

## **Dissociation of the behavioural and endocrine effects of lysine vasopressin by tryptic digestion**

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### **Summary**

1. Lysine vasopressin induces resistance to extinction of active avoidance behaviour (De Wied, 1971).
2. Digestion of lysine vasopressin with trypsin almost completely destroys the pressor-, antidiuretic-, oxytocic- and corticotrophin-releasing factor activities of lysine vasopressin, but does not materially influence its effect on the maintenance of an avoidance response.

### **Introduction**

Recent studies on the purification of pituitary peptides from hog pituitaries, capable of restoring the deficient learning behaviour of hypophysectomized rats, led to the isolation of (8-lysine)-de-9-glycinamide-vasopressin (DG-LVP) (Lande, Witter & De Wied, 1971). Since the posterior pituitary is implicated in the maintenance of a conditioned avoidance response (De Wied, 1965), and pitressin as well as lysine vasopressin (LVP) exerts a 'long term' effect on the maintenance of an avoidance response (De Wied & Bohus, 1966; De Wied, 1971), it was deemed of interest to study the effect of DG-LVP as compared to LVP on extinction of an avoidance response, and in addition to investigate the fate of the known biological activities of LVP after tryptic digestion.

### **Methods**

#### *Tryptic digestion of LVP*

DG-LVP was prepared from synthetic LVP (60 u/mg) (Ferring AB batch no. 12865) by tryptic digestion, since it is well known that this results in a specific cleavage of the C-terminal glycinamide residue (Lawler & du Vigneaud, 1953; du Vigneaud, Lawler & Popenoe, 1953). Paper chromatography of this synthetic LVP preparation demonstrated the presence of an immobile spot ( $R_f=0$ ), together with LVP ( $R_f=0.41$ ). Diphenyl carbamyl chloride (DDC)-treated trypsin (1 mg) was dissolved in 0.001 N HCl (0.1 ml) and incubated for 3 h at room temperature. From this solution, 50  $\mu$ l was added to a solution of 50 mg of LVP in 0.2 M ammonium acetate buffer at pH 7.8 (5 ml). The mixture was incubated for 16 h at 38° C and subsequently acidified to pH 4 with 0.5 ml acetic acid and centrifuged

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for 20 min at 10,000 *g* and 4° C; the precipitate (13.3 mg) was discarded. The clear supernatant was subjected to gel filtration on a 60 × 1 cm Sephadex G10 column. Elution was carried out with 0.1 M acetic acid at 10 ml/h and the extinction at 280 m $\mu$  was continuously recorded. Three peaks were obtained. The first peak contained ninhydrin positive material with  $R_f=0$  and  $R_f=0-0.09$ , which was also present in the original synthetic LVP. The second peak contained DG-LVP, as judged by its behaviour in paper electrophoresis and paper chromatography, and the third peak was negative to ninhydrin. The second peak was lyophilized (24.3 mg). Final purification was carried out by preparative paper chromatography in a solvent system of *n*-butanol:acetic acid:pyridine:water (15:3:10:24, by volume). A small spot with  $R_f=0$  was present, together with glycinamide ( $R_f=0.28$ ) and DG-LVP ( $R_f=0.43$ ). The DG-LVP spot was eluted and lyophilized (10.2 mg). Purity control was carried out by paper electrophoresis in a solvent system of pyridine:acetic acid:water (50:1:950, by volume) buffer pH 6.5 at 2,000 V for 1 h, which allows unequivocal identification of LVP and DG-LVP. The product so obtained showed the same analytical specifications and pharmacological activity as a synthetic preparation obtained by reduction of *s*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-s-benzyl-L-cysteinyl-L-prolyl-*N*-tosyl-L-lysine with sodium in liquid ammonia, followed by oxidative ring closure and isolation by countercurrent distribution (solvent system 2-butanol:0.1% acetic acid; 1:1) in 400 steps,  $r_{max}=44$ ,  $K=0.13$  and gel filtration over Biogel P6 in 0.1% acetic acid,  $V_e/V_o=3.7$ . Optical rotation, corrected for free peptide content, was  $[\alpha]_D-20.6^\circ$  ( $c=0.64$  in acetic acid:water, 1:9, by volume); amino acid analysis: aspartic acid, 1.02; glutamic acid, 1.04; proline, 1.00; cysteine, 1.96; tyrosine, 0.93; phenylalanine, 1.04 and lysine, 1.01.

#### *Conditioned avoidance behaviour*

The influence of DG-LVP as compared to LVP on the maintenance of an avoidance response was studied in male white rats weighing 120–140 grammes.

Avoidance conditioning was studied in a pole jumping test (De Wied, 1966). The conditioned stimulus (CS) was a light produced by a 40 W bulb placed on top of the box. The CS was presented 5 s before the unconditioned stimulus (US) of shock delivered through the grid floor. Animals could avoid the US by jumping on to the pole within the CS–US interval. Ten acquisition trials were given each day for three consecutive days. Animals which made more than 10 avoidances in 30 conditioning trials were injected subcutaneously with either of the two peptides, in various doses, immediately after the last of the acquisition trials. Extinction sessions during which the unconditioned stimulus was withheld were run 1, 2, 5 and 12 days after injection.

#### **Results**

As can be seen from Table 1, both peptides delay extinction of the avoidance response. The effect is dose-dependent. The highest dose of each maintained the avoidance response for at least 12 days. On a weight basis DG-LVP is approximately twice as active as the synthetic LVP preparation but the latter only contained 60 pressor units per milligramme. The purest synthetic lysine vasopressin preparations contain approximately 285 pressor units per milligramme. Accordingly, the DG-LVP is nearly half as potent as LVP. Calculation of potency of

the behavioural effect of DG-LVP against LVP from the results presented in Table 1, indicated that the latter had retained an activity of 106 u/mg (95% fiducial limits 101–108).

The pressor-, antidiuretic-, oxytocic- and corticotrophin-releasing factor activities of DG-LVP were subsequently investigated. The pressor activity was determined in the phenoxybenzamine-pretreated rat under urethane anaesthesia (Dekanski, 1952) by a 2+2 point assay.

The antidiuretic activity was measured in the alcohol-anaesthetized rat (De Wied, 1960). The oxytocic activity was determined *in vitro* on the uterus horn of an oestradiol (100 µg)-treated rat. Corticotrophin-releasing factor activity was measured in rats under pentobarbital anaesthesia treated with chlorpromazine (De Wied, Witter, Versteeg & Mulder, 1969). These three activities were determined by a 2+1 point assay.

The results, summarized in Table 2, are expressed in terms of potency of the standard preparations used. It is clear that the pressor-, antidiuretic-, oxytocic- and corticotrophin-releasing factor activities are almost completely destroyed by the removal of the glycinamide residue from LVP as a result of tryptic digestion.

## Discussion

Pitressin and LVP have been shown to facilitate retention of an avoidance response (De Wied & Bohus, 1966; De Wied, 1971). The present experiments indicate that tryptic digestion of LVP affects only slightly the influence of this nonapeptide on the retention of conditioned avoidance performance. In contrast, tryptic digestion destroys nearly all of the classical endocrine activities of LVP. This

TABLE 1. *Effect of a single injection of lysine vasopressin (LVP) or desglycinamide lysine vasopressin (DG-LVP) on the rate of extinction of a pole jumping avoidance response*

Treatment	Acquisition (30 trials)	Number of avoidances			
		Extinction sessions (10 trials each) (hours after injection)			
		24	48	120	268
LVP 12 mU s.c.	17±1.1*	9±0.5	2±0.6	—	— (4)
LVP 36 mU s.c.	15±0.3	8±0.0	6±0.5	5±0.3	5±0.3 (4)
LVP 108 mU s.c.	17±0.3	9±0.3	9±0.5	8±0.3	8±0.0 (4)
DG-LVP 0.1 µg s.c.	17±1.0	8±0.0	2±0.6	—	— (4)
DG-LVP 0.3 µg s.c.	15±0.4	8±0.3	5±0.3	6±0.5	5±0.0 (4)
DG-LVP 0.9 µg s.c.	15±0.7	9±0.5	10±0.3	10±0.3	9±0.5 (4)

\* Mean ± standard error of the mean. ( ) Number of animals used.

TABLE 2. *Pressor-, antidiuretic-, oxytocic- and corticotrophin releasing factor activity of desglycinamide lysine vasopressin*

	Potency u/mg†	95% Fiducial limits	Potency of synthetic LVP u/mg
Pressor activity	0.87	0.69–1.17	285*
Antidiuretic activity	2.60	1.07–6.31	260*
Oxytocic activity	0.16	0.13–0.20	5*
CRF activity	0.67	0.36–1.63	235‡

† Expressed in units of standard preparation; synthetic lysine vasopressin (60 u/mg) was used as a standard in assays 1, 2 and 4; synthetic oxytocin (Piton S) as the standard in assay 3. \* Berde & Boissonnas, 1968. ‡ Doepfner, Stürmer & Berde, 1963.

is in accord with findings of du Vigneaud *et al.* (1953) with respect to the oxytocic and pressor activity. The recent isolation of DG-LVP from hog pituitaries (Lande *et al.*, 1971) and its ability to restore the behavioural competence of hypophysectomized rats again reveals the importance of neurohypophysial and related hormones in the formation and maintenance of new behavioural patterns.

DG-LVP may be present as such in the pituitary, although the possibility that it represents an artefact of isolation cannot be excluded. It is also possible that it is formed from vasopressin in the circulation or in tissues by specific enzymes; part of it may then penetrate the brain and affect structures involved in the formation and maintenance of adaptive behaviour. Enzymes capable of releasing glycinamide from LVP include trypsin and also plasmin, as might be deduced from data on the proteolytic specificity of the latter (Groskopf, Hsieh, Summari & Robbins, 1968).

In this respect it is of interest to note that Glass, Schwartz & Walter (1969) found an enzyme in the kidney of rats which releases glycinamide from vasopressin and oxytocin; and a similar enzyme, more specific for oxytocin, in the uterus (Glass, Dubois, Schwartz & Walter, 1970). Accordingly, specific tissues may contain enzymes which destroy the target effect of LVP. Specific enzymatic release of the C-terminal tripeptide L-prolyl-L-leucylglycinamide (MSH-R-IF) of oxytocin by a microsomal preparation from stalk median eminence tissue (Celis, Taleisnik & Walter, 1971) also points to a possible dual function of neurohypophysial hormones: (1) as hormones with peripheral target organs (2) as 'prohormones' for oligopeptides, involved in regulation of central nervous processes. This may hold for the brain and/or the pituitary as well. However that may be, LVP and DG-LVP are unique in their 'long-term' influence on the maintenance of an avoidance response. Since DG-LVP lacks nearly all classical endocrine activities of vasopressin, this peptide has a great potential for further studies concerned with the underlying mechanism(s) of the retention of behavioural responses.

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