LEUCINE AMINOPEPTIDASE

IV. ISOLATION AND PROPERTIES OF THE ENZYME FROM SWINE KIDNEY*

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In the earlier studies (1-6) of leucine aminopeptidase, obtained from swine intestinal mucosa, a purification of 30- to 90-fold based on the activity of the crude aqueous extract was achieved. Attempts to obtain a greater purification were unsuccessful largely because of the instability of the enzyme. Studies of other tissues have shown that swine kidney is a more convenient and richer source of the enzyme. The finding that the stability is greatly increased in the presence of Mg++ has permitted an extensive purification by fractionation with acetone and ammonium sulfate and finally by electrophoresis on filter paper.

The most active preparations appear to be nearly homogeneous when studied by electrophoresis on paper and in the Tiselius cell and by sedimentation in the ultracentrifuge. A preliminary study of the amino acid composition shows that only the usual amino acids are present in this enzyme. The activation by Mn++ and Mg++ and the specificity of the aminopeptidase are presented elsewhere (7).

EXPERIMENTAL

Methods—Assays of enzyme activity were performed by the following procedure. Enzyme preparations were activated at 40° by incubation at pH 8.0 with 0.001 to 0.002 mM MnCl₂. The mixtures were buffered with 0.04 M tris(hydroxymethyl)aminomethane (Tris). An aliquot of the activated enzyme was added to a 2.5 ml. assay flask containing L-leucinamide hydrochloride buffered with Tris at pH 8.6. The final concentration of substrate was 0.05 M. The assay flask also contained sufficient MnCl₂ to give the same concentration as in the activation mixture.

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Hydrolysis rates were measured at 40° by titration of 0.2 ml. samples with alcoholic KOH (8). The complete hydrolysis of 1 amide bond is given as 100 per cent. Proteolytic coefficients \( C_1 \) were estimated in the usual manner from \( C_1 = K_1/E \), where \( K_1 \) is the first order velocity constant calculated in decimal logarithms and \( E \) is the protein concentration in mg. of protein N per ml. of assay solution. Assays were terminated after 60 to 90 minutes to avoid correction factors due to the slow oxidation of Mn++. Under these conditions, autolysis of enzyme and non-enzymatic hydrolysis of substrate were negligible. It has been reported (2, 3) that the aminopeptidase of intestinal mucosa requires incubation with the metal ion for maximal activation. This has also been observed with the enzyme from swine kidney, but it was found that shorter times were required as greater purification was achieved. This activation time with Mn++ decreased from 2 to 3 hours for preparations with a \( C_1 \) of 4 or less to 30 minutes for preparations with a \( C_1 \) of approximately 10. With purer preparations, there was little further change, and these required 15 to 20 minutes for optimal activation. Studies of the time-course of activation of the purified enzyme are presented elsewhere (7).

The number of units of enzyme activity was calculated from the \( C_1 \) value for L-leucinamide multiplied by the mg. of protein N in the preparation. Protein N was determined by a modification of the turbidimetric procedure of Bücher (9). The method was calibrated with human serum albumin of known nitrogen content. It is assumed that the kidney proteins have an average N content of 16 per cent. Protein N estimated by this method was in good agreement with values determined by a micro-Kjeldahl method.

**Purification**

Swine kidney is a richer source of leucine aminopeptidase than is the mucosa from swine intestine, the tissue used previously for several studies. An average \( C_1 \) of 0.055 has been found for an aqueous extract of fresh, ground kidney; this is nearly 3 times as high as that found for intestinal mucosa \( (C_1 = 0.02) \) (3).

In order to devise a satisfactory method of isolation, a large number of studies were made of the stability and other properties of the enzyme. A few of these are described, since these properties reflect important characteristics of the enzyme. Most of these studies were made with aqueous extracts of the acetone-dried powder obtained as Step 1 of the purification procedure given below.

Table I shows the effect of heating aliquots of the enzyme for 10 minutes at pH 8.0 (Tris buffer) in the presence of 0.01 M MgCl₂. The \( C_1 \) and yield were determined after removal of any formed precipitate. It is evident that little inactivation occurs after heating as high as 70° and that some
purification is obtained. This finding should be contrasted with earlier studies (3) on the leucine aminopeptidase of intestinal mucosa which is unstable even in the cold in the presence of Mn$^{2+}$ or in the absence of added metal ion. It should be noted that preparations of the kidney enzyme at various levels of purity ($C_1 = 1.3$ to 52) have been kept under toluene in

**Table I**

*Effect of Temperature on Stability of Leucine Aminopeptidase*

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$C_1$</th>
<th>Yield (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solution</td>
<td>1.7</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>1.7</td>
<td>96</td>
</tr>
<tr>
<td>50</td>
<td>1.7</td>
<td>104</td>
</tr>
<tr>
<td>60</td>
<td>1.9</td>
<td>96</td>
</tr>
<tr>
<td>70</td>
<td>2.5</td>
<td>88</td>
</tr>
<tr>
<td>80</td>
<td>1.3</td>
<td>34</td>
</tr>
</tbody>
</table>

**Table II**

*Precipitation of Leucine Aminopeptidase with Ammonium Sulfate*

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$C_1$</th>
<th>Yield (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract of acetone-dried powder</td>
<td>0.28</td>
<td>53</td>
</tr>
<tr>
<td>0-0.3 saturated (NH$_4$)$_2$SO$_4$</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
<tr>
<td>0.3-0.4 saturated (NH$_4$)$_2$SO$_4$</td>
<td>0.21</td>
<td>1.3</td>
</tr>
<tr>
<td>0.4-0.5 &quot; &quot;</td>
<td>0.27</td>
<td>7.9</td>
</tr>
<tr>
<td>0.5-0.6 &quot; &quot;</td>
<td>0.97</td>
<td>30</td>
</tr>
<tr>
<td>0.6-0.7 &quot; &quot;</td>
<td>0.92</td>
<td>46</td>
</tr>
<tr>
<td>0.7-0.8 &quot; &quot;</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

the refrigerator for as long as 15 months with little or no detectable loss of activity; this is also in striking contrast to the behavior of the intestinal enzyme.

Several studies at various pH values indicated that the stability of the enzyme is optimal near pH 8.0 and that heating at 70° causes progressively greater losses at lower pH values. At pH 5.5, 65 per cent of the enzyme is inactivated in 10 minutes, with no gain in purity.
Results of a study of the precipitation of the enzyme with ammonium sulfate appear in Table II. It is evident that most of the enzyme precipitates in the range from 50 to 70 per cent saturation and that more activity is recovered than was present in the initial extract. This was repeatedly observed and is probably due to the removal of inhibitors present in the acetone-dried powder. It should be noted that dialysis was always performed against Tris buffer at pH 8.0, since it was observed that dialysis at lower pH values caused appreciable losses of activity.

Fractionation with acetone or ethanol gave essentially similar results. Table III shows the results of a study of acetone fractionation at three pH values. It is evident that the bulk of the activity is precipitated in a narrow range of acetone concentrations.

A representative fractionation is given below. This procedure has been repeated many times with very consistent results. The range of Cl values for each step indicates assays in different runs. It was convenient to prepare the acetone-dried powder (Step 1) in lots of 2400 gm. of kidney and to perform the subsequent steps with powders obtained from two or three batches.

**Step 1. Preparation of Acetone-Dried Powder**—An acetone-dried powder of fresh, frozen swine kidneys was prepared essentially in the manner described earlier (9). After thawing to a semifrozen state, 2400 gm. of whole kidneys (freed of excess fat) containing 2450 units of enzyme were coarsely ground in a meat grinder and then treated in portions in a Waring blender for 1 minute with an equal volume (2400 ml.) of 53.3 per cent
ethanol at $-5^\circ$. One-fifth of the volume of the mixture or 960 ml. of 95 per cent ethanol at $-20^\circ$ were then added. After 30 minutes, the precipitate was collected by centrifugation for 1 hour at $1200 \times g$.\(^1\) The precipitate was then extracted with an equal volume of cold absolute ethanol ($-20^\circ$) for 30 minutes, and, after centrifuging as before, the precipitate was mixed with 2 volumes of cold acetone. After 2 hours, the material was collected on a Büchner funnel, and the acetone extraction was repeated. The solid material was again collected on a Büchner funnel and washed successively with acetone (0.5 volume), 50 per cent acetone-ether (0.25 volume), and finally with ether (0.25 volume). The fibrous material was first air-dried rapidly at room temperature and then in vacuo over sulfuric acid. The yield was 360 gm. of powder. A clarified aqueous extract of this powder had a $C_1$ of 0.2 to 0.3.

**Step 2. First Ammonium Sulfate Fractionation—**The acetone-dried powder, in lots of 600 to 800 gm., was extracted in convenient portions by treatment in a Waring blender for 1 minute with 7 volumes of water at room temperature. (All subsequent operations, except the heat treatment step were performed in the cold.) After 30 minutes, the mixture was centrifuged for 1 hour at $1200 \times g$ and the supernatant fluid saved. The precipitate was reextracted with 1 volume of water; the insoluble residue was removed by centrifugation and discarded. The two extracts were combined and the turbid solution was adjusted to pH 8.0 to 8.1 with $N\text{NaOH}$ (approximately 12 ml. per liter).

This solution was brought to 40 per cent saturation by addition of solid ammonium sulfate (242 gm. per liter), and after 30 minutes the precipitate (which was discarded) was removed either by filtration on fluted paper or under weak vacuum on a Büchner funnel (a filter aid such as Hyflo SuperCel is recommended). The supernatant solution was brought to 80 per cent saturation by addition of more ammonium sulfate (280 gm. per liter), and after 30 minutes the precipitate was collected and dialyzed in the manner described in Step 2. The $C_1$ was 1.5 to 2.4.

\(^1\) The supernatant fluid is a rich source of prolidase (10) and is routinely saved for the isolation of this enzyme.
Step 4. Precipitation with MgCl₂—The solution from Step 3 was brought to pH 7.0 by addition of N HCl and to 0.01 M MgCl₂ by addition of the solid salt. After 2 hours the inactive precipitate was removed by centrifugation and the supernatant fluid brought to pH 8.0 with N NaOH. The C₁ was 2.5 to 3.5.

Step 5. Heating—The solution was transferred to a stainless steel beaker and, with mechanical stirring, was heated in an 80° water bath until the temperature of the solution reached 70°. It was kept at 70° for at least 4 minutes or until a total of 10 minutes had elapsed from the start of the heating operation, whichever was longer. The preparation was then quickly cooled in an ice-water bath at 0°. The inactive precipitate was removed either by filtration or centrifugation. C₁ = 3.8 to 5.8.

Step 6. Acetone Fractionation—The solution from Step 5 containing about 2 per cent protein at 0°-5° was brought to pH 7.0 with N HCl. Acetone which had been cooled to -60° in a dry ice storage box was slowly added through a pipette to make the concentration 20 per cent. The inactive precipitate was removed by centrifugation for 10 minutes at 1200 × g. The supernatant solution was brought to 30 per cent acetone in the same manner and the precipitate collected by centrifugation. The precipitate was dissolved in a solution containing 0.005 M MgCl₂ and 0.005 M Tris at pH 8.0 and was dialedyzed at 5° for 16 to 18 hours against three more changes of the same solution. C₁ = 16 to 43.

In most cases, preparations were obtained with a C₁ of 30 to 43. In the few instances in which the purity was lower (C₁ = 16 to 18), the fractionation was repeated between 18 and 25 per cent acetone to bring the C₁ into the higher range (30 to 43). With preparations of C₁ = 35 to 43, repetition of the acetone fractionation was ineffective.

Step 7. Aging—Preparations from Step 6 were diluted to a concentration of 2 mg. of protein per ml. with a solution containing 0.005 M MgCl₂ and 0.005 M Tris at pH 8.0. These solutions were kept under toluene in the refrigerator for 6 to 10 weeks; a gradual precipitation occurred, with a small decrease in pH. The pH of the solution was checked at weekly intervals and brought to 8.0 as needed with N NaOH. The concentration of aminopeptidase remained constant, and, after about a 2-fold increase in purity, further storage had no effect. The C₁ = 55 to 83.

Paper Electrophoresis

For the experiments with paper electrophoresis, an assembly was used which was designed in this laboratory and was similar in construction to the equipment described by Flynn and de Mayo (11). A framework of glass rod was constructed to fit into an aquarium type tank, which was fitted with a tight glass cover. The protein solution was put on the center
of a section of Whatman No. 3 MM paper in a thin line so that, when the paper was placed on the support framework, the thin band of protein solution was at the apex directly over the uppermost glass supporting rod. The buffer was 0.08 M Veronal at pH 8.5 and contained 0.001 M MgCl₂. This was applied evenly to the paper on both sides of the protein band so that the buffer ascending to the apex would narrow further the band of protein solution. After allowing 15 minutes for the contents of the tank to come to equilibrium, current from a full wave rectifying power pack was switched on. The current density was adjusted to 0.2 to 0.6 ma. per cm. width and the resulting potential varied from 150 to 250 volts. For preparative runs the experiment was terminated after 16 hours. All operations were conducted in a cold room at 3-5°.

Protein was located on the paper by cutting test strips 1 cm. wide from the edges and the center of the sheet of paper and staining these with the bromophenol blue preparation described by Durrum (12). Horizontal strips containing the protein bands were then cut from the unstained portions of the paper and, after cutting to 10 cm. lengths, the protein was eluted by gravity flow at 5° with a buffer solution at pH 8.0 containing 0.005 M MgCl₂ and 0.005 M Tris.

With a preparation of leucine aminopeptidase which had a Cl of 25, three distinct bands were always visible. A heavy band was the fastest component, with a lighter band slightly more than midway between the origin and the fastest moving band; a third, very light band remained at the origin. A general background of color was present on the stained strips throughout the distance moved. Fig. 1, A presents the results of such an experiment. Table IV gives the enzymatic activity of the eluates from the sections shown in Fig. 1, A.

With a more active preparation of leucine aminopeptidase (Cl = 52), somewhat different results were obtained. The enzyme moved along the paper as one dense, homogeneous band; the only other visible band was a very light one close to the origin. The Cl of the eluates from several experiments with this preparation were 85 to 88; others ranged down to 70. Figs. 1, B and 1, C show the results obtained in two electrophoretic experiments with this more active enzyme preparation. Table IV gives the enzymatic activity of the eluates from the sections shown in Figs. 1, B and 1, C and for several additional experiments including some larger scale preparative runs (Step 8).

**Step 8. Electrophoretic Separation**—For the preparative electrophoresis on filter paper, the following procedure was adopted. Preparations from Step 7 were concentrated in a cellophane bag by blowing a stream of air against the bag. These solutions were then dialyzed against water containing 0.005 M MgCl₂ and 0.005 M Tris at pH 8.0. Aliquots of 0.5 ml.
containing 7.5 to 10 mg. of protein were placed on 45 X 57 cm. sheets of Whatman No. 3 MM filter paper, and purification was accomplished by electrophoresis with 0.08 M Veronal buffer at pH 8.5 and 0.001 M MgCl₂. A current density of 0.23 ma. per cm. at a potential of 150 volts was applied for 16 hours, after which test strips from the sides and center of the sheet were stained as described above. Eluates of the strips containing the most rapidly migrating protein band were obtained by the method described above to yield preparations with a C₁ of 70 to 88.

Table V presents a summary of the entire purification procedure for a representative run. From the starting material, an over-all purification of 1600-fold was achieved, calculated from the soluble protein N of the original extract. The highest activity achieved, C₁ = 88 for the hydrolysis of L-leucinamide, compares favorably with that of other proteolytic en-

![Representative electrophoretic runs on paper of preparations of the aminopeptidase. A, initial C₁ = 25 in 0.1 M Veronal buffer at pH 8.5 containing 0.005 M MgCl₂. The strip was stained after 15 hours (current density, 0.42 ma. per cm. at 190 to 220 volts). B and C were run for 16 hours with an enzyme preparation (C₁ = 52) under slightly different conditions and, as shown, were sectioned and eluted differently. B contained 0.002 M MgCl₂ in 0.08 M Veronal at pH 8.5, whereas C contained 0.001 M MgCl₂ in 0.1 M Veronal at pH 8.5. The current density in both runs was 0.66 ma. per cm. at 260 volts. Eluates were obtained from the unstained portions of the paper.](image-url)
zymes, being very much higher than those of the proteinases on synthetic substrates. Crystalline carboxypeptidase (13) has a $C_1 = 14$ for carbobenzoxyglycyl-L-phenylalanine at the same substrate concentration.

**Table IV**

*Activity of Eluates from Electrophoresis on Paper*

Activity ($C_1$) and units (in parentheses) for eluates after electrophoresis on paper. The units are those actually recovered and are uncorrected for losses on the strips which were stained as a guide for the elution. The fractions are numbered in order of increasing mobility. For Experiments A to C, the fractions cut are shown in Fig. 1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial $C_1$</th>
<th>$C_1$ Fraction 1</th>
<th>$C_1$ Fraction 2</th>
<th>$C_1$ Fraction 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Fig. 1, A)</td>
<td>25 (17)</td>
<td>16 (2)</td>
<td>56 (10)</td>
<td>28 (2)</td>
</tr>
<tr>
<td>B ( &quot; 1, B)</td>
<td>52 (15)</td>
<td>88 (3.0)</td>
<td>87 (3.5)</td>
<td></td>
</tr>
<tr>
<td>C ( &quot; 1, C)</td>
<td>52 (15)</td>
<td>70 (1.4)</td>
<td>81 (5.8)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>52 (15)</td>
<td>49 (1)</td>
<td>75 (5.5)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>52 (15)</td>
<td>16 (0.5)</td>
<td>76 (4.3)</td>
<td>67 (2.8)</td>
</tr>
<tr>
<td>F. Preparative</td>
<td>52 (61)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. &quot;</td>
<td>47 (81)</td>
<td>56 (6)</td>
<td>82 (43)</td>
<td></td>
</tr>
<tr>
<td>H. &quot;</td>
<td>47 (120)</td>
<td>48 (9)</td>
<td>70 (77)</td>
<td></td>
</tr>
</tbody>
</table>

* Only the major band was eluted.

**Table V**

*Purification of Leucine Aminopeptidase from Swine Kidney*

This is a representative run for 2400 gm. of kidney. The initial enzyme content is based upon the activity of a clarified aqueous extract; such extracts are not prepared for the purification procedure given.

<table>
<thead>
<tr>
<th>Step No.</th>
<th>$C_1$</th>
<th>Total protein (mg.)</th>
<th>Total units (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.055</td>
<td>293,000</td>
<td>2450</td>
</tr>
<tr>
<td>1. Acetone powder extract*</td>
<td>0.25</td>
<td>45,300</td>
<td>1700</td>
</tr>
<tr>
<td>2. 1st sulfate precipitation</td>
<td>1.1</td>
<td>11,600</td>
<td>1900†</td>
</tr>
<tr>
<td>3. 2nd &quot; &quot;</td>
<td>2.4</td>
<td>4,170</td>
<td>1500</td>
</tr>
<tr>
<td>4. $\text{MgCl}_2$ precipitation</td>
<td>3.6</td>
<td>2,640</td>
<td>1430</td>
</tr>
<tr>
<td>5. Heating</td>
<td>5.8</td>
<td>1,320</td>
<td>1120</td>
</tr>
<tr>
<td>6. Acetone fractionation</td>
<td>43</td>
<td>105</td>
<td>720</td>
</tr>
<tr>
<td>7. Aging</td>
<td>52</td>
<td>87</td>
<td>720</td>
</tr>
<tr>
<td>8. Electrophoretic separation‡</td>
<td>88</td>
<td>23</td>
<td>320</td>
</tr>
</tbody>
</table>

* From a yield of 360 gm. of powder.
† The high recovery in this step is presumably due to the removal of inhibitors.
‡ Only 82 mg. of protein from Step 7 were used, and the final yield is calculated from this quantity.
(0.05 M). Preparations of crystalline prolidase, another \( \text{Mn}^{++} \)-activated peptidase, have been obtained with \( C_1 = 130 \) for the scission of glycyl-L-proline (10, 14).

From the initial rate of hydrolysis of 0.05 M leucinamide, it may be computed that approximately 200,000 moles of substrate are hydrolyzed per minute per 100,000 gm. of enzyme. This high turnover number compares favorably with that of other hydrolytic enzymes. The aminopeptidase obtained by Robinson, Birnbaum, and Greenstein (15) from a particulate fraction of swine kidney has a very different specificity from that of leucine aminopeptidase and is reported to have a turnover number of about 60,000.

**Studies of Chemical and Physical Properties**

*Electrophoresis*—As purification progressed, it was useful to study the preparations in a Tiselius apparatus equipped with the Longsworth schlieren scanning device. Fig. 2 presents the electrophoretic patterns obtained with preparations of leucine aminopeptidase having a \( C_1 \) of 2.8 to 73. The data are given in Table VI. It appears that the major contaminating
protein traveled at pH 8.5, with a mobility very near that of the enzyme
\((u = -6.0 \times 10^{-5} \text{ sq. cm. per volt per second}).\)

In the preparation with \(C_1 = 73\) there is a slight asymmetry at the base
of the trailing edge. This may be an impurity or inactive enzyme, but,
in any case, represents less than 10 per cent of the total area. It is likely
that preparations with \(C_1 = 88\) will prove to be completely homogeneous,
but the small amount of such products at present available has prevented
more thorough studies of electrophoretic behavior in the Tiselius cell.
However, the best preparations were studied by electrophoresis on paper;
these always revealed only a single band. Attempts to fractionate this

| \(C_1\) | Protein concentration | Component 1 | | Component 2 | | Component 3 | | Component 4 |
|---|---|---|---|---|---|---|---|
| 2.8 | 1.36 | 14 | -2.6 | | 34 | -4.5 | 52 | -6.5 |
| 10.5 | 1.40 | 9 | -2.6 | | 21 | -4.1 | 70 | -5.6 |
| 22 | 0.82 | 5 | -1.7 | 23 | -3.4 | 16 | -4.7 | 56 | -6.2 |
| 43 | 1.00 | 3 | -1.8 | 0 | -2.0 | 21 | -4.2 | 67 | -5.0 |
| 73 | 0.52 | | | | <10 | -4.8 | >90 | -6.0 |
| 88* | | | | | | | 100 |

* Preparations at this level of activity were apparently homogeneous when run
by electrophoresis on paper. Insufficient material was available for a test in the
Tiselius cell.

band by cutting it and eluting the faster and slower portions yielded
preparations with identical activity at the highest levels reported; namely,
\(C_1 84\) to \(88.\)

A plot of \(C_1\) versus the percentage of the component
with \(u = -6.0 \times 10^{-5}\) indicates the presence of a large amount of inactive material
with essentially the same mobility as the enzyme. Sedimentation studies of the
partially purified preparations \((C_1 = 2\) to \(6)\) revealed a component with \(s = 4.3\) S
in the neighborhood of 4.3 to 4.4 S, which is near the values reported for the serum albumins
of various species. It should be noted, however, that no component with \(s = 4.3\) S
could be detected in our best preparations.

It was indicated earlier (14) that preparations of leucine aminopeptidase with
a \(C_1\) of 85 were only about 40 per cent pure as judged by electrophoresis. It has now
been found that the apparent electrophoretic heterogeneity is an artifact. In the
absence of added \(Mg^{+2}\) ions considerable inactivation of the enzyme occurs, and the
Sedimentation in Ultracentrifuge—Some of the purified enzyme with $C_1 = 73$ to 88 was used for sedimentation studies. These were performed in a Spinco ultracentrifuge. A brief description of this instrument and the controls and procedures as used in this laboratory have been reported previously (16). The studies were made at 59,780 r.p.m., which is equivalent to centrifugal fields of approximately $240,000 \times g$ and $300,000 \times g$ at the meniscus and base, respectively.

Measurements made in Veronal buffer containing 0.005 M MgCl$_2$ at portion of protein bound to the metal ion remaining after dialysis has a lower mobility than the active material. Separation of the more rapidly moving component from the Tiselius cell has given preparations with essentially the same $C_1$ that was obtained after electrophoresis on paper.
pH 8.5 showed a single, monodisperse sedimenting boundary, as illustrated in Fig. 3. The influence of protein concentration on the sedimentation constant \( s_{20,w} \) was studied at three concentrations; the results are presented in Fig. 4. The line drawn through the points gives an extrapolated value of \( s_{20,w} \) at zero protein concentration of 12.6 S. On the basis of this value and comparison with proteins having similar sedimentation constants, leucine aminopeptidase would appear to have a molecular weight in the neighborhood of 300,000.

### Table VII

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amino Acid Residue per 100 gm. Protein</th>
<th>Amino Acid</th>
<th>Amino Acid Residue per 100 gm. Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>10.2</td>
<td>Methionine</td>
<td>1.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.5*</td>
<td>Isoleucine</td>
<td>5.7</td>
</tr>
<tr>
<td>Serine</td>
<td>6.4*</td>
<td>Leucine</td>
<td>8.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.0</td>
<td>Tyrosine</td>
<td>2.7</td>
</tr>
<tr>
<td>Proline</td>
<td>4.3</td>
<td>Phenylalanine</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.3</td>
<td>Histidine</td>
<td>2.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.4</td>
<td>Lysine</td>
<td>8.3</td>
</tr>
<tr>
<td>Half cystine</td>
<td>2.4†</td>
<td>Ammonia</td>
<td>1.9*</td>
</tr>
<tr>
<td>Valine</td>
<td>6.6</td>
<td>Arginine</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Total: 97.5

* The actual recovery was 4.4 gm. of threonine and 4.3 gm. of serine (on a residue basis); these values have been corrected for the average destruction of these amino acids observed in 70 hours hydrolysis in this laboratory with other proteins (19, 20). Correspondingly, the ammonia recovery, on a residue basis, was 2.5 gm. and has been corrected for the ammonia liberated by the calculated serine and threonine destruction.

† This is probably a minimal value, since only incomplete recoveries are usually obtained under the conditions of this analysis.

Absorption Spectrum—The absorption spectrum of a highly purified preparation of leucine aminopeptidase is shown in Fig. 5. The main absorption band was in the region usually found for simple proteins and indicated the presence of the aromatic amino acids. No evidence was found for the presence of nucleic acid or other substances which absorb strongly in the near ultraviolet region.
Amino Acid Composition—The amino acid composition of leucine aminopeptidase was estimated by chromatographic separation of the constituent amino acids on the sulfonated polystyrene resin, Dowex 50 (17). A 0.9 X 100 cm. column was used for the acidic and neutral amino acids and a 0.9 X 15 cm. column for the basic amino acids and ammonia. The quantitative estimations were made by the ninhydrin procedure of Moore and Stein (17, 18). The hydrolysis and preparation of the samples were performed as described in earlier studies on carboxypeptidase (19) and papain (20). Since sufficient material was available for only a single run, the hydrolysis was performed for 72 hours at 105° in order to be certain of complete hydrolysis of the protein. The results are reported in Table VII.

The composition of this enzyme does not reveal any striking characteristics. Most noteworthy, perhaps, are the low tyrosine content and the high leucine content which, except for aspartic and glutamic acids, is the most abundant amino acid. This may be significant when it is recalled that leucine aminopeptidase shows its highest activity on aliphatic substrates and particularly compounds containing leucine (1, 6, 7).

Although the data in Table VII, which represent only a single run, are probably reliable only to the extent of 5 or 10 per cent for each value, they suggest that the protein contains only the usual amino acids. Tryptophan has not been determined. The data appear to account for 97.5 per cent of the weight of the protein and indicate that other constituents are probably absent or present in only minor amounts.

DISCUSSION

The isolation of leucine aminopeptidase in a state of apparent homogeneity now permits more definitive studies of the activation behavior and specificity of this metal ion-activated enzyme. Some first studies of this sort are reported in Paper V (7). Further study of the chemical and physical properties will depend on the development of methods which will permit the isolation of larger amounts of the enzyme.

The studies of the enzyme reported here indicate that leucine aminopeptidase is a typical protein with a molecular weight in the neighborhood of 300,000, which is large when compared with most of the enzymes obtained from animal tissues. However, since the studies were made in the presence of 0.001 M Mg++, which is essential for stabilization, it is possible that this high value may be due to aggregation of the protein. The high mobility of the enzyme at pH 8.5 suggests an acidic isoelectric point, probably in the region of pH 4 to 5. It is noteworthy that the enzyme is

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4 We are indebted to Mrs. Vina Buettner-Janusch for these analyses.
very unstable in this region and manifests its optimal stability near pH 8 to 8.5.

SUMMARY

1. Leucine aminopeptidase of swine kidney has been purified about 1600-fold by procedures involving fractionation with ammonium sulfate and acetone and by electrophoresis on paper.

2. The best preparations appear to be homogeneous, as judged by electrophoretic studies and by sedimentation in the ultracentrifuge. The absorption spectrum and amino acid composition indicate that the enzyme is a simple protein.

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