THE USE OF OPTICAL ROTATION IN THE STUDY OF PROTEIN HYDROLYSIS

By THEODORE WINNICK AND DAVID M. GREENBERG
(From the Division of Biochemistry, University of California Medical School, Berkeley)

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Change in optical rotation was first used as a measure of protein digestion by Schütz in 1885 (1). This investigator found that the amount of ovalbumin digested by pepsin, as measured by the optical rotatory power of the non-protein products, was proportional to the square root of the quantity of enzyme used. This relationship is commonly known as the Schütz or Schütz-Borissov rule. Schütz boiled the digestion mixture with ferric chloride to precipitate any protein residue, and found that the clear filtrate, which contained the hydrolytic products, was highly levorotatory.

Despite the simplicity and accuracy offered by optical rotation measurements, very little use has been made of this method for following protein hydrolysis. Gore (2) showed that when gelatin was digested by papain there was no longer a large change in specific rotation of the protein with temperature and that there was a regular relation between the activity of the enzyme used and the change in specific rotation of the substrate. Quick (3) utilized these facts to develop an empirical procedure for measuring the quantity of proteolytic enzyme present in various malts by the rate at which these enzymes decreased the specific rotation of gelatin solution.

The cleavage of synthetic peptides by trypsin and erepsin has been followed by change in optical rotation (4), but the accuracy was limited by the fact that, in most cases, the observed changes in rotation were rather small.

In the present study, optical rotation is shown to be a criterion of both the amounts and kinds of degradation products produced by either enzymic or acid hydrolysis of proteins. Only "com-
plete" proteins, precipitable by trichloroacetic acid, are used as substrates, so that, following enzymic digestion, measurements can be made exclusively upon the clear trichloroacetic acid filtrates, which contain only non-protein fractions, and not upon whole digestion mixtures.

Since total nitrogen analyses were always carried out on the non-protein fractions, the optical rotatory power of these solutions is expressed by an arbitrary term, which we define by analogy with specific rotation, as

\[ \alpha N^\circ_D = \frac{100 \times \text{observed rotation, degrees}}{\text{length of tube, dm.} \times \text{gm. total N per 100 ml.}} \]

\( \alpha N^\circ_D \) can be converted to ordinary specific rotation, \( \alpha^\circ \) (or vice versa), by multiplying the former quantity (or dividing the latter) by the fractional percentage of N which the optically active substances in solution contain. The method of obtaining this percentage will be indicated subsequently for specific cases.

When the hydrolysis is with mineral acid, samples of the protein hydrolysate are decolorized with carbon and filtered, and analyzed in the same manner as the above trichloroacetic acid filtrates.

It will be shown that \( \alpha N^\circ_D \) is related to the percentage of free amino groups in the degradation products of casein, so that the former quantity is a measure of the degree of splitting of the peptide bonds. This fact is utilized in the comparison of the action of different proteases on the same protein substrate.

An indication that enzymic hydrolysis proceeds by a somewhat different path than that followed during acid hydrolysis is obtained by correlating the changes in \( \alpha N^\circ_D \) of the different non-protein fractions with corresponding changes in the ratio of free amino N to total N, \(-\text{NH}_2:\text{N.}\)

EXPERIMENTAL

**Proteolytic Enzymes** The preparation of the plant proteases, papain, bromelin, asclepain, and solanain, in partially purified form has been described by us (5, 6). The solutions of these enzymes used in subsequent measurements contained 5 mg. of enzyme per ml. of 0.05 m NaCN, adjusted to pH 7. The animal proteases used were Armour's trypsin and Merck's pancreatin. These preparations are, of course, crude mixtures of enzymes, but
were quite satisfactory for the present purpose. Filtered, aqueous extracts were used for the digestions.

Sheep erepsin was salted-out of an aqueous extract of ground duodenal tissue by two-thirds saturation with solid \((\text{NH}_4)_2\text{SO}_4\). The precipitate was dialyzed, and an aqueous extract of this material was used. The hog erepsin used in certain experiments was a 30 per cent glycerol extract of hog intestinal mucosa, and was further activated by the addition of dilute \(\text{Mn}^{++}\). The yeast polypeptidase was a concentrate prepared by Dr. M. J. Johnson. It was further activated by dilute \(\text{Zn}^{++}\).

Proteins—The proteins used were ovalbumin, prepared by Sørensen’s method (7), twice recrystallized, and then dialyzed; Van Slyke casein (8); and edestin prepared according to Osborne (9).

Methods

For the enzymic digestions, 2 ml. portions of enzyme solution were added to 15 ml. volumes of protein solution, buffered at pH 7.5 to 8.0. After a specified time of digestion, usually at 37\(^\circ\), 5 ml. of 20 per cent trichloroacetic acid solution were added to each digest. The mixtures were filtered, and their optical rotations were measured in a 2 dm. polarimeter tube with a Schmidt and Haensch polarimeter. A General Electric sodium vapor lamp was the source of illumination. The temperature of the measurements was 25\(^\circ\), except when otherwise specified. Several readings were usually made on each solution, and the average value taken.

Following the rotation measurements, the same solutions were analyzed for total N by the micro-Kjeldahl method and for amino N by the micromethod of Van Slyke. In the latter method, the \(\text{HNO}_2\) was allowed to react for 5 minutes at about 20\(^\circ\).

In some instances the tyrosine color value was also measured, according to Anson’s method (10). In these latter measurements the intensity of the blue color produced with the phenol reagent was determined with an Evelyn photoelectric colorimeter. This

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1 We are greatly indebted to Dr. J. Berger and Dr. M. J. Johnson of the Department of Biochemistry, University of Wisconsin, for supplying us with this preparation.
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instrument was calibrated against a series of standard tyrosine solutions.

In certain cases in which the substrate was subjected to the action of two or three different enzyme preparations, the digestion mixture was briefly heated at 95–100° to inactivate each enzyme prior to the addition of the next one.

In all cases, control measurements were carried out in which the trichloroacetic acid solution was first added to the protein substrates and the enzyme solutions added afterwards. These corrections, which were subtracted from the experimental values, usually amounted to from 0° to 0.10° of optical rotation per 2 dm., 0.1 to 0.2 mg. of total N per ml., 0.05 to 0.1 mg. of amino N per ml., and 0 to 0.005 \times 10^{-2} \text{ milliequivalent of tyrosine color per ml.} for the digestions with the plant proteases. The corrections were about twice as large when pancreatin, trypsin, or the peptidase preparations were used.

The optical rotation readings made on each solution usually agreed to within 0.02° to 0.05°, so that, for an observed rotation of 2° to 5°, the relative error was about 1 per cent.

Effect of Varying Temperature and pH on Optical Rotation of Non-Protein Fraction—Measurements at different temperatures were made on the trichloroacetic acid filtrate from a papain digest of casein, a polarimeter tube with an outer metal jacket for water circulation and temperature control being used for this purpose. It was found that, between 16.6–37.5°, the greatest range in the observed rotations was from −3.04° to −3.12°, a variation corresponding to approximately twice the limit of the experimental error. Accordingly, it was not necessary to specify accurately the temperature at which all subsequently recorded measurements were made, and 25° (the usual temperature of the polarimeter room) is taken as the standard temperature.

In order to determine the effect of varying pH on optical rotation, 10 ml. portions of the trichloroacetic acid filtrate of a tryptic digest of casein were adjusted to different pH values with acid or alkali and made up to a 25 ml. volume with water. Between pH 1 and 7, the observed rotations increased gradually from −3.56° to −3.87° and then gradually fell to −3.76° at pH 10. In view of the great variation of rotation with pH in the case of many proteins and of all amino acids (11), this relative constancy in the
rotation values of the tryptic digestion products is surprising, and cannot be explained by us. When the final concentration of trichloroacetic acid was varied between 2 and 7.5 per cent, there was no appreciable effect produced on the optical rotation.

Optical Rotation Measurements Following Enzymic Digestion in Urea Solution—The digestion of most denatured proteins in concentrated urea solutions can be followed by optical rotation changes as readily as in the case of aqueous solutions, except that (owing to the presence of urea) amino and Kjeldahl N determinations cannot be made on the filtrates. Hemoglobin is not a suitable protein for these measurements, since, upon prolonged digestion, some of the red color due to heme is carried over into the trichloroacetic acid filtrate, and interferes with the optical rotation readings.

Concentrated urea is known to increase the specific rotation of ovalbumin (12). This was likewise observed to be true for casein and its intermediate digestion products. Thus we found \( \alpha^2_{25} = -144^\circ \) for casein in 6.6 \( \text{M} \) urea at pH 7.5. This figure is about 45 per cent higher than the value \( \alpha^2_{25} = -100^\circ \), reported by Almquist and Greenberg (13) for aqueous casein solutions at the same pH.

As an example of the measurement of the rotation of proteolytic digestion products in urea, 12 ml. of 3.12 per cent casein in 6.6 \( \text{M} \) urea\(^2\) were digested by 2 ml. of papain solution for 20 hours at 37\(^\circ\). 5 ml. of 20 per cent trichloroacetic acid were then added, and the resulting very slight precipitate filtered out. The filtrate, which contained all of the original casein N, and which was in 4 \( \text{M} \) urea, gave a value of \( \alpha_{N^2} = -655^\circ \). This value is about 10 per cent higher than that subsequently reported for aqueous casein digests.

Comparison of Optical Rotation with Other Methods for Measuring Rate of Enzymic Proteolysis—Fig. 1 illustrates the use of four independent methods for measuring the rate of digestion of casein by papain. The curves are all very much alike and all level out after about 2 hours. This is the time which was required for the complete conversion of the original casein to the non-protein form. This latter fact was ascertained by comparing the concentration of total N in the trichloroacetic acid filtrates with that in the ini-

\(^2\) The percentage of casein in the substrate was calculated from the total N content, determined prior to the addition of urea.
tial substrate, after correction for the dilution of the filtrates. Also, after about 2 hours, there was no appreciable precipitate when the trichloroacetic acid was added to the digestion mixture.

If the final tyrosine color value of $0.90 \times 10^{-2}$ milliequivalent per ml. is used to calculate the percentage of tyrosine in the original casein, a value of 7.1 per cent is obtained upon correction for the dilution with enzyme and trichloroacetic acid solutions.

This is in fair agreement with the value of 6.55 per cent found by Folin and Ciocalteau (14), considering that the former figure is uncorrected for tryptophane, which is present to about 2 per cent but which gives a much less intense color with the phenol reagent. All other proteases which were tested on casein produced essentially the same final tyrosine color value in the digestion products; namely, that corresponding to 6.8 to 7.5 per cent tyrosine (plus tryptophane) in the original casein.
Correlation of $\alpha N^25$ and $-\text{NH}_2: N$ with Time and Degree of Hydrolysis—Curves 1 and 2 of Fig. 2 show that, when casein is hydrolyzed by papain at 40°, the values of $\alpha N^25$ and $-\text{NH}_2: N$ in the non-protein filtrate do not change greatly throughout the whole time of the digestion. The final value, $\alpha N^25 = -600^\circ$, of the digestion products is only about 15 per cent lower than that of the original casein, $\alpha N^25 = -710^\circ$. Digestion with papain at a higher temperature, 60°, yielded essentially the same results.

Fig. 2. $\alpha N^25$ and $-\text{NH}_2: N$ as functions of the time of hydrolysis of casein. Hydrolysis with papain, Curve 1, $\alpha N^25$ versus time in minutes; Curve 2, $-\text{NH}_2: N$ versus time in minutes. These curves were calculated from the data of Curves 1, 2, and 4 of Fig. 1. Hydrolysis with HCl, Curve 3, $\alpha N^25$ versus time in minutes $\div 10$; Curve 4, $-\text{NH}_2: N$ versus time in minutes $\div 10$. These curves were obtained by hydrolyzing 12 gm. of casein with 300 ml. of 1.5 HCl, and analyzing aliquots, removed after different times, for optical rotation, amino N, and total N.

The final value, $-\text{NH}_2: N = 0.175$ (Curve 2), compared to the ratio 0.73, obtained by acid hydrolysis (Curve 4), indicates that about 24 per cent of all the peptide bonds are split by the papain.

3 This value, determined at pH 3.0, corresponds to $\alpha^25 = -111^\circ$, assuming 15.6 per cent N in the casein.

4 This value is about equal to the ratio 0.724 found by Hunter and Smith (15), and slightly higher than the ratio 0.70 reported by Dunn (16). Our amino N values may include a small percentage of ammonia N, since the
It is noteworthy that \( \alpha N_{D}^{25} \) and \(-\text{NH}_2\text{N} \) (for enzymic hydrolysis) become constant after about 2 hours. As previously mentioned, this is also the time required for all of the original casein to be converted to the non-protein form. Evidently longer times of digestion with the papain caused no further degradation of the non-protein substances.

With acid hydrolysis, the rotation is seen to change from the initial value for casein, \( \alpha N_{D}^{5} = -710^\circ \), to \( \alpha N_{D}^{4} = +88^\circ \) for the final hydrolysate; i.e., the amino acid mixture, after about 2000 minutes or 33 hours. With 13.7 per cent as the N content of this mixture (on a dry basis), \( \alpha N_{D}^{25} = +88^\circ \) corresponds to \( \alpha N_{D}^{20} = +12.0^\circ \). It is interesting that this last value agrees surprisingly well with the value, \( \alpha N_{D}^{20} = +10.8^\circ \), for the complete amino acid mixture (excluding tryptophane), calculated solely from a consideration of the optical form of the individual amino acids of casein (19), their specific rotations in HCl solution (11), and the percentage to which each is present in the protein (18). This agreement suggests that there is no extensive racemization of the amino acids during the hydrolysis of a protein with dilute acid.

Comparison of Paths of Acid and Enzymic Hydrolysis of Casein—The comparison is shown in Fig. 3. Curve A, which represents acid hydrolysis, is obtained from Curves 3 and 4 of Fig. 2 by ammonia due to amide N was not removed from the trichloroacetic acid filtrates. About 10 per cent of the total N of casein is amide N (16), and Van Slyke (17) has shown that a third of the NH\(_3\) of 0.1 N \((\text{NH}_4)_2\text{SO}_4\) reacts with HNO\(_3\) in 5 minutes at 24\(^\circ\). This last fact was confirmed by us. We did not determine whether papain liberates NH\(_3\) from casein, but it appears likely that this occurs, since tryptic hydrolysis liberates a fourth of the amide N of casein (15), and papain is known to split hippurylamide.

\( \text{NH}_2\text{N} \) reappears only after the first 30 minutes of boiling with HCl, the casein hydrolysate gives only a very slight precipitate with trichloroacetic acid, and after an hour none at all. This indicates that the protein is very rapidly degraded to the non-protein stage.

From the amino acid composition of casein (18), the average weight of the amino acid residues was calculated to be 123. Tryptophane, which is destroyed by acid hydrolysis, is not included in this calculation. Assuming the addition of 18 gm. of water during complete hydrolysis, the N content drops from 15.6 to 123/141 \(\times\) 15.6 = 13.8 per cent. Essentially the same value, 13.8 per cent, was calculated by considering the percentage of N in each amino acid and the percentage to which each (excluding tryptophane) is present in the casein.
plotting the $\alpha N^\circ_{28}$ values against the corresponding values of the $-\text{NH}_2:N$ ratios.

![Graph](image)

**Fig. 3.** $\alpha N^\circ_{28}$ as a function of $-\text{NH}_2:N$ in acid and in enzymic hydrolysis of casein. The points of Curve B represent digestions of 15 ml. portions of 4.0 per cent casein at 37° by 2 ml. portions of different enzyme solutions, in the order, and for the time specified below: (1) solanain, 20 hours; (2) papain, 20 hours; (3) bromelin, 20 hours; (4) asclepain, 20 hours; (5) papain and bromelin (acting simultaneously), 20 hours; (6) papain + solanain (acting simultaneously), 20 hours; (7) pancreatin, 18 hours; (8) papain, 18 hours, + pancreatin, 20 hours; (9) papain, 18 hours, + trypsin, 20 hours; (10) trypsin, 18 hours; (11) trypsin, 20 hours, + papain, 20 hours; (12) trypsin, 20 hours, + hog erepsin, 3 hours; (13) trypsin, 20 hours, + sheep erepsin, 18 hours; (14) trypsin, 20 hours, + hog erepsin, 9 hours; (15) trypsin, 20 hours, + yeast polypeptidase, 3 hours; (16) trypsin, 20 hours, + yeast polypeptidase, 26 hours; (17) trypsin, 20 hours, + hog erepsin, 9 hours, + yeast polypeptidase, 17 hours; (18) trypsin, 20 hours, + yeast polypeptidase, 26 hours, + papain, 2 hours (at 60°). 0.5 ml. volumes of yeast polypeptidase solution (instead of 2 ml.) were used for the digestions.

Curve B is the composite result of a number of separate digestion experiments made with different enzymes and enzyme combinations on the same casein substrate. The conversion of casein
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to non-protein material was 95 to 100 per cent complete in all these cases. The papainases, papain, bromelin, and asclepain, are seen to yield almost identical values (points 2 to 4 on Curve B), so that their mechanisms of action on casein are very likely the same. In agreement with this view is the observation that the combination of papain and bromelin (point 5) has no different effect than has either protease acting alone.

The non-protein product produced by solanain (point 1) appears to have approximately the same αN_d^{25} value, −580°, as in digestions with the above papainases, but the ratio −NH_2:N = 0.155 is slightly lower, indicating that this enzyme split only about 20 per cent of the peptide bonds of casein, whereas the papainases split about 25 per cent of the bonds. It is interesting that the behavior of solanain in the present experiments is so similar to that of the papainases, since it is not a member of this group of proteases (6). Point 6 shows that solanain is not able to further the degree of hydrolysis when acting in conjunction with papain.

Pancreatin (point 7) and crude trypsin (point 10) yield products which have lower αN_d^{25} values, and −NH_2:N ratios of about 0.35 to 0.38. These last values indicate that approximately 50 per cent of the peptide bonds of casein are broken, and compare very well with the value of 0.374, reported by Hunter and Smith (15), for a 24 hour digestion of casein with crude trypsin. These workers found the initial rate of splitting to be very rapid, but were able to obtain much higher amino N values by prolonging the digestion over many days. For example, Dauphinee and Hunter (20) obtained the ratio −NH_2:N = 0.466 for a 14 day digestion of casein with trypsin. In our experiments, digestions with different enzymes were not prolonged for more than a single day, and the amount of enzyme used was small in relation to the quantity of substrate.

Points 8 and 9 suggest that when papain acts on casein prior to the pancreatic proteases, the net hydrolysis is the same as when the latter enzymes act alone on casein. However, point 11 indicates that when papain acts on the tryptic digest, there is a certain amount of further hydrolysis.

Points 12 to 14 indicate the very similar action of sheep and hog erepsin on tryptic digests of casein. These peptidase mixtures,
like the plant proteases, complete their action in a short time, and yield no further hydrolysis over longer periods of time.

Yeast polypeptidase, acting subsequent to trypsin (points 15 and 16), and in combination with erepsin (point 17) and papain (point 18), yields a still higher degree of hydrolysis.

In view of the fact that optical rotation is a function of molecular structure, the spread between Curves A and B suggests that the proteolytic hydrolysis of casein proceeds through a somewhat different set of intermediate substances than with acid hydrolysis. It is significant that the solution corresponding to point 18 of Curve B gave a positive (pink) biuret test, although the ratio $\text{NH}_2:N = 0.62$ indicates that about 85 per cent of all the original peptide $N$ was in the free amino form. Apparently some tripeptides, and probably even higher polypeptides, were still present in the solution. However, a point on Curve A having approximately the same $\text{NH}_2:N$ ratio, 0.635, but a very different $\alpha_{N_{25}}$ gave a negative biuret reaction. This indicates that there were no molecules larger than dipeptides in the latter solution.

These observations confirm the above view that the enzymic and acid hydrolysis of casein follows different paths. Presumably Curve B should meet the lower tip of Curve A if the same final products (simple amino acids only) are obtained by either method.

Application of Optical Rotation Method to Ovalbumin and Edestin

—The results in Table I show that ovalbumin and edestin resemble casein in that the initial $\alpha_{N_{25}}$ and $\text{NH}_2:N$ values of the non-protein products are not greatly modified by prolonged digestion with papain.

It is interesting that the digestion products of ovalbumin have higher leverotation values ($\alpha_{N_{25}} = -415^\circ$ to $-360^\circ$) than the original native protein. Almquist and Greenberg (21) give $\alpha_{N_{22}} = -35.1^\circ$ for native ovalbumin at pH 1.72. This rotation corresponds to $\alpha_{N_{22}} = -225^\circ$, assuming 15.6 per cent N in ovalbumin. The higher rotation of the digestion products is probably related to the fact that the optical rotation of ovalbumin is greatly increased by denaturation (12, 22).

Osborne (23) gives the value $\alpha_{N_{2}} = -41.3^\circ$ for native edestin in neutral 10 per cent NaCl solution. Since this protein has 18.8
per cent N, \( \alpha N_d^{20} = -220^\circ \). This last value is considerably smaller than those reported for the digestion products in Table I.

Calvery (24) gives 0.728 as the value of \(-\text{NH}_2: \text{N}\) obtained by the complete acid hydrolysis of ovalbumin. Based on this figure, the value 0.175 reported in Table I for ovalbumin indicates that about 24 per cent of the peptide links were split by papain. This former value is much lower than the ratio \(-\text{NH}_2: \text{N} = 0.51\) reported by Calvery for the digestion of ovalbumin by papain for a period of days. Calvery found that papain split two-thirds of the peptide bonds of ovalbumin, while pepsin and trypsin each split only a third of the bonds. He was able to obtain com-

TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Time of digestion</th>
<th>( \alpha N_d^{20} )</th>
<th>(-\text{NH}_2: \text{N})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>hrs.</td>
<td>degrees</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-415</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>-360</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td>Edestin</td>
<td>hrs.</td>
<td>degrees</td>
<td></td>
</tr>
<tr>
<td>½</td>
<td>-325</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-320</td>
<td>0.115</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-300</td>
<td>0.125</td>
<td></td>
</tr>
</tbody>
</table>

15 ml. portions of 5 per cent solutions of ovalbumin and edestin, partially denatured by brief heating at 100\(^\circ\), were digested by 2 ml. of papain solution at 37\(^\circ\). The edestin solution contained 10 per cent NaCl to assist in keeping the protein in solution.

The ratio \(-\text{NH}_2: \text{N} = 0.125\) reported in Table I for edestin is comparable to the value 0.147 reported by Dauphinee and Hunter (20) for an 8 hour digestion of edestin with crude trypsin. These workers obtained the ratio \(-\text{NH}_2: \text{N} = 0.531\) when this digestion was prolonged for 35 days. The value 0.125 indicates a splitting of 20 per cent of the peptide bonds of edestin, the ratio 0.628 being taken to indicate the complete hydrolysis (20).

It is interesting that the \(-\text{NH}_2: \text{N}\) ratios, resulting from the action of papain, are almost the same for the three different proteins, casein, ovalbumin, and edestin. This fact suggests that
papain hydrolyzes certain proteins to an approximately equal degree.

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SUMMARY

1. A quantitative method is described for measuring the amount of proteolytic digestion by determining the degree of optical rotation of the non-protein products. Casein, ovalbumin, and edestin are used to illustrate the method.

2. Changes in optical rotatory power are used in conjunction with the increase in free amino groups to follow the course of the acid hydrolysis of casein. The final value of the specific rotation of the hydrolysate agrees closely with the theoretical value calculated from data given in the literature for the different amino acids of casein.

3. By measuring the optical rotation and percentage of free amino nitrogen of the products resulting from the action of different plant and animal proteases and peptidases on casein, evidence is obtained which indicates that the enzymic and acid hydrolysis of this protein proceeds by somewhat different paths.

4. Evidence from optical rotation and amino nitrogen measurement with different plant enzymes suggests that these proteases hydrolyze casein in a very similar manner and yield the same non-protein products.

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