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# DECREASE OF SUBSTANCE P IN PRIMARY AFFERENT NEURONES AND IMPAIRMENT OF NEUROGENIC PLASMA EXTRAVASATION BY CAPSAICIN

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**1** Rats were pretreated with capsaicin (50 mg/kg, s.c.) on the 2nd, 10th, or 20th day of life. Three months later immunoreactive substance P (I-SP) was determined in skin, sensory nerves and the central nervous system. Neurogenic plasma extravasation was also examined.

**2** Pretreatment at the age of 2 or 10 days resulted in a decrease (26 to 69%) of I-SP in skin, saphenous and vagus nerve, dorsal roots, dorsal half of the spinal cord, and medulla oblongata. The I-SP content of the ventral half of the spinal cord, of midbrain, hypothalamus, striatum, cortex, and cerebellum remained unchanged. Neurogenic plasma extravasation was inhibited by more than 80%.

**3** In contrast to this irreversible effect of capsaicin on newborn rats, pretreatment of 20 day old rats led to reversible depletion of I-SP and to reversible impairment of neurogenic plasma extravasation.

**4** Capsaicin pretreatment of adult rats caused a marked depletion of I-SP in the skin of the hind paw and an impairment of neurogenic plasma extravasation. A similar decrease of I-SP was seen after chronic denervation of the skin.

**5** Intra-arterial infusion of substance P (threshold dose $5 \times 10^{-13}$ mol/min) or physalaemin induced dose-dependent plasma extravasation. Somatostatin, vasoactive intestinal polypeptide, caerulein and the enkephalin-analogue FK 33-824 were ineffective in doses 100 fold higher.

**6** The results indicate that the action of capsaicin on substance P neurones is restricted to primary sensory neurones. Since in every case a decreased substance P content of the skin was associated with impaired neurogenic plasma extravasation, it is suggested that release of substance P is involved in neurogenic plasma extravasation.

## Introduction

Cutaneous application of chemical irritants like capsaicin or mustard oil causes stimulation of chemosensitive pain receptors and plasma extravasation (Jancsó, Jancsó-Gábor & Szolcsányi, 1967). This plasma extravasation can also be evoked by antidromic nerve stimulation and is due to release from sensory nerves of a neurogenic factor (Jancsó *et al.*, 1967). The nature of this factor is still unknown. The initial stimulation of pain fibres by capsaicin is followed by insensitivity. Systemic capsaicin pretreatment of adult rats leads to a similar insensitivity, which is associated with a decrease of microvesicles in sensory nerve endings (Szolcsányi, Jancsó-Gâbor & Joó, 1975). In contrast, capsaicin pretreatment of newborn rats results in irreversible degeneration of B-type neurones and their axon terminals (Jancsó, Kiraly & Jancsó-Gábor, 1977).

Substance P is present in a population of unmyelinated primary sensory neurones, terminating in the skin and in the substantia gelatinosa of the spinal cord (Hökfelt, Kellerth, Nilsson & Pernow, 1975; Cuello, Polak & Pearse, 1976; Cuello, Del Fiacco & Paxinos, 1978). Thus the distribution of substance P neurones and that of capsaicin-sensitive neurones is similar. Substance P can be released by antidromic nerve stimulation from sensory nerves (Olgart, Gazelius, Brodin & Nilsson, 1977) and it can induce plasma extravasation (Lembeck, Gamse & Juan, 1977). This led us to investigate the effect of capsaicin pretreatment at various ages on the substance P content of the peripheral and central nervous system. In parallel experiments, neurogenic plasma extravasation was examined. A capsaicin-induced decrease of substance P was described by Gasparović, Hadzović,

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Huković & Stern (1964) and by Jessell, Iversen & Cuello (1978). In addition some of the peptides present in peripheral nerves were infused intra-arterially into the hind legs of rats to see whether they induced plasma extravasation.

## Methods

### *Animals*

Sprague Dawley rats (strain OFA, SD) of either sex were used and reared under controlled temperature and lighting conditions. The young rats were separated from their mother at the age of 6 weeks.

### *Capsaicin pretreatment*

Capsaicin solutions were prepared according to Janscó *et al.* (1967): 50 mg/kg capsaicin or solvent was injected subcutaneously into 2, 10, 15, or 20 day old rats. The injection volume did not exceed 0.1 ml. The capsaicin injection was followed by severe impairment of respiration. Respiration had to be assisted manually for up to 5 min in 2 day old rats. Adult rats (200 to 250 g) were pretreated with 50 mg/kg capsaicin (10 mg/ml, s.c.) on two consecutive days; control rats received solvent only.

### *Neurogenic plasma extravasation*

Unless otherwise stated, rats were tested 3 months after pretreatment. Under pentobarbitone anaesthesia (50 mg/kg, i.p.) the trachea was cannulated. Rectal temperature was monitored and kept at 37°C by a heating lamp.

In one set of experiments, the saphenous nerves were carefully exposed, cut in the thigh and the distal ends were placed on bipolar platinum electrodes. The nerves were covered with paraffin oil. Thirty min after the electrodes had been placed in position, Evans Blue (50 mg/kg i.v.) was injected and 5 min afterwards one nerve was stimulated for 15 min (2V, 10 ms, 20 Hz). At the end of stimulation the rats were killed by bleeding and the skin area innervated by the saphenous nerve was removed.

In the other set of experiments the trachea was cannulated and Evans Blue was injected as described above; 5 min later the dorsal side of one hind paw was painted with 5% (w/w) mustard oil in liquid paraffin (Jancsó *et al.*, 1967). Paraffin oil alone was applied to the control paw. Painting was repeated every 5 min, and 15 min after the first application the rats were killed and the skin of the dorsal paw side was removed.

Extravasated Evans Blue in the skin was extracted with 4 ml formamide for 24 h at 60°C for colorimetric determination at 620 nm. The amount of Evans Blue exceeding that of the control paw was used to quantify neurogenic plasma extravasation.

### *Intra-arterial infusion into the hind paw*

Rats were anaesthetized with pentobarbitone (50 mg/kg, i.p.) and the superficial epigastric artery was canulated for retrograde infusion. Evans Blue was injected intravenously 15 min later. After another 5 min, infusion into the femoral artery was started at a rate of 0.06 ml/min for 5 min. The peptides were dissolved in isotonic phosphate buffer pH 7.4, containing 1 g/l gelatine and 25 mg/l Cialit. Solvent only was infused into control rats. Extravasated Evans Blue was extracted as above. The amount of Evans Blue exceeding that of control rats was used to measure plasma extravasation caused by the substances infused.

### *Chronic denervation of the skin*

The saphenous nerve of one side was cut in the thigh under pentobarbitone anaesthesia (50 mg/kg, i.p.). Eight days later, the skin area innervated by the nerve was removed from the denervated and the control paw. The skin of 5 rats was pooled and used for determination of substance P.

### *Determination of substance P*

Rats were killed by a blow on the neck. The brain was removed and chilled on ice. Brain regions were dissected on an ice-cold steel plate according to Glowinski & Iversen (1966), except that the hippocampus was not isolated. Six regions were separated: cerebellum, medulla oblongata, hypothalamus, striatum, midbrain, and cortex. They were immediately frozen on dry ice. The lumbar spinal cord was removed, chilled and the dorsal roots were dissected. The spinal cord was then frozen on dry ice and cut into thin slices which were divided into a dorsal and ventral half. Finally, the vagus nerve of the neck, the saphenous nerve and the skin of the dorsal side of the hind paw were removed. The whole dissection did not take more than 20 min.

The dorsal roots and the nerves were extracted by boiling in 0.01 N HCl as described by Gamse, Lembeck & Cuello (1979). Tissue from brain and spinal cord was homogenized by sonification in 10 volumes (w/v) of 0.1 N HCl, boiled for 10 min and centrifuged. The pellet was washed with 1 ml 0.01 N HCl and the combined supernatants were lyophilized. By this method of extraction 74 $\pm$ 1.3% ($n$ = 3) of 1.2 ng synthetic substance P could be recovered. Chromatography on Sephadex G-25 of an extract from the medulla oblongata showed a single peak of immuno-

reactivity, co-chromatographing with synthetic substance P.

The skin was pulverized in liquid nitrogen and extracted with acetone-HCC, according to Chang & Leeman (1970). Recovery of synthetic substance P by this method was more than 95%. Substance P was measured with a sensitive and specific radioimmunoassay, as described by Gamse *et al.* (1979). The minimum detection limit was 25 pg substance P per sample. The antibody showed less than 0.01% cross-reactivity for any of the known neuropeptides. The term 'immunoreactive substance P' (I–SP) is used for substance P measured by radioimmunoassay. The values of I–SP were not corrected for loss of substance P during extraction and are given in ng I–SP per g wet wt.

*Substances*

The following substances were used: capsaicin (Merck, Darmstadt); caerulein (Takus, Farmitalia, Milano); Cialit (2-ethyl-mercury-mercapto-benzoxazol-5-Na-carbonic acid, Asid, München); FK 33-824 (D-ala$^2$-mephe$^4$-met(O)$^5$-ol-enkephalin, Sandoz, Basel); physalaemin (Farmitalia, Milano); somatostatin and substance P (Peninsula, San Carlos); vasoactive intestinal polypeptide (gift of Dr V. Mutt, Stockholm).

## Results

*Capsaicin pretreatment of 2 and 10 day old rats*

*Effect on I–SP in the peripheral nervous system* The animals did not show obvious changes in their appearance, behaviour or motility. Three months after pretreatment, I–SP was markedly reduced in the skin of the hind paw (Table 1). A similar decrease of the I–SP content was found in the saphenous and vagus nerve and in the dorsal roots. In rats pretreated on the 2nd day of life, the % depletion of I–SP was greater than in rats pretreated on the 10th day (Table 1).

*Effect on I–SP in the central nervous system* A decrease of I–SP in the dorsal half of the spinal cord was found in both groups of animals (Table 1). In rats pretreated on the 2nd day of life, the decrease of I–SP in the dorsal roots exceeded that of the dorsal half of the spinal cord. In contrast to the dorsal half, the I–SP content of the ventral half of the spinal cord was not different from that of control animals. In the medulla oblongata, capsaicin pretreatment led to a small, but significant decrease of I–SP. Pretreatment had no effect on I–SP levels in midbrain, hypothalamus, striatum, cortex, and cerebellum (Table 1).

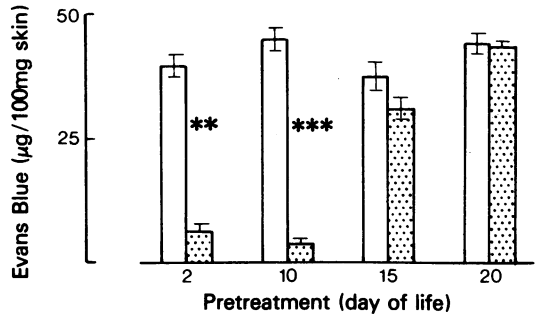


**Figure 1** Evans Blue content (µg/100 mg) of the skin of the rat hind paw painted with 5% mustard oil 3 months after pretreatment with capsaicin at the ages indicated. Open columns: controls; stippled columns capsaicin-treated. Values are mean ± s.e. mean, $n = 6$. ** $P < 0.01$; *** $P < 0.001$.

*Effect on plasma extravasation* Neurogenic plasma extravasation was tested 3 months after capsaicin pretreatment. In agreement with Jancsó *et al.* (1977), the reaction to mustard oil was reduced by 84% ($n = 6$) in rats pretreated on the 2nd day of life (Figure 1). In the same group of animals, neurogenic plasma extravasation induced by antidromic nerve stimulation was inhibited by 97% ($n = 6$). This shows that the impairment of neurogenic plasma extravasation achieved by neonatal capsaicin pretreatment can be measured by electrical, as well as by chemical (mustard oil) nerve stimulation. Mustard oil was used for all further experiments. Capsaicin pretreatment on the 10th day of life caused a somewhat, but not significantly, greater impairment of neurogenic plasma extravasation than the pretreatment on the 2nd day of life (Figure 1).

*Capsaicin pretreatment of 15 and 20 day old rats*

Neurogenic plasma extravasation in rats, 3 months after pretreatment with capsaicin on the 15th or 20th day of life was not different from that in control rats. (Figure 1).

When tested 4 days after capsaicin pretreatment on the 20th day of life, the I–SP in the skin was decreased by 58% ($n = 6$, $P < 0.001$) and neurogenic plasma extravasation was reduced by 77% ($n = 6$, $P < 0.001$) (Figure 2). Also 11 days after pretreatment, neurogenic plasma extravasation was reduced by 40%, whereas no difference from control animals was seen 3 months after pretreatment (Figure 2). At this time the I–SP content of the skin, as well as of the saphenous and vagus nerves did not differ from that of controls (Table 1). However, in the dorsal roots, the dorsal half of the spinal cord and in the medulla oblongata, the I–SP content remained significantly

**Table 1** Substance P content (ng/g wet wt.) of skin, some peripheral nerves and central nervous system of rats, 3 months after pretreatment with capsaicin (50 mg/kg, s.c.) on the 2nd, 10th or 20th day of life

| | 2nd day | | | | | 10th day | | | | | 20th day | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Control* | n | *Capsaicin* | n | %† | *Control* | n | *Capsaicin* | n | %† | *Control* | n | *Capsaicin* | n | %† |
| Skin | 1.91 ± 0.51 | 6 | 0.61 ± 0.10* | 8 | 68 | 1.90 ± 0.18 | 5 | 1.06 ± 0.13** | 5 | 45 | 1.86 ± 0.22 | 7 | 1.79 ± 0.14 | 8 | 4 |
| Vagus nerve | 51.0 ± 5.8 | 6 | 19.6 ± 3.5*** | 5 | 62 | 76.6 ± 6.2 | 5 | 41.6 ± 6.1** | 5 | 44 | 59.6 ± 5.8 | 7 | 63.5 ± 6.8 | 5 | — |
| Saphenous nerve | 55.6 ± 10.8 | 6 | 19.2 ± 3.6** | 6 | 65 | 63.5 ± 7.2 | 7 | 40.5 ± 7.1* | 5 | 36 | 48.9 ± 7.8 | 7 | 45.1 ± 4.6 | 4 | 8 |
| Dorsal roots | 24.5 ± 1.1 | 5 | 7.6 ± 0.7*** | 6 | 69 | 21.0 ± 1.3 | 5 | 11.0 ± 0.7*** | 5 | 48 | 19.9 ± 0.8 | 6 | 14.0 ± 1.0** | 6 | 30 |
| Spinal cord Dorsal | 242 ± 17 | 6 | 115 ± 19*** | 6 | 52 | 223 ± 14 | 4 | 130 ± 8.5*** | 5 | 42 | 240 ± 4.0 | 6 | 165 ± 7.2*** | 6 | 31 |
| Spinal cord Ventral | 53.5 ± 6.3 | 6 | 49.5 ± 5.3 | 4 | 7 | 50.0 ± 1.9 | 5 | 57.7 ± 3.0 | 5 | — | 51.3 ± 4.0 | 4 | 57.9 ± 4.2 | 6 | — |
| Medulla obl. | 139 ± 5.1 | 4 | 103 ± 3.5*** | 6 | 16 | 129 ± 2.2 | 5 | 107 ± 5.1** | 5 | 17 | 137 ± 4.2 | 4 | 111 ± 4.4** | 7 | 9 |
| Midbrain | 133 ± 12.9 | 6 | 128 ± 11.8 | 5 | 5 | 165 ± 10.8 | 4 | 169 ± 7.0 | 5 | — | 163 ± 4.5 | 4 | 147 ± 3.4 | 8 | 10 |
| Hypothalamus | 229 ± 7.6 | 5 | 231 ± 21.6 | 6 | — | 273 ± 17.6 | 5 | 278 ± 5.9 | 5 | — | 258 ± 9.6 | 7 | 258 ± 6.5 | 7 | — |
| Striatum | 110 ± 10.9 | 6 | 93.4 ± 10.1 | 6 | 15 | 109 ± 3.1 | 5 | 102 ± 8.9 | 5 | 6 | 113 ± 7.6 | 4 | 107 ± 4.6 | 6 | 6 |
| Cortex | 19.2 ± 1.3 | 6 | 20.1 ± 2.7 | 6 | — | 19.0 ± 0.4 | 4 | 20.8 ± 1.3 | 5 | — | 21.1 ± 0.7 | 4 | 20.9 ± 0.7 | 7 | — |
| Cerebellum | 4.08 ± 0.25 | 6 | 3.16 ± 0.45 | 5 | 22 | 2.70 ± 0.31 | 4 | 3.62 ± 0.46 | 5 | — | 3.75 ± 0.75 | 4 | 3.72 ± 0.25 | 8 | 3 |

Values are mean ± s.e. mean. †% decrease compared to controls.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

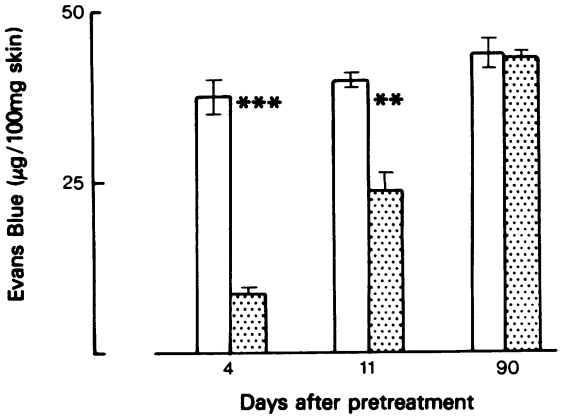


**Figure 2** Evans Blue content (μg/100 mg) of the hind paw skin after painting with 5% mustard oil. Rats were pretreated with capsaicin at the age of 20 days and tested later on the days indicated. Open columns: controls; stippled columns: capsaicin-treated. Values are mean ± s.e. mean, $n = 6$. ** $P < 0.01$; *** $P < 0.001$.

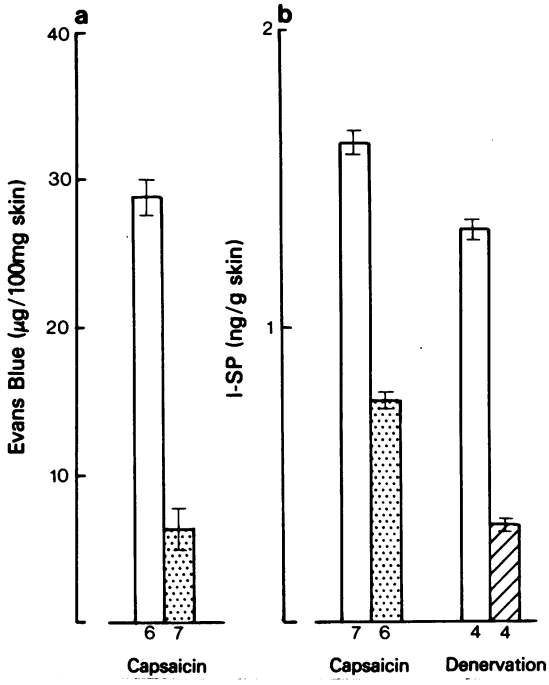


**Figure 3** (a) Evans Blue content (μg/100 mg) of the hind paw skin following painting with 5% mustard oil 4 days after capsaicin pretreatment of adult rats (stippled column); open columns: controls. (b) Immunoreactive substance P (I-SP) content (ng/g) of the hind paw skin of adult rats, 4 days after capsaicin pretreatment (stippled column) or 8 days after denervation (hatched column); controls: open columns. Values are mean ± s.e. mean of $n$ experiments, $P < 0.001$.

reduced. Even 5 months after pretreatment, the I-SP content of the dorsal roots and the dorsal half of the spinal cord were still 16 and 15% respectively ($n = 5$) below controls (not significant) and were 17% below controls in the medulla oblongata (17%, $n = 5$, $P < 0.05$). No changes of the I-SP content were found in the other brain regions examined.

*Capsaicin pretreatment of adult rats*

Neurogenic plasma extravasation induced by mustard oil was reduced by 78% 4 days after the last capsaicin injection (Figure 3). At the same time, the I-SP content in the skin of the hind paw was depleted by 54%. In comparison, 8 days after cutting the saphenous nerve, I-SP in the skin, innervated by this nerve, was found to be reduced by 76% (Figure 3).

*Intra-arterial infusion of neuropeptides*

Intra-arterial infusion of neuropeptides into the femoral artery was used to examine their ability to induce plasma extravasation in the hind paw. Substance P was found to induce plasma extravasation dose-dependently with a threshold dose of about $5 \times 10^{-13}$ mol/min. Physalaemin, another tachykinin, was 3 times more potent than substance P. No plasma extravasation was induced by infusions ($n = 3$) of caerulein ($3.8 \times 10^{-11}$ mol/min), vasoactive intestinal polypeptide ($5.2 \times 10^{-11}$ mol/min), somatostatin ($1.2 \times 10^{-10}$ mol/min), and the enkephalin analogue FK 33-824 ($2.4 \times 10^{-10}$ mol/min). Plasma extravasation by infusions of substance P was also tested 4 months after capsaicin pretreatment of 2 day old rats. The effect of substance P was not significantly different from that in control rats.

## Discussion

The investigation shows that neonatal capsaicin pretreatment leads to a substance P decrease in areas containing primary sensory fibres. In agreement with previous findings of Gasparović *et al.* (1964) and Jessell *et al.* (1978), made on adult rats, neonatal capsaicin pretreatment caused a marked decrease of substance P in the dorsal half of the spinal cord. However, no change was found in the ventral half of the spinal cord.

In rats, pretreated on the second or tenth day of life, a similar substance P decrease was found in the skin, in the saphenous nerve and in the dorsal roots. Concomitantly with this decrease, neurogenic plasma extravasation was irreversibly impaired, confirming

the findings of Jancsó *et al.* (1977). The smaller decrease of substance P in the dorsal half of the spinal cord suggests that substance P neurones originating in the spinal cord (Hökfelt, Ljungdahl, Terenius, Elde & Nilsson, 1977) are not affected by capsaicin. Some afferent substance P neurones terminate in the ventral horn (Otsuka, 1978). The failure of capsaicin to reduce substance P in the ventral horn may indicate that these neurones are structurally different from those terminating in the dorsal horn. The substance P content of the vagus nerve was decreased to a similar degree as that of the saphenous nerve. Therefore, the decrease of the substance P content in the medulla oblongata may be explained by a substance P decrease in terminals of cerebral nerves. Capsaicin led to a decrease of substance P only in those regions which contain primary afferent neurones. In addition, no change of the substance P content could be detected in the gastro-intestinal tract after neonatal capsaicin pretreatment (unpublished results). In so far as substance P neurones are concerned, this indicates a selective action of capsaicin on primary afferent substance P neurones. Whether other afferent neurones besides those containing substance P are also affected, remains to be investigated.

Capsaicin selectively excites and subsequently desensitizes $C_2$ fibres, which conduct impulses from polymodal nociceptors (Szolcsányi, 1977). The capsaicin-induced substance P depletion indicates that these afferent substance P neurones belong to the $C_2$ fibre group. This is in agreement with immunohistochemical findings of unmyelinated substance P neurones (Hökfelt *et al.*, 1975; Cuello *et al.*, 1976; Gamse *et al.*, 1979). Some substance P neurones seem to be myelinated (Gamse *et al.*, 1979). Such myelinated, capsaicin-insensitive substance P neurones could terminate in the ventral half of the spinal cord, where no substance P depletion was found.

Pretreatment on the 20th day of life caused a marked substance P decrease in the skin, 4 days after pretreatment; however, 3 months later the substance P content was fully restored. This can be assumed also for the saphenous and vagus nerve. At that time restoration of the substance P content of dorsal roots was not completed. A possible explanation is that in bifurcating axons less material is transported into the central than into the peripheral branch (Komiya & Kurokawa, 1978). Neurogenic plasma extravasation in the same group of animals was also impaired 4 and 11 days after pretreatment, but fully recovered within 3 months. A similar degree of recovery was also found in rats pretreated on the 15th day of life. The results taken together indicate an irreversible substance P decrease associated with an irreversible impairment of neurogenic plasma extravasation, by capsaicin pretreatment up to the 10th day of life. According to Jancsó *et al.* (1977), capsaicin pretreatment of 2 day old rats leads to a substantial degeneration of unmyelinated nerve fibres. The irreversible decrease of substance P in this group of rats suggests a degeneration of substance P neurones. At this age the rat nervous system is not fully developed, since maximum brain growth occurs during the first three postnatal weeks (Dobbing, 1968). This perhaps explains why capsaicin pretreatment on the 20th day of life leads to a reversible depletion, but not to degeneration of substance P neurones. Similarly, neurogenic plasma extravasation is reversibly impaired. In adult rats also, the effect of capsaicin is reversible within 3 months (Jancsó & Jancsó-Gábor, 1959). In contrast to neonatal capsaicin pretreatment, pretreatment of adult rats does not cause axonal degeneration, but leads to a decrease of microvesicles (Szolcsányi *et al.*, 1975). Our experiments show that the reversibility of the capsaicin effect in 20 day old rats is similar to that in adult rats. Like capsaicin pretreatment, denervation also causes inhibition of neurogenic plasma extravasation (Jancsó *et al.*, 1967). Parallel to this inhibition, substance P is markedly decreased in the skin.

All the results taken together reveal a parallelism between the reduction of substance P in primary sensory neurones and impairment of neurogenic plasma extravasation. This allows the assumption that substance P may be involved in neurogenic plasma extravasation. According to Jancsó *et al.* (1967) plasma extravasation following cutaneous application of capsaicin is caused by release of a still unknown factor from sensory neurones. In favour of substance P being this factor is its ability to induce plasma extravasation on intra-arterial infusion (Lembeck *et al.*, 1977). Since neither chronic denervation (Lembeck *et al.*, 1977) nor neonatal capsaicin pretreatment reduce this effect of substance P, any indirect action via release of a neurogenic factor from capsaicin-sensitive neurones can be excluded. Substance P is the only neuropeptide known so far to be present in afferent neurones (Lundberg, Hökfelt, Schulzberg, Norell, Nilsson, Uvnäs-Wallenstein, Terenius, Dahlström, Rehfeld, Elde & Said, 1978) to induce plasma extravasation. The fact that the non-mammalian peptide, physalaemin, shares this property of substance P indicates that the permeability increasing activity is located in the C-terminal part of the peptides.

Substance P is present in unmyelinated sensory neurones (Hökfelt *et al.*, 1975, Cuello *et al.*, 1976, Gamse *et al.*, 1979), it can be released from the peripheral endings of trigeminal neurones by antidromic nerve stimulation (Olgart *et al.*, 1977), it can induce plasma extravasation and there exists a highly effective inactivating system for substance P in peripheral vascular beds (Lembeck, Holzer, Schweditsch & Gamse, 1978). Recently a release of substance P from spinal cord slices by capsaicin was shown (Gamse &

Molnar, 1979). All these facts together with the present results are in favour of a mediator role of substance P in neurogenic plasma extravasation.

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