koning, H. Wisman and R. Geiger, Chem. Ber. *107*, 215 (1974).
10 H-Leu-Met-NH_2 was obtained by deprotection of Boc-Leu-Met-NH_2; B. Mehlis, H. Apelt, J. Bergmann and H. Niedrich, J. prakt. Chem. *314*, 390 (1972).

Brain catecholamines and sleep states in offspring of caffeine-treated rats

M. Enslen, H. Milon and H.P. Würzner

Nestlé Products Technical Assistance Co. Ltd, Biological Experimentation Services, CH-1350 Orbe (Switzerland), 13 December 1979

Summary. Caffeine was administered in the diet to rats throughout gestation. In the 2 consecutive untreated generations, an increase of parodoxical sleep was observed at maturity. In the 1st generation, the dopamine level was markedly reduced in the locus coeruleus, whereas that of noradrenaline remained constant. The effect was less pronounced in the 2nd generation.

Caffeine, probably the most widely consumed alkaloid, is a central nervous system (CNS) stimulant and acts principally on the brain cortex[1,2]. Kinetic studies have shown that it is very rapidly distributed in the body[3] and via the placenta is transferred to the foetus[4,5]. It also freely passes the blood-brain barrier[6].

Since it has been reported that chlorpromazine[7] and amphetamine[8] induce changes in behaviour[7] and catecholamine metabolism[7,8] in offspring of treated rats, we were interested to investigate possible transmissible effects of caffeine in the CNS. Therefore, caffeine was administered in the diet to rats during their entire gestation. The sleep patterns were analyzed in the offspring of the 1st and 2nd generation. Catecholamines were determined in discrete brain nuclei which it has been suggested play a role in the regulation of sleep[9].

Material and methods. A total of 120 male and female Sprague-Dawley rats (Iffa Credo - St-Germain-sur-Arbresle, France), weighing 235-240 g, were mated 1 male and 2 females per cage. The mating period was terminated after 48 h. The females were then randomly assigned to 4 groups, in which group A was the control and the test groups received diets containing caffeine at 0.0125% for group B, 0.025% for group C and 0.1% for group D admixed in a commercial standard diet (Nafag 850, Gossau, Switzerland). These diets were given ad libitum with tap water during gestation only.

In the 1st generation (F_1), due to the large number of animals, only females were studied. To obtain the 2nd generation (F_2), adult F_1 females of groups A and D were mated with males of group A. Both adult male and female rats of this 2nd generation were studied.

Electrodes were implanted in the cerebral cortex and neck muscles and sleep pattern parameters were measured in 14 rats per group of the 1st generation and 10 rats per group of the 2nd generation when at a weight of 240 g. Recordings of sleep states were performed after an adaptation period of at least 20 days in an airconditioned, sound-proof room (22 ± 1 °C, $50 \pm 5\%$ relative humidity). A light-dark cycle of 12 h was maintained, starting at 07.00 h. Waking (W), slow wave sleep (SWS) and paradoxical sleep (PS) were estimated by a double blind visual reading of the cortical electroencephalogram and the electromyogram from the neck muscles over a period of 7 h (from 08.30 to 15.30 h). PS and SWS phases were considered and counted if they lasted more than 10 and 30 sec, respectively.

Each locus coeruleus of 5 animals from each group was excized according to the method of Palkovits[10]. It was then homogenized in 100 µl n-butanol-HCl. The amine derivatives were formed in 20 µl of trifluoroacetic anhydride and acetonitrile (1:1). The derivatives were analyzed by a mass-fragmentographic method, using deuterated analogues as internal standards[11].

Results. The average daily caffeine intake per rat was 0, 2.9 ± 0.1, 5.7 ± 0.1 and 21.5 ± 0.5 mg for groups A, B, C and D, respectively. There were no significant differences in the number of pups per litter, the sex ratio per litter, the mortality or body weight gain of the pups. However, the