Evidence has been presented earlier (2, 3) that leucine aminopeptidase can degrade about two-thirds of the molecule of mercuripapain, liberating free amino acids, without altering the enzymic activity of the papain when it is reactivated. Although several experimental results supported this conclusion, the best evidence came from two types of observations: first, new amino end groups different from the end group of intact papain, were found in the degraded enzyme, and second, amino acid analysis of degraded mercuripapain revealed that it was different from the native enzyme only by those amino acids liberated by the aminopeptidase (3).

Experiments reported in this paper provide additional evidence that mercuripapain can be degraded to an enzymically active fragment. Chromatographic procedures were developed which have allowed the separation of the active fragment in homogeneous form. The active substance contains approximately 76 residues in contrast to the 185 residues of intact papain. The fragment also differs from intact papain in chromatographic behavior, in ultraviolet absorption spectrum, amino end group, electrophoretic mobility, and molecular weight. Although the specific activity of the degraded enzyme is about 3 times that of intact papain, both kinds of enzyme have an identical substrate specificity and show similar behavior with respect to denaturation by heat, acid, or urea.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Leucine aminopeptidase was isolated from swine kidney (4, 5). Activity was estimated at 40° with 0.05 m leucinamide as substrate at pH 8.5 in Tris buffer containing 0.005 m MnCl₂ (5). All activities are expressed in terms of the proteolytic coefficient, \( C_1 \), calculated as previously described (5). Before they were used for degradation of mercuripapain, all aminopeptidase preparations were treated with diisopropylfluorophosphate to inhibit traces of kidney cathepsins which might be present (4). The molar quantity of active enzyme in all preparations was estimated on the assumption that pure enzyme has a \( C_1 \) = 88 and a molecular weight of 300,000 (6).

The limiting factor in the extent to which experiments with aminopeptidase can be performed is the availability of this enzyme. Preliminary studies showed that mercuripapain could be degraded to the same extent by aminopeptidase preparations with a \( C_1 \) of 30 to 90, if aminopeptidase was purified at the final step by zone electrophoresis on starch columns (4, 5). This finding was useful in that the relatively large quantities of aminopeptidase required for maximal degradation of mercuripapain could be obtained by pooling fractions from the starch column which yielded final preparations with \( C_1 \) of 35 to 40.

Mercuripapain was prepared by a modification of the procedure of Kimmel and Smith (7). Enzymic assays were performed at pH 5.2 in 0.01 m acetate buffer with 0.05 m \( \alpha \)-benzoyl-L-argininamide as substrate. Activities are expressed as \( C_1 \) and were calculated in the same manner as described for leucine aminopeptidase. Assay for papain in fractions obtained from chromatographic columns was performed by a ninhydrin procedure (8) under essentially the same conditions as described for the titrimetric assay. It should be re-emphasized that aminopeptidase does not hydrolyze benzoyl-L-argininamide, and that assays performed with this substrate reflect only the presence of papain or its active degradation products.

**RESULTS**

**Extensive Degradation of Mercuripapain**

In previous studies (2, 3) it was shown that mercuripapain could be hydrolyzed to varying degrees in 24 hours, the extent depending on the molar ratio of the mercuripapain to the aminopeptidase in the hydrolysis reaction. At ratios between 145 and 165, approximately 20 amino acid residues were liberated per mole of mercuripapain, whereas at ratios between 20 and 30, 50 to 70 residues were removed. At ratios of 20 or less, more extensive hydrolysis was observed. Thus, it seemed desirable to examine the nature of the digestion at even lower molar ratios in order to determine whether extensive degradation resulted in either a decrease in the activity of the degraded material or could produce a fully active, small molecule. For this purpose experiments were performed under digestion conditions in which the molar ratio of mercuripapain to leucine aminopeptidase was less than 20.

In a typical experiment, 80 mg of aminopeptidase (\( C_1 = 40; 0.133 \mu \text{mole} \)) were incubated at 40° with 0.005 m MgCl₂ in 0.01 m Tris buffer at pH 8.5. A solution containing 12 mg (0.6 \( \mu \text{mole} \)) of mercuripapain was added and the mixture was incubated for 24 hours at 40°. Hydrolysis was estimated by analyzing aliquots of the reaction mixture with the ninhydrin reagent of Moore and Stein (9). Other aliquots were activated with cysteine and ethylenediaminetetraacetate and assayed with \( \alpha \)-benzoyl-L-argininamide. A summary of the results of this ex-
Purification of Degraded Mercuripapain

In order to characterize further the active fragment, it was necessary to develop methods for separating it from the reaction mixtures described in Table I. In the development of these methods, advantage was taken of observations that either papain (11) or degraded papain (3) is absorbed by IRC-50 which has been equilibrated with 0.1 M sodium phosphate buffer at pH 6.2, whereas aminopeptidase is not held by the resin under these conditions. Fig. 1 shows for Preparation 3 the three stages of the chromatographic isolation based on these considerations.

Before chromatography, the reaction mixture was exhaustively dialyzed against distilled water in order to remove amino acids, salts, etc. The solution was then equilibrated by dialysis against 0.1 M sodium phosphate buffer at pH 6.2. The sample was applied to a 1.3 × 73-cm column of IRC-50 previously equilibrated with the same buffer, and the column was developed by gravity flow with the above phosphate buffer at room temperature at a rate of 5 to 6 ml per hour. Eluate fractions of 1 to 3 ml were collected per tube.

Although earlier analyses (10) indicated that papain contains 180 amino acid residues, more recent studies have demonstrated that there are probably 185 residues in the protein, viz. one more residue each of histidine, arginine, lysine, leucine, and isoleucine than reported earlier. R. L. Hill, J. R. Kimmel, W. R. Schmidt, and E. L. Smith, unpublished studies.

We do not feel that the liberation of excessive numbers of amino acid residues, i.e. numbers greater than 109, the difference between 185 and 76 (the average number of residues in the active fragment, see below), can be derived primarily from papain because of the excellent recovery of papain activity in the experiments listed on Table I. Moreover, analysis was performed in a few cases on the total amino acids liberated. There appears to be no need to present these results other than to note that the quantity of certain amino acids was greater than expected from complete digestion of papain.

### Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Ratio</th>
<th>MP</th>
<th>C1</th>
<th>Extent of hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/mole</td>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.6</td>
<td>12</td>
<td>40</td>
<td>1.42 1.25</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>12</td>
<td>35</td>
<td>1.42 1.26</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>20</td>
<td>40</td>
<td>1.70 1.70</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>20</td>
<td>40</td>
<td>1.20 1.20</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>90</td>
<td>40</td>
<td>1.20 1.20</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>12</td>
<td>35</td>
<td>1.55 1.55</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>12</td>
<td>35</td>
<td>1.65 1.45</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>10</td>
<td>35</td>
<td>1.43 1.38</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>40</td>
<td>35</td>
<td>1.4 1.3</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>40</td>
<td>40</td>
<td>1.6 1.6</td>
</tr>
</tbody>
</table>

Mg++-activated leucine aminopeptidase (LAP) was incubated at 40° in 0.01 M Tris buffer at pH 8.5 with mercuripapain (MP), at the molar ratio of aminopeptidase to mercuripapain indicated. Aliquots of the reaction mixture were analyzed with ninhydrin reagent throughout the 24-hour incubation in order to determine the extent of hydrolysis as leucine equivalents per mole of mercuripapain. Additional aliquots were activated with cysteine and ethylenediaminetetraacetate and assayed with benzoyl-L-argininamide.
enzyme emerges only after the buffer change. The protein emerging with the first buffer appears to be derived from the leucine aminopeptidase, although this cannot be judged by assay since the conditions of chromatography inactivate this enzyme. The irregular curve of papain activity is undoubtedly the result of some contamination by inert protein and of distortion present in the enzyme preparation. The protein emerging only after the buffer change. The protein fraction was collected per tube. Two separate experiments were performed on the same column, one with the isolated fragment and the other with intact enzyme.

Further purification was achieved by pooling the fractions between Tubes 100 and 260 (Fig. 1A) and dialyzing to remove salts. The preparation was lyophilized, equilibrated against 0.2 M phosphate buffer at pH 6.0, and chromatographed on an IRC-50 column of the same dimensions as before. The protein emerging from the column was collected and assayed as described above. The results are shown in Fig. 1B. It is apparent that additional inert material can be removed and that activity is present in two discrete peaks. Furthermore, activity of the fractions in Tubes 40 to 80 is almost directly proportional to the protein concentration. This suggests that these fractions contain nearly homogeneous material.

Rechromatography of fractions from Tubes 36 to 60 under the same conditions results in a single peak emerging at the same position as found at the preceding chromatographic step (Fig. 1C). Furthermore, there is a constant ratio between protein concentration and hydrolytic activity toward benzoyl-L-arginine-4-nitroanilide characteristic of a highly purified enzyme. The final yield of enzymic activity was 17% of that of the papain used for degradation by aminopeptidase.

In other experiments the yield ranged from 10 to 20%.

Fig. 2 shows for comparison the chromatographic behavior of the extensively degraded active material and of intact papain on the column equilibrated with 0.2 M sodium phosphate buffer at pH 6.0. The observed difference in the point of emergence of the two proteins demonstrates clearly that they do not have the same behavior and that intact papain emerges later than the active fragment from such a column. In the experiments illustrated in Fig. 1, B and C, as well as in all others performed under the same conditions, no significant amount of enzymic activity could be detected in the region where intact papain would emerge. Hence it is evident that essentially all of the papain is degraded by the aminopeptidase. It is also noteworthy that the small active peak, present in Fig. 1B, must represent a different stage of degradation than the major peak of activity. Because insufficient amounts of material were available, this was not studied further. It may be noted that the secondary active peak was present in variable amount in most but not all preparations.

Many preparations, including all of those listed in Table I, were chromatographed by these procedures. In each case a similar protein concentration-activity pattern was obtained, regardless of the apparent extent of hydrolysis estimated during the degradation by the aminopeptidase.

Chemical Properties of Degraded Mercuriapapain

Amino Acid Composition—The amino acid composition of several different preparations of the chromatographically purified, active fragment has been determined. Hydrolysis was performed under reduced pressure for 24 hours at 110°C in 3 times glass-distilled 6 N HCl. Table II presents the results of these analyses.

Each analysis represents a single determination on the preparation indicated. Hydrolysates of Preparations 3 to 6 were analyzed by the ion exchange chromatographic procedure of Moore et al. (18), as modified by Kimmel and Smith (14), whereas Preparations 8 and 9 were analyzed on the automatic recording analyzer of Spackman et al. (15). The latter method was performed with the Spinco model MS automatic amino acid analyzer. These two methods of analysis, although inherently the same, have somewhat different levels of precision for small quantities of amino acids. For example, the system of Kimmel and Smith (14) often results in errors caused either by base-line irregularities, or by insufficient and variable resolution of individual amino acids. Also, errors can be introduced during the manual method of ninhydrin development. The automatic recording system does not have these limitations and is more accurate. For these reasons, the average results for those analyses performed in the two different systems are reported separately.

Some of the data obtained in these analyses require additional comment. Preparations 3 to 6 were oxidized with performic acid before hydrolysis. This permitted cysteine and cystine to be estimated as cysteic acid. Because the recovery of cysteic acid is only 85% of theory in the case of intact papain (12), a 15% correction has been applied to the values found. None of the other values was corrected for destruction which might have occurred during the acid hydrolysis. Low recoveries might be expected for serine and threonine which are particularly labile during acid hydrolysis; however, because the analyses, when compared to one another, showed average deviations which are of the same order of magnitude as any correction which might be applicable, it was felt to be unnecessary to employ such factors.

Insufficient material was available for moisture, nitrogen, and ash analyses of the various preparations. Consequently, the number of residues of each amino acid per mole of enzyme was calculated on the assumption that the degraded enzyme contained only one histidine residue. Thus, the micromolar yield of histidine in each analysis was arbitrarily taken as equivalent to one residue. This should give a reasonable estimate since it is now recognized that papain contains two residues of histidine, one of which is removed during the degradation by the aminopeptidase. This method of calculation also provides good reason for reporting the analyses in two groups in that histidine is very susceptible to the problems of resolution indicated above.

![Fig. 2. Chromatography of intact papain and of active fragment on IRC-50. The chromatographic procedures were identical to those described in B with the exception that 1.8-ml fractions were collected per tube. Two separate experiments were performed on the same column, one with the isolated fragment and the other with intact enzyme.](image-url)
Amino acid composition of extensively degraded mercuripapain

Each value given for Preparations 3 to 9 represents a single determination. Values for cysteic acid were obtained after oxidation with performic acid and are corrected for the 15% loss which is estimated to occur (12). Where values are omitted either no determination was made (cysteic acid and tryptophan) or no estimate was possible because of poor resolution or accidental loss as in the case of four amino acids with Preparation 4. Average values are given with average deviations. The single tryptophan determination was made spectrophotometrically (see below).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Preparation</th>
<th>Average preparation 3 to 6</th>
<th>Average preparation 8, 9</th>
<th>Assumed number residues</th>
<th>Intact papain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td>2.7</td>
<td>2.9 ± 0.2</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.5</td>
<td>6.1 ± 0.5</td>
<td>6</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>2.1</td>
<td>2.3 ± 0.5</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>4.2</td>
<td>4.5 ± 0.6</td>
<td>4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.3</td>
<td>7.0 ± 0.2</td>
<td>7</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Proline</td>
<td>5.5</td>
<td>4.4 ± 0.7</td>
<td>4</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.3</td>
<td>9.0 ± 0.3</td>
<td>10</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.2</td>
<td>5.1 ± 0.3</td>
<td>5</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>5.7</td>
<td>5.3 ± 0.9</td>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>3.9 ± 0.3</td>
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<td>10</td>
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<tr>
<td>Leucine</td>
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<td>4.8 ± 0.7</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.7</td>
<td>3.7 ± 0.1</td>
<td>3</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.8</td>
<td>1.8 ± 0.3</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>3.5</td>
<td>3.8 ± 0.4</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>3.0</td>
<td>2.4 ± 1.1</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>1.0</td>
<td>1.0 ± 0.0</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.1</td>
<td>2.1 ± 0.0</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Total number 76 185

This reason, the analyses of Preparations 3 to 6 might be less accurate than those for Preparations 8 and 9.

Both methods of analysis gave identical results for several amino acids, viz. serine, alanine, isoleucine, phenylalanine, and lysine. Values for other amino acids agree to within one residue per mole with the exception of those for proline and arginine. These last amino acids are especially difficult to estimate by the less precise manual ninhydrin method because estimates for both are particularly susceptible to errors introduced by base-line irregularities, especially when they are present in small amount. In addition, proline gives a low color yield with the ninhydrin reagent used for its estimation. Arginine values are often low because this amino acid emerges as a broad peak which is difficult to measure precisely. For these reasons, values for proline and arginine obtained with Preparations 8 and 9 are believed to be more nearly correct. Cysteic acid was estimated only by the less precise method; however, estimates of this amino acid are not subject to most of the analytical difficulties discussed above, and indeed the yield shows an average deviation of only 0.2 residue per mole in the four analyses.

By taking these factors into account it has been possible to arrive at a reasonable estimate of the number of amino acid residues per mole of degraded papain. These values and the number of residues per mole for intact papain are given in the last two columns of Table II. Thus the enzyme fragment contains 76 residues representing a calculated molecular weight of 8,375. This is in contrast to intact papain which has a molecular weight of 20,583, calculated from the estimated 185 residues per mole.

Amino End-group Analysis—Thompson (16) has shown that diinitrophenylisoleucine is the only α-dinitrophenyl amino acid obtained in stoichiometric amount after treatment of mercuripapain with fluorodinitrobenzene. When mercuripapain which had been degraded by leucine aminopeptidase to the extent of 20 to 36 residues per mole was examined, no diinitrophenylisoleucine could be detected although new amino end groups appeared (3).

With the limited supply of active fragment available, the phenylisothiocyanate method of Edman (17) was used in an attempt to obtain information concerning the amino-terminal end of the fragment. The paper strip modification of this method (18) was used on Preparation 9. Only the phenylthiohydantoin derivative of aspartic acid could be identified when an aliquot was examined by paper chromatography in Solvent A of Edman and Sjöquist (19), although some additional iodine acid reactive material remained at the origin of the chromatogram.

After the first step of analysis, the degraded enzyme was carried through the same procedure as before for identification of the second amino acid in the fragment. The only compound present was proline phenylthiohydantoin which was identified by paper chromatographic analysis in Solvents A (19) and F (20). When the third step of the Edman analysis was performed there were several phenylthiohydantoin derivatives present in small amount; however, the derivative of glycine seemed to be present in largest quantity when Solvents A and F were used.

These results suggest that the amino-terminal sequence of the fragment is Aep.Pro.Gly.. This finding is regarded as tentative because of the limited quantity of fragment used and because it has not been possible as yet to perform such a study on another preparation.

In a parallel experiment, performed at the same time, intact papain was examined by the paper strip method. Clearly the
formed at pH 4 in 0.1 acetate buffer and at pH 8.5 in 0.1 M Veronal buffer yielded molecular weights in the range of 4,500 to 9,000. Each analysis, however, was performed at a protein concentration of 0.5% or less, concentrations which do not permit precise analysis of the patterns. Instead of the optimal 80° angle for the schlieren diaphragm, usually recommended (22), the concentration gradients had to be estimated at 60° and 70°.

Although these results did not yield a precise measurement of the weight of the fragment, the values obtained are in the range calculated from the amino acid composition and the specific activity determination (see below). Clearly these results definitely show that the fragment is of much lower molecular weight than is the intact enzyme.

The method of ultracentrifugal analysis used above, when performed at optimal concentrations (1 to 2%), yields excellent molecular weight estimates. For example, at pH 8.5, the oxidized A-chain of insulin (23) gave a value of 2,380 compared to a theoretical molecular weight of 2,532; crystalline glucagon (24), at pH 9.5 in glycine buffer, gave a molecular weight of 3,440 compared to a theoretical value of 3,482.

The authors wish to thank Mr. Douglas M. Brown for performing the ultracentrifugal analyses.

**Electrophoretic Analysis**—Analysis was performed by paper electrophoresis in a Spinco model R apparatus on 3 cm wide strips of Whatman 3MM paper at 5 mA and 50 volts for 17 hours at room temperature. Intact mercuropapain was run simultaneously for comparison. Univalent buffers at pH values of 4.0, 7.5, 8.4, and 9.6 were used at 0.1 ionic strength. The results are shown in Fig. 3 which gives the net migration of both intact papain and the fragment in centimeters per hour as a function of pH.

Three features of these results deserve comment. First, at the four pH values used, papain and the fragment migrated as single bands; this provides additional evidence as to the homogeneity of the fragment. Second, papain and the fragment have nearly the same isoelectric range although exact isoelectric points cannot be obtained by this technique. Third, both kinds of enzyme have nearly the same net charge at pH values above the isoelectric region, but not in the acidic range. This indicates small but significant differences in the acid-base properties of the two enzymes, and, indeed, aids in explaining why the fragment does not behave in the same manner as intact papain when it is chromatographed on IRC-50.

**Physical Properties of Extensively Degraded Mercuropapain**

**Ultracentrifugal Analysis**—Because the solubility of the active fragment was very low, ultracentrifugal analysis could not be made at favorable protein concentrations. In spite of this difficulty, the molecular weights of Preparations 3 and 4 were estimated by the approach to sedimentation equilibrium method of Archibald (21), as described by Schachman (22). These studies were conducted in a Spinco model E ultracentrifuge equipped with a slow speed attachment and a special phase plate as the schlieren diaphragm. Several experiments which were performed at pH 4 in 0.1 M acetate buffer and at pH 8.5 in 0.1 M Veronal buffer yielded molecular weights in the range of 4,500 to 9,000. Each analysis, however, was performed at a protein concentration of 0.5% or less, concentrations which do not permit precise analysis of the patterns. Instead of the optimal 80° angle for the schlieren diaphragm, usually recommended (22), the concentration gradients had to be estimated at 60° and 70°.

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**Ultraviolet Absorption Spectrum: Tryptophan Analysis**—The ultraviolet absorption spectra of intact papain and of fragment (Preparation 9), measured at pH 5 and pH 12.4 in an automatic recording Cary spectrophotometer model 14, are shown in Fig. 4, A and B. There appears to be no difference between the spectra obtained at pH 5, both curves showing a maximal absorption at 2784 Å; however, the spectra obtained at pH 12.4 (0.02 N NaOH) are markedly different from each other. Both spectra show a shift to higher wave lengths in alkali, the intact papain exhibiting a maximum at 2895 Å with a shoulder near 2850 Å, whereas the fragment shows a maximum at 2800 Å with a shoulder near 2900 Å. The absorption at 2895 to 2900 Å is due to tyrosine and that at 2800 to 2850 Å to tryptophan.

When the curve for papain, obtained after 1 hour at pH 13, was analyzed by the method of Beneze and Schmid (25), it was found that the amount of tyrosine was 16.7 ± 0.2 residues per mole and the amount of tryptophan was 4.90 ± 0.05 residues per mole. These results are in excellent agreement with earlier studies which showed that papain contains 17 tyrosine and 5 tryptophan residues (10).

Since the absolute extinction coefficient of the fragment of papain is unknown, only a ratio could be computed from the absorption curves. The results indicated the presence of almost exactly twice as much tyrosine as tryptophan. The average results in Table II show that the fragment contains 4 residues of tyrosine; hence, 2 residues of tryptophan are present in the fragment. This result is consistent with the observation (3) that only 1 residue of tryptophan is liberated from mercuropapain when about 12 residues per mole are removed from the amino-terminal end of the molecule.

**Enzymic Properties of Active Fragment**

**Specific Activity**—Estimation of the specific activity of the fragment was made by titrimetric or ninhydrin procedures. Preparation 9 was purified as rapidly as possible and care was taken to avoid all possible inactivating conditions. The data given in Table III indicate that hydrolysis of the substrate follows first order kinetics and the average C, is 3.4. Although this

*We wish to thank Mr. A. N. Glazer for his assistance in performing these determinations.

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**Fig. 3. Electrophoretic migration on paper of intact mercuropapain and fragment as a function of pH.**

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assay is probably accurate only to the extent of ±10%, it is sufficiently precise for comparative purposes with intact papain assayed in the same manner. A specific activity of 3.4 corresponds to a molecular weight of 8,500 since undegraded Preparation 9 had a C1 = 1.4 and a calculated molecular weight of 20,083. Two other preparations, 3 and 5, for which similar assays were performed, gave C1 values of 3.7 and 2.4, respectively; these values yield calculated molecular weights of 9,500 and 10,300 by the method given above for Preparation 9. The average molecular weight for the three preparations is 9,400. This estimate for the fragment is in reasonable accord with the value 8,400 calculated from the amino acid composition.

It should be recalled, however, that the C1 for papain reflects not only the size and purity of the preparation but also inactivation caused by alteration of the active site. Indeed, such inactivation has been reported for native papain (11). Major physical and chemical parameters such as molecular weight, amino acid composition, antigenicity, and electrophoretic mobility all appear to be the same for papain preparations with a range of C1 values from 0.7 to 1.8 (11, 27). Hence, the observations of an average molecular weight for the fragment higher than expected, or of an average C1, somewhat lower than expected, are not inconsistent with other observations, but are probably due to inactivation produced during the handling required for purification.

Substrate Specificity—The relative activity of the degraded enzyme compared with that of intact papain has been estimated with four synthetic substrates. The results obtained with Preparation 10 are given in Table IV. It is evident that within the precision of these measurements, no alteration of the specificity of the degraded enzyme could be found. Similar results have been reported earlier for enzyme preparations which were less extensively degraded (3).

Stability to Heat, Acid, and Urea—It has been shown that papain is inactivated at high temperatures (27), at low pH (28), and at high urea concentrations (29). The effect of these conditions on the fragment in comparison with intact papain has been determined; the results are summarized in Table V. No signif-

TABLE III
Hydrolysis of α-benzoyl-L-argininamide by extensively degraded papain

Substrate at 0.05 M acetate buffer at pH 5.2 containing 0.01 M cysteine and 0.001 M ethylenediaminetetraacetate was incubated at 40° in a final volume of 2.5 ml with 0.7 μg protein N per ml (Preparation 9). Protein content was estimated by a turbidimetric method (26).

<table>
<thead>
<tr>
<th>Concentration of Cysteine</th>
<th>Time</th>
<th>Hydrolysis</th>
<th>K, X 10⁻⁸</th>
<th>C,</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg protein N/ml</td>
<td></td>
<td>%</td>
<td>sec⁻¹</td>
<td></td>
</tr>
<tr>
<td>0.000/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>10.2</td>
<td>2.5</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>18.5</td>
<td>2.3</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>29</td>
<td>2.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>36</td>
<td>2.4</td>
<td>3.3</td>
<td></td>
</tr>
</tbody>
</table>

* Substrates were prepared as previously described (3).

TABLE IV
Substrate specificity of degraded mercuripapain

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Intact mercuripapain</th>
<th>Degraded papain (Preparation 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Benzoxy-L-argininamide</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbobenzoxy-L-glutamic acid diamide</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td>Benzoyglycinamide</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Carbobenzoxy-L-histidinamide</td>
<td>5.4</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* Substrates were prepared as previously described (3).

TABLE V
Stability of degraded and intact papain to heat, urea, and acid

The enzymes were assayed with α-benzoyl-L-argininamide as substrate as described in Table IV. Aliquots of either papain or fragment (Preparation 10) were treated in parallel as follows. Heat inactivation was performed by incubating solutions separately in a boiling water bath (95°) for 2 minutes, followed by chilling immediately to 5°. The enzymes were assayed immediately. Acid inactivation was achieved by adding 0.1 N HCl to aliquots of the enzyme solutions so as to bring them to pH 2. After 2 minutes at room temperature, the solutions were neutralized to pH 7 with 0.1 N NaOH and assayed. Urea inactivation was produced by adding the enzymes to an assay reaction mixture which contained sufficient urea to bring the urea concentration to either 0.5 M or 1.0 M. Activities are expressed as the relative rate of hydrolysis compared to that of the untreated controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact papain</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5 M urea</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>1.0 M urea</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>Heating to 95°</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acid</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4. Ultraviolet absorption spectra of intact mercuripapain and fragment at pH 5 and pH 12.4. A. The spectra of the fragment. B. The spectra of intact mercuripapain (0.34 mg per ml).
cent differences were found for the stability of the fragment and intact enzyme.

These data are significant in that they indicate a requirement for a three-dimensional structure for the degraded enzyme despite its smaller size.

**DISCUSSION**

With the lesser amounts of aminopeptidase which were used in earlier studies (3) it was demonstrated that the liberated amino acids plus the composition of the residual papain added up to the original composition. In the present study the objective was to degrade the mercuripapain as extensively as possible. In order to achieve this, the amount of protein in the aminopeptidase preparation was 2 to 10 times the amount of mercuripapain used as substrate. Under these circumstances, as already mentioned above, large amounts of amino acids were derived from the aminopeptidase preparations. Nevertheless, this did not interfere with the isolation of an active fragment whose properties appear to be independent of the conditions of the experiment. This is shown most strikingly by the fact that the major amount of active material exhibited identical chromatographic behavior and, most important, in the six cases in which the purified fragment was analyzed, the amino acid composition was found to be the same within the experimental limits of these determinations.

These results demonstrate both the adequacy of the method used for purification and its reproducibility. Moreover, one must conclude that, whatever the features of the papain molecule which prevent further degradation by aminopeptidase, this action appears to stop at a very definite place in the single peptide chain of the papain molecule. This is indicated not only by the aforementioned identity of amino acid composition, but by the finding that it has been impossible to effect any substantial decrease of papain activity. The small losses occasionally encountered are no greater than would be expected when mercuripapain is incubated for 24 hours at 40°.

There appear to be two possible reasons that the action of the aminopeptidase would cease. First of all, the only known type of amino acid sequence which effectively impedes the action of the aminopeptidase, other than the presence of a t-amino acid residue, a possibility which cannot be seriously considered, is the presence of a peptide bond involving the nitrogen of a proline residue. The results indicate that the residue next to the amino-terminal residue of the fragment is indeed a proline residue. We cannot be certain, however, that this is a satisfactory explanation inasmuch as the data indicate that the aminopeptidase has been able to liberate three proline residues before its action has ceased at the fourth one. Indeed, it may be recalled that the second residue from the amino-terminal end of the papain molecule is proline (38) and the enzyme has no difficulty with this portion of the molecule. Furthermore, Residue 24 from the amino-terminal end is also proline* and the aminopeptidase can readily penetrate this position also.

A second possible reason that the action of the aminopeptidase ceases where it does may be found in earlier observations in which many proteins are not susceptible to hydrolysis by the aminopeptidase unless the three-dimensional structure of these proteins is altered in such a way that the molecules are effected unfolding (4). It is possible, therefore, that the folded structure of this portion of the native papain molecule prevents further action by the aminopeptidase.

The high proline content (6 of 76 residues or 7.0 weight per cent) in the fragment is particularly interesting. One might expect this amount of proline, if distributed statistically in the peptide chain, to limit the helical content of the fragment. Indeed, it has been estimated that proline, when present in a protein to the extent of about 8%, deforms the primary structure into a random coil (31). Certainly little evidence is available that a helical conformation is necessary for enzymic activity, but on the other hand, these results, as well as those of Dixon et al. (32) with trypsin suggest that a helical conformation might not be necessary in an active site of an enzyme.

A major concern in this investigation has been to obtain as much proof as possible that the isolated fragment has been obtained in pure form and is different from the intact papain molecule. There is no need to recapitulate all of the evidence which has been presented above in the experimental section. Although the very small quantities of available material have limited these studies, the results reported above indicate that our objectives have been reasonably fulfilled. Among the more important results is the amino acid composition of the active fragment which shows certain striking features. First of all, the presence of only three half cystine residues in the fragment as compared to six in the intact molecule is of interest since clearly one of these three residues must provide the thiol group which is known to participate in the active site of the enzyme (11, 27, 33). The amino acid present in smallest amount in the fragment compared to intact enzyme is tyrosine. It has been suggested many times that the bonding of the phenolic side chain of this amino acid contribute greatly to the stabilization of proteins. Although it is unknown whether tyrosine residues play such a role in papain, it is clear that the majority of these residues is not essential.

Work has been in progress in this laboratory for some time in an attempt to determine the complete amino acid sequence of papain (34–36). Although this work is still incomplete, its successful conclusion should permit, in conjunction with studies of the active fragment, a careful assessment of dispensable and indispensable features of the enzyme molecule. Indeed, knowledge of the composition of the fragment is aiding in preliminary assignment of certain major peptide sequences to the amino- or carboxyl-terminal regions of the molecule.

The sequence tentatively identified as being amino terminal in the fragment is Asp-Pro-Gly... From a chymotryptic digest of oxidized papain a tetrapeptide has been isolated which possesses the sequence, Asp-NH₂-Pro-Gly-Tyr... Unfortunately, we do not know as yet from structural studies where this tetrapeptide occurs in the complete sequence of papain. When this information becomes available, it should be possible to assess more critically the identification of the amino-terminal sequence of the fragment.

In contrast to the different physical and chemical properties of the fragment and of the intact enzyme are the enzymic properties which seem to be much the same regardless of size. Not only is the specificity of the fragment the same, as judged with four synthetic substrates, in agreement with earlier results (3), but the two forms of enzyme exhibit the same susceptibility to denaturing conditions.

Although the above studies do not provide information as to what parts of the primary structure are essential for catalytic

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* A. Light and E. L. Smith, unpublished observations.
activity, it does indicate that the active site of papain is contained within the approximately 76 residues in the carboxyl-terminal position of the molecule. Conversely, it is evident that the 109 residues liberated from crystalline mercuripapain play no significant role in the catalytic activity of the papain molecule. These findings indicate that events which occur at the catalytic site during interaction with substrate must involve a limited region of the enzyme which determines not only specificity but catalytic efficiency as well. In addition, the results with denaturing conditions suggest that the structure of the catalytic region of the enzyme cannot be visualized in simple terms of linear primary structure.

SUMMARY

1. Mercuripapain can be degraded by large amounts of leucine aminopeptidase to an enzymically active fragment. Chromatographic procedures were developed which permit isolation of the fragment in homogeneous form as judged by its chromatographic behavior, amino acid composition, and electrophoretic behavior over a wide pH range.

2. Amino acid analysis showed that the isolated active fragment contains an average of 76 residue in contrast to intact papain which contains 186 residues per mole. The molecular weight calculated from these analyses is 8,375, a value which is in accord with estimates obtained from ultracentrifugal analysis and measurement of specific activity.

3. The α-amino end group of the fragment was shown by the phenylisothiocyanate method to differ from the α-amino end group of intact papain.

4. On the basis of paper electrophoretic analysis, the fragment has an isoelectric point near that of intact papain and nearly the same mobility above the isoelectric region, but different mobilities in the pH range acidic to the isoelectric region.

5. Both intact papain and the fragment have a maximal absorption at 2784 A at pH 5, whereas spectral analysis at alkaline pH showed distinctly different absorption curves. Calculation from the absorption spectrum indicates that the fragment contains two tryptophan residues per mole.

6. The active fragment is identical to intact enzyme in substrate specificity as judged with four synthetic substrates.

7. Both intact papain and active fragment possess a similar sensitivity to denaturation by heat, urea, and acid. These results indicate the requirement of an organized three-dimensional structure in the active fragment.

REFERENCES

Isolation and Characterization of an Enzymically Active Fragment of Papain
Robert L. Hill and Emil L. Smith