We have shown that ^{3}H-*N*-SP when applied extracellularly in vivo can label intracellular proteins with no apparent deleterious effects on cell function. In addition to providing a unique view of axonal transport, ^{3}H-*N*-SP and other related agents (*7*, *8*) can be used as radioactive vital stains to investigate a variety of biological phenomena. This vital stain has the additional unique feature of potentially providing information about the nature of the proteins involved.

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7. Covalent protein labels have traditionally been used to distinguish between membrane proteins that face the cytoplasmic versus the extracellular surface [see K. L. Carraway, *Biochim. Biophys. Acta* **415**, 379 (1975) for a review]. In previous studies, we found that ^{125}I-labeled Bolton-Hunter reagent [*N*-succinimidyl-3-(4-hydroxy, 5-[^{125}I]iodophenyl propionate] effectively labeled all the proteins in isolated squid axoplasm [T. Yoshioka, H. C. Pant, I. Tasaki, J. Baumgold, G. Matsumoto, H. Gainer, *Biochim. Biophys. Acta* **538**, 616 (1978); H. C. Pant, S. Terrakawa, J. Baumgold, I. Tasaki, H. Gainer, *ibid.* **513**, 132 (1978)]. The intra-axonal (axoplasmic) proteins were labeled equally well when the reagent was applied extracellularly to intact axons with no deleterious effects on the action potential mechanism or general cellular vitality (H. C. Pant and H. Gainer, unpublished observations).
8. The ^{3}H-*N*-SP is one of a class of *N*-acylating reagents, which covalently attach to the free α- and ϵ-amino groups in proteins [H. Boyd, I. C. Calder, S. J. Leach, B. Milligan, *Int. J. Pept. Protein Res.* **4**, 109 (1972); S. J. Leach and H. Boyd, *ibid.* **5**, 239 (1973)]. This reagent reacts rapidly with protein substrates (half-time is 2 to 3 minutes) at physiologic *p*H and is soluble in both aqueous and organic solvents. It rapidly penetrates cell membranes and labels proteins bound to cytoplasmic and intracellular membranes equally well. Exposing intact rat posterior pituitaries to ^{3}H-*N*-SP labeled cytoskeletal proteins as well as intragranular neurosecretory proteins (neurophysins) [(D. Fink and H. Gainer, *Brain Res.* **177**, 208 (1979)]. The label spontaneously hydrolyzes to inactive products in aqueous solution in the absence of substrate. This hydrolysis is relatively slow (half-time about 90 minutes), and hence, the ^{3}H-*N*-SP is maintained in a small volume of toluene, which is added to the aqueous solution just before use. The residual toluene in the solution is then blown off by a stream of nitrogen.
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10. Deparaffinized sections were coated with Kodak NTB2, diluted in a ratio of 1:1, dried upright, and stored at 4°C in lightproof boxes for 7 days. Radioautograms were developed in Dektol (Kodak) diluted in a ratio of 1:1 for 3 minutes at 16°C, fixed, washed, and stained with 0.1 percent toluidine blue in 1 percent sodium borate.
11. The labeling profile of the proteins (Fig. 1B) resembles the slow component of anterograde transport in its molecular weight distribution [R. J. Lasek and P. Hoffman, in *Cell Motility*, R. Goldman, T. Pollard, J. Rosenbaum, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976), p. 1021; P. Hoffman and R. J. Lasek, *J. Cell Biol.* **66**, 351 (1975)]. The profile at 8 mm contains both slow-component a (Sc_a) and slow-component b (Sc_b) proteins. At later times after injection (for example, 10 days) and longer distances from the injection site, we have been able to resolve the Sc_a component (that is, the neurofilament triplet and tubulin) and the complex Sc_b component separately (D. Fink and H. Gainer, *J. Cell Biol.*, in press).
12. Immediately after injection of ^{3}H-*N*-SP, the injection site contained three prominent labeled proteins with molecular weights of 68,000, 27,000 and 16,000. The 27,000 and 16,000 peaks are identical in size to those of myelin proteins previously described in the rat sciatic nerve [S. Greenfield, S. Brostoff, E. H. Eylar, P. Morrell, *J. Neurochem.* **20**, 1207 (1973); S. Micko and W. Schlaepfer, *ibid.* **30**, 1044 (1978)]. From 2 to 45 days after labeling, the injection site still contained the 27,000 and 16,000 proteins, but the 68,000 protein was absent (D. J. Fink and H. Gainer, *J. Cell. Biol.*, in press) (see Fig. 1B for the location of the 68,000 peak after 5 days).
13. The ^{3}H-*N*-SP labeling procedure is not restricted to the study of slowly transported proteins. We have recently applied this approach to study fast axonal transport in the rat sciatic nerve and hypothalamo-neurophyophysial system.
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25 June 1979

Substance P: Does It Produce Analgesia or Hyperalgesia?

Abstract. *In the hot plate test, substance P given intravenously at doses of 5 × 10^{-5} and 5 × 10^{-4} gram per kilogram caused analgesia, while lower doses caused hyperalgesia. The influence of substance P on nociception depended on the individual mouse's sensitivity to pain (control response latency). Analgesia was produced by substance P administered to mice with high sensitivity to thermic stimulation, whereas hyperalgesia occurred in mice whose control latencies were longer than normal. This result is interpreted as an indication that substance P is capable of normalizing responsiveness to pain and could be classified as a regulatory peptide.*

Substance P (SP) produces potent, long-lasting analgesic activity in mice as determined by the hot plate technique (*1*, *2*). Substance P was found to be a long-lasting analgesic also in the tail-flick test of rats to which it had been intracerebrally administered (*3*). Oehme *et al.* (*4*) reported, in contrast, that SP produced hyperalgesia. Frederickson *et al.* (*2*) have partly resolved those conflicting observations by showing that SP has a dual action on nociception; their studies indicated that SP in small doses produced analgesia in mice and that this analgesic effect was blocked by naloxone. With higher doses of SP the analgesic activity was lost and hyperalgesia occurred when the higher doses were given in combination with naloxone. Frederickson *et al.* (*2*) concluded that very small doses of SP probably released endorphins, while higher doses caused direct excitation of neuronal activity in nociceptive pathways. Our experiments suggest that the capability of SP to produce analgesia or hyperalgesia may depend also on the responsiveness of the animals to pain (as indicated by response latency) before the SP is administered.

We tested SP for its action on nociception by means of the hot plate procedure and acetic acid–induced writhing (*4*). ICR standard mice (18 to 22 g) were used. The temperature of the hot plate was controlled thermostatically at 57°C. A plexiglass cylinder (10.0 cm in height, 20.5 cm in inner diameter, and with open top) was used to confine the mice to a restricted area of the plate surface. Substance P was administered intraperitoneally or intravenously. Control mice were given isotonic saline. The time, in seconds, from first contact with the plate to first hind-paw licking was recorded as response latency. Control latencies were estimated 1 day before testing and also 15 minutes before SP treatment. The values 15 minutes before SP

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treatment were averaged to provide the mean control latency. For writhing studies, mice received intraperitoneal injections of acetic acid. The saline-treated control mice writhed 30 times on average during the first 12 minutes after injection of acetic acid. In this test, analgesic activity was indicated by inhibition of writhing during the same period. The SP was injected intravenously 20 minutes before acetic acid treatment. The *P* values were calculated by Student's *t*-test for the hot plate experiment and by the Mann-Whitney U test for the writhing experiment. Earlier experiments showed that, in hot plate testing, response latencies were reduced (hyperalgesia) by SP (*4*). When conducting those experiments (*4*), we were under the impression that the action of SP differed in mice depending on whether they had shorter or longer control response latencies. Therefore, a selection procedure was applied in our experiments whereby mice were grouped according to their control response latencies.

Mice with control response latencies below 15 seconds were put together in group 1; group 2 included mice with latencies between 15 and 29 seconds, and group 3 included those with latencies between 30 and 44 seconds. The response latencies of the three groups were measured again 30, 60, 90, and 120 minutes after injection of SP. The latencies recorded 60 minutes after SP treatment are given in Table 1. The response latencies of the three groups were altered in different ways by SP treatment. In group 1 (short control response latencies) SP caused analgesia and the latency between first contact and first hind-paw licking was prolonged. In group 3 (longest control response latencies) the response latency was reduced by SP, a sign of hyperalgesia. In group 2 (control response latencies between those of groups 1 and 3) response latencies were also depressed by SP treatment, although less than with group 3. These results suggest that the action of SP on nociception depends on the responsiveness of the mice prior to SP administration. Madden *et al.* (*5*) presented evidence that the level of endogenous opioids in the brain can be correlated with responsiveness to pain. It has been postulated (*2*), furthermore, that SP produces analgesia by the release of endorphins. Applying the above to our results, we suggest that SP was capable of releasing endorphins only in mice of group 1, that is, those with short control response latencies. For the other two groups, those with longer control response latencies, SP may have produced hyperalgesia through direct excitation of neuronal activity in nociceptive pathways as suggested also by Frederickson *et al.* (*2*). Thus, the same dose of SP would be capable of producing different effects, analgesia or hyperalgesia, depending on the responsiveness, probably related to endorphin levels, of the mice involved. We observed, furthermore, that 60 minutes after SP administration the mean response latencies of all three groups were almost identical (Table 2). Thus, SP was capable of modulating or normalizing responsiveness to pain.

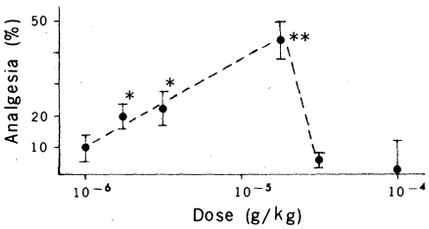

Fig. 1. Analgesic activity of SP in the writhing test.

Frederickson *et al.* (*2*) reported that SP produced a bell-shaped dose-response curve in the hot plate test. Maximum analgesia was observed after administration of 2.5 ng per mouse (1.25×10^{-7} g/kg). Analgesia was also found (*2*) to occur after administration of 50 ng per mouse ($\approx 2.5 \times 10^{-6}$ g/kg). In our present experiments, we found the capability of SP to produce analgesia or hyperalgesia to be dose-dependent, as did Frederickson *et al.* (*2*). Yet there were differences between the two works since in our experiments higher SP doses (5×10^{-5} and 5×10^{-4} g/kg or 1 and 10 μg per mouse) produced analgesia and lower doses (5×10^{-7} to 2.5×10^{-5} g/kg or 10 to 500 ng per mouse) produced hyperalgesia. This difference may be attributed, however, to differences in experimental conditions. For our experiment, we used mice with control response latencies below 15 seconds while Frederickson *et al.* used mice with an average latency of 23 seconds and we used a 57°C hot plate, whereas Frederickson *et al.* used a 52°C hot plate.

We also used acetic acid–induced writhing to assess the action of SP on nociception. A bell-shaped dose-response curve to SP was also obtained with this test (Fig. 1). We attributed this again to the dual action of that peptide, release of endorphins, and direct neuronal excitation as first postulated by Frederickson *et al.* (*2*). In an attempt to test this, mice were first treated with naloxone (0.4 mg/kg, given intraperitoneally, for 30 minutes) before evaluation of the analgetic effect of SP. Indeed, naloxone antagonized SP analgesia, as would be predicted by the above postulation.

Thus, in confirmation of the earlier report of Frederickson *et al.* (*2*), SP has two actions on nociception; it can cause either analgesia or hyperalgesia depending on the dose of SP and the initial responsivity of the animal. High responsiveness to thermal stimuli can be reduced, and low responsiveness can be increased. Such action results in "normalization" of individual responsiveness to pain. In rats under stress by immobilization or noise, SP restored disorders in blood pressure, sleep, or avoidance

Table 1. Changes in mean response latency (seconds) of mice with different control values prior to intravenous injection of SP (500 μg/kg). N.S., not significant.

Group	*N*	Change in latency (mean ± S.E.M.)	*P*
Latency < 15 seconds			
Control	40	− 0.9 ± 5.9	N.S.
SP-treated	74	+ 5.0 ± 20.1	< .05
Latency 15 to 29 seconds			
Control	40	− 0.7 ± 21.5	N.S.
SP-treated	75	− 6.7 ± 14.1	< .001
Latency ≥ 30 seconds			
Control	20	− 7.2 ± 32.3	N.S.
SP-treated	50	− 16.6 ± 19.1	< .001

Table 2. Mean response latencies of mice before and 60 minutes after treatment with SP. The test procedures were as in Table 1.

Group	Response latency (seconds)			
	Before SP		After SP	
	Mean ± S.E.M.	*N*	Mean ± S.E.M.	*N*
Shorter	11.53 ± 0.27	74	16.47 ± 2.29	74
Medium	22.28 ± 0.53	75	16.00 ± 1.59	75
Longer	34.32 ± 0.68	50	17.10 ± 2.54	50

learning (*6*, *7*). Different doses of SP can produce different effects also. Frederickson found that smaller SP doses, alone, produced analgesia, while higher doses, given after prior treatment with naloxone, resulted in hyperalgesia. We also observed dose-dependent analgetic or hyperalgetic effects of SP. We found, furthermore, as did Frederickson *et al.* and others, that SP analgesia was reversible by naloxone. This supports a role for endogenous opioid peptides in this action. A dual action of SP on nociception suggests that there might be more than one receptor type for SP (*2*). Some further evidence for the possible existence of such different receptors has been provided by Bergmann *et al.* (*7*) who investigated SP action on smooth muscle. The presence of several signatures in one molecule determining different functions is referred to in information theory as the principle of ambiguity or indeterminancy of effect. That principle can be biologically important when molecules have conformationally flexible structures, such as the linear flexible SP undecapeptide (*4*).

Thus we suggest that the dual actions of SP result from this flexibility of the molecule and that both the dose of the peptide and condition of the animal determine which configuration and effect predominate. Thus, the resultant effect is a "normalization" of the responsivity of the animal to painful stimuli. Accordingly, we propose that the term regulatory peptides or "regulides" be applied to such peptides.

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8. We thank Dr. R. C. A. Frederickson for suggestions and assistance in the preparation of this manuscript.

23 August 1979; revised 21 January 1980

Cadmium Increases Tissue Factor (Coagulation Factor III) Activity by Facilitating Its Reassociation with Lipids

Abstract. *The coagulant activity of partially purified and delipidated tissue factor (TF) (coagulation Factor III) has previously been recovered by dialysis of the apoprotein after addition of mixed brain lipids and deoxycholate. Inclusion of cadmium chloride in the relipidation mixture greatly increases the recovered activity of highly purified TF from human placenta by promoting incorporation of TF into phospholipid vesicles; TF that had not been incorporated into vesicles showed no coagulant activity. Thus, TF must be present in a lipid bilayer for expression of coagulant activity. In vitro, cadmium induces fusion of lipid vesicles and may contribute to the incorporation of proteins in membranes.*

The proteolytic activation of coagulation Factor X by Factor VIIa (*1*) is dependent on the presence of calcium and is accelerated by tissue factor (TF) (coagulation Factor III) complexed with phospholipids (*2*). The activity of TF purified from human placenta (*3*, *4*) is lost as lipids are removed during fractionation, but can be recovered by relipidation of the apoprotein. The coagulant activity of TF (*5*), reactivated by dialysis with a lipid preparation from bovine brain in deoxycholate (*6*), is increased by adding $CdCl_2$ to the protein-lipid mixture. The $CdCl_2$ does not replace calcium, which is required for the interactions of vitamin K–dependent clotting enzymes with phospholipids.

Since the TF-accelerated cleavage of Factor X is the first of several enzymatic steps leading to coagulation in the two-stage clotting assay (*5*), we determined the rate of activation of Factor X (*7*) by measuring the time-dependent release of the activation peptide. When TF was relipidated in the presence of cadmium, the rate of Factor X activation was greater than it was in the absence of cadmium (Fig. 1). Furthermore, the difference in activation rate increased as TF was added to the reaction, demonstrating that cadmium had specifically increased the TF activity.

Tissue factor activity recovered after relipidation depends on the amount of phospholipid present in the relipidation mixture (*2*). We therefore conducted experiments to determine whether cadmium affected the optimum concentration of mixed brain lipids for recovery of TF coagulant activity. In the absence of cadmium, the optimum lipid concentration was near 0.3 m*M* [determined as phosphate (*6*)], resulting in a TF activity of 6.8 U/ml. This lipid optimum was not measurably different when $CdCl_2$ was included at 10m*M*, 15 m*M*, or 20 m*M*, although the TF coagulant activity was increased tenfold after relipidation with 10 m*M* $CdCl_2$. In addition, the $CdCl_2$ optimum remained constant between 5 m*M* and 7 m*M* when the relipidation mixture contained (determined as phosphate) 0.1 m*M*, 0.2 m*M*, 0.3 m*M*, or 0.4 m*M* mixed brain lipids. Thus, lipids and cadmium have distinct effects on the recovery of TF activity which are amenable to independent optimization.

The three primary components of the relipidation mixture (TF, mixed brain lipids, and $CdCl_2$) were dialyzed together in various combinations to ascertain whether cadmium was acting on the apoprotein or on the lipids. Incubation and dialysis of $CdCl_2$ with either TF or mixed brain lipids, prior to addition of the third component and a second dialysis, failed to increase the TF activity. Thus cadmium was not removing potential inhibitors (such as sulfhydryl compounds) from either component, nor was it selectively activating the apoprotein or altering the state of the lipids to increase their ability to promote coagulation. Furthermore, functionally significant amounts of $CdCl_2$ were not being retained by either the lipids or TF apoprotein, since dialysis of $CdCl_2$ with either component failed to increase TF activity. Increased activity was observed when $CdCl_2$ and TF were added to dialyzed lipids, or when $CdCl_2$ was added to TF dialyzed with lipids, followed by additional dialysis. This series of experiments indicated that the increased activity was the result of interactions between apoprotein, cadmium, and lipids.

The conditions we use for TF relipidation produce phospholipid vesicles (*8*); we therefore used sucrose density-gradient ultracentrifugation to examine the effect of $CdCl_2$ on the TF-phospholipid complexes. After centrifugation, activity in the preparations relipidated with and without cadmium was present in the top half of the density gradient (Fig. 2). However, the TF relipidated with $CdCl_2$ produced a sharp peak of activity between 13 and 16 percent sucrose, which was not observed in the sample relipidated without $CdCl_2$. Relipidation of each density-gradient fraction with additional lipids and $CdCl_2$ produced addi-