LHRH had no effect on circulating cholesterol levels (data not shown).

The present results demonstrate that NT and SP, 2 closely related peptides sharing certain properties, have opposite effects on circulating cholesterol levels. Several explanations may be proposed for the hyper- and hypocholesterolemic effect of NT and SP, respectively. Elevation of plasma cholesterol after i.v. NT suggests that the peptide may be involved at some step in the transport of cholesterol.

Considerable insight into the importance of blood cholesterol for regulating sterol metabolism in several tissues has come from studies using rats in which lipoproteins were reduced by 4-APP[15]. This model cannot, however, discern the mechanism(s) by which substance P mimics the action of 4-APP. Since NT antagonizes the effect of substance P and of 4-APP and 4-APP is known to selectively inhibit hepatic secretion of lipoproteins[15], we suggest that substance P, and also NT, may act on the liver.

A considerable concentration of NT and SP has been demonstrated in the small intestine of the rat[16], and some lipoproteins active in cholesterol transport synthesized in this segment of the gut are transported rapidly into the plasma via the mesenteric lymph[17,18]. Thus hypercholesterolemia may be induced by either intestinal overproduction or hypothalamic hypersecretion of NT followed by an increased delivery of NT to the gut or other tissues active in cholesterol transport. Determination of the physiological significance of these actions will require further studies, but the fact that both peptides are found in the brain and small intestine suggests that they may have some physiological role as humoral agents responsible for the occurrence of neurogenic hyper- and hypocholesterolemia. To our knowledge, these are the 1st results implicating substance P in the induction of hypocholesterolemia and in an antagonistic effect on NT-induced hypercholesterolemia. However, plasma levels of SP and NT have been reported to be far below the concentrations achieved by the injection of 5–20 µg peptide. It may be added that the doses used are rather high and bound to produce hypotensive effects. The precise point at which SP and NT act in producing these effects is difficult to identify. Since the liver is one of the sites of NT action[19], it is possible that NT acts to stimulate and SP acts to inhibit hepatic secretion of lipoproteins.

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2 R. Carraway and S.E. Leeman, J. biol. Chem. *248*, 6854 (1973).
3 M.M. Chang and S.E. Leeman, J. biol. Chem. *245*, 4784 (1970).
4 M. Brown and W. Vale, Endocrinology *98*, 819 (1976).
5 R. Carraway, L.M. Demers and S.E. Leeman, Endocrinology *99*, 1452 (1976).
6 Y. Kato, K. Chihara, S. Ogo, Y. Iwasaki, H. Abe and H. Imura, Life Sci. *19*, 441 (1976).
7 C. Rivier, M. Brown and W. Vale, Endocrinology *100*, 751 (1977).
8 E. Vijayan and S.M. McCann, Endocrinology *105*, 64 (1979).
9 E. Vijayan and S.M. McCann, Life Sci. *26*, 321 (1980).
10 E.D. Bransome, Jr, A. Rev. Physiol. *30*, 171 (1968).
11 E. Vijayan and S.M. McCann, Neuroendocrinology *25*, 150 (1978).
12 E. Vijayan and S.M. McCann, Brain Res. *155*, 35 (1978).
13 T.S. Shiff, P.S. Roheim and H.A. Eder, J. Lipid Res. *12*, 596 (1971).
14 L.L. Rudel and M.D. Morris, J. Lipid Res. *14*, 364 (1973).
15 M.H. Christie, J.F. Strass and G.L. Flickinger, Endocrinology *105*, 92 (1979).
16 R. Carraway and S.E. Leeman, J. biol. Chem. *251*, 7035 (1976).
17 R.W. Mahley, B.D. Bennett, D.J. Moore and M.E. Gray, Lab. Invest. *25*, 435 (1971).
18 P.R.H. Green, A.R. Tall and R.M. Glickman, J. clin. Invest. *61*, 528 (1978).
19 K. Nagai and L.A. Frohman, Life Sci. *19*, 273 (1976).

Increase in pyridinoline cross-linking of mouse bone collagen induced by estrogen

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Summary. Pyridinoline, a non-reducible cross-link of collagen, was measured in the cortical bone of testectomized mice after repeated s.c. injections of estradiol benzoate. Pyridinoline content was increased significantly whereas the contents of hydroxylysine, and reducible cross-links remained unchanged.

A fluorescent cross-linking amino acid was recently isolated from collagen of ox tendon and bone and named pyridinoline[3]. Pyridinoline gradually increases with age in several tissues[4]. Aldimine cross-links, which are detectable after chemical reduction with $NaBH_4$ and are therefore called reducible cross-links decrease with age[5]. The decrease of solubility of collagen with age, therefore, is not explained by the stabilization of these cross-links in most species of animals, and they are considered as intermediates in the production of mature and stable cross-links[6,7]. Pyridinoline may possibly be one of the long-anticipated 'mature' cross-links of collagen, and 2 hypotheses are suggested about the pathway of its formation, which begin with hydroxylysine or its derivatives[8,9].

Steroid hormones, in particular estrogen, reportedly have various effects on connective tissue metabolism[10,11]. Estrogen affects the metabolism of connective tissues in vivo[12–15], and in vitro[16] suggesting that steroid hormones regulate the synthesizing process of fibrous proteins in fibrogenic cells. Moreover estrogen seems to accelerate extracellular maturation of collagen which was evidenced by decrease of solubility in skin collagen and an increase in the lysyl oxidase activity of collagen in bone, skin[17] and uterine cervix[18]. In the present experiment the effect of estrogen on the changes in the amount of cross-links of bone collagen, especially pyridinoline, was studied using mice.

Materials and methods. Male DDD mice[19] were fed a commercial diet and testectomized under ether anesthesia

at 28 days of age. After operation, estradiol benzoate (2 µg per mouse), dissolved in sesame oil, was given s.c. twice weekly for 7 weeks. Control mice were testectomized and injected with sesame oil. Animals were killed by cervical dislocation and diaphyses of long bones (humerus, femur and tibia) were dissected. Bone cortices were pulverized in liquid nitrogen and decalcified in 0.5 M EDTA in 0.05 M Tris-HCl buffer, pH 7.4. Acid-soluble collagen was removed in sufficient 0.5 M acetic acid. Defatting and dehydration were performed in a mixture of ethanol and ether (1:1). The remaining insoluble collagen was lyophilized. Insoluble collagen and reduced insoluble collagen were hydrolyzed in 6 N HCL at 110 °C for 24 h under N_2 in sealed glass tubes. The HCl was removed by evaporation in vacuo. Amino acid analysis was performed after acid hydrolysis of collagen using a JEOL JLC-6AH amino acid analyzer. Bone insoluble collagen was reduced with $NaBH_4$[20]. The hydrolysate of reduced collagen was applied to a JEOL JLC-6AH amino acid analyzer and the amount of reducible cross-links were determined according to the method of Masuda et al.[21]. The hydrolysate of insoluble collagen was applied to a P-cellulose column and the pyridinoline content was determined according to the method of Fujimoto and Moriguchi[22] by fluorometry using JASCO FP-550 fluorescence spectrophotometer. Collected fractions of pyridinoline had fluorescence and uV-absorption spectra identical with those previously reported[3]. An aliquot of the hydrolysate that was used in the determination of pyridinoline was used for the assay of hydroxyproline content according to the method of Woessner[23]. The amount of pyridinoline was expressed as the number of residues/100 hydroxyproline residues by comparison of fluorescence intensity with that of a known amount of pure pyridinoline.

Results and discussion. Table 1 shows the amino acid composition of the insoluble bone collagens of the estrogen-treated and control mice. No difference was found between the 2 collagens even in hydroxylysine content. The elution profile of the reducible cross-links of insoluble collagen in mouse bone is shown in the figure. DHLNL was the major reducible cross-link of this collagen and a trace amount of hydroxylysinonorleucine was also detected. Changes in the amount of DHLNL in estrogen treated and control collagens are shown in table 2. There are no significant difference in the amount of this cross-link between the 2 collagens. The amount of pyridinoline is shown in table 2. Estrogen treatment resulted in a significant increase of pyridinoline in the bone collagen of mice. Our present study has shown that reducible cross-link DHLNL remained unchanged and that pyridinoline increased significantly after estrogen treatment. In this study, soluble collagen, which constitutes a low proportion (2–4%) of total bone collagen[17] was not analyzed for the measurement of cross-links. The amount of reducible cross-links, which was not influenced by estrogen in the insoluble fraction of bone collagen, may be changed in the soluble fraction. It is also probable that acid treatment of the collagen resulted in a loss of labile reducible cross-links. We are now carrying out an investigation to elucidate this point. Lysyl oxidase is known to convert peptidyl lysine and hydroxylysine to the aldehydes allysine and hydroxyallysine respectively by oxidative deamination[24]. It is generally accepted that this is the only enzyme-dependent step of cross-link formation, and subsequent reactions occur spontaneously[24]. We have already shown that estrogen increased lysyl oxidase activity in the bones of mice using the same experimental model[17]. Thus estrogen seems to accelerate maturation of collagen cross-link in mouse bone by increasing the amount of pyridinoline through enhanced activity of lysyl oxidase.

Table 1. Amino acid composition of bone insoluble collagen of testectomized and estrogen treated mouse (residues/1000 total residues)

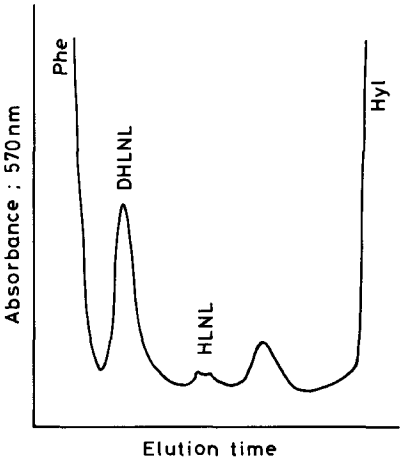
	Tx+EB	Tx+oil
Hyp	92.4	93.7
Asp	47.6	47.1
Thr	20.7	20.2
Ser	35.7	35.2
Glu	77.0	76.1
Pro	134.0	130.1
Gly	314.2	317.2
Ala	104.1	103.9
Cys	-	-
Val	26.8	27.0
Met	6.9	6.9
Ile	11.1	10.8
Leu	27.1	26.9
Tyr	5.7	5.7
Phe	11.3	12.8
Hyl	12.5	11.6
Lys	19.8	20.4
His	5.3	5.1
Arg	47.7	49.2

Tx, testectomy; EB, estradiol benzoate dissolved in sesame oil; oil, sesame oil.

Table 2. Effect of estrogen treatment on the content of reducible cross-link dihydroxylysinonorleucine (DHLNL) and non-reducible cross-link, pyridinoline

	Tx+EB	Tx+oil
DHLNL (residue/1000 residue of amino acids)	0.104 ±0.019 (3)	0.102 ±0.008 (3)
Pyridinoline (residue/100 residue of Hyp)	0.1146±0.00365 (6)*	0.0993±0.00192 (6)

Tx, testectomy; EB, estradiol benzoate dissolved in sesame oil; oil, sesame oil. The number of determination is indicated in parentheses. Each value represents the mean±SE. * $p<0.005$ Tx+EB vs Tx+oil (Student's t-test).



Elution profile of $NaBH_4$-reduced mouse bone insoluble collagen by long column of JEOL JLC-6AH automatic amino acid analyzer. Dihydroxylysinonorleucine (DHLNL) is the major reducible cross-link of mouse bone and a small amount of hydroxylysinonorleucine (HLNL) also exsists.

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3 D. Fujimoto, K. Akiba and N. Nakamura, Biochem. biophys. Res. Commun. *76*, 1124 (1977).
4 T. Moriguchi and D. Fujimoto, J. Biochem., Tokyo *84*, 933 (1978).
5 M.L. Tanzer, in: Biochemistry of collagen, p. 137. Eds G.N. Ramachandran and A.H. Reddi. Plenum Press, New York 1976.
6 A.J. Bailey and M.S. Shimokomaki, FEBS Lett. *16*, 86 (1972).
7 K. Fujii and M.L. Tanzer, FEBS Lett. *43*, 300 (1974).
8 D.R. Eyre and H. Oguchi, Biochem. biophys. Res. Commun. *92*, 403 (1980).
9 D. Fujimoto, T. Moriguchi, T. Ishida and H. Hayashi, Biochem. biophys. Res. Commun. *84*, 52 (1978).
10 G. Asboe-Hansen, Physiol. Rev. *38*, 446 (1958).
11 T.F. Dougherty and D.L. Berliner, in: Treatise on collagen, vol. 2, p. 367. Ed. G.N. Ramachandran. Academic Press, New York 1968.
12 T. Takeda, Y. Suzuki and C.S. Yao, Acta path. jap. *25*, 135 (1975).
13 K.Y.T. Kao, W.E. Hitt, A.T. Bush and T.H. McGavack, Proc. Soc. exp. Biol. Med. *117*, 86 (1964).
14 H. Hama, T. Yamamuro and T. Takeda, Acta orthop. scand. *47*, 473 (1976).
15 J. Shikata, H. Sanada, T. Yamamuro and T. Takeda, Connect. Tissue Res. *7*, 21 (1979).
16 M. Hosokawa, M. Ishii, K. Inoue, C.S. Yao and T. Takeda, Connect. Tissue Res. *9*, 115 (1981).
17 H. Sanada, J. Shikata, H. Hamamoto, Y. Ueba, T. Yamamuro and T. Takeda, Biochim. biophys. Acta *541*, 408 (1978).
18 H. Ozasa, T. Tominaga, T. Nishimura and T. Takeda, Endocrinology *109*, 618 (1981).
19 J. Staats, Cancer Res. *32*, 1609 (1972).
20 M.L. Tanzer and G. Mechanic, Biochem. biophys. Res. Commun. *32*, 885 (1968).
21 M. Masuda, S. Karube, Y. Hayashi, H. Shindo and M. Igarashi, FEBS Lett. *63*, 245 (1976).
22 D. Fujimoto and T. Moriguchi, J. Biochem., Tokyo *83*, 863 (1978).
23 J.F. Woessner, Jr, Archs Biochem. Biophys. *93*, 440 (1961).
24 R.C. Siegel, in: International review of connective tissue research, vol. 8, p. 73. Eds D.A. Hall and D.S. Jackson. Academic Press, New York 1979.

Morphological aspects of chemically stimulated bovine chromaffin cells

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Summary. Isolated bovine chromaffin cells were chemically stimulated. The resulting appearance of local changes in membrane structure were compared with the integration of granular proteins into the cell membrane as seen after binding of fluorescence labeled anti-dopamine-β-hydroxylase. Protuberances of the cell membrane which can be perceived by Nomarski contrast optics are largely congruent with local tracer accumulations at the cell surface.

In isolated chromaffin cells exocytotic release of catecholamines (CA) can be made visible immunochemically by observing the appearance of membrane vesicular antigens at the surface of the cell membrane by means of fluorescence microscopy[1]. Recently, structural changes were observed at the cell surface of chemically stimulated rat chromaffin cells by means of Nomarski contrast optics[2]. Here we try to correlate these 2 results.

Materials and methods. a) Preparation of cells: Bovine chromaffin cells were isolated as described recently[1]. 2 or 3 drops of cell suspension which contained $0.8\text{–}1.2\times10^7$ cells/ml isotonic standard buffer (i.e. 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.135 M NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$ and 9 mM D-glucose) were dropped on to a slide and incubated for at least 45 min at 36 °C in a moist chamber. After washing out residual suspended cells, the cells adhering to the glass were stimulated at 21 °C with either 3.5 mM Ba^{2+}, with black widow spider venom (BWSV; extract of 1 gland in 2.5 ml standard buffer) or with 1.0 mM La^{3+} in standard buffer. CA secretion of an aliquot of cell preparation was assayed radioenzymatically by a catechol-O-methyltransferase method. During these stimulations, antiserum raised in rabbits against bovine membrane integrated dopamine-β-hydroxylase (amphiphilic DBH) was present in a dilution of 1:20. The antiserum was highly specific for bovine DBH but did not bind to DBH of other species[1]. After repeated washings the cells were incubated with fluorescein-conjugated γ-globulin (20 min at 21 °C, dilution 1:10). Thereafter, cells were washed 3 times.

The living cells were observed in a chamber consisting of 2 circular slides (1 slide used for application of the cells), an aluminium frame and a silicone gasket. The length of the beam through the chamber was approximately 1 mm.

b) Optics: A Zeiss Standard miscroscope was used, equipped with Nomarski contrast optics (objective $\times$ 100) and a condenser for incident fluorescent illumination. Cells which were brought into the chamber were observed and photographed both by means of Nomarski optics and by fluorescence microscopy.

Photographic prints were made on Ilford HP5 400 ASA black/white film. For fluorescence pictures exposure time was 25 sec. Prints were made on hard paper. Nomarski photographs were exposed automatically and printed on normal paper.

Results. By stimulation with Ba^{2+} during 30 min, bovine chromaffin cells can be induced to form several protuber-

Increase in percent of CA secreted by an aliquot of the same cell preparation stimulated for 10 min at 21 °C by various stimuli over non-stimulated controls incubated for 10 min in standard buffer. Control samples of approximately 5×10^5 cells released 3.8 µmoles CA, i.e. 3.3 µmoles adrenaline + 0.4 µmoles noradrenaline + 0.06 µmoles dopamin into the supernatant

	Dopamine	Adrenaline	Noradrenaline
La^{3+}	310 ± 46	567 ± 100	424 ± 78
Ba^{2+}	238 ± 43	474 ± 119	616 ± 91
BWSV	236 ± 58	528 ± 112	493 ± 103