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Inhibition by Converting Enzyme Inhibitors of Pig Kidney Aminopeptidase P

Nigel M. Hooper, John Hryszko, Sylvester Y. Oppong, and Anthony J. Turner

Several inhibitors of angiotensin converting enzyme were also found to inhibit aminopeptidase P, whereas inhibitors of other mammalian aminopeptidases were ineffective. Aminopeptidase P purified from pig kidney cortex was found to contain one atom of zinc per polypeptide chain, confirming its metalloenzyme nature. The concentrations of converting enzyme inhibitors required to cause 50% inhibition (I_{50}) of aminopeptidase P were in the low micromolar range. The most potent converting enzyme inhibitors toward aminopeptidase P were the carboxylalkyl compounds, cilazaprilat, enalaprilat, and ramiprilat (I_{50} values of 3–12 μM). The sulfhydryl compounds captopril (I_{50} 110 μM) and YS980 (I_{50} 20 μM) were slightly less potent at inhibiting aminopeptidase P. In contrast, the carboxylalkyl compounds benazeprilat, lisinopril, and pentoprilat; the sulfhydryl compound rentiapril; and the phosphoryl compounds ceranopril and fosinoprilat had no inhibitory effect against aminopeptidase P. This compares with I_{50} values in the 1–6 nM range for these inhibitors with angiotensin converting enzyme. Inhibition of aminopeptidase P may account for some of the effects or side effects noted with the clinical use of converting enzyme inhibitors. These results may provide the basis for the design of more selective inhibitors of angiotensin converting enzyme or mixed inhibitors of aminopeptidase P and angiotensin converting enzyme, or both. (*Hypertension* 1992;19:281–285)

KEY WORDS • aminopeptidases • angiotensin converting enzyme inhibitors • captopril • enalaprilat

Angiotensin converting enzyme (ACE) (EC 3.4.15.1) is a zinc metallopeptidase that catalyzes the removal of C-terminal dipeptides from susceptible substrates. ACE is probably best known for its role in the renin-angiotensin system where it catalyzes the conversion of the inactive decapeptide angiotensin I to the octapeptide angiotensin II a potent vasoconstrictor and salt-retaining agent.¹ In addition, ACE inactivates the vasodilatory and natriuretic peptide bradykinin. Thus, ACE has a dual role in the maintenance of blood pressure and fluid and electrolyte homeostasis, and inhibitors of ACE have been used successfully in the treatment of hypertension and congestive heart failure.² In recent years, numerous potent and apparently selective converting enzyme inhibitors have been developed (see References 3–5 for review). These inhibitors can be divided into three classes depending on the group that coordinates to the active site zinc atom: 1) carboxylalkyl, 2) sulfhydryl, and 3) phosphoryl. Several untoward side effects have been noted with the clinical use of some converting enzyme inhibitors,^{2,5,6} some of which may be due to the involvement of ACE in other physiological processes, since the enzyme has a wide tissue and cellular distribution and is not always colocalized with other components of the renin-angiotensin sys-

tem.^{7–9} Angiotensin I and bradykinin are also not the only regulatory peptides cleaved by ACE.⁹ Alternatively, or in addition, some of the side effects or effects, or both, observed with the use of converting enzyme inhibitors may be due to the inhibition of other enzymes.

Aminopeptidase P (AP-P) (EC 3.4.11.9) catalyzes the removal of N-terminal amino acids from oligopeptides containing a penultimate prolyl residue.¹⁰ The enzyme has been shown to cleave several biologically active peptides, including bradykinin and substance P,^{11,12} and there is strong evidence to indicate that, together with ACE, AP-P in the lung is responsible for the inactivation of circulating bradykinin (see Reference 13 for review). Recently, we identified mammalian AP-P as being anchored at the extracellular side of the plasma membrane by a covalently attached glycosyl-phosphatidylinositol moiety,¹⁴ and we have subsequently purified the enzyme from pig kidney to apparent homogeneity after its release from the membrane by phosphatidylinositol-specific phospholipase C (PI-PLC).¹⁵ Purified AP-P is a metalloenzyme; its activity is inhibited by chelating agents and is stimulated by Mn^{2+} or Co^{2+} ions.¹⁵ In the present study, we confirm that AP-P is a zinc-containing metallopeptidase and show that the enzyme is not inhibited by compounds that display a relatively broad specificity toward other members of the mammalian aminopeptidase superfamily, suggesting different active site requirements for AP-P. Further, we show that AP-P is inhibited by several converting enzyme inhibitors that are currently in clinical use or undergoing preclinical and clinical trials. Such inhibition may explain some of the effects or side-effects, or both, noted with the use of converting enzyme inhibitors

From the Membrane Peptidase Research Group, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, UK.

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Address for correspondence: Dr. N.M. Hooper, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT UK.

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and could lead to the development of more selective inhibitors of ACE or mixed inhibitors of ACE and AP-P.

Methods

Materials

AP-P was purified from pig kidney cortex after release from the membrane by bacterial PI-PLC as described in Hooper et al.¹⁵ ACE was purified from pig kidney cortex by affinity chromatography on lisinopril-2.8 nm-Sepharose as described previously.¹⁶ Both enzyme preparations were apparently homogeneous as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had specific activities of 7.2 μmol Pro-Hyp produced per minute per milligram for AP-P and 20.0 μmol His-Leu produced per minute per milligram for ACE.

Enalapril (MK 421), enalaprilat (MK 422), lisinopril (MK 521), benazeprilat (L 155,360), and L 155,212 were gifts from Merck Sharp & Dohme Research Laboratories, Rahway, N.J. Spiraprilat (SCH 33861) was a gift from Schering-Plough Research, Bloomfield, N.J. Pentopril (CGS 13945) and pentoprilat (CGS 13934) were gifts from CIBA-GEIGY Corporation, Summit, N.J. Quinapril (PD 109452-2Q), quinaprilat (PD 109548-1), indolapril (PD 109763-2), and indolaprilat (PD 110021-0) were gifts from Parke-Davis Pharmaceutical Research Division, Ann Arbor, Mich. Cilazapril (Ro 31-2848/006) and cilazaprilat (Ro 31-3113/000) were gifts from Roche Products Ltd., Welwyn Garden City, U.K. Rentiapril (SA 446) and YS 980 were gifts from Santen Pharmaceutical Co., Ltd., Osaka, Japan. Ramiapril (Hoe 498) and ramiprilat were gifts from Hoechst Pharmaceutical Research Laboratories, Milton Keynes, UK. Zofenoprilat (SQ 26,703), fosinoprilat (SQ 27,519), ceranopril (SQ 29,852), and captopril (SQ 14,225) were gifts from Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J. Kelatorphan was a gift from Dr. B. Roques (Paris). Arphamenines A and B were obtained from Sigma Chemical Co., Poole, UK. All other materials were from sources previously noted.

Determination of Zinc

All buffers were made up with deionized H₂O. Dialysis tubing was boiled in the presence of ethylenediaminetetraacetic acid and was washed extensively with deionized H₂O. All glassware was acid-washed in 6M HCl and was rinsed with deionized H₂O before use. The enzyme sample (0.2 mg protein/ml) was dialyzed against three changes of 5 mM Tris/HCl, pH 7.9 at 4°C to remove any nonspecifically bound zinc. After determining the protein concentration of the dialyzed sample, duplicate 0.4-ml aliquots were analyzed on an Instrumental Laboratories IL 157 atomic absorption spectrometer (Thermo Electron Ltd., Birchwood, UK), ZnSO₄ (0–6 μM) was used as standard.

Enzyme Assays

AP-P was assayed using Gly-Pro-Hyp (1 mM) as substrate in 0.1 M Tris/HCl, 4 mM MnCl₂, pH 8.0 at 37°C. The product, Pro-Hyp, was separated from the substrate and was quantitated by reverse-phase, high-performance liquid chromatography.¹⁴ ACE was assayed fluorimetrically with BzGly-His-Leu (1 mM) as substrate.¹⁷ Incubations were performed in duplicate

TABLE 1. Effect of Metallopeptidase Inhibitors on the Activity of Aminopeptidase P

Class of peptidase	Inhibitor	Concentration (mM)	Relative activity (%)
	None	...	100.0
Aminopeptidases	Actinonin	0.01	100.4
	Amastatin	0.01	91.5
	Arphamenine A	1.0	143.0
	Arphamenine B	1.0	127.0
	Bestatin	0.1	84.5
	Puromycin	0.1	111.3
Endopeptidases	Kelatorphan	1.0	108.3
	Phosphoramidon	1.0	83.3

Purified aminopeptidase P (AP-P) (100 ng) was incubated with Gly-Pro-Hyp as described in the "Methods" section. Results are the mean of duplicate determinations, which did not differ by more than 10%.

with each concentration of inhibitor, and the enzyme concentration was kept constant in the inhibition studies. Enzyme and inhibitors were preincubated for 5 minutes at 37°C. In the absence of inhibitor, there was maximally 20% substrate breakdown.

Results

Metal Ion Content of Aminopeptidase P

AP-P purified from pig kidney cortex was examined for its content of Zn²⁺ by atomic absorption spectroscopy as described in the "Methods" section. Purified AP-P was found to contain 0.97 mol Zn²⁺ per mole protein. Parallel determinations on another zinc metallopeptidase, membrane dipeptidase, revealed the presence of 0.98 mol Zn²⁺ per mole protein, in agreement with previous results.¹⁸

Effect of Aminopeptidase and Endopeptidase Inhibitors on Activity of Aminopeptidase P

The effect on the activity of AP-P of specific inhibitors of aminopeptidases and endopeptidases was examined (Table 1). Of the typical aminopeptidase inhibitors, only bestatin caused slight (16%) inhibition of the activity of AP-P. The other aminopeptidase inhibitors and the two endopeptidase inhibitors kelatorphan and phosphoramidon failed to inhibit AP-P significantly.

Effect of Converting Enzyme Inhibitors on Activity of Aminopeptidase P

The effect on the activity of AP-P of converting enzyme inhibitors at a concentration of 1 mM was examined (Table 2). None of the esterified pro-drugs caused more than 25% inhibition of AP-P. However, a number of the active drugs caused substantial (more than 85%) inhibition of AP-P. A more detailed examination of the effect of these inhibitors on AP-P was undertaken, and the inhibition of AP-P was directly compared with the inhibition of ACE (Figure 1 and Table 3). All of the converting enzyme inhibitors, apart from ceranopril and pentoprilat, displayed 50% inhibition (I₅₀) values toward ACE in the nanomolar range, in

TABLE 2. Effect of Inhibitors of Angiotensin Converting Enzyme on the Activity of Aminopeptidase P

Class of inhibitor	Inhibitor	Relative activity (%) at an inhibitor concentration of 1 mM
	None	100
Carboxylalkyl	Benazeprilat	109.7
	Cilazapril*	75.0
	Cilazaprilat	0.0
	Enalapril*	96.2
	Enalaprilat	0.0
	Indolapril*	162.0
	Indolaprilat	7.0
	Lisinopril	105.2
	L155,212	0.0
	Pentopril*	108.0
	Pentoprilat	79.5
	Quinapril*	159.0
	Quinaprilat	5.5
	Ramipril*	85.0
Ramiprilat	0.0	
Spiraprilat	11.0	
Sulfydryl	Captopril	2.3
	Rentiapril	65.7
	YS 980	0.0
	Zofenoprilat	13.7
Phosphoryl	Ceranopril	46.3
	Fosinoprilat	160.5

Purified aminopeptidase P (AP-P) (100 ng) was incubated with Gly-Pro-Hyp as described in the "Methods" section. The results are the mean of three independent duplicate determinations with each inhibitor.

*Pro-drug.

agreement with published results.⁴ Several of the carboxylalkyl converting enzyme inhibitors (L155,212, enalaprilat, cilazaprilat, and ramiprilat) displayed I_{50} values toward AP-P in the low micromolar range (Table 3). The structurally related compounds benazeprilat, lisinopril, and pentoprilat had no inhibitory effect on AP-P, whereas indolaprilat, quinaprilat, and spiraprilat were intermediate in their inhibitory action on AP-P (Table 3). Of the sulfydryl converting enzyme inhibitors, YS 980 was the most potent at inhibiting AP-P with an I_{50} of 20 μ M (Table 3). Captopril and zofenoprilat were fivefold to 20-fold less potent at inhibiting AP-P, and rentiapril had negligible inhibitory effect. Neither of the two phosphoryl converting enzyme inhibitors, ceranopril and fosinoprilat, inhibited AP-P (Table 3).

Discussion

Aminopeptidase P was, until recently, a poorly characterized enzyme mainly due to difficulties encountered in solubilizing the protein from other membrane components. With the observation that AP-P was a member of the glycosyl-phosphatidylinositol-anchored membrane protein family (see References 19 and 20 for

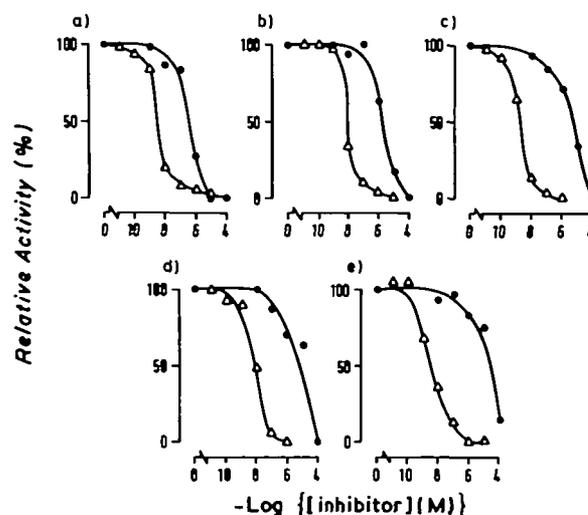


FIGURE 1. Line graphs show inhibition curves of aminopeptidase P (AP-P) and angiotensin converting enzyme (ACE). Pig kidney AP-P (100 ng) or pig kidney ACE (100 ng) were incubated with the appropriate substrate, and the reaction products were quantitated as described in the "Methods" section. Inhibitors used were L155,212 (panel a), enalaprilat (panel b), cilazaprilat (panel c), ramiprilat (panel d), and YS 980 (panel e). Each point is the mean of duplicate determinations. ●, AP-P; △, ACE.

review), we were able to solubilize AP-P in a hydrophilic form from pig kidney membranes with bacterial PI-PLC before its purification to apparent homogeneity.^{14,15} Purified AP-P was inhibited by chelating agents, and its activity was stimulated by Mn^{2+} or Co^{2+} ions, indicative of a metalloenzyme.¹⁵ In the present study, we have confirmed the metalloenzyme nature of AP-P by demonstrating the presence of one atom of zinc bound per polypeptide chain. Although AP-P is classified as an aminopeptidase, removing N-terminal residues from tripeptides and oligopeptides when there is a penultimate prolyl residue,^{21,22} inhibitors of other members of the mammalian aminopeptidase superfamily (aminopeptidase N, EC 3.4.11.2; aminopeptidase B, EC 3.4.11.6; aminopeptidase A, EC 3.4.11.7; and aminopeptidase W, EC 3.4.11.16) were ineffective at blocking the activity of AP-P (Table 1). This suggested that the active site of AP-P differs from these other zinc aminopeptidases. In addition, the endopeptidase inhibitor phosphoramidon and the mixed aminopeptidase/endopeptidase inhibitor kelatorphan had no significant inhibitory effect on AP-P (Table 1).

Surprisingly, purified pig kidney AP-P was inhibited, with I_{50} values in the low micromolar range, by a number of "selective" inhibitors of ACE (Table 3 and Figure 1). Although these converting enzyme inhibitors were at least 100-fold more potent at inhibiting ACE than AP-P, an I_{50} value in the micromolar range *in vitro* may be significant in causing inhibition of AP-P *in vivo*. For example, teprotide, the first effective inhibitor of ACE *in vivo*, displays an I_{50} toward the enzyme of 1.0 μ M.²³ Also, the inhibitor of renal dipeptidase, cilastatin, which is coadministered with the β -lactam antibiotic imipenem to prevent its breakdown by dipeptidase in the kidney, displays an I_{50} value of 0.1 μ M *in vitro*.²⁴

TABLE 3. Comparison of the Potency of Converting Enzyme Inhibitors on the Activities of Aminopeptidase P and Angiotensin Converting Enzyme

Class of inhibitor	Inhibitor	I_{50}	
		AP-P (μ M)	ACE (nM)
Carboxylalkyl	L155,212	0.3 \pm 0.2	2.7
	Enalaprilat	2.9 \pm 1.4	6.0
	Cilazaprilat	6.5 \pm 3.4	3.0
	Ramiprilat	12.0 \pm 5.6	2.0
	Quinaprilat	266	0.5
	Indolaprilat	485	3.0
	Spiraprilat	560	1.0
	Benazeprilat	>1,000	5.6
	Lisinopril	>1,000	1.0
	Pentoprilat	>1,000	81.5
Sulfydryl	YS 980	19.5 \pm 6.4	3.5
	Captopril	109.8 \pm 52.3	4.0
	Zofenoprilat	440	3.0
	Rentiapril	>1,000	2.0
Phosphoryl	Ceranopril	\sim 1,000	63.0
	Fosinoprilat	>1,000	2.0

The I_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity under the assay conditions described in the "Methods" section. The results for aminopeptidase P (AP-P) are either the mean \pm SEM of four separate determinations or the mean of duplicate determinations. The results for angiotensin converting enzyme (ACE) are the mean of duplicate determinations.

The binding of the synthetic assay peptide Gly-Pro-Hyp to the active site of AP-P is represented in Figure 2. As with the binding of enalaprilat to ACE,²³ the carboxyl group of the inhibitor probably coordinates to the active site zinc ion of AP-P; thus the esterified pro-drugs were ineffective at inhibiting AP-P (Table 2). The carboxylalkyl inhibitors that caused substantial inhibition of AP-P (Table 3) are structural analogues of enalaprilat.³ Ramiprilat is a bicyclic derivative with a five-membered ring fused to positions 4 and 5 of the prolyl ring, whereas cilazaprilat is a bicyclic lactam derivative and L155,212 is a bicyclic lactam, thiaproline derivative (compound 11a in Wyvratt et al²⁵). Of the carboxylalkyl converting enzyme inhibitors that do not inhibit AP-P with I_{50} values in the low micromolar range, pentoprilat has an aromatic ring and indolaprilat a six-membered ring fused to positions 4 and 5 of the prolyl ring, benazeprilat has an aromatic ring fused to the lactam ring, quinaprilat has an aromatic ring fused to positions 4 and 5 of its six-membered prolyl substitute, and spiraprilat has a five-membered ring containing two sulfur atoms fused to position 4 of the prolyl ring.³ Thus, with each of these five inhibitors, the presence of a relatively bulky substituent on the prolyl ring, or its equivalent, diametrically opposite the free carboxyl group leads to decreased inhibition of AP-P, suggesting that the S_2' site in AP-P cannot accept such bulky groups (Figure 2). The difference in the inhibitory potencies of enalaprilat and lisinopril toward AP-P is particularly significant (Table 3). Lisinopril has a lysyl side chain in place of the alanyl side chain in enalaprilat. This change prevents binding of lisinopril to AP-P, presumably because the S_1' site in the enzyme cannot

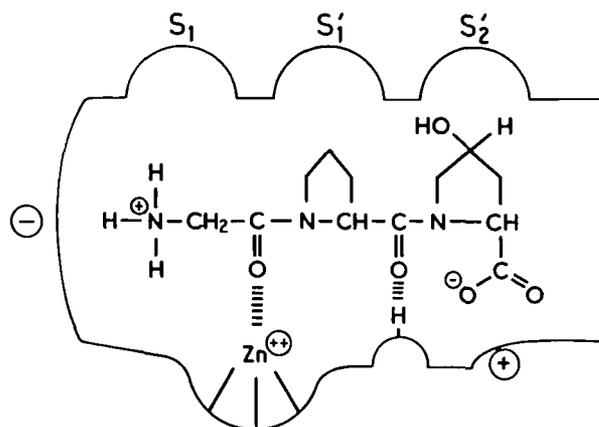


FIGURE 2. Schematic diagram shows hypothetical model of the binding interactions between the synthetic assay substrate Gly-Pro-Hyp and the active site of aminopeptidase P.

accept such a large positively charged side chain (Figure 2). Therefore, lisinopril is much more selective for ACE.

The sulfydryl inhibitors captopril and YS 980, which also inhibited AP-P (Table 3), are structurally identical apart from a sulfur atom in position 4 of the prolyl ring of YS 980,³ a feature that appears to enhance the binding of YS 980 to the S_2' site (Figure 2). Zofenoprilat differs from captopril in possessing an aromatic ring attached to position 4 of the prolyl ring via a sulfur bridge, whereas rentiapril differs from YS 980 in having a hydroxylated aromatic ring attached to position 5 of the prolyl ring,³ features that decrease the binding of inhibitors to AP-P (see above). Neither of the phosphoryl converting enzyme inhibitors are effective on AP-P (Table 3). This may be either a function of the phosphoryl group or because ceranopril has a lysyl side chain (as in lisinopril) or because fosinoprilat has a six-membered ring attached to position 4 of the prolyl residue; either of these features appear to prevent binding of inhibitors to AP-P (see above). In summary, the features that make an inhibitor selective for ACE over AP-P are the presence of a large positively charged side chain (e.g., lysyl) in the S_1' position, or a large (six-membered) ring attached to the prolyl ring diametrically opposite the free carboxyl group in the S_2' position, or both (Figure 2).

AP-P has a wide tissue distribution. Enzyme activity has been detected in human and pig kidney, pig and bovine lung, and pig and rat intestine.^{11,14,26} However, the enzyme has only been purified from pig kidney¹⁵ and rat brain.¹² Recently, immunocytochemical studies have colocalized AP-P with ACE in pig kidney, intestine, and brain microvessels (K. Barnes and N.M. Hooper, unpublished observations). In addition a similar activity has been identified in human serum.²⁷ The precise role (or roles) of AP-P, however, is unknown. Several biologically active peptides have a penultimate Pro residue at the N-terminus that renders the peptide resistant to attack by most aminopeptidases and dipeptidyl aminopeptidases.²⁸ In vitro, AP-P is capable of releasing the N-terminal amino acid from substance P, corticotropin-like intermediate lobe peptide, β -casomorphin, [Tyr¹]-melanostatin, morphiceptin, and diprotin A¹² (N.M. Hooper, unpublished observations). Thus, AP-P may have an important role in initiating the metabolism of a

number of bioactive peptides. Because of its specificity, AP-P had been suggested to play a role in the breakdown of collagen or other proteins with repeating Gly-Pro-Xaa sequences.¹⁰ In support of this proposal is the recent observation that excessive urinary excretion of imino-oligopeptides is due to a deficiency in small intestine AP-P.²⁹ Probably the best-documented function of AP-P is its role in pulmonary bradykinin metabolism (see Reference 13 for review). Together with ACE, AP-P inactivates this potent vasodilator by removal of the N-terminal Arg, allowing another cell surface peptidase, dipeptidyl peptidase IV (EC 3.4.14.5), to subsequently remove the Pro-Pro sequence. Thus, the wide tissue distribution and potential range of substrates suggests that inhibition of AP-P by converting enzyme inhibitors may cause widespread disruption of normal physiological processes.

Two scenarios can be hypothesized in view of the interaction of certain converting enzyme inhibitors with AP-P. First, some of the side effects that have been noted with the use of converting enzyme inhibitors in the treatment of hypertension and congestive heart failure^{2,5,6} may be due to inhibition of AP-P. It is possible that a comparison of the side effects noted with the use of, for example, enalapril (which also inhibits AP-P) and lisinopril (which does not inhibit AP-P), may indicate the physiological processes in which AP-P has a role. Alternatively, considering the participation of both AP-P and ACE in the metabolism of bradykinin, mixed inhibitors of these two enzymes may be more effective in potentiating the effects of bradykinin. This approach has recently been applied to the development of mixed inhibitors of ACE and endopeptidase 24.11 (enkephalinase, EC 3.4.24.11).³⁰ The involvement of endopeptidase 24.11 in the inactivation of the vasodilatory, diuretic, and natriuretic peptide atrial natriuretic factor suggested that mixed inhibitors of ACE and endopeptidase 24.11 might have potential in the treatment of certain cardiovascular and salt-retention disorders.

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