

# Further Studies on Aminopeptidase-M in Blood in Children with Cholestatic Liver Diseases and Viral Hepatitis

ROMAN M. JANAS, PhD, JERZY SOCHA, MD, PhD, KRZYSZTOF WARNAWIN, MSc,  
and JOLANTA RUJNER, MD

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Aim of this study was to determine and further characterize the serum aminopeptidase-M in children with liver diseases. Based on our new assay, we have shown two fractions of the enzyme. Activity of the first fraction is expressed in undiluted serum at pH adjusted from 8.5 (pH of stored serum) to 7.4. Activity of the second fraction (cryptic activity) appears in the serum (pH 7.4) as a result of dilution and/or addition of aniline naphthalene sulfonic acid. In children with Alagille syndrome, extrahepatic biliary duct atresia, Byler's disease, and acute hepatitis due to hepatitis B virus infection, activities of both fractions are highly elevated as compared to healthy children or those with chronic viral hepatitis. Moreover, serum aminopeptidase-M seems to reflect other aspects of the pathological process than those reflected by the alanine aminotransferase and  $\gamma$ -glutamyltranspeptidase. Due to increased activity and broad substrate specificity, the enzyme seems to be also a cofactor of cholestasis and hepatitis.

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**KEY WORDS:** cholestasis; viral hepatitis; Alagille syndrome; Byler's disease; biliary duct atresia; enkephalins; serum aminopeptidase.

Membrane-bound aminopeptidase-M (EC 3.4.11.2) functions as an ectopeptidase at the cell surface in many tissues and there is also a soluble form of this enzyme in serum and other body fluids (1–3). Serum aminopeptidase-M activity is markedly increased in patients with cholestasis and was shown to be a useful marker of hepatobiliary disease (4–7). The enzyme is thought to originate from the liver (2). However, its multiple molecular forms present in serum may arise from other organs and tissues (6, 8).

In hematology, aminopeptidase-M (cluster of dif-

ferentiation 13) is a well-recognized marker of myeloid cell leukemia (9–11). Interestingly, it has been found that aminopeptidase-M on alimentary or respiratory tract epithelial cells is a receptor for human coronavirus 229E (12). In a large population of apparently healthy humans, the enzyme activity seems to exhibit some variations that are dependent on age, sex, alcohol consumption, smoking, and drug intake (13).

Among the substrates of aminopeptidase-M, including those involved in growth and differentiation regulation, phagocytosis, and bactericidal and tumoricidal activity (3, 4, 9, 14–16), both methionine (met) and leucine (leu) enkephalins are rapidly degraded after their administration to humans or animals (17–28). In circulation, the endogenous enkephalins seem to be protected from degradation through binding to carrier proteins (19). However, a free fraction of the enkephalins would be susceptible to the enzyme.

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From the Department of Radioimmunology, Molecular Endocrinology Laboratory, and Department of Gastroenterology and Nutrition, The Children's Memorial Health Institute, Warsaw, Poland.

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Address for reprint requests: Dr. Roman M. Janas, Department of Radioimmunology, Molecular Endocrinology Laboratory, The Children's Memorial Health Institute, Al. Dzieci Polskich 20, 04-736 Warsaw, Poland.

Enkephalins appear to be implicated in cholestatic liver disease and may be an important pathogenic factor in such complications as pruritus or encephalopathy. Indeed, plasma enkephalin concentrations are highly elevated in adult patients with cholestasis of differing aetiologies (7, 29–32), and in rats with an experimental model of acute cholestasis (33, 34). Accumulating in blood, enkephalins may cross the blood–brain barrier and evoke, via their increased availability in the central nervous system, a syndrome of chronic opiate-receptor stimulation (35, 36). Preliminary experiments have shown that naloxone or nalmefene, opiate-receptor antagonists, ameliorated or reversed pruritus in patients with chronic cholestatic liver disease and, in some patients, precipitated a syndrome that resembles the withdrawal reaction of opiate addiction (37, 38).

Recently, we have shown that in children with cholestasis of differing etiologies increased serum met-enkephalin occurs concomitantly with highly elevated serum aminopeptidase-M activity and that both parameters were apparently not affected by a short-term treatment with ursodeoxycholic acid (39, 40). Alternations in the aminopeptidase-M activity as well as their relevance to the situation in cholestasis and viral hepatitis are not fully understood yet, and further systematic studies seem to be worthwhile. Therefore, in this study we attempted to further characterize and compare the serum aminopeptidase-M activity in healthy children, children with cholestatic liver disease, and children with chronic or acute hepatitis due to hepatitis B virus (HBV) infection.

## MATERIALS AND METHODS

**Patients.** The protocol of this study was approved by the hospital ethics committee. The following groups of patients were included in the study: group 1, eight children, 8–14 years old (mean 10 years), with Alagille syndrome (arteriohepatic dysplasia), alanine aminotransferase (ALAT):  $158 \pm 69$  units/liter,  $\gamma$ -glutamyltranspeptidase ( $\gamma$ GTP):  $248 \pm 57$  units/liter; group 2, eight children, 3 months–2.5 years old (mean 1.5 year), with extrahepatic biliary duct atresia, ALAT:  $198 \pm 49$  units/liter,  $\gamma$ GTP:  $301 \pm 109$  units/liter; group 3, seven children, 6–12 years old (mean 8 years), with Byler's disease (progressive intrahepatic familial cholestasis), ALAT:  $91 \pm 18$  units/liter,  $\gamma$ GTP:  $15.8 \pm 6.0$  units/liter; group 4, seven children, 5–14 years old (mean 9 years), with chronic active hepatitis due to hepatitis B virus (HBV) infection, ALAT:  $69 \pm 15$  units/liter,  $\gamma$ GTP:  $27.5 \pm 8.9$  units/liter; group 5, six children, 11–17 years old (mean 14 years), with acute hepatitis due to HBV infection, ALAT:  $1145 \pm 450$  units/liter,  $\gamma$ GTP:  $438 \pm 112$  units/liter in four patients and 63 and 74 units/liter in two; and group 6, nine apparently healthy children, 6–14 years old (mean

10 years), ALAT:  $21 \pm 8.4$  units/liter,  $\gamma$ GTP:  $13.8 \pm 7.2$  units/liter.

**Blood Sampling.** Venous blood was sampled without anticoagulants for the diagnostic examinations; it was possible to use part of each sample in this study. Blood samples were centrifuged at 3000g for 15 min at 4°C, and the sera filtered through a 0.2- $\mu$ m filter (Sartorius-Membranfilter, Gottingen, Germany) to exclude all residual cell debris, and stored at  $-30^{\circ}\text{C}$ . Serum pH was measured using a pH microelectrode (Sigma Chemical Co., St Louis, Missouri).

**Assay of Aminopeptidase-M Activity.** Equal aliquots (500  $\mu$ l) of the sera were pooled in groups 1–6, respectively, and used for characterization of the aminopeptidase-M. The enzyme activity was also determined in the individual serum samples from patients from groups 1–6. Dilution of the serum was performed using 25 mmol/liter bisTris–Tris–acetic acid buffer with or without 0.15 mol/liter NaCl, at pH range 4.5–9.0. To maintain the serum pH at a desired level, the endogenous  $\text{HCO}_3^-$  was hydrolyzed with a negligible volume of 1 mol/liter HCl (final concentration 25–30 mmol/liter). Resulting  $\text{CO}_2$  was removed under low pressure, and pH was adjusted with a small volume of 1 mol/liter NaOH, HCl, or bisTris–Tris–acetic acid under the control of a pH microelectrode.

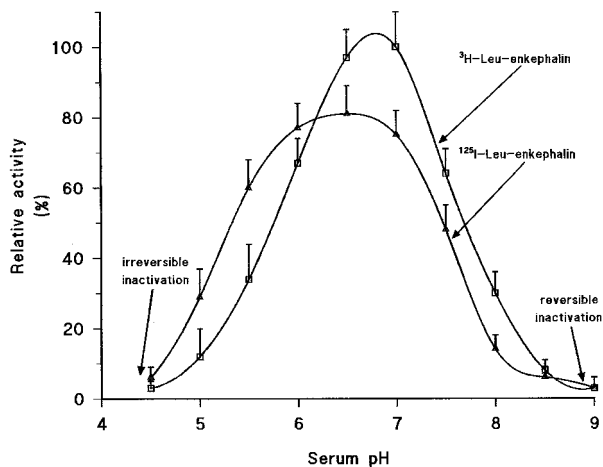
The incubation mixture consisted of 100  $\mu$ l of undiluted or diluted serum and 5  $\mu$ l of [ $^{125}\text{I}$ ] or [ $^3\text{H}$ ]leu-enkephalin as substrate (100 pmol/liter), from Incstar, Stillwater, Minnesota and DuPont NEN, Boston, Massachusetts, respectively, with specific activities  $\sim 500$  Ci/mmol and  $\sim 80$  Ci/mmol, respectively.

Incubation was performed at 37°C for 0 min (control) to 15 min, and terminated by acidification with 100 mmol/l acetic acid. Degradation of the substrates was quantified by the aluminum silicate binding assay as recently described (41) with slight modifications. A homogenous suspension (50 mg/ml) of aluminum silicate powder (Lloyd reagent, Pfaltz and Bauer, Waterbury, Connecticut) was prepared in 1% acetic acid. Aliquots of 2 ml were added to the tubes at the end of each incubation period. After vortexing for 1 min, the tubes were centrifuged at 3000g for 10 min. For [ $^{125}\text{I}$ ]leu-enkephalin, the supernatants were discarded by aspiration, and the silicate pellets counted for the  $\gamma$ -radioactivity. For the [ $^3\text{H}$ ]leu-enkephalin, 1.5-ml aliquots of the supernatants were mixed with 5 ml aliquots of the scintillation cocktail and counted for the  $\beta$ -radioactivity. Intra- and interassay coefficients of variation were 4.9% and 10.5% for iodinated and 8.9% and 14.5% for tritiated enkephalins, respectively.

The enzyme activity was expressed as picograms of degraded substrate per minute per milliliter whole serum.

Intact [ $^{125}\text{I}$ ]– or [ $^3\text{H}$ ]leu-enkephalin was almost completely adsorbed by the aluminum silicate ( $93 \pm 3\%$ , mean  $\pm$  SEM,  $N > 30$ ). Reverse-phase high-performance liquid chromatography showed that silicate pellet-bound radioactivity represented the intact labeled peptide, whereas 85–90% of the radioactivity appearing in the supernatants was due to [ $^{125}\text{I}$ ]– or [ $^3\text{H}$ ]tyrosine. Unidentified, two to three amino acid fragments constituted about 10–15% of the radioactivity and did not react with the specific leu-enkephalin antibody (data not shown).

**Effect of Inhibitors and Other Substances on Aminopeptidase-M.** Degradation of the labeled leu-enkephalin by



**Fig 1.** Effect of serum pH on the aminopeptidase-M activity. Undiluted, pooled sera from groups 1–6 were used in this experiment. Serum pH was adjusted at a desired, experimental level after previous hydrolysis of endogenous  $\text{HCO}_3^-$  as described in Materials and Methods. The enzyme activity was determined in duplicate in each serum pool using 100 pmol/liter [ $^{125}\text{I}$ ]- and [ $^3\text{H}$ ]leu-enkephalin. Data are presented as mean  $\pm$  SEM.

serum aminopeptidase-M was quantified by determining the percentage of intact label in the presence of increasing concentrations of specified substances. The extent of the label degradation in the control probes, no substance added (35–40% degradation after a specified incubation period) was taken as 100% relative activity. Before the assay, the inhibitors to be tested were preincubated with the serum at  $\sim 20^\circ\text{C}$  for 15 min. The inhibitory constants ( $K_i$ ) and Michaelis constant ( $K_M$ ) towards unlabeled leu-enkephalin were determined as previously described (39).

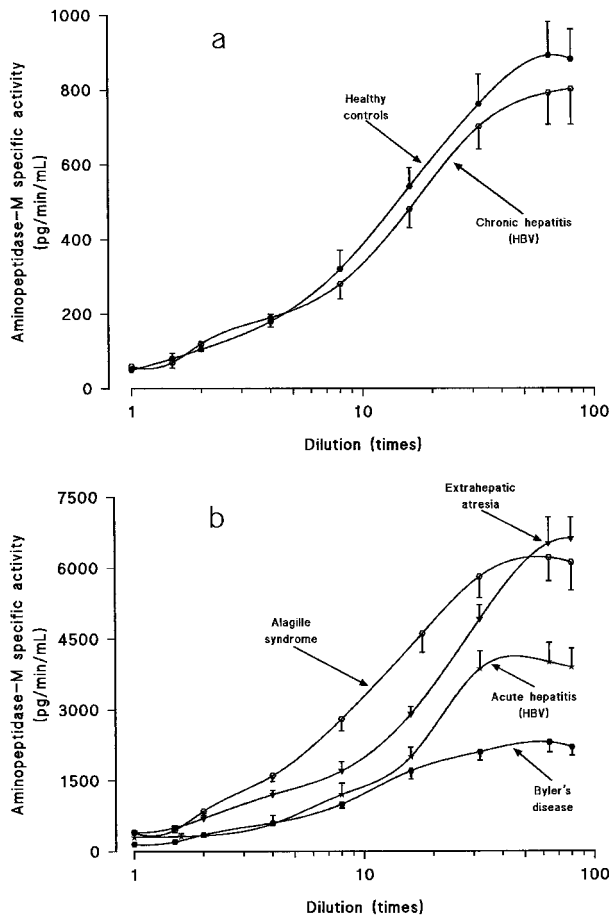
The following substances used in the present study were purchased from Sigma: 1,10-phenanthroline, bestatin, puromycin, leu-enkephalin, met-enkephalin, bovine serum albumin, 8-aniline naphthalene sulfonic (ANS) acid, taurocholic acid, ursodeoxycholic acid, and cholic acid. Other chemicals were of analytical grade.

**Statistical Analysis.** Values are expressed as means  $\pm$  SEM unless otherwise specified. The Mann-Whitney U test was used to determine the significance of differences between means;  $P < 0.05$  was considered significant.

## RESULTS

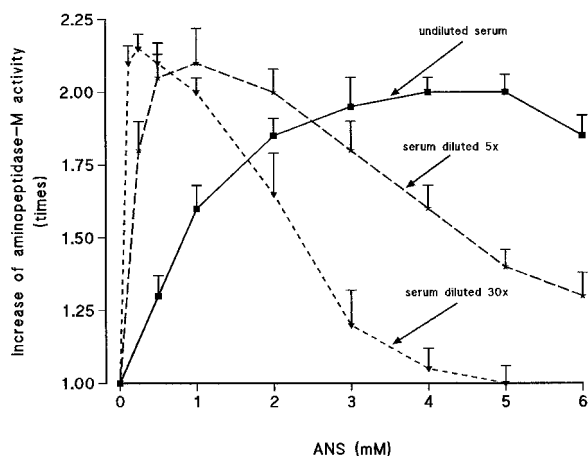
During storage, serum pH rises to 8.5. The effect of pH on aminopeptidase-M activity in undiluted serum pools from groups 1–6 is shown in Figure 1. The pH curves were symmetric and dynamic with an optimum at about 6.5–7.3 for both iodinated and tritiated leu-enkephalin. The same data were obtained in the experiments with diluted sera.

The increase in serum aminopeptidase-M activity as a result of dilution with 25 mmol/liter buffer, pH 7.4, is shown in Figure 2a,b. As shown in Figure 2a, the enzyme activity increased from  $45 \pm 5$  (undiluted



**Fig 2.** Effect of serum dilution on the aminopeptidase-M activity (a and b). Pooled sera (groups 1–6) were used in this experiment. Serum pH was adjusted to 7.4, dilutions were performed with 25 mmol/liter bisTris–Tris–acetic acid, pH 7.4, and the enzyme activity determined in triplicate for each serum pool and for each dilution using 100 pmol/liter [ $^{125}\text{I}$ ]leu-enkephalin as described in Materials and Methods. Data are presented as means  $\pm$  SEM.

serum, pH 7.4) up to maximal values of  $880 \pm 90$  pg/min/ml (80-fold diluted serum; values are means  $\pm$  SEM). There were no statistically significant differences in the aminopeptidase-M activity between healthy children and those with chronic active hepatitis due to HBV infection ( $P > 0.05$ ). As shown in Figure 2b, the enzyme activity in the undiluted sera, pH 7.4, from children with Alagille syndrome, extrahepatic biliary duct atresia, Byler's disease, and acute hepatitis due to HBV infection was:  $411 \pm 55$ ,  $480 \pm 39$ ,  $198 \pm 25$ , and  $300 \pm 69$  pg/min/ml, respectively. In the samples diluted 50- to 80-fold, the activity reached maximal values of  $6100 \pm 800$ ,  $6900 \pm 950$ ,  $2150 \pm 280$ , and  $3880 \pm 410$  pg/min/ml, respectively. Negligible dilution, up to 1.5-fold, was without effect (Figure 2a,b). The same data were obtained in the exper-



**Fig 3.** Effect of 8-aniline naphthalene sulfonic acid on the serum aminopeptidase-M activity. The pH of pooled sera from groups 1–6 was adjusted to 7.4, dilutions were performed with 25 mmol/liter bisTris–Tris–acetic acid, pH 7.4, and the enzyme activity was determined in duplicate for each serum pool and each dilution using 100 pmol/liter [ $^{125}$ I]leu-enkephalin as described in Materials and Methods. Data are means  $\pm$  SEM.

iments where buffered saline, pH 7.4, served as a serum diluent (data not shown).

The effect of the increasing concentrations of ANS acid on aminopeptidase-M activity is shown in Figure 3. In the undiluted and 5- and 30-fold diluted sera, the enzyme activity increased about twice in the presence

of 5, 1 and 0.1 mmol/liter ANS acid, respectively. For any dilution, an optimal concentration ( $C$ ) of the ANS acid may be estimated from the following empirical formula:  $5/A = C$  (in millimoles per liter), where  $A$  is the -fold dilution of the serum. Higher ANS acid concentrations were inhibitory, but the enzyme activity did not decrease below its basal level (no ANS acid added). Inhibition and substrate specificity data of serum aminopeptidase-M in the serum pools in groups 1–6 are shown in Table 1. In both undiluted and diluted sera, puromycin, bestatin, and amastatin inhibited the enzyme with  $K_i$  values of about 70  $\mu$ mol/liter, 1.8  $\mu$ mol/liter, and 70 nmol/liter, respectively. In the presence of ANS acid, the enzyme sensitivity to the inhibitors was changed to a certain degree; however, potency and selectivity ranges of the inhibitors remained unchanged. The  $K_M$  value, determined with leu-enkephalin as substrate, was  $359 \pm 62$   $\mu$ mol/liter in both undiluted and diluted sera from groups 1–6 (Table 1).

Aminopeptidase-M activity in the individual sera from groups 1–6 was evaluated under different assay conditions, and the results are shown in Table 2. In the undiluted samples with pH 8.5, the enzyme activity was statistically significantly underestimated ( $P < 0.005$ ) as compared to the same samples with pH adjusted to the physiological level. Maximal enzyme

**TABLE 1.** INHIBITION AND SUBSTRATE SPECIFICITY CHARACTERISTICS OF SERUM AMINOPEPTIDASE-M IN HEALTHY CHILDREN AND CHILDREN WITH CHOLESTASIS AND ACUTE OR CHRONIC HEPATITIS DUE TO HBV INFECTION\*

Serum pools (pH 7.4)	$K_i$ ( $\mu$ mol/liter)			$K_m$
	Puromycin	Bestatin	Amastatin	Leu-enkephalin
Undiluted	$72.0 \pm 8.3$	$1.80 \pm 0.20$	$0.070 \pm 0.011$	$305 \pm 71$
Diluted 50-fold	$69.0 \pm 4.9$	$2.10 \pm 0.14$	$0.066 \pm 0.012$	$312 \pm 83$
Diluted 50-fold + 0.1 mmol/L ANS	$78.0 \pm 9.5$	$0.59 \pm 0.09$	$0.150 \pm 0.021$	$292 \pm 42$

\* For each serum pool, (groups 1–6), determinations were performed in triplicates. Data are presented as means  $\pm$  SEM.

**TABLE 2.** SERUM AMINOPEPTIDASE-M ACTIVITY IN HEALTHY CHILDREN AND CHILDREN WITH LIVER DISEASES

Serum sample	Serum aminopeptidase-M activity (pg/min/ml)					
	Healthy subjects (N = 9)	Chronic hepatitis (N = 7)	Acute hepatitis (N = 6)*	Alagille syndrome (N = 8)*	Extrahepatic atresia (N = 8)*	Byler's disease (N = 7)*
Undiluted, pH 8.5	$9 \pm 5$	$9 \pm 8$	$69 \pm 11$	$79 \pm 15$	$142 \pm 18$	$44 \pm 14$
Undiluted, pH 7.4	$59 \pm 12$	$89 \pm 20$	$305 \pm 175$	$356 \pm 110$	$427 \pm 140$	$240 \pm 90$
80-fold diluted, pH 7.4	$890 \pm 205$	$805 \pm 283$	$3275 \pm 1410$	$6285 \pm 1456$	$6580 \pm 1928$	$1975 \pm 190$
80-fold diluted, pH 7.4 + 0.06 mmol/L ANS†	$1621 \pm 392$	$1503 \pm 409$	$6058 \pm 1899$	$14281 \pm 3500$	$15127 \pm 4025$	$3830 \pm 490$

\*  $P < 0.001$  versus healthy children and those with chronic active hepatitis. Values are expressed as means  $\pm$  SEM.

† 8-aniline naphthalene sulfonic acid.

activity was observed in the samples that were diluted 80-fold. The serum aminopeptidase-M activity was statistically significantly higher in children with cholestasis and acute hepatitis as compared to healthy children or those with chronic active hepatitis ( $P < 0.005$ ).

## DISCUSSION

We attempted to determine and characterize the expression and properties of serum aminopeptidase-M activity in children with liver diseases and in healthy children. We have shown the presence of two fractions of the enzyme. Activity of the first fraction (basic activity) is expressed in the undiluted or negligibly diluted serum at pH 7.4. Activity of the second fraction (cryptic activity) appears in the serum as a result of dilution and/or addition of ANS acid. In children with cholestasis and in children with acute hepatitis due to HBV infection, both fractions of the enzyme are highly elevated as compared to healthy children or those with chronic active hepatitis due to HBV infection.

Since enkephalins are among the well-characterized, natural aminopeptidase-M substrates (2, 3, 17, 19–28), in our assay we used radiolabeled leu-enkephalin at a concentration close to the peptide level in circulation. [ $^3\text{H}$ ]Leu-enkephalin is biologically indistinguishable from the native peptide whereas substitution of the iodine seemingly influenced the substrate–enzyme interaction, resulting in a slightly decreased rate of hydrolysis (Figure 1). The enzyme pH optimum was narrow, about 6.5–7.3, and the activity dramatically decreased outside that range. After one freeze–thaw cycle, the pH of serum increases to 8.5 due to near complete  $\text{CO}_2$  evaporation. To avoid a serious underestimation of the enzyme activity (20–24, 26, 39, 40), serum pH must be carefully adjusted to 7.4 or, alternatively, to the enzyme pH optimum. Maintaining the serum pH may be achieved by dilution of serum with an appropriate buffer. However, as we have shown, a dilution is another factor affecting the serum aminopeptidase-M activity. Whether pH plays a role in *in vivo* regulation of the enzyme activity remains to be determined. Aminopeptidase-M activity dynamically increased as a result of dilution of the serum with simple buffer or buffered saline. The plateau of the activity was found in the samples diluted 50- to 80-fold, whereas a negligible dilution, up to 1.5-fold, was without effect. In healthy children and children with chronic active hepatitis due to HBV infection, enzyme activity increased

from 70 (undiluted serum) to 900 pg/min/ml (80-fold diluted serum). In children with Byler's disease, the respective values were 240 and 2000 pg/min/ml. The highest increases were found in children with Alagille syndrome (from 360 to 6300 pg/min/ml), extrahepatic biliary duct atresia (from 430 to 6600 pg/min/ml), and those with acute hepatitis due to HBV infection (from 300 to 4000 pg/min/ml). In many reports (6, 7, 11, 13, 19, 23, 27, 33), including ours (39, 40), serum dilution was not taken into account as a factor that may affect aminopeptidase-M activity. An increase of enzyme activity as a result of dilution suggests that a large portion of the enzyme molecules is not hydrolytically active and is present in the serum as a cryptic fraction. This may be due to presence of the enzyme natural inhibitor(s) that dissociate as a result of dilution. If this assumption is true, then an increase of the serum aminopeptidase-M in cholestasis and acute hepatitis is not due to a deficiency of such an inhibitor since both fractions of the enzyme are highly elevated. Activation of the enzyme may occur *in vivo*. Recently, Martinez and coworkers (20, 26) have shown that serum aminopeptidase-M activity in rats is capable of changing rapidly in response to environmental experience. However, the mechanism of enzyme activation as a result of serum dilution remains to be determined. Addition of ANS acid, capable of disrupting weak bonds, increased serum aminopeptidase-M activity in a dose-dependent manner (Figure 3). However, the mechanisms of increasing enzyme activity by ANS acid and by dilution seem to be different, and both factors appear to act independently on the enzyme structure, on regulatory factors, or both. Our inhibition and specificity data have shown substantially the same characteristics of the enzyme both in the undiluted and diluted sera determined with or without the presence of ANS acid. Our data suggest that a reliable aminopeptidase-M assay may be performed using a picomolar concentration of the enkephalin labeled with iodine or tritium, added at a negligible volume to an aliquot of the undiluted serum with pH adjusted to the physiological level. Maximal enzyme activity may be determined using 50- to 80-fold-diluted serum. Hemolysis must be avoided since erythrocytes contain an abundance of enkephalin-degrading activity (40). Use of EDTA plasma, phosphate, citrate, or barbital buffers markedly decreases the enzyme activity. For the monitoring of aminopeptidase-M activity, a variety of substrates (6–9, 11, 13, 18–26, 28, 33, 42) and techniques may be applied (7–11, 20–22, 24, 27, 42); however, we have shown here that a sensitive, fast, and inexpensive

aluminum silicate binding assay is an excellent method for detection of degradation of  $^3\text{H}$ - or  $^{125}\text{I}$ -labeled enkephalins.

Cholestasis is associated with an accumulation of enkephalins and other opiate peptides in blood that may contribute to pathogenesis of pruritus or encephalopathy (7, 29–39). Whether the opioid system is altered in acute viral hepatitis remains to be determined. Although the origin of enkephalins in cholestasis is unknown (35), it seems that an impairment of the hepatic degradation of these peptides does not occur (40). Therefore, an increase of serum aminopeptidase-M in cholestasis may be speculated to be a homeostatic attempt to prevent unlimited accumulation of opiate peptides in circulation. However, due to increased activity, the enzyme seems to cause substantial changes in the turnover of other, biologically active peptides in the blood (43). Therefore, we suggest that the enzyme is not only a marker but also a cofactor of the disease. The source of serum aminopeptidase-M was not proven, but the enzyme is thought to originate from the liver (2), where it is a hepatocyte-membrane-bound metalloproteinase virtually absent in the hepatocyte cytosol. The enzyme may reflect other pathways or aspects of the pathological process than those reflected by serum ALAT or  $\gamma\text{GTP}$ . Indeed, our patients with Byler's disease (see Materials and Methods) had a normal level of the serum  $\gamma\text{GTP}$ , moderately increased ALAT, and high levels of aminopeptidase-M. In contrast, children with active chronic hepatitis had elevated levels of  $\gamma\text{GTP}$  and ALAT but normal aminopeptidase-M activity. Among our six patients with acute hepatitis due to HBV infection (ALAT > 600 units/liter), two patients had moderately elevated  $\gamma\text{GTP}$  and highly increased aminopeptidase-M activity whereas four other patients exhibited high increase of both parameters.

Aminopeptidase-M in the serum exhibits a high amplitude of changes both in cholestasis and acute viral hepatitis. However, its physiological and pathophysiological role, as well as its relevance to the situation in cholestatic liver diseases and viral hepatitis will require further studies. Our data seem to provide new, important insights for further studies on a role of this enzyme.

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