

THE DECARBOXYLATION OF AMINO ACIDS, PROTEINS, AND PEPTIDES BY *N*-BROMOSUCCINIMIDE

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One of the most widely used methods for the determination of amino acids is the ninhydrin technique perfected by Van Slyke and his coworkers (1). This permits the measurement of amino acid concentration either colorimetrically or by the volume of carbon dioxide released during the reaction. The measurement of carbon dioxide by the Van Slyke manometric apparatus is capable of great precision but is not amenable to the simultaneous analysis of many samples. The colorimetric assay, although not as accurate, makes simultaneous determinations feasible. One disadvantage of both techniques is that they require heating to 100°. The colorimetric procedure suffers from the added disadvantage of sensitivity to atmospheric ammonia. Another method for the decarboxylation of amino acids was recently described (2). This involved heating the amino acids to approximately 150° in the presence of aromatic aldehydes as catalysts. The yield of the reaction varied from 25 to 70 per cent of theory.

In the present paper, we describe a decarboxylation method, based upon work by Barakat *et al.* (3-6), which lends itself to the determination of amino acids and offers considerable promise in the determination of end group carboxyls in peptides and proteins. The decarboxylating agent is *N*-bromosuccinimide. This compound has had extensive use as a reagent for the bromination of unsaturated compounds and, with suitable catalysts, for other compounds as well (7). These bromination reactions are carried out in organic solvents, usually carbon tetrachloride. Aside from the observation that *N*-bromosuccinimide is hydrolyzed to hypobromous acid and succinimide in the presence of water, no serious investigation of the properties of *N*-bromosuccinimide in an aqueous medium had been undertaken until the work of Barakat. He observed that certain α -substituted carboxylic acids were decarboxylated with a concomitant evolution of bromine when allowed to react with an aqueous *N*-bromosuccinimide solution. Among the various α -substituted carboxylic acids which were susceptible to this reagent was alanine. It is pertinent to note that the reaction went to completion at room temperature.

Apparatus and Reagents

The *Barcroft-Warburg apparatus* was used with constant volume manometers. Distilled water containing a trace of detergent was used as manometer fluid.

N-Bromosuccinimide and succinimide were purchased from the Distillation Products Industries of the Eastman Kodak Company, and were found to be of sufficient purity to be used without recrystallization. *Duponol-Me 1 (dry)* was obtained from E. I. du Pont de Nemours and Company. Duponol consists primarily of sodium lauryl sulfate along with small quantities of other long chain alcohols. *Palladium chloride* was purchased from the Fisher Scientific Company. The *amino acids* used were obtained from the California Foundation for Biochemical Research. Glycylserine and glutathione were also obtained from this company. The *peptides*, L-arginyl-L-glutamic acid, L-leucylglycylglycine, glycyl-L-asparagine, glycylglycylhydroxyproline, carnosine, and glycylglycyl- β -alanine, were generously contributed by Professor Emil Smith. Histidylhistidine, glycylphenylalanine, and glycylaspartic acid were purchased from the Nutritional Biochemicals Corporation. Lysozyme, papain, trypsin, and chymotrypsinogen were purchased from the Worthington Biochemical Corporation. The sample of mercuripapain was donated by Professor Emil Smith. Zinc insulin was contributed by Eli Lilly and Company, while chymotrypsinogen, trypsinogen, trypsin, α -chymotrypsin, and δ -chymotrypsin were kindly provided by Professor Hans Neurath. β -Lactoglobulin and α -lactalbumin were generously contributed by Dr. William G. Gordon of the Eastern Regional Research Laboratory.

Methods

In aqueous solutions containing only *N*-bromosuccinimide and an amino acid, there is an evolution of both carbon dioxide and bromine. The production of carbon dioxide is stoichiometric, being directly proportional to the amino acid concentration, while the bromine liberated results from both the decarboxylation reaction and the hydrolysis of *N*-bromosuccinimide by water. It has been found, however, that the evolution of bromine may be reduced to negligible amounts by carrying out the reaction in 1 M sodium acetate-acetic acid buffer at pH 4.7 in the presence of succinimide. It is believed that the effect of succinimide is to decrease the hydrolysis of *N*-bromosuccinimide. To insure the complete absence of bromine within the reaction vessel, 40 per cent potassium iodide is added to a side arm as a trapping agent.

The *N*-bromosuccinimide reagent used in the decarboxylation of amino acids and peptides is prepared by adding 2.5 gm. each of *N*-bromosuccinimide and succinimide to 25 ml. of 1 M sodium acetate-acetic acid buffer

at pH 4.7. Because of its very low solubility, most of the *N*-bromosuccinimide remains in suspension. The use of a suspension of *N*-bromosuccinimide rather than a dilute solution is necessary to maintain a sufficient concentration of the reagent throughout the reaction period. It should be mentioned, however, that with amino acids which react rapidly it is possible to obtain quantitative results with a freshly prepared dilute solution.

The reaction of *N*-bromosuccinimide with amino acids does not proceed at pH values less than 3. The choice of pH 4.7 is dictated by the fact that carbon dioxide is retained by the reaction medium above pH 5. Above pH 8 there is decomposition of *N*-bromosuccinimide into bromine and succinimide. The rate of the reaction increases with an increase of the temperature, but the rate of hydrolysis of *N*-bromosuccinimide increases also. It was found that 30° was the optimal temperature.

The reaction of peptides with *N*-bromosuccinimide is much slower than that of amino acids. It is possible to increase the rate of the reaction by the addition of palladium chloride. The rate of reaction is maximal when palladium chloride is present in the molar ratio of 5:1 with respect to the substrate.

The decarboxylation of amino acids and peptides is carried out in the following manner. The *N*-bromosuccinimide suspension (0.5 ml.) is pipetted into one side arm of the reaction vessel and 0.5 ml. of the 40 per cent potassium iodide solution into the other. The amino acid or peptide, in solution, is added to the main compartment. In the peptide reaction, 0.1 ml. of PdCl₂ of a suitable concentration is added to the main compartment. The total volume of solution in the main compartment is brought to 3 ml. with 10 per cent succinimide in 1 M sodium acetate-acetic acid buffer at pH 4.7. After a 20 minute equilibration period, the reaction is initiated by tipping the *N*-bromosuccinimide into the main compartment. Controls without substrate are run at the same time. The amino acid reaction reaches completion in 30 minutes, while the peptides require from 30 minutes to 3 hours.

By the use of a differential technique with saturated barium hydroxide as a carbon dioxide trapping agent, it has been verified that carbon dioxide is the only gas evolved.

The precision of a method which utilizes the Warburg apparatus is dependent upon the accuracy with which the reaction vessels are calibrated. The flask constants obtained by the usual mercury calibration technique are, among other things, a function of the solubility in the reaction medium of the gas being determined. Because of the inherent difficulties in the accurate determination of carbon dioxide solubility, a different method of calibration was devised. With alanine as the standard amino acid, each

vessel was calibrated by determining the volume of gas which results from the decarboxylation of 5 μ moles of alanine.

Proteins—Proteins, when treated under the above conditions, are readily precipitated. This can be prevented by the use of the cationic detergent Duponol (sodium lauryl sulfate), which was found to be most effective when added to the proteins before their treatment with *N*-bromosuccinimide.

The *N*-bromosuccinimide reagent is also modified by the inclusion of Duponol. It is prepared by adding 2.5 gm. of *N*-bromosuccinimide, 2.5 gm. of succinimide, and 2.5 gm. of Duponol to 25 ml. of 1 M sodium acetate-acetic acid buffer.

The state of the protein when added to the reaction vessel depends to a large degree upon the quantity necessary for an accurately measurable evolution of carbon dioxide. In the case of high molecular weight proteins, it may be found most feasible to weigh the protein directly into the main compartment of the reaction vessel where it may either dissolve or remain suspended.

The reaction mixture for the decarboxylation of proteins consists of the protein in the main compartment to which 10 per cent Duponol-10 per cent succinimide in 1 M sodium acetate-acetic acid buffer is added to bring the volume to 3 ml. One side arm contains 0.5 ml. of the *N*-bromosuccinimide reagent, and the other contains 0.5 ml. of 40 per cent potassium iodide. Most of the proteins we have studied react more slowly than amino acids and peptides. At 30° the decarboxylation reaction requires 30 minutes to 6 hours to reach completion.

Results

Amino Acids—The following amino acids were investigated: alanine, glycine, serine, threonine, leucine, isoleucine, valine, methionine, phenylalanine, cystine, cysteine, histidine, lysine, tryptophan, arginine, tyrosine, glutamic acid, aspartic acid, proline, asparagine, glutamine, and β -alanine. 5 μ moles were used in each determination except with cystine and tyrosine, of which 1.25 μ moles were employed. Four analyses were run on each amino acid.

In every case except four, 5 μ moles of CO₂ were evolved. The four exceptions were tyrosine and cystine, 1.25 μ moles of CO₂; aspartic acid, 10 μ moles; β -alanine, no reaction.¹ Aspartic acid gives rise to 2 moles of CO₂ per mole of amino acid.

The sensitivity of the reaction is illustrated in Fig. 1. The lower limit

¹ β -Alanine evolved 5 μ moles of CO₂, the theoretical amount, when the reaction was allowed to continue for 6 hours.

of amino acid concentration which may be used is dictated by the accuracy with which one may read the Warburg manometer. It was found possible to measure, with a fair degree of accuracy, volumes of carbon dioxide as small as 5 μ l. (equivalent to 0.223 μ mole of amino acid, except aspartic acid).

An indication of the reproducibility and the spread of values obtained on a typical amino acid (alanine) is given by the results obtained from eighteen reactions, carried out simultaneously, each with 5 μ moles of ala-

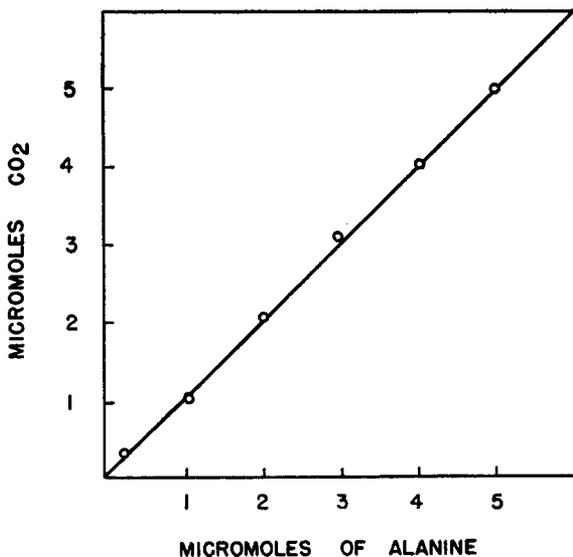


FIG. 1. Sensitivity of *N*-bromosuccinimide reaction. The evolution of carbon dioxide as a function of the amount of alanine.

nine. The mean of the values was 5.04 μ moles of carbon dioxide with an estimated standard deviation of 0.243.

Proteins and Peptides—The reactions of *N*-bromosuccinimide with proteins and peptides are described in Tables I and II, respectively.

DISCUSSION

The present results indicate that, with the exception of aspartic acid and β -alanine, the *N*-bromosuccinimide reaction is specific for carboxyl groups adjacent to an α -substituted carbon atom. It is of interest to note that aspartic acid behaves in a similar fashion when treated with ninhydrin (1). β -Alanine is quantitatively decarboxylated, though quite slowly. The reaction requirement for an α -substituted carbon atom is further indicated by the results obtained with proteins. Although these

TABLE I
Evolution of CO₂ from Proteins When Treated with N-Bromosuccinimide

| Protein analyzed | Micrograms protein Micromoles CO ₂ | Mol. wt. | No. of terminal COOH groups (adjacent column) | |
|------------------------|--|-------------|---|-------|
| | | | Reported | Found |
| Insulin | 3,100 | 5,733 (12) | 2 (12) | 2 |
| | 3,000 | 6,000 (13) | | |
| | 3,100 | | | |
| Chymotrypsinogen | 29,300 | 25,000 (14) | 0 (9) | 1 |
| | 27,700 | | 1 (11) | |
| | 25,300 | | | |
| α -Lactalbumin | 18,200 | 15,500 (15) | 1 (16) | 1 |
| | 17,300 | 16,500 (17) | | |
| β -Lactoglobulin | 38,500 | 35,400 (18) | 2 (19) | 1 |
| | 37,200 | | | |
| Papain | 11,500 | 20,700 (7) | 1 (7) | 2 |
| | 11,100 | | | |
| Mercuripapain | 11,200 | 43,500 (7) | 2 (7) | 4 |
| | 10,800 | | | |
| α -Chymotrypsin | 22,100 | 21,600 (20) | 2 (9, 10) | 1 |
| | 23,000 | | | |
| δ -Chymotrypsin | 12,200 | 21,600 (21) | 2 (9, 10) | 2 |
| Lysozyme | 14,400 | 17,200 (22) | 1 (23) | 1 |
| | 15,000 | 14,900 (24) | | |
| | 14,600 | | | |
| Trypsin | 20,200 | 23,800 (25) | 0 (26) | 1 |
| | 24,500 | | | |

The figures in parentheses are bibliographic references.

TABLE II
Evolution of CO₂ from Peptides When Treated with N-Bromosuccinimide

| Peptide analyzed | Micrograms peptide Micromoles CO ₂ | Mol. wt. |
|--|--|----------|
| Glycylserine | 161.5; 161.5; 162.0 | 162.2 |
| Histidylhistidine | 293.0; 293.5; 293.0 | 292.4 |
| Glycyl-L-aspartic acid | 95.8; 95.2; 95.6 | 190.2 |
| Glycylphenylalanine | 220.9; 221.0; 219.8 | 222.3 |
| L-Arginyl-L-glutamic acid H ₂ O | 323.2; 324.1 | 321.3 |
| Glycylglycylhydroxyproline | 246.2; 243.8; 242.9 | 245.3 |
| L-Leucylglycylglycine | 241.8; 242.3; 242.5 | 245.4 |
| Carnosine nitrate (β -alanyl-L-histidine) | 145.8; 146.7; 147.0 | 286.3 |
| Glycylglycyl- β -alanine | 203.8; 204.2; 204.6 | 203.3 |
| Glycyl-L-asparagine | 191.6; 189.9; 193.5 | 189.2 |
| Glutathione | 305.4; 304.8; 305.3 | 307.3 |

studies were not carried out under the most precise conditions (the proteins were not corrected for moisture content), the molecular weights that may be deduced from the results are, in most cases, acceptable in magnitude. The values are minimal since only one reactive carboxyl group per mole is postulated. In the case of insulin in which the existence of a two chain molecule has been established, the molecular weight obtained by this method must be multiplied by 2 to give the accepted value. Papain and mercuripapain evolve a quantity of carbon dioxide also indicative of two reactive carboxyl groups per molecule. This assumes a true molecular weight of approximately 21,600 (8). These two carboxyl groups could either arise from a single dicarboxylic amino acid such as a terminal aspartic acid, or from two monocarboxylic amino acids if the protein is composed of two chains.

The studies upon chymotrypsinogen, α -chymotrypsin, and δ -chymotrypsin indicate the presence of one *C*-terminal group in chymotrypsinogen,² one *C*-terminal group in α -chymotrypsin, and two *C*-terminal groups in δ -chymotrypsin. Gladner and Neurath (9, 10) through the use of carboxypeptidase report the absence of a *C*-terminal group in chymotrypsinogen and two *C*-terminal groups in both α -chymotrypsin and δ -chymotrypsin. Recently, however, Meedom (11) has reported the existence of a *C*-terminal tyrosine residue in chymotrypsinogen from studies in which oxidation procedures were used. The observation of a molecular weight of 23,800 for trypsin is of interest in view of the lack of any conclusive evidence regarding the presence of a *C*-terminal amino acid in this protein.

The positive results obtained with proteins and peptides point out a major difference between the action of ninhydrin and that of *N*-bromosuccinimide. Van Slyke *et al.* (1) reported little or no evolution of CO₂ when proteins and peptides were treated with ninhydrin.

It would be of little value at this time to speculate upon the reaction mechanism without a thorough knowledge of the reaction products. This phase of the study is under way. Preliminary studies of the products from amino acids indicate the formation of aldehydes which, in view of analogous reactions, is to be expected. The effect of PdCl₂ upon the rate of the reaction is not yet clearly understood. Preliminary studies of its

² Earlier studies with chymotrypsinogen in which a much lower concentration of *N*-bromosuccinimide was used indicated the absence of terminal carboxyl groups. This lower concentration was adequate for all other proteins studied, although the rate of reaction was somewhat slower. This discrepancy in the case of chymotrypsinogen might possibly be of significance in view of the disagreement with regard to the presence or absence of a *C*-terminal amino acid (9, 11). Another factor which may explain this discrepancy is that the earlier work carried out in this laboratory with chymotrypsinogen was done with a different preparation of the protein. Investigations now under way may resolve this discrepancy.

effect upon the rate of decarboxylation of insulin indicate that the rate of reaction of proteins may be increased in its presence.

The value of this technique in the quantitative determination of amino acids lies primarily in its simplicity and ease. The fact that the reaction proceeds at moderate temperatures (30–37°) may render it especially useful in the study of enzymatic reactions in which amino acids are involved.

The application of this reaction to the study of proteins has ramifications of possibly great importance. It offers a means of chemically modifying protein structure by a relatively mild procedure and provides a basis for obtaining supplementary information on the molecular weights of proteins.

SUMMARY

A method is described for the quantitative decarboxylation of amino acids, proteins, and peptides with use of *N*-bromosuccinimide as a decarboxylating agent. The quantity of carbon dioxide evolved is measured manometrically in the Warburg apparatus.

The optimal reaction conditions, along with the results obtained with several amino acids, proteins, and peptides, are described.

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