tocytes isolated from clofibrate-fed rats [24].

The drugs probucol and pantethine did not affect the parameters of the metabolism of hepatic CoA shown in Tables 1 and 2. In contrast to the remaining drugs, probucol does not lower the level of total triglycerides in serum when given to humans, but may lower LDL-cholesterol by 10–15% [21]. This relationship may also suggest that the effect on the metabolism of CoA is related to the triglyceride lowering effect of clofibrate, the clofibrate analogs and tiadenol.

Summary. The hypolipidemic drugs bezafibrate, ciprofibrate, clofibrate, fenofibrate, pantethine, probucol and tiadenol were supplied during 9 days to male rats weighing 100 g. The drug dosage was about 185 μmoles in 24 hr. Increased biosynthesis and content of CoA, and increased activity of pantothenate kinase and carnitine palmitoyltransferase were found in the livers of rats fed bezafibrate, ciprofibrate, clofibrate, fenofibrate and tiadenol. A role of CoA-SH in the serum triglyceride lowering mechanism(s) of these drugs is discussed.

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Demonstration of dipeptidyl carboxypeptidase activity in rat brain stem synaptosomes using first-order kinetic analysis

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Dipeptidyl carboxypeptidase (DCP; angiotensin-converting enzyme; kininase II; EC 3.4.15.1) is a ubiquitously distributed enzyme that cleaves dipeptide residues from the carboxyl terminal of distinct peptides. It is involved in the synthesis of angiotensin II and the catabolism of bradykinin [1]. There is also a growing body of evidence that indicates that DCP might be responsible for the synthesis [2, 3] or degradation [4–6] of met- and leu-enkephalin. Although the presence of DCP has been reported in various brain regions [7–11], the subcellular localization of this enzyme in distinct brain areas has not been studied in detail. Yang and Neff [7] reported that 32% of whole brain DCP was localized to the crude mitochondrial fraction, which contains a number of organelles, including nerve terminals [12]. Arregui *et al.* [9], on the basis of intrastriatal kainic acid lesions, concluded that DCP in the substantia nigra was localized primarily to nerve endings. However, to our knowledge, direct localization of DCP to enriched synaptosomes, which are believed to be composed primarily of nerve terminals [12], has not been reported in the literature. Furthermore, the precise role of DCP in brain is unknown, and it is also unclear whether DCP is specific for a few biologically active peptides or whether it is a general, non-specific peptidase. Recently, Chevillard and Saavedra [11] reported a heterogenous distribution of DCP in rat brain stem. This uneven distribution of DCP is consistent with the hypothesis that the enzyme participates in the processing of as yet unidentified, but biologically

active, peptides in specific neurons. A second criterion that is consistent with a role for DCP in the processing of biologically active peptides is the presence of enzyme activity in nerve terminals. We report the presence of DCP in enriched rat brain stem synaptosomal preparations using first-order enzyme kinetic analysis. First-order kinetic conditions allow for the most sensitive and physiologically relevant assay of DCP and were used to calculate percent substrate utilization, which is proportional to V_{max}/K_m for single substrate reactions. We believe that these studies represent the first published evidence for localization of DCP to rat brain synaptosomes.

Male Sprague–Dawley rats (200–250 g) were decapitated and the brain stem at the level of the tegmentum was dissected free from higher brain regions. Brain stems were repeatedly washed with ice-cold 0.32 M sucrose/20 mM Tris-acetate (pH 7.4) to remove all adhering blood, which contains high DCP activity. Subcellular fractionation of brain stem tissue was carried out using modifications of procedures previously described [12, 13]. Tissue was homogenized in 10 vol. of ice-cold 0.32 M sucrose/20 mM Tris-acetate (pH 7.4) using a Thomas Type A Teflon–glass homogenizer. Sucrose/Tris buffer was used unless noted otherwise. In some studies, the homogenizing buffer contained 30 mM 1-*O*-*n*-octyl-β-D-glucopyranoside, a non-ionic detergent. The homogenate was centrifuged at 1000 *g* for 10 min (4°) and the 1000 *g* pellet was resuspended. The 1000 *g* supernatant fraction was centrifuged at 10,000 *g* for 20 min (4°) and the resulting pellet was resuspended. This suspension was either assayed directly or layered on top of a discontinuous sucrose density gradient as described by Gray and Whittaker [12]. The gradient was centrifuged in a SW41 rotor at 81,500 *g* for 120 min (4°); the mitochondrial, synaptosomal and myelin bands were collected and individually re-centrifuged at 100,000 *g* for 30 min (4°); and the pellets were resuspended. In those studies employing a lysed mitochondrial preparation, the 10,000 *g* pellet was resuspended in 20 mM Tris-acetate (pH 7.4), vortexed vigorously, and allowed to sit on ice for 30 min. The suspension was then centrifuged at 50,000 *g* for 30 min (4°) and the pellet was resuspended. Lastly, the 10,000 *g* supernatant fraction was centrifuged at 50,000 *g* for 30 min, and the resulting pellet was resuspended. The 50,000 *g* supernatant fraction was also kept for assay. Protein content in all subcellular preparations was determined using the method of Bradford [14].

The assay system was a modification of the method of Ryan *et al.* [15], which employs the substrate [^{3}H]benzoyl-phenylalanyl-alanyl-proline ([^{3}H]BPAP, Ventrex Inc., Portland, ME). DCP has a K_m of 12.5 μM for [^{3}H]BPAP that compares favorably with its K_m for angiotensin I and bradykinin, 30 μM and 0.85 μM, respectively [1], and that is orders of magnitude lower than its K_m for other artificial substrates, which ranges between 2 and 5 mM [1, 15]. The assay buffer was ordinarily 20 mM Tris-acetate/100 mM NaCl (pH 7.4). When intact synaptosomes were used, the assay buffer contained 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose and 50 mM Tris-acetate (pH 7.4; 326 mOsM). Tissue protein per assay tube ranged between 25 and 100 μg. Product formed and percent substrate utilization were linear with protein content up to 125 μg. Test drugs were added in final concentrations as described in the tables. Assays were initiated by the addition of [^{3}H]BPAP (9 pmoles; 20 Ci/mmole) and proceeded at 37° for 10 min in a final incubation volume of 200 μl. The reaction was terminated by the addition of 1 ml of 0.1 N HCl. Blank values were determined from samples to which HCl was added prior to incubation. Product was extracted by the addition of 1 ml toluene to samples, which were vortexed for 10 sec and centrifuged at 1000 *g* for 15 min to break the emulsion. Five hundred microliters of the upper organic phase was counted with 10 ml of scintillation fluid for 2–5 min. Partitioning of product and substrate into the organic phase was 48–51% and less than 2% respectively. Percent substrate utilization was calculated as described by Ryan *et al.* [15] using the following formula:

$$\frac{\text{Product formed} \times 4.4 \times 100}{\text{Total substrate} \times \mu\text{g protein}}.$$

The correction factor of 4.4 was used to correct for sampling only half of the product dispersed in the upper organic layer and for the partitioning part of product in 1.2 ml of aqueous phase and 1 ml of organic phase. Percent substrate utilization varied with enzyme concentration in different subcellular fractions as depicted in tables.

Reaction kinetics were demonstrated to be first-order with respect to substrate concentration, as percent conversion of substrate to product was constant and independent of the substrate concentration employed in these studies. First-order kinetic conditions simulate the presumed endogenous concentrations of substrates for DCP in plasma, which are around 5–50 pM [16]. More importantly, these conditions also allow for the most sensitive assay of DCP because the substrate maintains the highest possible specific radioactivity at low substrate concentrations. Furthermore, less chance of substrate cross-reactivity with non-specific peptidases exists. Cross-reactivity might be a problem when artificial substrates such as hippuryl-histidyl-leucine are employed in the millimolar range to detect tissue DCP activity. Hippuryl-histidyl-leucine has been used by investigators to study brain DCP activity [7, 10, 11]. Lastly, for a single substrate enzymatic reaction, the percent substrate utilization using first-order conditions can be shown to be directly proportional to V_{max}/K_m and is a good index of specific enzymatic activity [17].

Subcellular distributions were calculated both in terms of specific enzyme activity (as reflected in the percent substrate utilization) and as total enzyme activity (total units of enzyme per subcellular fraction). The highest specific enzyme activity of DCP was present in the enriched synaptosomal fraction (Table 1). The synaptosomal activity was 3.6 times greater than the specific enzymatic activity of the whole homogenate. The myelin and the mitochondrial subfractions had the next highest activities, being 203 and 161% respectively. Total enzyme activity was highest in the nuclei/unbroken cell fraction, followed by the crude mitochondrial pellet (Table 1). Microsomes and cytosol had minimal total enzyme activity. These results are in contrast to the subcellular fractionation study of Yang and Neff [7], who reported the highest specific activity of DCP in the microsomal pellet. The conflicting results may be explained by our use of a different substrate and kinetic conditions to assay DCP. Furthermore, the present study employed preparations from a discrete brain region, whereas Yang and Neff [7] used preparations derived from whole brain. Whether heterogeneity in specific activity of DCP in the subcellular fractions is due to different concentrations of enzyme molecules per unit protein, or to changes in the distribution of endogenous DCP inhibitory factors, cannot be addressed in this study. The presence of endogenous inhibitory factors for DCP has been reported in plasma and aorta [18, 19]. Nevertheless, the identification of DCP of high specific activity in enriched synaptosomes is consistent with the hypothesis that the enzyme is involved in the processing of biologically active peptides.

DCP appeared to be membrane-bound, as lysed mitochondrial pellets contained virtually the same enzyme activity as intact pellets (Table 2). The presence of DCP on membranes was further confirmed by homogenizing brain stems in buffer containing 30 mM 1-*O*-*n*-octyl-β-D-glucopyranoside, which increased the yield of DCP activity in cytosol to 789% of control (Table 2). The detergent had no effect on plasma DCP activity, indicating that the increased activity in the cytosol was due to solubilization and not activation of the enzyme (data not shown). How-

Table 1. Subcellular distribution of DCP activity in rat brain stem*

Fraction	Percent substrate utilization per μg protein in 10 min	Percent specific activity	Percent total activity
Whole homogenate	0.34	100	100
Nuclei and unbroken cells (1000 *g*)	0.43	126	89
Intact crude mitochondrial pellet (10,000 *g*)	0.55	161	10
Myelin band	0.69	203	
Synaptosomal band	1.21	356	
Mitochondrial band	0.55	161	
Microsomal pellet (50,000 *g*)	0.10	30	2
Cytosol (50,000 *g* supernatant)	0.06	18	3

* Subcellular distribution is expressed as percent substrate utilization, which for single substrate reactions is proportional to V_{max}/K_m. Values for the major subcellular fractions represent the mean of three experiments in which quadruplicate samples were used to generate data points. Values for the subfractions of the mitochondrial pellet represent the mean of two experiments in which quadruplicate samples were used to generate data points.

Table 2. Localization of rat brain stem DCP activity to synaptosomal membranes*

	Percent substrate utilization per μg protein in 10 min	Percent control activity
Intact crude mitochondrial pellet	0.55	100
Lysed mitochondrial pellet	0.50	91
50,000 *g* Supernatant fraction Standard homogenization	0.09	100
50,000 *g* Supernatant fraction Homogenization with 30 mM 1-*O*-*n*-octyl-β-D-glucopyranoside	0.71	789

* Each value represents the mean of two experiments in which quadruplicate samples were used to generate data points.

ever, the localization of the enzyme to the outer or inner layer of the vesicles in the intact mitochondrial pellet cannot be ascertained as the permeability of vesicular membranes for [^{3}H]BPAP is unknown. Information concerning the localization of DCP on synaptic membranes will be important to the development of an understanding of the role of the enzyme in peptide processing.

Various treatments were studied *in vitro* to determine which factors might alter synaptosomal DCP activity. Activity was inhibited by captopril and EDTA in a dose-dependent manner (Table 3). The majority of DCP activity was shown to be chloride-dependent (Table 3). However, in the absence of added NaCl, DCP activity was still present (23% of DCP activity in the presence of 100 mM NaCl). The residual activity might be due to contaminant chloride from tissue or to the presence of a second carboxypeptidase that is inhibited by captopril but that is not chloride-dependent. The residual activity in the absence of chloride might also be due to the observation that DCP possesses different requirements for the halide depending on the substrate employed [15]. The fact that DCP activity was inhibited by captopril and EDTA, and that approximately 80% of the activity required chloride, indicates that the vast majority of apparent enzyme activity satisfies the criteria that identify the enzyme as DCP (EC 3.4.15.1) [16].

A variety of conditions that have been shown to alter synaptosomal tyrosine hydroxylase activity acutely were studied to determine whether DCP activity could be similarly affected. The rationale for these experiments was based on the hypothesis that catecholamines and enkephalins might exist as cotransmitters in the same nerve terminal [20]. However, depolarizing conditions using 65 mM KCl, presynaptic adrenergic agonists and antagonists, and phosphorylating conditions, all of which have been reported to alter synaptosomal tyrosine hydroxylase activity [21, 22], modified DCP activity by only ± 10% (data not shown). Hence, although high DCP activity was detected in enriched synaptosomes, no mechanism of acute regulation could be distinguished in the present study. This apparent lack of acute regulation employing procedures that are thought to affect catecholamine biosynthesis is by no means inconsistent with a role for DCP in peptide processing in neuronal tissue. Novel means of regulation for peptidase enzymes are possible and are presently being explored.

In summary, DCP of high specific activity was localized to enriched synaptosomes prepared from rat brain stem. This report is the first to appear in the literature presenting direct evidence for the subcellular localization of DCP activity in synaptosomes. This finding lends credence to the hypothesis of a neuronal renin-angiotensin system in brain and is also consistent with a role for DCP in the processing of other biologically active peptides such as enkephalins. The ability of 1-*O*-*n*-octyl-β-D-glucopyranoside to solubilize the enzyme coupled to the presence of DCP activity in both intact and lysed synaptosomes suggests that neuronal DCP is membrane-bound. DCP was characterized by its dependency on NaCl and the ability of EDTA and captopril to inhibit the enzyme. No mechanism of short-term regulation was observed when procedures that affect tyrosine

Table 3. Effect of various treatments on rat brain stem synaptosomal DCP activity*

Treatment	Concn (M)	Percent substrate utilization per μg protein in 10 min	Percent control activity
Control		0.89	100
NaCl	0	0.20	23
	25×10^{-3}	0.40	45
	50×10^{-3}	0.78	88
	75×10^{-3}	0.85	96
	100×10^{-3}	0.92	103
Captopril	1×10^{-13}	0.67	75
	1×10^{-11}	0.74	83
	1×10^{-9}	0.36	40
	1×10^{-7}	0.12	13
	1×10^{-5}	0.10	11
EDTA	1×10^{-6}	0.62	70
	1×10^{-5}	0.30	34
	1×10^{-4}	0.16	18
	1×10^{-3}	0.13	15

* Control sample contained 100 mM NaCl. Each value represents the mean of two experiments in which quadruplicate samples were used to generate data points.

hydroxylase activity were employed. Long-term mechanisms by which DCP in neuronal tissue can be regulated are presently being studied.

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