Leucine Aminopeptidase Fragments from an Ascites Tumor*

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By 1940 (4) about 35 enzymes had been isolated and crystallized, and the controversy over their chemical nature was resolved with the conclusion that all enzymes contained a protein component essential for their activity. In 1951, however, Binkley (5-7) claimed to have purified highly active peptidases which analyzed as polynucleotides and were free from protein. His procedure was designed to eliminate protein in direct contrast to the classical methods of protein isolation used by earlier workers on peptidase purification. For example, Anson (8) had crystallized carboxypeptidase as a water insoluble protein, and Johnson (9) had isolated yeast polypeptidase as a single ultracentrifugal and electrophoretic component. Of the aminopeptidases and imidopeptidases, Smith and Bergmann (10) had reported partial purification of leucine aminopeptidase and prolidase from hog intestinal mucosa. More recently these enzymes (11, 12) have been highly purified by classical as well as modern methods of protein separation, and they analyzed as typical proteins. Other aminopeptidases (13) and tripeptidases (14, 15) have similarly been isolated. It seems unlikely that some small contaminant in these protein preparations could be responsible for their high enzymatic activity.

Nevertheless Binkley et al. (6, 16) continue to report the isolation of preparations of peptidases by a method of protein elimination which involves an initial autolysis or prolonged proteolytic digestion of acetone powder extracts followed by salt-precipitation and chloroform-octanol shaking. In an abstract published in 1955, Binkley (17) stated that several specific protein-free aminopeptidases of swine kidney contained uridine diphosphate, guanosine diphosphate, glucosamine, and simple peptides which differed in amino acid content in various peptidases. In a recent communication Binkley (18) reported the composition of a highly active cysteinyl glycine as an equimolar guanosine diphosphate-amino acid complex.

This paper describes the results obtained by the application of Binkley's (6) method to the purification of leucine aminopeptidase from a mouse ascites carcinoma. The procedures were adapted for use with small quantities of material. It was found that the resulting enzymatically active protein breakdown products were associated with either or both polynucleotides and polysaccharides. Whether this association is an artifact of the method or reflects the conditions in the cell remains to be determined. In contrast to usual methods of purification where the number of components is kept to a minimum, this method therefore has a tendency to increase this number. Although there is no direct evidence of the production of enzyme fragments, the results suggest that the proteolytic breakdown products of peptidase molecules are associated with other substances which, under certain conditions, may have a stabilizing function. Biologically active protein fragments were studied as early as 1936 (see table in (19)) and the peptide fragments produced by Binkley's method of purification add to the constantly increasing number of these interesting substances.

It has now been shown, however, both by the work of Semenza (20) and that reported here, that the protein or polypeptide components of these enzyme complexes can be separated from the polynucleotide carriers and that the enzyme activity is associated with the protein. Semenza purified cysteinyl glycine from hog kidney with the use of Binkley's (6) autolytic and precipitation methods and by column chromatographic fractionation separated the inactive polynucleotides from the enzymatically active protein fraction. Enzymatic and chemical methods were used in the present work to separate nucleotides from the ascites cell leucine aminopeptidase fragments. As yet complete removal of carbohydrate has not been achieved in the ascites material. The resulting protein-containing preparations represent a 2000-fold purification of leucine aminopeptidase and have an average specific activity, C, 23, about one-fourth the highest, C, 88, obtained by Spackman et al. (11) for leucine aminopeptidase from hog kidney.

EXPERIMENTAL

Growth and Preparation of Ascites Cells—The Lettré Ehrlich hyperdiploid mouse ascites carcinoma provided a material with a relatively homogeneous population of actively growing cells, which were known to have a high peptidase content (21). The ascites was maintained by 0.2 ml of serial intraperitoneal transplantation into adult (2 to 2½ month) female mice of the ICR albino strain. Tumor samples were taken at 9 days' growth in the asymptotic portion of the growth curve (22). Ascites fluid (about 80 ml) was aspirated by sterile syringes from 20 to 25 mice and collected in tubes in an ice bath. All subsequent operations were carried out in a cold room. After centrifugation for 20 minutes the serum was discarded, and the cells twice subjected for 2 minutes to hypotonic shock with about half their volume of water to cytolyze the few contaminating red blood cells (23).

Homogenization of Ascites Cells and Preparation of Supernatant
Fraction—The packed washed cells suspended in one-fourth their volume of 0.9 per cent NaCl-KCl (10 Na:1 K) and 0.01 mM NaHCO₃ were transferred to a Servall omnimixer and homogenized at full speed for 3 minutes. Whole cells, nuclei, and mitochondria were removed by centrifugation in a Spinco model L centrifuge for 15 minutes at 105,000 × g, and microsomes removed from this supernatant fluid by 1 hour of centrifugation at 105,000 × g. The resulting supernatant fractions were stored frozen at -30°.

Chemical Determinations: Peptidase Assay—The use of ultramicrochemical methods permitted purification of an amount of material containing initially only 3 to 6 enzyme units (30 to 60 ml. supernatant fraction). A total of 12 μl. of a given preparation sufficed for the routine analyses at each step. Peptidase assay was carried out by the Linderström-Lang and Holter (24) acetone-HCl titration of amino groups. L-Leucinamide, 0.05 mM final concentration (pH 8 (22°) with NaOH), was used as substrate. Dilutions of the concentrated enzyme preparations were made so that enzyme activity was proportional to concentration (less than 50 per cent hydrolysis was attained in 1 hour). Determinations, enzyme and reagent blanks were all run in triplicate at 40°. Activation with 0.002 mM MnCl₂ was used at all times, preincubation (1 hour at 22°) being necessary only through the first three steps of the preparation. Enzyme activities were calculated in terms of the first order velocity constant K₁. This was expressed in decimal logarithms for comparative purposes. Specific activity was designated by the proteolytic coefficient C₁, that is K₁ per mg. of protein N per ml. of reaction mixture. The number of units of enzyme activity was calculated from the C₁ value multiplied by the mg. of protein N in the preparation. Enzyme units were used for computation of yield. It should be noted that the C₁ values in preliminary abstracts (1, 2) were calculated on the basis of log, rather than log₁₀ as is customary.

Protein—Protein nitrogen was determined by the sensitive and specific bromsulfalein method of Nayyar and Glick (25). The dye quantitatively precipitates protein down through a molecular weight of 3000 (subtilin) and does not precipitate nucleic acids. Therefore it is an excellent procedure to use with mixtures of these substances, especially in this case where the proteins are very low in aromatic amino acids. The method was standardized by Kjeldahl digestion and nesslerization of chloroacetic acid precipitates of ascites supernatant fractions where nucleic acid made up less than 2 per cent of the nucleoprotein precipitates. At the various steps of purification, the bromsulfalein protein N values agreed with the total N values obtained on residues in which nucleotides and polynucleotides had been removed by hot trichloroacetic acid extraction of cold chloroacetic acid precipitates. By volume reduction, the protein concentration was kept sufficiently high to insure complete precipitation of even the most purified materials with equal volumes of cold 20 per cent, although not by 10 per cent chloroacetic acid.

Nucleotides, Hexosamines, and Sugars—Quantities of nucleotides were calculated from absorbancy values at 255 μμ, with factors derived from the base composition of the nucleotides. In all cases complete absorption curves were plotted. Micro-modifications (26) of the Mejbaum orcinol and King methods were used respectively for ribose and phosphorus determination. Hexosamine was determined by the Rondle-Morgan (27) method on an ultramicro-scale. Hexuronic acid was estimated by the carbazole reaction of Dische (28).

RESULTS

Purification

Step 1. Preparation of Supernatant Fraction—Initial elimination of more than half of the cell protein was achieved by differential centrifugation (see “Experimental”), the soluble peptidases being recovered entirely in the supernatant fraction. It was therefore unnecessary to use Binkley’s first step (6, 16), preparation of an acetone powder, which caused great losses in activity in the ascites material.

The average specific activity, C₁, of homogenates was found to be 0.013 and that of the supernatant fractions 0.028, the latter value being comparable to that, 0.02, of aqueous extracts of fresh hog intestinal mucosa and less than that, 0.055, of hog kidney (11).

Step 2. Proteolytic Digestion and Salt-Alcohol Precipitation—Binkley’s next step, digestion with proteolytic enzymes followed by salt-alcohol precipitation, gave excellent yields of leucine aminopeptidase activity with elimination of more than 99 per cent of the protein. Table I gives the results of digestion of the ascites supernatant fraction with trypsin and chymotrypsin with recovery of the enzyme in the fraction soluble in 1 mM NaCl and 0.5 volume of alcohol, and insoluble in 1.1 volumes of alcohol. As emphasized by Binkley (6), it was necessary to prevent a drop in pH. The bicarbonate buffer aided in maintaining a pH over 6.5. Addition of solid bicarbonate to give pH values over 7 failed to increase enzyme yield. Other methods of differential precipitation of peptidases described by Binkley (6, 16) as those with barium acetate, magnesium acetate, or 0.2 mM NaCl with various proportions of alcohol were less satisfactory than 1 mM NaCl alcohol.

With trypsin purified by the method of McDonald and Kunitz (29) in combination with chymotrypsin (Worthington Biochemicals), 4 to 8 hours of digestion at 22° sufficed to lower the protein concentration to 0.1 to 0.4 per cent of that of the supernatant fraction with little loss in enzyme activity and great increase in specific activity. With Worthington trypsin 48 hours was chosen as optimal for subsequent experiments. Trypsin appeared to be the active enzyme. Longer digestion or addition of carboxypeptidase seemed to give little increased protein hydrolysis. It will be noted that recoveries of over 100 per cent were obtained, indicating elimination or destruction of an inhibitor in the supernatant fraction. Malmgren et al. (22) have reported an inhibitor of peptidases in ascites serum. Binkley et al. (16) have also found increases in activity of peptidases during proteolytic digestion of hog kidney preparations. Spackman et al. (11) noted an increased yield of leucine aminopeptidase at the first ammonium sulfate precipitation, presumably due to the removal of inhibitors.

In recent experiments, Series B, additional protein was eliminated at this stage by centrifuging off some material insoluble in the buffer. The recoveries ranged from 77 to 148 per cent, and the C₁ values of 9 to 22 were comparable to those reported by Binkley et al. (16) for hog kidney leucinamidase (to use Binkley’s terminology) at this stage of purification (1st ethanol, 111 per cent yield, C₁ 0.9 based on total N).

Redigestion with trypsin, chymotrypsin, and carboxypeptidase activated by MnCl₂ was used at all times, preincubation (1 hour at 22°) being necessary only through the first three steps of the preparation.
in Series A gave some further reduction in protein content but
decreased yields of peptidase and no significant increases in C1.
In Series B, the decrease in protein was negligible, no change
occurred in C1 values, but the yield decreased 25 per cent.
Proteolytic digestions at later stages of purification invariably
led to lowered yields of peptidase probably in part due to losses
in reprecipitation. It was concluded that the remaining protein
could not be digested by these proteolytic enzymes.

Effect of Guanidine-HCl—The essential nature of protein for
enzyme activity was indicated by several experiments on the
effect of 2 M guanidine-HCl on digestion of the ascites cell supernatant
fraction. Binkley et al. (16) had observed no effect on the
peptidase activity of proteolytically digested hog kidney ex-
tacts in the presence of 1 M guanidine-HCl. Semenza (20), how-
ever, found that although the activity of highly purified cysteiny1
glycinase of hog kidney was lost reversibly in 6 M urea, it was
lost irreversibly in treatment with trypsin or chymotrypsin in
the presence of urea, although stable to these enzymes in the
absence of urea. Therefore, the denatured protein was in some
way changed by the trypsin and chymotrypsin.

Table II gives the results on the effect of 2 M guanidine-HCl
on the digestion of the ascites cell supernatant fraction by trypsin
and chymotrypsin. In the Series A experiments, both the
amount of protein and yield of enzyme were reduced in the pres-
ence of this protein denaturant, but the specific activities re-
maind similar to those in the control experiments indicating an
effect on precipitability of the peptidase but not its activity. On
the other hand, in the Series B experiments where insoluble inert
protein was removed, the specific activity of the preparations
without guanidine-HCl was an order of magnitude higher than
those digested in the presence of guanidine-HCl. In the latter,
the lower protein recovery was due to appreciably greater
digestion of protein by the enzymes, but to denaturation of
protein as seen by the greater volume of precipitate obtained on
addition of a half-volume of alcohol. This denaturation, as
might be expected, separated protein from nucleic acid, and the
guanidine-treated preparations contained about twice the con-
tent of nucleotides that was found in the controls, although pepti-
dase activity was reduced 20-fold. In addition, before precipita-
tion, the peptidase activities of the brines were reduced about
8 fold by the presence of guanidine-HCl. Further purification
(Steps 3 and 4) of the guanidine-HCl-treated material resulted in
little increase in specific activity. Therefore in this ascites
cell material, it appeared that the peptidase activity was asso-
ciated with a protein fragment which might be partially protected
from complete denaturation by association with polynucleotides
and, as will be seen later, polysaccharides.

Composition of Leucine Aminopeptidase Preparations at Step 2
—As might be expected, the material soluble in 1 M NaCl and
insoluble in 0.5 to 1.1 volumes of alcohol was a complex contain-
ing low molecular weight nucleic acids, proteins, and polysac-
charides, and possibly other unrecognized substances. Fig. 1
gives the ultraviolet absorption spectrum (---) of a preparation
at Step 2. The strong absorption with a peak at 258 mw and
the 280:260 ratio of 0.51 provided good evidence for polynucleo-
tides. From the absorption curves the presence of protein was
not immediately evident since the content in aromatic amino acid
was low after proteolytic digestion. The absorption spectrum of Binkley’s (9) cysteiny1 glycinase has a 280:260 ratio of about
0.55 and a 260:230 ratio of 1.57 comparable to the 1.53 of the
curve in Fig. 1. As reasonably pure preparations of RNA have

### Table I

<table>
<thead>
<tr>
<th>Enzyme* (0.25 mg./ml.)</th>
<th>Time</th>
<th>Series</th>
<th>Recovery of protein</th>
<th>Leucine aminopeptidase</th>
<th>Yield</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(21-25)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin (K-M)</td>
<td>4-8</td>
<td>A</td>
<td>0.11-0.43</td>
<td>57-95</td>
<td>3-19</td>
<td></td>
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<tr>
<td>Chymotrypsin (W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin (W)</td>
<td>8</td>
<td>A</td>
<td>0.58-1.30</td>
<td>68-107</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin (W)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase (W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin (W)</td>
<td>8</td>
<td>A</td>
<td>1.16-1.54</td>
<td>43-107</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin (W)</td>
<td>24</td>
<td></td>
<td>0.26-0.54</td>
<td>60-133</td>
<td>5-9</td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase (W)</td>
<td>48</td>
<td></td>
<td>0.25-0.99</td>
<td>61-144</td>
<td>3-9</td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase (W)</td>
<td>72</td>
<td></td>
<td>0.36-0.98</td>
<td>55-100</td>
<td>2-7</td>
<td></td>
</tr>
<tr>
<td>Trypsin (W)</td>
<td>45</td>
<td>B</td>
<td>0.14-0.28</td>
<td>77-148</td>
<td>9 22</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin (W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Redigestion† with tryp-
| sin (W)                | 2-4   | A       | 0.07-0.14           | 44-90                  | 12-14 |                  |
| Chymotrypsin (W)       |       |         |                     |                        |       |                  |
| Carboxypeptidase (W)   |       |         |                     |                        |       |                  |
| Redigestion† with tryp-
| sin (W)                | 2     | D       | 0.11                | 62                     | 13    |                  |

* K-M refers to trypsin purified by Dr. Margaret McDonald
and kindly given to us by Dr. B. Kaufmann. W refers to
enzymes from the Worthington Biochemical Corporation.
† In these experiments, after 48 hours of digestion followed by
salt-alcohol precipitation, the suspensions A or solutions B were
redigested with proteolytic enzymes and reprecipitated.

Fresh proteolytic enzyme solutions were added to the ascites cell supernatant fractions at 0 and 3 hours, and, in the case of the
48 and 72 hour digestions, also at 24 and 48 hours, respectively.
A few drops of chloroform-toluene were added to prevent bacterial
action. After the given digestion periods at 22°C, the preparations
were stored at 2°C for 18 to 45 hours before precipitation. The
solutions were then cooled in an ice bath and made 1 M to NaCl
and 0.5 volume of cold 100 per cent alcohol saturated with NaHCO3
added. After 30 minutes in an ice bath, the precipitate was
centrifuged down at 1500 r.p.m. in a cold room and 1.1 volumes
cold 100 per cent alcohol added. After 30 minutes precipitation
at 0°C and centrifugation at 1500 r.p.m., the supernatant fluid was
discarded and the precipitate suspended in one-tenth the original
volume of 0.9 per cent NaCl-KCl (10 Na;1 K), 0.01 M NaHCO3,
0.005 M MgCl2. Series A. Series B, the final suspensions were
allowed to stand for 2.5 hours at 2°C and 18 hours at 2°C, then
were centrifuged and the clear supernatant fluids assayed.
TABLE II

Effect of 2 M guanidine-HCl on digestion of supernatant fraction with proteolytic enzymes

Solid guanidine-HCl was stirred into the supernatant solutions to make them 2 M in guanidine-HCl and simultaneously sufficient solid NaHCO$_3$ was added to bring the pH to 8. After proteolytic enzyme digestion, the preparations were salt-alcohol precipitated and dissolved in buffer containing 0.005 M MgCl$_2$ as described in the legend of Table I.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Time (hrs.)</th>
<th>Series</th>
<th>2 M guanidine-HCl, pH 8</th>
<th>Recovery of protein</th>
<th>Leucine aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>From supernatant fraction</td>
<td>Relative</td>
</tr>
<tr>
<td>Trypsin (W)*</td>
<td>7</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>1.30</td>
</tr>
<tr>
<td>Chymotrypsin (W)</td>
<td></td>
<td></td>
<td>+</td>
<td>0.44</td>
<td>34</td>
</tr>
<tr>
<td>Carboxypeptidase (W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin (W)</td>
<td>48</td>
<td>A</td>
<td>-</td>
<td>0.50</td>
<td>100</td>
</tr>
<tr>
<td>Chymotrypsin (W)</td>
<td></td>
<td></td>
<td>+</td>
<td>0.11</td>
<td>11</td>
</tr>
<tr>
<td>Trypsin (W)</td>
<td>48</td>
<td>B</td>
<td>-</td>
<td>0.09</td>
<td>100</td>
</tr>
<tr>
<td>Chymotrypsin (W)</td>
<td></td>
<td></td>
<td>+</td>
<td>0.05</td>
<td>53</td>
</tr>
</tbody>
</table>

* Enzymes from Worthington Biochemical Corporation.

TABLE III

Effect of pH and Mg$^{++}$ ions on stability of leucine aminopeptidase at stage 2

The alcohol precipitates were dissolved in the various buffer-salt mixtures and assayed after 18 to 21 hours at 2$^\circ$.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Molarity</th>
<th>pH</th>
<th>MgCl$_2$, 0.005 M</th>
<th>Leucine aminopeptidase yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO$_3$</td>
<td>0.01</td>
<td>8.4</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Tris</td>
<td>0.04</td>
<td>8.3</td>
<td>+</td>
<td>99</td>
</tr>
<tr>
<td>Tris</td>
<td>0.04</td>
<td>8.1</td>
<td>-</td>
<td>34</td>
</tr>
<tr>
<td>Tris</td>
<td>0.04</td>
<td>7.3</td>
<td>+</td>
<td>73</td>
</tr>
</tbody>
</table>

The effect of different buffers, pH, and anions on enzyme stability was tested. The concentration of Na and K was without significant effect but as seen in Table III, addition of Mg$^{++}$ ions greatly increased stability. Tris$^3$ (Sigma 121) and bicarbonate buffers were equally good but stability at pH 8.3 was better than at 7.3. These data are in agreement with the experience of others (11, 16) in purification of these enzymes.

Treatment with Chloroform-Octanol—Table IV gives the data from experiments in which Binkley's (6) next step, treatment with chloroform-octanol, was tried on the ascites cell peptidase preparations. As expected from this Sevag (30) procedure, the protein was reduced in amount but not completely eliminated. At Step 2, higher peptidase specific activities were found only in Series A preparations known to contain excess protein. The increases were very small and in no way comparable to the great (10-fold) increases found by Binkley et al. (6, 16) with hog kidney peptidase preparations. The lability of the ascites cell leucine aminopeptidase fragments was such that, regardless of medium or nucleotide to protein ratio, the yields of enzyme were decreased, the smaller values (9 per cent of control) corresponding from analyses, but also from the effect of treating with enzymes specific for these substances. Glucosamine occurred in a molar concentration of about one-tenth that of nucleotides, and hexoses and hexuronic acids were also present. These sugars, probably in the form of polysaccharides since they were precipitable by alcohol, could be reduced in amount by treating the original supernatants with a-amylase and hyaluronidase before trypsin and chymotrypsin were added. An observed drop in viscosity indicated breakdown of high molecular weight compounds.

Stability of Leucine Aminopeptidase at Step 2.—It was observed in the earliest experiments that the stability of the enzymes at this step was very poor, but improved by addition of 0.005 M MgCl$_2$. The effect of different buffers, pH, and anions on enzyme stability was tested. The concentration of Na and K was without significant effect but as seen in Table III, addition of Mg$^{++}$ ions greatly increased stability. Tris$^3$ (Sigma 121) and bicarbonate buffers were equally good but stability at pH 8.3 was better than at 7.3. These data are in agreement with the experience of others (11, 16) in purification of these enzymes.

* The abbreviation used is: Tris, tris(hydroxymethyl)amino-methane.
The preparations were shaken for 15 minutes with one half their volume of saturated bicarbonate and water washed (16) chloroform-octanol (3:1 or 55:5), centrifuged at 1500 r.p.m. for 15 minutes, and the supernatant fluid pipetted off the emulsion. This process was repeated until no precipitate formed on the meniscus. The medium was 0.9 per cent NaCl-KCl and 0.01 M NaHCO₃ except in two cases where 0.005 M MgCl₂ was present, and in one of which the preparations were dissolved in water. Ranges of values from six experiments are given, as no effect of medium was observed.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein*</th>
<th>Nucleotide:protein ratio</th>
<th>Specific activity</th>
<th>Yield with respect to untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Supernatant fraction</td>
<td>4870-7300</td>
<td>1.9-7.7</td>
<td>0.013-0.014</td>
<td>100</td>
</tr>
<tr>
<td>2. Trypsin, chymotrypsin: salt-alcohol precipitation</td>
<td>3-26</td>
<td>5.7-12.9</td>
<td>0.023-0.035</td>
<td>60-98</td>
</tr>
<tr>
<td>3. RNase treatment of 2: salt-alcohol precipitation</td>
<td>0.4-1.3</td>
<td>1.4-6.1</td>
<td>16.4-24.8</td>
<td>10-44</td>
</tr>
<tr>
<td>3a. Chloroform-octanol (4 times) treatment of 3</td>
<td>0.11-0.14</td>
<td>7.3-7.6</td>
<td>0.1-0.5</td>
<td>0.01-0.7</td>
</tr>
</tbody>
</table>

* Protein N × 6.25.
† Weight for weight.
† An aliquot of the preparation at stages 2 and 3 standing at room temperature for the same time as another aliquot was treated with chloroform-octanol.

Mean values from 8 experiments, Series B, in which nucleotides were removed from the preparations. In four experiments hyaluronidase (Worthington Biochemicals) (0.1 mg. per ml., 2 hours, 22°) digestion of the supernatant fraction preceded Step 2, trypsin-chymotrypsin treatment, and in a single experiment α-amylase (Mann Laboratories, 0.2 mg. per ml., 2 hours, 22°) was used before hyaluronidase. Recovery of peptidase by salt-alcohol precipitation was carried out after each Step 2 to 6. The yields of peptidase, protein, and nucleotide were reset at 100 per cent at Step 2 in order that the effect of Steps 3 to 6 could be seen relative to the composition of the preparations at Step 2.

Steps 3 to 6. Removal of Nucleotides from Nucleoprotein Complexes—The next steps involved a study of the effect on the enzyme activity of progressive removal of nucleotides from the preparations by means of RNase, snake venom esterase, 2 M guanidine-HCl, and ATP and Norit A. Table V summarizes all the purification steps including the following Steps 3 to 6 which were employed for the removal of nucleotides. In four of the eight preparations, hyaluronidase and in two α-amylase plus hyaluronidase were used before Step 2 for breakdown of polysaccharides. At the concentrations and times given, these enzymes had no effect on peptidase activity or recovery. As will be seen below, greater concentrations, however, did have an effect on recovery.

Table V

<table>
<thead>
<tr>
<th>Steps</th>
<th>Leucine aminopeptidase Specific activity Yield</th>
<th>Protein</th>
<th>Nucleotide</th>
<th>Nucleotide:protein ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Supernatant fraction</td>
<td>0.03</td>
<td>100</td>
<td>0.03</td>
<td>100</td>
</tr>
<tr>
<td>2. Trypsin-chymotrypsin</td>
<td>15</td>
<td>111</td>
<td>0.22</td>
<td>4.0</td>
</tr>
<tr>
<td>3. Ribonuclease</td>
<td>16</td>
<td>72</td>
<td>62</td>
<td>21</td>
</tr>
<tr>
<td>4. Snake venom esterase</td>
<td>18</td>
<td>51</td>
<td>40</td>
<td>7</td>
</tr>
<tr>
<td>5. 2 M guanidine-HCl</td>
<td>30</td>
<td>25</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>6. ATP-Norit</td>
<td>23</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

* Weight for weight.
† Yields reset at 100 per cent (see legend above).
losses occurred in the salt-alcohol precipitation. At this and succeeding steps after 1 M NaCl and 0.5 volume of alcohol precipitation, 1.2 volumes of alcohol were used for the second precipitation of the peptidase from the supernatant alcohol. The precipitates were made up in the above mentioned buffer at a concentration of 0.9 per cent and 0.01 M NaHCO₃, 0.005 M MgCl₂ to a volume $T$ of the original. At Step 4, some precipitation occurred in an ice bath, salt-alcohol precipitated, and made up in buffer-MgCl₂ to a volume $T$ of the original. As seen from the mean values in Table V, the specific activity was somewhat higher, but the enzyme yield and protein concentration were reduced to 51 and 40 per cent, respectively, of those at Step 2; the nucleotides dropped to 7 per cent and the nucleotide to protein ratio to 0.6 (range 0.34 to 1.63). Reduction of nucleotide was far more complete if treatment with RNase and esterase was carried out in two separate steps rather than simultaneously (nucleotide to protein ratios, 1.5 to 10.0).

Incubation with 0.3 M alkali at 37° for 18 hours removed no further nucleotide, as checked by paper electrophoresis."\* The nucleotide and protein-containing complex moved as a single spot 1 cm. towards the anode in 1 hour (1000 volts, 3 ma., pH 3.5).

**Step 5.** 2 M Guanidine HCl—Many methods of separating nucleic acid and protein involve the use of protein adsorbants and denaturants. The adsorbants tried, Dowex 2 and Ksoolin, caused great losses in enzyme activity. Since the size of the protein fragment is probably relatively small, it seemed possible that a mild denaturant, as 2 M guanidine-HCl might be effective. Solid guanidine-HCl sufficient to give a 2 M solution and solid sodium bicarbonate to give pH 8, were stirred into the solutions from Step 4. The preparations were then allowed to remain at room temperature (22°) for 15 minutes. The enzyme material was cooled in an ice bath, salt-alcohol precipitated, and made up in buffer-MgCl₂ to a volume $T$ of the original. At Step 4, some of the precipitates had a slight yellow cast but all were completely white at Step 5. The guanidine-HCl was effective in removing a contaminating protein, as also seen from the increase in specific activity (Table V). The nucleotide to protein ratio, however, increased although only 2 per cent of the nucleotide at Step 2 remained, along with 25 per cent of the peptidase activity.

The specific activities of the preparations at this stage ranged from 21 to 60 with a mean value of 30. Compared to the mean value of 0.0135 for the homogenates this represented a 2200-fold purification.

**Fig. 2** shows the ultraviolet absorption spectra of preparations after esterase treatment, Step 4, and after guanidine-HCl, Step 5. The maxima are about the same, 257 μ, and the minimum after guanidine-HCl shifts from 234 to 236 as expected with selective removal of nucleotides.

*Table VI gives the composition of the nucleotide component at Step 5.*

Preparations were hydrolyzed for 1 hour at 100° in sealed tubes with an equal volume of concentrated hydrochloric acid. The hydrolysates were chromatographed by two dimensional paper chromatography (isopropanol-HCl and water) and the eluted bases identified by ultraviolet spectrophotometry. The calculations of quantities of bases were made from published (33) molar absorbancies.

![Graph](image_url)

**Fig. 2.** Absorption spectra of preparations at Steps 4, esterase; 5, guanidine-HCl; and 6, ATP-Norit (3 times).

![Graph](image_url)

**FIG. 2. Absorption spectra of preparations at Steps 4, esterase; 5, guanidine-HCl; and 6, ATP-Norit (3 times).**

**TABLE VI Composition of nucleotide component at step 5**

<table>
<thead>
<tr>
<th>Pooled samples</th>
<th>Guanine*</th>
<th>Adenine*</th>
<th>Cytosine*</th>
<th>Uracil*</th>
<th>Purine</th>
<th>Base analysis</th>
<th>Ribose (orcinol)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.476</td>
<td>0.100</td>
<td>0.279</td>
<td>0.144</td>
<td>0.614</td>
<td>58</td>
<td>70</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>0.614</td>
<td>0.614</td>
<td>0.224</td>
<td>0.161</td>
<td>0.598</td>
<td>61</td>
<td>74</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Values given as moles per mole base. We are indebted to Elizabeth C. Travaglini for carrying out these experiments.

It can be seen from Table V that the average yield was 72 per cent of that at Step 2, the $C_1$ showing only a small increase. The nucleotide, however, was reduced to 21 per cent of that at Step 2 and the resulting nucleotide-to-protein weight ratio dropped from 4.0 to 1.3.

**Step 4. Snake Venom Esterase—Snake venom esterase (Crotalus adamanteus from Ross Allen’s Reptile Institute, Silver Springs, Florida) was added to the preparation from Step 3 in a concentration of 120 μg. per ml. The enzymes were allowed to act for 4 hours at 22° and 17 hours at 2°, followed by salt-alcohol precipitation. The precipitates were made up in the above mentioned buffer at a concentration of $\rho_0$ the original. As seen from the mean values in Table V, the specific activity was somewhat higher, but the enzyme yield and protein concentration were reduced to 51 and 40 per cent, respectively, of those at Step 2; the nucleotides dropped to 7 per cent and the nucleotide to protein ratio to 0.6 (range 0.34 to 1.63). Reduction of nucleotide was far more complete if treatment with RNase and esterase was carried out in two separate steps rather than simultaneously (nucleotide to protein ratios, 1.5 to 10.0).

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**Table VI gives the composition of the nucleotide component.**

Two pooled samples were analyzed for base composition,\* ribose, and phosphorus content. Guanine was the predominant base with cytosine next and adenine and uracil relatively low. The per cent purine by base analysis was 58 and 61 per cent, the ribose analyses giving higher values, 70 and 74 per cent. The discrepancy was probably caused by the presence of hexuronic acids which with the orcinol reaction have a maximum absorption at 670 μ, as does ribose (31). Hexosamine, if present, was below the sensitivity of our method of measurement (27). The finding of 2 moles of phosphorus per mole of base could be interpreted as indicating the presence of mixtures of nucleotides or nucleoside diphosphates. The latter compounds were reported

\* We are indebted to Elizabeth C. Travaglini of this Institute for carrying out these experiments.
by Binkley et al. (17) to be components of the peptidases along with glucosamine and a simple peptide. The enzyme activity, however, seemed clearly associated with the protein at Steps 4 and 5. Fig. 3 shows the relationship of the peptidase activities of 35 separate preparations (plotted as $K_1$), to the protein $N$ and nucleotide contents of the individual reaction mixtures. The scatter diagram on the right shows no relationship of nucleotide to activity whereas the trend with the protein is clear.

**Step 6: ATP-Norit**—Removal of the remaining nucleotide was achieved with the procedure used by Davie et al. (34) with the tryptophan-activating enzyme. The preparations resulting from Step 5 were treated for 20 minutes at 22° with 0.005 m mole per ml. of potassium-ATP (Sigma) and its equivalent of MgCl₂ in salt bicarbonate buffer, followed by salt-alcohol precipitation (2°). The precipitates were dissolved in 1/10 of the original volume of either 0.04 M Tris buffer (pH 8.1, 0.005 m MgCl₂) or bicarbonate buffer (0.9 per cent NaCl-KCl, 0.02 m NaHCO₃, 0.005 m MgCl₂). Tris buffer gave better pH control and consequently more stable preparations. The solutions were then shaken for 10 minutes with 17 μg. per ml. Norit A, (Pfanstiehl, washed with HCl and water, and then with Tris buffer, until free from chlorine and orcinol negative), centrifuged 10 minutes, and the supernatant fluids pipetted off. This process was repeated four to six times or until the ultraviolet absorption curves stabilized, and showed nucleotides had been removed.

No significant reduction in nucleotide to protein ratio was found in experiments in which preparations at earlier stages of purification, Steps 2 and 3, were treated with ATP, reprecipitated, and shaken with Norit as well as being submitted to each of these processes alone. Apparently ATP was effective only when the polynucleotide content had been reduced by treatment with nucleases, as after Step 4, when the full procedure removed nucleotides but gave preparations of low specific activity, $C_I$ 7 to 8. At Step 5, addition of Norit alone eliminated nucleotides but resulted in excessive reduction of protein and enzyme activity. ATP followed directly by Norit without reprecipitation also caused great losses in activity. The purification steps as outlined in Table V were chosen for routine preparation of the ascites leucine aminopeptidase fragment.

No studies have been made thus far of the specific effect of ATP in this reaction. It is possible that other nucleotides or pyrophosphate might give the same result, that is the separation of the nucleotide from the protein in the enzyme complex without undue damage to the enzyme.

As seen from Table V, the leucine aminopeptidase preparations resulting from Step 6 had an average $C_I$ of 23 which was lower than at the previous step. The yield and protein contents had been reduced to 5 and 3 per cent, respectively, of the values at Step 2 but the material contained no detectable nucleotide. It should be emphasized that the volume reduction gave solutions of sufficient concentration for accurate measurement of protein content and enzyme activity.

**Composition of Ascites Cell Leucine Aminopeptidase Preparations at Step 6**

**Nucleotides**—It is obviously impossible to demonstrate the complete absence of a substance. In the case of nucleotide, in the minute amounts available of these purified peptidase preparations, it is even difficult to define the highest amounts that would not be measurable by the methods employed. Assays for ribose, and purine and pyrimidine were used for detection of nucleotide as these tests were more sensitive and specific than phosphorus determination.

Accurate ribose determination could not be achieved with the orcinol reaction (26) which with this material gave a broad absorption curve consistent with the presence of polysaccharide constituents as hexoses, uronic acids, and sialic acid, in a total sugar concentration of about 100 times the possible ribose level. The carbasole reaction (28) showed sufficient hexuronic acid present to account for all material absorbing at 670 mp in the orcinol reaction thereby theoretically eliminating the possible presence of ribose. Initial treatment with hyaluronidase reduced the uronic acids 10-fold but did not completely eliminate this polysaccharide component except possibly in one case. Without positive identification of the sugars in these preparations, however, it is difficult if not impossible to state that ribose was absent.

The possible amounts of purine and pyrimidine in these preparations were below the limits of detection by microbiological assay, therefore the nonspecific but very sensitive method of ultraviolet absorption had to be used for testing for the presence of nucleotides. The lowest curve in Fig. 2 shows the ultraviolet absorption of the material at Step 6 in relation to Steps 5 and 4. Fig. 4 gives the absorption spectrum on a larger scale of a typical preparation after ATP-Norit treatment. Further exposure to Norit removed more protein and did not change the shape of the curves. The 260:280 ratio is now 1.0 and the 260:230, 0.1.

The curve is typical of proteins low in aromatic amino acids. These amino acids were probably removed by the proteolytic enzyme treatment as the hog kidney leucine aminopeptidase of Spackman et al. (11) shows a high peak at 280 μ, The pH of the material as diluted in the microcell was about 6.1. Addition of alkali to bring the pH to 10.6 caused a change in the spectrum typical of aromatic amino acids (32) and opposite to that of nucleotides (33), i.e. a slight rise in the region 240 to 270, and 285 to 300 μ. The absorption curves of the enzyme preparations could be closely matched by mixtures of amino acids in which the aliphatic were present in a molar ratio of 500 times the aromatic (2 phenylalanine:2 tyrosine:1 tryptophane). It...
was concluded therefore that if nucleotides were present, the amounts were very low compared to protein.

Protein—Calculating from the maximum possible nucleotide in a preparation reading zero at 670 mp in the orcinol reaction, and assuming a 1:1 molar nucleotide to protein ratio, gave a molecular weight of about 85,000 for the protein. Similar calculations made from maximum nucleotide contribution to the ultraviolet absorption curves gave comparable values. These molecular weights seem very high for protein that is not completely precipitatable in 5 per cent cold trichloroacetic acid and are much greater than the values Binkley has mentioned for his purified peptidase fragments. Although the ascites material at Step 6 was nondialyzable through 18/32 Visking tubing, it is possible that the enzyme molecules were associated in the presence of magnesium giving particles too large to pass through the membrane. Complete precipitability with an equal volume of 20 per cent trichloroacetic acid would indicate a molecular weight at least in the protein and not the polypeptide range. But certainly the molecular size could not be great enough to allow for a mole of nucleotide per mole of protein.

The small scale paper chromatographic method of Redfield (35) was used for separation of amino acids in hydrolysates (formic-HCl (36)) of the ascites preparations. In spite of the small size of the samples, about 10 μg. of protein, twelve amino acids could be clearly identified: aspartic and glutamic acids, leucine or isoleucine or both, alanine, threonine, serine, histidine, glycine, lysine, phenylalanine, valine, and methionine. From the ultraviolet absorption spectra it was suspected that cysteine, tryptophan, and tyrosine were also present. Therefore, other than proline and arginine which could not be detected in the

Approximate values of 30,000 to 50,000 were calculated by Dr. Sam Sorof of this Institute for the molecular weights of peptidases in dialysed ascites cell supernatant solutions from unpublished data obtained by velocity ultracentrifugation. Localization of the enzymes within the various components was determined by the use of the analytical filter paper partition cell.

Carbohydrate—Polysaccharides occurred in these enzyme preparations at approximately half the weight of the protein. No attempt has been made to identify the specific sugars but efforts have been directed toward elimination of these components. Initial treatment of supernatant fractions with hyaluronidase (0.1 mg. per ml.) gave 10-fold reduction of uronic acids but still left enough of these compounds to interfere with ribose determination. α-Amylase (0.2 mg. per ml.) seemed to have little effect in reducing hexoses, but use of higher concentrations (0.3 to 0.4 mg. per ml.) resulted in a striking effect on the precipitability of the enzyme complex after treatment with guanidine-HCl, Step 5. The higher concentrations of alcohol (1.5 volumes) needed for complete enzyme recovery led to preparations with low specific activity. The material precipitable with the usual 1.2 volumes of alcohol contained (mean of four experiments) 14 per cent of the enzyme activity at Step 2, and 7 per cent of the protein in contrast to the 25 per cent and 11 per cent, respectively, obtained when lower concentrations of amylase were used. When the nucleotide was removed by treatment with ATP, reprecipitation, followed by Norit, only 0.8 per cent of the enzyme activity remained and the protein amounts were too low for accurate determination, although pointing to the presence of enzyme of high specific activity. The material was not precipitable by 20 per cent trichloroacetic acid and lost activity very rapidly. The ultraviolet absorption curves were flat from 260 to 300 mp indicating a very low content of aromatic amino acids and no nucleotide. Sugars, although reduced in quantity, were still present in these preparations. Apparently, polysaccharide was necessary in the enzyme complex in order to recover stable enzyme by these methods.

Stability of Ascites Cell Leucine Aminopeptidase Fragment—Preparations at Step 6 (in Tris buffer, pH 8, 0.005 m MgCl₂) were relatively stable when stored frozen but lost activity at refrigerator temperatures. After 7 days (2–4°) the C₁ dropped to 50 per cent, then fell more slowly, and after 18 days 33 to 50 per cent of the original activity remained. The decrease in C₁ was greatest when the initial specific activity was exceptionally high as in the case of material with a C₁ of 60 dropping to 57 in 3 days at 2°. There was no change in the protein content of the preparations, but those with higher protein concentration tended to be more stable. An exceedingly low carbohydrate content also seemed to lead to instability. Further work, however, would be necessary to determine the role, if any, of sugars in these enzyme systems. The instability at refrigerator temperatures of the ascites cell leucine aminopeptidase fragment is in contrast to the great stability of the all-protein hog kidney enzyme (11). The latter has a molecular weight in the presence of magnesium of 300,000 and a high content of aromatic amino acids. It is not surprising that the ascites enzyme fragment of far lower molecular weight should readily lose the conformation necessary for activity especially in view of its low content of aromatic amino acids.

Characterization of Ascites Cell Leucine Aminopeptidase Fragments

Variation of Activity with pH—The pH activity curve for the ascites cell aminopeptidase fragment is given in Fig. 5. The peak is at pH 8.0 to 8.4, 22°, (7.5 to 7.9, 40°) in contrast to the enzyme purified from hog kidney by Spackman et al. (11) which
showed peak activity at pH 8.8 to 0.1 and the "leucine amidase" purified from the same tissue by Binkley et al. (16) which had increasing activity through pH 9.2 (37). The significance of this difference is obscure but might possibly have to do with amino acid composition.

Activity with Different Substrates—Table VII gives the relative rates of hydrolysis of different substrates by the purified leucine aminopeptidase from hog kidney (38) and the fragments from ascites cells. It is clear that the behavior of the two enzymes is quite similar. L-Leucylglycylglycine and L-leucylglycine were hydrolyzed at rates comparable to L-leucinamide whereas L-aminoglycine and glycyl-L-leucine were cleaved at about one-tenth the rate of L-leucinamide. This is quite a different situation from that in whole ascites cells or homogenates where the latter two substrates were hydrolyzed at many times the rate of leucinamide (21). The cleaving of diglycine by the purified tumor enzyme was not increased in the presence of cobalt as it was in cell extracts. The weak activity toward this substrate was therefore not due to a contaminating glycyglycine peptidase.

The zero value for the last two substrates indicates the virtual absence of prolidase and cathepsin in the preparations. Unfortunately, the small amount of the ascites enzyme purified has precluded trying its activity on proteins as the amount of enzyme necessary is of the same order of magnitude as the weight of protein substrate (39). Possible esterase (37) activity of these preparations has also not as yet been investigated.

DISCUSSION

It now seems clear both from this work on ascites tumor cell leucine aminopeptidase and from Somenza's (20) studies of cysteinyl glycinate, as well as earlier work previously cited, that the peptidases are not exceptional and are proteins as are all known enzymes. Nucleotides are unnecessary for enzymatic activity but under certain circumstances may contribute to stability. It is not impossible, however, that in the cell the peptidases exist in the form of nucleoproteins or mucoo- or glycoproteins. Binkley's (5, 6) finding that after exhaustive digestion with proteolytic enzymes, the resulting peptidase fragments were bound to polynucleotides is most interesting. Further work will be needed to determine whether this combination is the result of the chemical techniques used, or whether it reflects true conditions in the cell.

The specific activities reported by Binkley et al. (16) for "crude concentrates" of "leucine amidase" (C₁₄) 450 from hog kidney are an order of magnitude greater than the highest (C₁₂) 88 found by Spackman et al. (11) for their all-protein leucine aminopeptidase from the same organ, and the highest recorded for the leucine aminopeptidase (C₁₀) 60 fragment from ascites tumor cells. The possibility exists that with the more stable kidney enzyme, a smaller peptidase fragment may be isolated when protected by polynucleotide than can be done with the more labile ascites enzyme. The inaccessibility to chloroform-octanol shown by the ascites preparations is in great contrast to Binkley's experience (16) with kidney enzymes. Leucine aminopeptidase from swine intestinal mucosa was found (11) to be less stable than that from hog kidney, and has been purified only to a C₁₀ of 3.1 (40). The question arises whether peptidases in rapidly growing cells, as tumor and intestinal mucosa cells, are associated with less stable lower molecular weight complexes than they are in mature cells as in the kidney.

FIG. 5. The effect of pH on the activity of ascites cell leucine aminopeptidase fragments. The substrate, leucinamide (0.05 M final concentration) was in 0.10 M Tris buffer at the given pH values measured at 22°. Due to the instability of the enzyme, each point is plotted as per cent of the activity of a preparation run simultaneously at pH 8.

<table>
<thead>
<tr>
<th>Substrate (0.05 M)</th>
<th>Leucine aminopeptidase from hog kidney, (38), pH 8.8</th>
<th>Leucine aminopeptidase from ascites tumor, pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucinamide</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L-Leucylglycylglycine</td>
<td>120</td>
<td>69</td>
</tr>
<tr>
<td>L-Leucylglycine</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>L-Alanylglutamine</td>
<td>9.4 10*</td>
<td>10</td>
</tr>
<tr>
<td>Glycyl-L-leucine</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Triglycine</td>
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<td>2.0</td>
</tr>
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<td>Glycylglycine</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Glycyl-L-proline</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Benzoyl-L-argininamide</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* DL-Alanylglutamine.

SUMMARY

Leucine aminopeptidase from mouse ascites carcinoma cells has been purified about 2000-fold by small scale procedures which included some of the initial steps of Binkley's method including proteolysis and salt-alcohol precipitation. The nucleotides were separated from the resulting nucleoprotein-containing complexes by means of nucleases, guanidine-HCl, adenosine triphosphate and Norit. Some carbohydrate remained but the enzyme activity resided in the protein components.

The mean specific activity of the resulting ascites cell enzyme fragment was about half that of the all protein leucine aminopeptidase purified from hog kidney by Spackman et al. and about one-tenth that of the nucleotide-containing "leucine ami-
The nucleotide-free ascites leucine aminopeptidase fragment has a substrate specificity very similar to the protein hog kidney enzyme but differs from the latter enzyme in being less stable and containing fewer aromatic amino acids.

Acknowledgments—The author is indebted to Dr. Jack Schultz for his continued interest and support. The encouragement to undertake this work given by the late Dr. Jesse Greenstein is gratefully acknowledged.

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Leucine Aminopeptidase Fragments from an Ascites Tumor
Elizabeth K. Patterson


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