LIBERATION OF AMINO ACIDS FROM RAW AND HEATED CASEIN BY ACID AND ENZYME HYDROLYSIS*

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Earlier work showed that the nutritive value of casein was decreased by dry heat and that the addition of lysine restored its nutritive value (1). The lysine was not damaged materially by the heat treatment, since analyses of acid hydrolysates of heated casein with lysine decarboxylase (2) and chemical isolation as the picrate (3) showed no decrease in the lysine content. Eldred and Rodney (2), using the lysine decarboxylase method, and Pader, Melnick, and Oser (4), using the \textit{Streptococcus faecalis} assay, found that heating casein in a dry state at 150° for a few hours decreased the quantity of lysine liberated by enzyme hydrolysis \textit{in vitro}. Block, Jones, and Gersdorff (3) reported that the lysine content of casein was not affected by exposure to dry heat at a temperature at 150°, but that enzymatic liberation of the amino acid was decreased.

Melnick, Oser, and Weiss (5) pointed out that factors known to increase the nutritive value of soy bean protein also increase its \textit{in vitro} digestibility. In a recent report, Riesen \textit{et al.} (6) showed that the degree of liberation of the ten essential amino acids from soy bean oil meal by pancreatin was increased when the meal had been autoclaved for 4 minutes at 15 pounds pressure. When the period of autoclaving was extended to 4 hours, the liberation of these amino acids was decreased below that obtained with the raw meal. The amino acid content was unaffected by the short autoclaving procedure; after prolonged heat treatment, the lysine, arginine, and tryptophan values found by microbiological assay of acid or alkaline hydrolysates were decreased.

To determine whether casein was altered similarly, the effect of heat treatment on the amino acid composition and the extent of liberation of amino acids by enzymes were measured microbiologically. Since preliminary experiments indicated that this protein was much more resistant to changes in digestibility by moist heat than soy bean protein, the longer period of autoclaving was extended from 4 to 20 hours. In this work, the

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release of amino acids by successive treatment with a number of mammalian digestive enzymes was investigated.

EXPERIMENTAL

Preparation of Samples—Pyrex trays were filled to a depth of 0.5 inch with vitamin test casein (Smaco) and heated in an autoclave at 15 pounds pressure (121 °C) for 4 minutes and for 20 hours. The heated casein was then dried in a stream of air at 65 °C for 24 hours. Moisture and Kjeldahl nitrogen determinations were made on each sample.

Acid and Alkaline Hydrolysis—The conditions of acid hydrolysis which released maximum quantities of amino acids from casein, as measured by the formol titration, were determined in preliminary experiments. Casein was autoclaved with 25 volumes of 3 to 5 N hydrochloric acid at 15 pounds pressure for 13 to 18 hours; maximum liberation of amino groups was obtained with 3 N acid for 10 hours. Since longer periods of hydrolysis caused a slight reduction of the formol titration value, the 10 hour period with 3 N HCl was adopted for the assay of all amino acids except cystine, tryptophan, and tyrosine.

Riesen (7) found that free cystine was destroyed by the acid hydrolysis procedure used for the release of other amino acids and that maximum cystine values were obtained when the casein was autoclaved with 30 volumes of 2 N hydrochloric acid for 3 hours. This procedure, used in these studies for the hydrolysis for cystine analysis, should give comparable values for the three casein samples, though they may be somewhat lower than the true cystine content.

Alkaline hydrolysates for tryptophan and tyrosine assays were prepared by autoclaving samples of casein with 20 volumes of 5 N sodium hydroxide for 15 hours at 15 pounds pressure. Complete racemization was assumed.

Enzyme Hydrolysis—In enzyme digestion studies, commercial preparations of pancreatic and ereptic enzymes from several sources were assayed for their relative proteolytic or peptidase activities by measuring with formol titration the amino groups liberated from unheated casein. For proteinase activity determinations, 1 gm. of casein was shaken at 37 °C for 4 hours with 20 mg. of the preparation to be tested at pH 8 with 50 ml. of carbonate buffer. The substrate for peptidase activity determination was prepared by digesting casein for 2 days in this manner with the most active pancreatic enzyme preparation tested. For these assays, 20 mg. of the crude peptidase preparation were incubated at pH 7 for 1 hour with the pancreatic digest of 1 gm. of casein. Pepsin (Difco), whole pancreas (Uvicin), and erepsin (Difco) were selected for this work. By employing these enzymes successively for short periods of incubation, the rates of the digestion of the raw and heated casein samples were determined with periodic α-amino nitrogen and microbiological amino acid determinations.
10 gm. each of the raw and the two heated casein samples were placed in 2 liter Erlenmeyer flasks with 500 ml. of 0.1 N hydrochloric acid and shaken at 37° overnight. 10 ml. of enzyme solution containing 50 mg. of pepsin (Difco) were then added to each flask. A fourth flask containing 500 ml. of 0.1 N hydrochloric acid and 50 mg. of pepsin, but no substrate, served as a blank. After 40, 70, and 100 minutes, pH measurements were made and 2 ml. aliquots were removed from each flask for Van Slyke α-amino nitrogen determinations, to measure the progress of the digestion. At 100 minutes, the rate of digestion was decreasing rapidly; therefore, at 120 minutes a 50 ml. aliquot was removed from each flask. These aliquots were heated in a boiling water bath for 15 minutes to inactivate the pepsin, and were stored at −4° for amino acid assays.

Immediately after removal of the 50 ml. aliquots, 8 ml. of 5 N sodium hydroxide were added to each digestion flask to neutralize the solutions partially and to arrest the peptic activity. The solutions were then adjusted to pH 8.2 with 5 N sodium hydroxide and 10 ml. of toluene added. 10 ml. of pancreas (Viobin) solution (filtered water extract containing 10 mg. per ml.) were then pipetted into each flask. The course of the digestion was again followed with pH measurements and Van Slyke α-amino nitrogen determinations; after 1 hour the solutions were readjusted to pH 8.2. After 2 hours 50 ml. aliquots were removed and treated as before.

The contents of each flask were then adjusted to pH 7.0 and 10.0 ml. of erepsin (Difco, filtered water extract containing 5 mg. per ml.) were immediately added. The progress of digestion was again determined by periodic pH measurements and α-amino nitrogen determinations. After 2 hours, 50 ml. aliquots were removed and treated as above. The digestion was allowed to continue for 5 days longer, at which time 50 ml. aliquots were again removed.

Amino Acid Assays—Sixteen amino acids were determined microbiologically on the acid and enzyme hydrolysates with the following organisms for the amino acids indicated: Lactobacillus arabinosus 17-5 for glutamic acid, leucine, tryptophan, valine, and phenylalanine; Leuconostoc mesenteroides P-60 for aspartic acid, cystine, glycine, histidine, isoleucine, lysine, proline, and tyrosine; Streptococcus faecalis R for methionine and threonine; and Lactobacillus delbrueckii 3 for arginine. All amino acids except cystine were determined by the methods of Henderson and Snell (8). Cystine was determined with an oxidized peptone medium as described by Riesen et al. (9).

RESULTS AND DISCUSSION

Amino Acid Content of Raw versus Heated Casein—The data presented in Table I show that, with the exception of cystine, the amino acid content of casein as measured microbiologically after acid hydrolysis (alkaline hydroly-
### Table I

Liberation of Microbiologically Available Amino Acids from Raw and Heated Casein by Acid and Enzymes

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid content of acid hydrolysates†</th>
<th>Percentage liberation by digestive enzymes‡</th>
<th>Pepsin, 2 hrs.</th>
<th>Pepsin + pancreas, 2 hrs. each</th>
<th>Pepsin + pancreas + erepsin, hrs. each</th>
<th>Same, continued 5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
<td>4 min.§</td>
<td>20 hrs.§</td>
<td>Average</td>
<td>Raw</td>
<td>4 min.§</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>Raw</td>
<td>per cent</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.20</td>
<td>3.18</td>
<td>3.14</td>
<td>3.17</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Aspartic</td>
<td>6.54</td>
<td>6.34</td>
<td>0.17</td>
<td>0.35</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cystine‖</td>
<td>0.50</td>
<td>0.62</td>
<td>0.14</td>
<td>0.51‖</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Glutamic</td>
<td>19.27</td>
<td>19.07</td>
<td>18.95</td>
<td>19.10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.69</td>
<td>1.56</td>
<td>1.71</td>
<td>1.65</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.60</td>
<td>2.54</td>
<td>2.49</td>
<td>2.54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.34</td>
<td>5.61</td>
<td>5.42</td>
<td>5.46</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.44</td>
<td>8.61</td>
<td>8.81</td>
<td>8.62</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.54</td>
<td>6.59</td>
<td>6.33</td>
<td>6.49</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.13</td>
<td>2.11</td>
<td>2.26</td>
<td>2.17</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.70</td>
<td>4.49</td>
<td>4.65</td>
<td>4.61</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Proline</td>
<td>10.69</td>
<td>9.56</td>
<td>9.34</td>
<td>9.86</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.58</td>
<td>3.69</td>
<td>3.50</td>
<td>3.59</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Tryptophan‖</td>
<td>1.03</td>
<td>1.09</td>
<td>1.03</td>
<td>1.05</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Tyrosine‖</td>
<td>4.51</td>
<td>4.19</td>
<td>4.38</td>
<td>4.36</td>
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<td>1</td>
</tr>
<tr>
<td>Valine</td>
<td>6.72</td>
<td>6.62</td>
<td>6.68</td>
<td>6.67</td>
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<td>2</td>
</tr>
<tr>
<td>α-Amino N**</td>
<td>73‖</td>
<td>76‖</td>
<td>77‖</td>
<td>76‖</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Amino acid totals††</td>
<td>87.48</td>
<td>85.77</td>
<td>85.00</td>
<td>86.20</td>
<td>5</td>
<td>5</td>
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</tbody>
</table>
The amino acid figures represent the average of from three to five microbiological assays of two different hydrolysates. The total nitrogen in the raw casein was 13.51, 4 minute casein 13.66, and the 20 hour casein 13.50 per cent; the moisture contents were 8.00, 8.14, and 6.50 per cent, respectively.

† See the text for the conditions of hydrolysis. The acid hydrolysis values are expressed as per cent (gm. per 100 gm. of protein) amino acid yielded by casein, the values for the heated caseins were adjusted to the nitrogen content of the raw casein.

‡ See the text for the conditions of enzyme hydrolysis. The amino acid values were computed by dividing the amount of amino acid liberated by enzymatic hydrolysis by the amount liberated from casein by acid hydrolysis (average of raw and heated caseins) and multiplying by 100. The enzyme α-amino nitrogen figures were computed similarly.

§ The casein was heated by autoclaving for 4 minutes or 20 hours at 15 pounds.

‖ The acid hydrolysis average figure was computed from the liberation from the raw and 4 minute heated casein. Enzymatic liberation values for 20 hour heated casein were computed with the acid liberation figure for this casein sample instead of the average figure.

¶ Since alkaline hydrolysis was used, complete racemization was assumed and figures represent twice the actual amounts measured.

** Determined by the semimicro nitrous acid method of Van Slyke.

†† The figures represent the percentage of the total nitrogen released in the Van Slyke procedure.

‡‡ Acid hydrolysis totals are the sums of the percentages of the amino acids liberated. Enzyme hydrolysis totals were obtained by dividing the total amount of microbiologically available amino acids liberated enzymatically by the total amount liberated from the corresponding casein sample by acid hydrolysis (or alkaline hydrolysis) and multiplying by 100.
sis for tyrosine and tryptophan) was not affected significantly by autoclaving at 15 pounds pressure (121°) for 4 minutes or 20 hours. The cystine value was reduced to one-fourth that of the raw casein by autoclaving for 20 hours, but was unchanged by autoclaving for 4 minutes.

Liberation of Amino Acids by Digestive Enzymes—In Fig. 1 are shown typical hydrolysis curves of raw casein and casein autoclaved for 4 minutes and 20 hours at 15 pounds when subjected to successive digestion with pepsin, pancreas enzymes, and erepsin. Heated casein was digested more rapidly in the initial stages by pepsin; the extent of digestion was about the same at the end of 2 hours, regardless of the heat treatment. The rate of release of α-amino nitrogen during pancreatic and ereptic digestion of casein was slightly increased by autoclaving for 4 minutes and decreased by autoclaving for 20 hours. A disproportionately large reduction in pH in relation to the release of α-amino nitrogen occurred during the 1st hour of pancreatic digestion.

The interpretation of the data on the extent of amino acid liberation by enzymes when measured by microbiological procedures is complicated by...
the probable utilization of peptides by the microorganisms commonly used for the assay of amino acids. Peptides that have been investigated thus far show variable activity, ranging from 0 to 100 per cent when assayed for the amino acids which they contain (10–13). In view of this variation in response of microorganisms to peptides, the term “microbiologically available” amino acids will be used in the discussion of Table I. The above objection invalidates these values for other than gross comparative purposes.

**Pepsin Digestion**

The liberation of α-amino nitrogen and microbiologically available amino acids from casein by pepsin was unaffected by heat treatment. There was considerable variation in the extent of liberation of the individual amino acids; a relatively large percentage of the arginine and much lower percentages of glutamic acid, glycine, leucine, methionine, phenylalanine, threonine, and tryptophan were released. No significant quantities of the other amino acids were liberated. The percentage of arginine that became microbiologically available was about 8 times as great as the average1 percentage of all sixteen amino acids. Half of the α-amino nitrogen liberated (measured by the Van Slyke method) could be accounted for by microbiologically available amino acids.

**Pepsin Plus Pancreas Digestion**

The liberation of α-amino nitrogen and the “average” liberation of amino acids from casein by pepsin followed by pancreas were slightly increased by autoclaving for 4 minutes and decreased by autoclaving for 20 hours. All amino acids except proline were released by pancreas enzymes. The extent of liberation of aspartic acid, cystine, histidine, and isoleucine was less than 10 per cent, while the total amino acid liberation was approximately 20 per cent and was equal to the percentage release of α-amino nitrogen.

The percentage of the arginine which became microbiologically available was approximately 4 times as great as the average of the other amino acids. Hunter and Dauphinee (14) have also reported a rapid cleavage of this amino acid from casein and gelatin by trypsin. It is not known, however, whether arginine is liberated in the form of peptides having high activity for Lactobacillus delbrueckii or as free arginine.

In general the results obtained with pancreas enzymes agree with those of other workers. Abderhalden (15) found more rapid liberation of tyrosine than of glutamic acid from casein by pancreatin. Hunter (16) found that a proline fraction exists in casein which is comparatively resistant to tryptic

1 See Table I, foot-note ‡‡.
digestion. It is possible that the low values obtained in the present study with enzyme digests are due to the absence of proline-releasing enzymes in the preparations used.

Digestion with Pepsin, Pancreas, and Erepsin

The extent of liberation of microbiologically available amino acids from casein was determined after following the pepsin-pancreatic digests prepared in the above manner with digestion by erepsin for 2 hours and 5 days. The extent of liberation of aspartic acid, cystine, histidine, isoleucine, and proline after 2 hours was below 10 per cent, while the total liberation of amino acids was about 40 per cent. Arginine, methionine, and tryptophan were nearly entirely liberated. After 5 days, most amino acids were completely available to the microorganisms used for assay. The liberation of cystine, which had been low throughout the digestion, increased considerably after 5 days. The release of aspartic acid and proline remained low, i.e. 10 to 20 per cent, while the liberation of methionine was approximately 150 per cent. The latter result could indicate activity of peptides above that expected on the basis of methionine content, or destruction of methionine during acid hydrolysis. The values for acid hydrolysates reported here are lower than those obtained by many other workers, a result which supports the latter explanation. In a previous study, an average value of 2.69 ± 0.26 per cent in the dried, ash-free protein was reported, while six values cited from the literature averaged 2.85 ± 0.21. The value for raw casein reported in Table I is 2.33 corrected for moisture and ash.

The amino acid totals exceeded the α-amino nitrogen values after digestion by erepsin for 2 hours or for 5 days. The average peptide size at the end of 5 days was 2 amino acid residues.

It should be pointed out that the extent of liberation of glutamic acid from proteins by enzymes is not strictly comparable to that obtained on acid hydrolysates, since in enzyme hydrolysates any glutamine released would have been converted to pyrrolidonecarboxylic acid by the heating to inactivate the enzymes, and the subsequent sterilization of the assay tubes by autoclaving. According to Hamilton (17) glutamic acid is relatively stable to acid hydrolysis and to autoclaving, whereas glutamine is converted to glutamic acid when heated below pH 3 and to the inactive pyrrolidonecarboxylic acid during autoclaving at pH 6.5. Work in this laboratory has shown that sterilization of the medium by autoclaving for 10 minutes at 12 pounds pressure at neutral pH causes complete or nearly complete loss of activity of free glutamine for Lactobacillus arabinosus. It appears from Table I that glutamic acid was quantitatively liberated by enzymes in 5 days or less. This may be accounted for by assuming (a) deamidation of the glutamine during enzyme hydrolysis, (b) release of
glutamine in peptide combinations which are not cyclized by heat, or (c) release of peptides of glutamic acid possessing more activity than glutamic acid itself, thus compensating for the glutamine destroyed during autoclaving.

The liberation of aspartic acid, in contrast to glutamic acid, was low throughout the 5 day digestion period. Whereas glutamine is fully as active as glutamic acid for Lactobacillus arabinosus (18), asparagine is much less active than aspartic acid for Leuconostoc mesenteroides P-60 (19). The low values obtained may be due to the liberation in the form of asparagine or peptides.

Casein appears to be less affected by heat than soy bean protein. Very little increase in digestibility was noted after 4 minutes of autoclaving; some destructive effects occurred after 20 hours. In both soy bean and casein, lysine was among the amino acids whose rate of release by enzymes after heat treatment was affected most adversely.

**SUMMARY**

1. Autoclaving casein at 15 pounds pressure for 4 minutes had no effect on the amino acid composition as measured by microbiological determinations after acid hydrolysis. Autoclaving for 20 hours reduced the cystine content, but did not affect the amounts of other amino acids.

2. The rate of release of α-amino nitrogen and of microbiologically available amino acids during a 2 hour digestion of casein with a limited quantity of pepsin was unaffected by the heat treatments. The release of amino acids from the pepsin digests after treatment with desiccated pancreas and then with erepsin was higher in the casein autoclaved for 4 minutes and lower in casein autoclaved for 20 hours, compared to unheated casein.

3. The release of amino acids from raw casein by pepsin was 5 per cent with 10 per cent liberation of α-amino nitrogen. 40 per cent of the arginine became microbiologically available.

4. Pancreatic digestion for 2 hours released approximately 22 per cent of the amino acids and 19 per cent of the α-amino nitrogen and, after an additional 2 hour digestion with erepsin, 39 per cent of the amino acids was available to the lactic acid bacteria and 27 per cent of the α-amino nitrogen was released. Continued digestion with no additional enzymes for 5 days released 50 per cent of the α-amino nitrogen and 77 per cent of the amino acids in microbiologically available form. Only small amounts of aspartic acid and proline were liberated.

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