THE FORMATION OF AMMONIA FROM PROTEINS IN ALKALINE SOLUTION

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Considerable amounts of ammonia may be formed during the alkaline hydrolysis of proteins. Osborne, Leavenworth, and Brautlecht (1) were of the opinion that the amide and guanidine groups of the protein were the sole source of this ammonia. In the alkaline hydrolysis of wheat gliadin, however, Vickery (2) observed a slow formation of ammonia from unidentified sources. The significance of the observation was masked by the unusually large amount of amide nitrogen in this protein. In the report which follows, we present evidence that considerable amounts of ammonia may originate in groups other than amide and guanidine.

A part of this may plausibly be attributed to partial deamination of cystine (3, 4), histidine (5), and serine (6). The evidence indicates, however, that a notable part—and that liberated under quite mild conditions of hydrolysis—originates in some structural feature of the protein itself, rather than in any specific amino acid component. The proteins studied were crystalline preparations of egg albumin, edestin, and \( \beta \)-lactoglobulin.\(^2\)

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1 These investigators studied four proteins. They concluded that the ammonia liberated was approximately equal to the sum of the amide nitrogen and of half the arginine nitrogen. Under the conditions of hydrolysis which they employed, arginine actually yields only 35 to 42 percent of its nitrogen as ammonia. Moreover, their method of determining amide nitrogen would tend to yield high values. The origin of significant amounts of ammonia from unidentified sources is not excluded by their observations.

2 This is the name which has been suggested for the crystalline globulin isolated by Palmer (7) from the albumin fraction of cow's milk.
Ammonia Formation from Proteins

EXPERIMENTAL

Stock solutions of the proteins were prepared and their protein content calculated from determinations of dry weight (110°) and ash. Reaction mixtures were then prepared from measured amounts of these and of a standard solution of NaOH. Samples of each reaction mixture were sealed in a series of small Pyrex tubes which were then heated in a boiling water bath (100°) or a constant

Fig. 1. Ammonia formation from egg albumin. Curve 1, 4.3 N NaOH at 68°; Curve 2, 1.4 N NaOH at 68°; Curve 3, 0.28 N NaOH at 68°; Curve 4, 4.3 N NaOH at 35°; Curve 5, 1.4 N NaOH at 35°; Curve 6, 1.2 N NaOH at 25°.
temperature water bath (25°, 35°, or 68°). Tubes were removed at intervals, chilled in ice water, and the contents analyzed for ammonia by the method of Conway and Byrne (8).

The Conway and Byrne method requires a 2 to 3 hour distillation period at room temperature. During this time there is a slight liberation of ammonia from the amide groups. The short time observations at 25° and 35° are therefore of only qualitative significance. At higher temperatures the amide hydrolysis is substantially complete even at the shortest reaction times employed.

In a few cases urea was estimated after the ammonia determination by titrating the reaction mixture with H₃PO₄ to pH 7.2, digesting with urease, and determining the ammonia formed.

Ammonia Formation from Egg Albumin—Representative data on egg albumin are shown graphically in Figs. 1 and 2. Inspection of the curves reveals the following characteristics.

At 35° the curves show an initial rapid formation of ammonia,
Ammonia Formation from Proteins

followed by a slow increase which continues throughout the period studied. Extrapolation of the slow rise back to zero time yields a value for the labile ammonia of 0.70 milliequivalent per gm. This is consistent with the maximum attained by the 25° curve and is identical with the value for amide nitrogen reported by Shore, Wilson, and Stueck (9). Their value was obtained by extrapolation of the time curves of ammonia formation in acid hydrolysis. It is interesting to note that \(\text{M NaOH}\) hydrolyzes the amide groups at 35° at a rate comparable to that of \(\text{M HCl}\) at 100°.

Our results for the three proteins studied suggest that a simple and precise determination of amide nitrogen may be made in the following manner. Set up a series of Conway vessels containing a solution of the protein in \(\text{M NaOH}\). Allow the spontaneous distillation to proceed at 25-35°. At chosen periods varying from 12 to 60 hours take one of the vessels and titrate the ammonia which has distilled. If the values at the successive reaction times are in close agreement, the mean may be taken as the amide nitrogen. If a progressive increase with time is observed, a linear extrapolation to zero time will give a close approximation to the true amide nitrogen.

At 68° the curves rise very rapidly to a value somewhat above the amide and then assume a fairly constant slope. With increasing concentration of \(\text{NaOH}\) this slope increases slightly and is displaced upward on the ordinate. The hydrolysis of arginine can be expected to contribute to the ammonia formed under these conditions (10).

At 100° the curves in 1.4 and 0.28 \(\text{N NaOH}\) rapidly reach a maximum of about 2.0 milliequivalents per gm. and show a slow rise above this figure with increasing time. In 4.3 \(\text{N NaOH}\) a maximum of only 1.8 to 1.9 milliequivalents is attained, following which there is a decrease in the amount of ammonia present. Extrapolation of the decreasing slope to 2.0 milliequivalents at zero time is not unreasonable (Fig. 2).

The maximum value of 2.0 milliequivalents per gm. is further confirmed by an experiment in 2.3 \(\text{N Ba(OH)}_2\) at 100°, also shown in Fig. 2.

*Ammonia Formation from Edestin and \(\beta\)-Lactoglobulin*—More limited data have been obtained on the other two proteins, the graphs of which, in Figs. 3 and 4, show characteristics similar to
the curves for egg albumin. In Table I the values for the total alkali-labile ammonia as determined in 1.4 M NaOH at 100° and for the ammonia produced at 35° or 25° are compared with the analytical data on these proteins. The amide nitrogen estimated by extrapolation of the low temperature curves to zero time shows satisfactory agreement with the amide nitrogen determined by other methods. In Column 6 are the values for the ammonia in excess of the sum of the amide and one-half of the arginine nitrogen.

The slow increase above the plateau in the curves at 100° in M NaOH probably represents contributions from such amino acids as cystine, histidine, and serine. Any ammonia that these acids may yield to the unknown fraction is neglected in the analysis of the rate
FIG. 4. Ammonia formation from edestin in 1.4 n NaOH. Curve 1, 100°; Curve 2, 68°; Curve 3, 35°.

TABLE I
Ammonia Formation from Three Proteins in Alkaline Solution

In Columns 1 and 2 are given data from the literature or from Appendix II. In Column 3 are the values for amide groups obtained by extrapolation of the experiments at 25° or 35°. Column 4 is calculated from Columns 1 and 3. The values obtained are subtracted from the total ammonia at 100° (Column 5) to give the ammonia from unidentified sources in Column 6.

The results are expressed in mM per gm.

<table>
<thead>
<tr>
<th></th>
<th>Arginine (1)</th>
<th>Amide groups (2)</th>
<th>Amide groups, NaOH experiments (3)</th>
<th>Amide + arginine N (4)</th>
<th>Total alkali-labile NH₃ (5)</th>
<th>Unidentified NH₃ (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg albumin</td>
<td>0.32 (11)*</td>
<td>0.70 (9)*</td>
<td>0.70</td>
<td>1.34</td>
<td>2.03</td>
<td>0.69</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>0.16†</td>
<td>0.77‡</td>
<td>0.78</td>
<td>1.10</td>
<td>1.77</td>
<td>0.67</td>
</tr>
<tr>
<td>Edestin</td>
<td>0.96 (11)*</td>
<td>1.37‡</td>
<td>1.34</td>
<td>3.25</td>
<td>3.71</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Bibliographic reference number.
† Vickery, H. B., personal communication.
‡ See Appendix II.
of ammonia formation which follows. An attempt to estimate the magnitude of such contribution is made later.

*Rate of Formation of Unidentified Ammonia*—An analysis of the rates of ammonia formation under various conditions of temperature and hydroxyl ion concentration suggests that two types of reaction are involved. Let us, first, correct the experimental curves for the contribution of amide and arginine groups. The total of the former can merely be subtracted at all points on the curves for 68° and 100°. Corrections for the ammonia formed from arginine were made on the basis outlined in Appendix I.³

³ The chief primary products of the hydrolysis of arginine are urea and ornithine, most of the ammonia being formed secondarily from the urea. There should thus be an accumulation of urea in the reaction mixtures during the early stages of the hydrolysis. This was demonstrated by analysis in which urease was employed. The amounts of urea found agreed well with those calculated from the equation in Appendix I, assuming 0.32 mm of arginine per gm. of egg albumin.
Ammonia Formation from Proteins

The application of the corrections to the ammonia data obtained at 68° is shown in Figs. 5 and 6. The experimental values for the ammonia formed less the amide ammonia were plotted, and values were interpolated at intervals from a smooth curve drawn through the data. The arginine corrections calculated from the equation in Appendix I were subtracted and the values obtained were plotted as the discrete points on the lower curves of the graphs.

Fig. 6. Rate of formation of unidentified ammonia from β-lactoglobulin and edestin. The curves are theoretical plots obtained from the rate constants in Table II. Discrete points are the corrected values for the unidentified ammonia obtained as explained in the text. Curve 1, β-lactoglobulin in 1.4 N NaOH at 100°; Curve 2, edestin in 1.4 N NaOH at 100°; Curve 3, β-lactoglobulin in 1.4 N NaOH at 68°; Curve 4, edestin in 1.4 N NaOH at 68°.

Examination of the trend of these points shows a comparatively rapid initial reaction followed by a slower rise which is almost linear with respect to time. A curve of this type can be most simply accounted for as the sum of two independent first order reactions, having rates differing by a factor of about 10. The data have been treated on this assumption. Extrapolation of the linear portion of the curves to zero time should give an approximate value for the extent of the more rapid process. This value will be re-
ferred to as $A_1$. The extent of the slower process ($A_2$) will be taken as equal to the total unknown ammonia minus $A_1$.

First order constants, $k_1$ and $k_2$, for the two reactions and values for $A_1$ and $A_2$ have been derived to give the best fit with the data and are shown in Table II. The smooth curves in Figs. 5 and 6 are theoretical plots calculated from these constants.

This method of treatment was extended to the data at 100°. There are insufficient points on the rising portion of the curve to define the trend very accurately. The final corrected values for the ammonia are shown as the discrete points in the upper curves of the graphs (Figs. 5 and 6). The smooth curves through the data

| Table II |
| Values for Extent of Two Processes, $A_1$ and $A_2$, and for Respective First Order Rate Constants Used in Plotting Theoretical Curves in Figs. 5 and 6 |

The rate constants are calculated in terms of common logs and reciprocal hours.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$k_1$</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg albumin</td>
<td>0.29</td>
<td>0.40</td>
<td>0.06</td>
<td>0.004</td>
</tr>
<tr>
<td>68°, 4.3 N NaOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68°, 1.4 °° °°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100°, 1.4 °° °°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>0.23</td>
<td>0.44</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>68°, 1.4 N NaOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100°, 1.4 °° °°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edestin</td>
<td>0.17</td>
<td>0.29</td>
<td>0.05</td>
<td>0.005</td>
</tr>
<tr>
<td>68°, 1.4 N NaOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100°, 1.4 °° °°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

are theoretical plots obtained with the $A_1$ and $A_2$ values for each of the proteins derived from the data for 68° and with the average values of $k_1$ and $k_2$ which give the best fit with the data for all three proteins. The values used for $A$ and $k$ are shown in Table II. The derived curves adequately describe the experimental data. It should be noted that the errors in the case of edestin are relatively larger than for the other proteins because the amounts of amide and arginine present make the corrections large as compared with the unknown ammonia.

In fitting any individual curve it is not surprising that satisfactory values for $A$ and $k$ can be found, since variation can be
made in any of the four parameters which describe the curve
\((A_1, A_2, k_1, k_2)\). However, some confidence can be placed in this
method of treating the data in view of the fact that constants of
the same order of magnitude describe the curves for all of the
proteins at the same NaOH concentration and that the same values
for \(A_1\) and \(A_2\) give satisfactory results for egg albumin at different
temperatures and NaOH concentrations.

Reaction \(A_1\) can be followed to completion at 68° and the agree-
ment is good enough to place some reliance in the statement that
this is a first order reaction which proceeds to a definite maximum
independently of the ammonia formation from other sources. It
is quite possible that \(A_2\) is a composite of several reactions, since
it cannot be followed to completion under conditions which enable
it to be considered separately from \(A_1\).

Source of Unidentified Ammonia—If the ammonia is formed as
a result of the decomposition of some specific amino acid, it should
be possible to obtain it in the same quantity by acid hydrolysis of
a protein followed by treatment of the hydrolysate with alkali
under the conditions which defined the maximum ammonia pro-
duction in the previous experiments. Data are given below on
this type of experiment on egg albumin.

The maximum amount of ammonia obtained in excess of amide
and arginine ammonia was only about 0.15 milliequivalent per gm.
after 20 hours at 100° in m NaOH. Under these conditions the
processes \(A_1\) and \(A_2\) should be substantially complete. This 0.15
milliequivalent per gm. is probably included in \(A_2\) and may be
composed of contributions from histidine, cystine, serine, and possi-
ably other amino acids. No trace of the remaining 0.25 milli-
equivalent of \(A_2\) or of the more labile 0.29 milliequivalent of \(A_1\)
could be found in these experiments.

The estimate of the unidentified ammonia should thus be reduced
to 0.54 milliequivalent per gm. for egg albumin. This represents
about 5 per cent of the total nitrogen in the protein. No data of
this type were obtained on the other proteins studied, but the am-
onia that cystine, histidine, and serine will yield cannot be much
greater than for egg albumin. None of the proteins used contains
much cystine, and the ammonia from histidine is small in any case.
There are no satisfactory analytical data on the serine content of
these proteins. However, the amounts which have been isolated
from most proteins are small, and under the conditions of these experiments it was found that the ammonia obtained from serine is equal to about 30 per cent of its nitrogen.

It can be concluded that the 0.54 milliequivalent of ammonia from egg albumin is not a result of the decomposition of amino acids as such. However, it probably comes chiefly from amino groups, since it is difficult to account for this amount of nitrogen from any other source. Hence, this ammonia must be referable to some structural feature of the protein itself, perhaps to certain peptide bonds which yield amino acids on acid hydrolysis and ammonia on treatment with alkali. There has appeared, after the completion of the manuscript of this paper, a note by Nicolet and Shinn (12) on the lability of serine and threonine when in peptide combination. It is probable that part of the unidentified ammonia arises from these sources. This question will be further investigated.

Experiment I—A sample of egg albumin was hydrolyzed for 24 hours with 20 per cent H₂SO₄ under a reflux. Aliquots of this hydrolysate were sealed in Pyrex tubes with a sufficient excess of NaOH to give a final concentration of 1.0 N. The tubes were heated in a boiling water bath and ammonia was determined on samples removed at intervals. The ammonia present in an unheated sample amounted to 0.813 milliequivalent per gm. or a little in excess of the amide ammonia. This was subtracted from the ammonia found in the heated samples. The results are summarized in Table III.

Experiment II—The basic amino acids were removed from a H₂SO₄ hydrolysate with phosphotungstic acid. The excess phosphotungstic acid was removed with Ba(OH)₂ and the aliquots of the filtrate were sealed in tubes with excess NaOH and were treated as above. The ammonia present in an unheated sample was 0.038 milliequivalent per gm. This has been subtracted from the results in Table III.

Appendix I. Derivation of Arginine Corrections—Corrections were made above for the amount of ammonia formed from arginine under various conditions. The equations from which these corrections were calculated were obtained in the following manner from a study of the kinetics of hydrolysis of arginine and urea (10). *

* References to equation numbers are from this paper.
Ammonia Formation from Proteins

The urea formation during the course of arginine hydrolysis can be described by combining Equations 3, 20, and 22. Since only alkaline hydrolyses are being considered, the $k_{xy}$ term in Equation 3 can be neglected. The equation

$$\frac{du}{dt} = \frac{k}{n} x - k_1 n$$

**Table III**

Ammonia Obtained by Treating $H_2SO_4$ Hydrolysate of Egg Albumin with Alkali

Experiment I shows the ammonia formed in excess of that present in the acid hydrolysate. Two other similar experiments showed 0.803 and 0.805 milliequivalents per gm. in 26 and 22 hours respectively. Experiment II shows the ammonia formed after removing the arginine. Another similar experiment yielded 0.18 milliequivalent per gm. in 21 hours.

After allowance was made for the arginine present in Experiment I, an average of 0.15 milliequivalent per gm. of ammonia in excess of the amide and arginine ammonia was titrated in 20 to 25 hours.

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>Ammonia</th>
<th>Experiment II</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hrs.)</td>
<td>m.eq. per gm.</td>
<td>Time (hrs.)</td>
<td>m.eq. per gm.</td>
</tr>
<tr>
<td>3.1</td>
<td>0.313</td>
<td>19.4</td>
<td>0.148</td>
</tr>
<tr>
<td>15.9</td>
<td>0.461</td>
<td>98</td>
<td>0.177</td>
</tr>
<tr>
<td>23.7</td>
<td>0.759</td>
<td>162</td>
<td>0.191</td>
</tr>
<tr>
<td>40.5</td>
<td>0.794</td>
<td>282</td>
<td>0.214</td>
</tr>
<tr>
<td>63.6</td>
<td>0.806</td>
<td></td>
<td></td>
</tr>
<tr>
<td>111.5</td>
<td>0.800</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

was obtained. This was combined with Equation 23 and integrated to give

$$u = \frac{k}{a} \left( e^{-kt} - e^{-kt_1} \right)$$

To obtain the corrections for the ammonia formed from arginine, Equations 4, 5, 25, and the above equation were combined, integrated, and solved for total ammonia ($\Sigma NH_3$). The $k_{xy}$ term in Equations 4 and 5 was neglected as before. The following equation was obtained.

$$\frac{\Sigma NH_3}{a} = 2 + Ae^{-kt} + Be^{-kt_1} + Ce^{-kt_3} + De^{-kt_4}$$
where

\[ A = \frac{k_1}{n(k_1 - k)} \frac{2k_3 - k}{k_3 - k} - \frac{1}{p} \left( \frac{2k_4 - k}{k_4 - k} \right) \]

\[ B = \frac{k}{n(k_1 - k)} \frac{2k_3 - k_1}{k_3 - k_1} \]

\[ C = -\frac{k_1 k}{n(k_3 - k_1)(k_3 - k)} \]

\[ D = \frac{k}{p(k_4 - k)} \]

\[ p = m + 1 \]

\[ n = \frac{m + 1}{m} \]

\[ a = \text{initial concentration of arginine} \]

The constants have the same significance as in the previous paper (10).

The values for the constants required in the calculations are presented in Table IV. At 100° they were obtained by graphical interpolation from the data previously recorded. No systematic determinations on arginine hydrolysis were made at 100°. A single determination in NaOH indicated that the value of \( k \) must be about 3.5. The reaction is thus so rapid that errors in \( k \) even of the order of 50 per cent will have relatively little effect on the calculations.

At 66° the values of the constants were obtained in the same way and were then corrected to 68° on the basis of approximate critical increments calculated between 66–100° for \( k_1, k_3, \) and \( k_4 \) and between 35–66° for \( k \). Since the corrections are small, errors in the critical increments used are of little significance. It was assumed that \( m \) does not change with the temperature, although there is some indication that it is higher at 35° than at 66°. The calculations are here applied to proteins on the assumption that the rates of the several processes involved will not be affected by the incorporation of arginine in a peptide chain.

The only other simplification made was in neglecting reactions of OCN− with amino acids, such as were described by Boon and Robson (13). By analogy with the formation of urca from NH₄⁺
Ammonia Formation from Proteins

and OCN⁻ these would not be expected to occur in strongly alkaline solution.

\( \Sigma \text{NH}_3/a \) was calculated for various time intervals by means of these constants. From these values, \( \Sigma \text{NH}_3 \) was obtained, as-

**Table IV**

<table>
<thead>
<tr>
<th>Values for Velocity Constants Used in Calculating Ammonia Formed from Arginine during Hydrolysis of Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m = 5.5 ).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature, °C.</th>
<th>100</th>
<th>68</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH, w.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k )</td>
<td>3.5</td>
<td>0.240</td>
<td>0.262</td>
</tr>
<tr>
<td>( k_1 )</td>
<td>0.234</td>
<td>0.00815</td>
<td>0.0525</td>
</tr>
<tr>
<td>( k_2 )</td>
<td>0.170</td>
<td>0.0066</td>
<td>0.0066</td>
</tr>
<tr>
<td>( k_4 )</td>
<td>0.0258</td>
<td>0.0013</td>
<td>0.0047</td>
</tr>
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</table>

**Table V**

<table>
<thead>
<tr>
<th>Ammonia Formation during Acid Hydrolysis of ( \beta )-Lactoglobulin and Edestin</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta )-Lactoglobulin</td>
</tr>
<tr>
<td>0.25 M HCl at 85°</td>
</tr>
<tr>
<td>Time (hrs.)</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.75</td>
</tr>
<tr>
<td>1.0</td>
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<tr>
<td>24.0</td>
</tr>
<tr>
<td>47.0</td>
</tr>
<tr>
<td>49.0</td>
</tr>
</tbody>
</table>

assuming \( a \) to be 0.32 milliequivalent per gm. for egg albumin, 0.96 for edestin, and 0.16 for \( \beta \)-lactoglobulin (see Table 1).

**Appendix II. Amide Nitrogen of \( \beta \)-Lactoglobulin and Edestin—**
The methods used were those described by Shore, Wilson, and Stueck (9). These involve measurements of the time course of
ammonia production during acid hydrolysis. A summary of the results is given in Table V. The results with $\beta$-lactoglobulin show a rapid deamidation followed by a slow increase in ammonia formation at longer time intervals. In M HCl at 100° a linear extrapolation of this rise to zero time yields a value of 0.77 mM per gm. for the amide nitrogen of $\beta$-lactoglobulin. This value is confirmed by the experiment at the lower temperature. The secondary rise in the case of edestin is negligible. The results in several experiments, two of which are quoted here, are consistent with a value of 1.37 mM of amide per gm., substantially confirming values recorded in the literature (14).

SUMMARY

Data are presented on the rate and extent of the formation of ammonia at 25°, 35°, 68°, and 100° in various concentrations of NaOH from egg albumin, $\beta$-lactoglobulin, and edestin.

2. In all three proteins the amount of ammonia formed exceeded that due to the known sources which are amide groups and arginine.

3. The rate of formation of this excess ammonia can be most simply accounted for by assuming that it is produced from two independent sources by first order reactions.

4. Only a fraction of the unknown ammonia can be obtained by treating an acid hydrolysate of egg albumin with alkali. It is concluded that the ammonia is not formed by the decomposition of any amino acid as such, but it is referable to alkali-labile groups that exist in the protein.

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