"B_{12} enzyme," is itself a DNA-protein, it could play the role of an *auto catalyst* in the biosynthesis of new DNA-protein. Such an autocatalytic mechanism would be, of course, in agreement with the observed exponential kinetics of this biosynthetic process.

Further work, now in progress, will test the validity of our hypothesis, which for the time being may be regarded as a tentative explanation of the experimental facts presented in this paper.

Acknowledgment.—The authors wish to express their appreciation to Drs. J. P. Dailey, R. T. Rapala and E. E. Hays for their helpful interest in this work.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

A Method for the Quantitative Determination of C-Terminal Amino Acid Residues[1]

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RECEIVED DECEMBER 27, 1954

A carboxoid method which is analogous to a previously described aminoid method is reported. This C-terminal method has been evaluated on amino acids, dipeptides, tripeptides, lysozyme and in other work on lysozyme proteolyzates. The method appears to offer a number of advantages over other C-terminal procedures which have been described. Among these are stereospecificity, quantitative applicability, and ease of operation such as freedom from the need for fractionation. Principal discernible limitations are behavior which is less quantitative with dipeptides than with larger peptides and incomplete reactivity of terminal aspartic acid, glutamic acid and proline residues. When applied to lysozyme, one C-terminal residue of L-leucine was found. Of fourteen other types of residue assayed in the experiment designating C-L-leucine no other was significantly altered by the treatment with ammonium thiocyanate and acetic anhydride.

A stepwise N-terminal method, representing a modification of the original Abderhalden[3] and Edman[4] methods has been applied with quantitative results to synthetic peptides[5] and to subtractive structural studies of peptides and mixtures of peptides.[6,7] These modifications have been useful also in qualitative structural studies.[8,9] The utility of such a method would be greatly extended if it became possible to determine what proportions and types of C-termini are free, or the proportions and types of carboxoid residue liberated simultaneously with the aminoid, particularly in fragmentation studies.

A number of methods of assignment of C-termini has been reported.[10–21]

It should be stated parenthetically that, aside from chemical methods, carboxypeptidase has been employed already for C-terminal studies.[22,23,16] Uncertainties[10,24,25] in the application of proteases to prior fragmentation for structural studies, which might however be resolved by use of model synthetic peptides, yet cloud the picture. A study of proteolysis made possible in part by the carboxoid method described in this paper indicates, in fact, that concordance of sequential assignments, after fragmentation with different proteases, does not assure freedom from transpeptidation.[26] This follows inasmuch as results show that the substrate(s) is itself a primary determinant of the nature of the reaction.[26,27] Difficulties of an incompletely defined sort have been recorded for the carboxypeptidase technique.[28] However the discrepancies with enzymes are resolved,[21] the need for a quantitative carboxoid method is further emphasized.

The type of method which appeared to offer the most promise for quantitative and stepwise adaptations was that developed in its original qualitative form by Schlack and Kumpf[29] from the Johnson reaction[30,31] and applied extractively to proteins in later work.[15,18,19,21] This method was selected also because of the likelihood that some of the accumulated information on the similar phenyl isocyanate[10] and phenyl isothiocyanate methods

(1) Journal Paper No. J-2678 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 863. Supported by Grant G-3025 (C) from the National Institutes of Health, U. S. Public Health Service. Largely from the Ph.D. thesis, in Food Technology, of Thomas L. Hurst (1953).

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would be of direct help. In addition, the similar Dakin–West reaction which employs acetic anhydride and pyridine[32] was studied in this connection.

As in the application of the subtractive aminoid method, the carboxoid method relies upon hydrolysis of a terminally treated peptide. The results obtained suggest that substantial decomposition of the carboxoid thiohydantoin usually occurs, with minor or no recovery of the amino acid. This paper describes application of the method, with modifications, to amino acids, dipeptides, three tripeptides and to lysozyme.

Materials and Methods

Amino Acids.—The amino acids employed in the studies summarized in Table II were U. S. P. standard L samples.

DL-Leucine was used to prepare 5-isobutyl-2-thiohydantoin by the method of Johnson and Nicolet.[30]

Peptides.—Preparation and constants of leucylvaline, valylleucine and valylglycylphenylalanine have been reported.[5] Leucylglycylvaline,[33] leucylglycylphenylalanine,[34] benzoylleucylglycylphenylalanine[35] were prepared by K. F. Itschner according to the published directions. Benzoylglycylphenylalanine[36] was prepared by K. F. Itschner by benzoylation of glycylphenylalanine,[34] which was also used as such. Prolylleucine has been described in earlier work[37] and benzoylleucylvaline, m.p. 218–220° uncor., was synthesized by L. C. Carpino.[38] All peptides were composed of DL residues with the exception of the prolylleucine.[5,37]

Lysozyme was the crystalline chicken egg white lysozyme, lots 003L and 003L1, and was a gift from Armour and Company. Electrophoretic homogeneity was established in the laboratories of Dr. Joseph Foster. The moisture content was determined by drying to constant weight at 105°.

Microbiological Assays.—These were conducted with the media and organisms employed in related studies.[5] Each result was determined from averages of either triplicated tubes at each of two levels or duplicated tubes at each of three levels.

Arbitrary criteria were adopted in order to permit relatively objective rejection of aberrant values. When two of the triplicate values agreed within 5 relative % and the third differed by as much as 20%, the third was discarded. When the assay values in one of the duplicated tubes at each of three levels differed by as much as 20% from the averages of three levels, the aberrant value was discarded. Otherwise all values were averaged and the amount of assayed substance in the unknown was read from the standard graph, also obtained from triplicated points. When the average value of a sample at a lower concentration differed from the upper by as much as 10%, the assay was repeated.

Experimental and Results

One crystalline thiohydantoin, 5-isobutyl-2-thiohydantoin, was checked for microbiological utilizability following hydrochloric acid treatment of the type used in assay hydrolyses. The leucine recovered was assayed with *Lactobacillus arabinosus*. The hydrolyses were performed at 120° for 16 hr. in the autoclave. Direct determinations of the leucine equivalent of the thiohydantoin, without hydrolytic treatment, gave results in the range of 30–60%. The recoveries obtained from the "hydrolyzed" thiohydantoin are recorded in Table I.

In order to simulate more closely the process used with peptides, amino acids were treated with reagents in the excess employed with peptides. In each case, 2.0 mg. of the amino acid dissolved in 2.0 ml. of water was placed in a weighing bottle, and the contents were evaporated in a desiccator. To the dry residue was added 2.0 ml. of acetic anhydride containing 10% of acetic acid and 10 mg. of ammonium thiocyanate. The bottles were closed and heated for 30 min. on a steam-bath. After being cooled, each bottle was treated with 1.0 ml. of water and the bottles were set in an evacuated desiccator over sulfuric acid and solid sodium hydroxide until dry. To each was next added 5.0 ml. of 3 *N* hydrochloric acid and the samples were autoclaved in the open bottles at 120° for 16 hr. The aqueous acid, having undergone some dilution, was dispelled over a steam-bath. When superficially dry the bottles were finally desiccated in the presence of a little ammonium hydroxide to neutralize any free acid. The material was made up to a measured volume and assayed in duplicate at three different levels.[5] The results obtained are presented in Table II. Individual values represent entirely separate assays. In those cases in which the recoveries significantly exceeded the variation attributable to variation in microbiological assay (10%) the reaction and assay was again replicated.

Table I

Leucine Available to *Lactobacillus arabinosus* 17-5 from 5-Isobutyl-2-thiohydantoin after Acid Hydrolytic Treatment

Vessel	Initial *N* of HCl	Final *N* of HCl	Assayable leucine equiv., %
Beaker	3.0	1.2	41
Beaker	4.0	1.0	42
Beaker	6.0	1.4	35
Beaker	8.0	1.9	30
Sealed tube	3.0	3.0	30
Sealed tube	4.0	4.0	39
Sealed tube	6.0	6.0	58
Sealed tube	8.0	8.0	57

The results in Tables I and II may be compared for leucine. Recoverability of leucine following blocking treatment and hydrolysis is seen to be negligible in contrast to the substantial recoveries from hydrolyzed preformed thiohydantoin.

Table II

Recovery of Amino Acids after Carboxoid and Acid Hydrolytic Treatments

Amino acid	Organism and medium (See[6] for abbreviations)	Recovery, %
L-Alanine	cx[a]	14, 28
L-Arginine	fr	12, 29, 22
L-Aspartic acid	mq	89, 94
L-Cystine	aq	1, 12, 17
L-Glutamic acid	ax	51, 65
Glycine	bt	8, 24, 3, 1
L-Histidine	mr	20, 17, 14
L-Isoleucine	aq	9, 12, 4
	fq	6, 6
L-Leucine	aq	3, 1
	fq	3, 0
L-Lysine	mq	32, 33, 25
L-Methionine	fq	6, 9
L-Phenylalanine	bt	5, 15
L-Proline	bt	51, 47, 39
L-Serine	my	2, 3
L-Threonine	fz	19, 16
L-Tryptophan	aq	0, 0
L-Tyrosine	bq	5, 14, 3
L-Valine	fq	13, 14, 4, 0, 0, 24

[a] c = *L. citrovorum*, not symbolized in the earlier paper.[6]

Carboxoid Treatment of Peptides.—Many different kinds of treatment were tested. Some of the best results, some of the poorest results, and some of the methodologically most representative types are indicated in Table III. The treatments were as follows:

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TABLE III

PERCENTAGE AMINO ACIDS RECOVERED IN ASSAY AFTER VARIOUS TREATMENTS OF PEPTIDES

Treatment type	no.	1	2	3	1a	4	5	6	7	8
Leu-val	Leu	75	68	77					101 ± 8[a]	96 ± 12[a]
	Val	4	24	29					36 ± 1[a]	50 ± 3[a]
Val-leu	Val	31	45	28					36 ± 1[a]	
	Leu	80	12	3						
Val-gly-phe	Val		67	64						
	Gly		70	88						
	Phe		3	11						
Leu-gly-val	Leu		93	83						
	Gly		95	98						
	Val		2	33						
Pro-leu	Pro		60	67						
	Leu		16	2						
Leu-gly-phe	Leu				96	98	105			
	Gly					94	99			
	Phe				29	21	12			
Bz-leu-gly-phe	Leu				89					
	Gly				26					
	Phe									
Bz-gly-phe	Gly					91	90			
	Phe					35	12			
Gly-phe	Gly					95	95	97		
	Phe					34	18	12		
Bz-leu-val	Leu								88 ± 5[a]	28 ± 2[a]
	Val								104 ± 5[a]	51 ± 3[a]

[a] Standard deviations of six assays. For these assays only, the hydrolyzed peptide was used, instead of amino acid, for the standard curve.

No. 1.—Peptide in a 2.00-mg. portion was heated in a weighing bottle on the water-bath with 1.0 mg. of ammonium thiocyanate and 2.0 ml. of acetic anhydride for 30 min. The anhydride was evaporated and the residue treated with 2.0 ml. of concentrated ammonium hydroxide for 4 hr. at room temperature and again dried. The residue was hydrolyzed with 2.0 ml. of 2 *N* hydrochloric acid at 15 lb. steam pressure in an autoclave for 12 hours.

No. 1a.—Same as 1 except 4.0 mg. of ammonium thiocyanate instead of 1.0 mg.

No. 2.—Peptide in a 2.00-mg. portion was heated on the water-bath for 10 min. with 2.0 ml. of acetic anhydride containing 10% acetic acid. Three mg. of ammonium thiocyanate was added and heating was continued for 50 min. After evaporation to dryness, 2.0 ml. of concd. ammonium hydroxide and 0.1 ml. of 0.1 *N* sodium hydroxide were added and allowed to react for 4 hr. After drying again, the residue was hydrolyzed with 2.0 ml. of 6 *N* hydrochloric acid at 120° in the autoclave for 16 hr.

No. 3.—Two mg. of peptide in 2.0 ml. of water was benzoylated by 0.002 ml. of benzoyl chloride in 2.0 ml. of pyridine. The *p*H was maintained at 7.5–8 with brom thymol blue and 0.2 *N* sodium hydroxide, for 3 hr. at 37°. The liquid was evaporated and then treated as in No. 2 to form the thiohydantoin and hydrolyzed similarly.

No. 4.—Because results with large molecules appeared to be more quantitative (*cf.* Discussion) hydrogen peroxide was employed in some comparisons.

Two mg. of peptide was heated with 30 mg. of ammonium thiocyanate and 5.0 ml. of acetic anhydride (10% acetic acid) on a boiling water-bath for 30 min. After cooling, 1.0 ml. of water was added and the mixture was evaporated. Then were added 1.0 ml. of water and 0.1 ml. of 3 vol. % hydrogen peroxide and the sample stood for 3 hr. before evaporation and hydrolysis.

No. 6.—Same as No. 5 except that 1.0 ml. of concd. 2 *N* ammonium hydroxide and 0.1 ml. of 3 vol. % hydrogen peroxide were added instead of the ammonium hydroxide alone.

The utility of phenyl isothiocyanate as a carboxoid reagent was tested by No. 7 and 8.

No. 7.—DL-Leucyl-DL-valine, 2.3 mg., was dissolved in 4.3 ml. of water and 3.4 mg. of benzoyl-DL-leucyl-DL-valine was dissolved in 0.7 ml. of 1 *N* ammonium hydroxide and 3.5 ml. of water. Four ml. of each sample was placed in a weighing bottle and dried under reduced pressure. A 50 molar excess of ammonium thiocyanate in acetic acid: acetic anhydride (1:9) was added and the mixture heated on a boiling water-bath for 30 min. The reaction mixture was dried down and the residue was hydrolyzed for 10 hr. with 6 *N* hydrochloric acid at 120°. The resultant assays present the standard deviations for six microbiological assays.

No. 8.—Same as No. 7, with phenyl isothiocyanate replacing ammonium thiocyanate.

Qualitative chromatograms following the Dakin–West reaction[32] indicated that 15 min. of heating leucylvaline with pyridine and acetic anhydride resulted in virtually complete loss of valine. After 1.5 hr. of heating, however, the leucine had almost entirely disappeared.

Carboxoid Treatment of Lysozyme.—To check on the effect of carboxoid treatment on the C-terminal residue and on others, 50 mg. of lysozyme and 0.5 ml. of acetic anhydride were placed in a weighing bottle in a vacuum desiccator over sodium hydroxide flakes and sulfuric acid. After evaporation was complete (8–10 hr.), 25 mg. of ammonium thiocyanate, 4.5 ml. of acetic anhydride and 0.5 ml. of acetic acid were added. The bottles were closed and heated with frequent agitation in boiling water for 30 minutes. After cooling, 0.5 ml. of water was added, and the liquid was evaporated in a desiccator.

Hydrolysis was accomplished by adding 5 ml. of 6 *N* hydrochloric acid and autoclaving for 16 hr. at 120°. The acid was evaporated over steam, and the dried residues were neutralized with sodium hydroxide, made to volume and assayed. Results of the two analyses performed on lysozyme are presented in Table IV.

TABLE IV

AMINO ACID COMPOSITION OF LYSOZYME BEFORE AND AFTER CARBOXOID TREATMENT

Amino acid	Mean compositional value, residues/ 14,700 g.	Standard dev.	Carboxoid treated sample 1, residues/ 14,700 g.	Carboxoid treated sample 2, residues/ 14,700 g.
Arginine	11.2	±0.3	11.0	10.8
Aspartic acid	19.8	± .2	19.9	..
Glutamic acid	4.1	± .1	4.2	4.2
Glycine	11.1	± .3	11.1	11.3
Histidine	1.0	± .0	0.9	0.9
Isoleucine	6.9	± .2	6.4	6.8
Leucine	7.0	± .2	5.9	6.0
Lysine	5.7	± .1	5.8	5.9
Methionine	2.1	± .1	1.7	2.1
Phenylalanine	3.0	± .0	2.7	2.9
Proline	2.0	± .1	1.9	1.9
Serine	9.6	± .2	9.2	9.4
Threonine	6.7	± .2	6.7	6.3
Tyrosine	3.0	± .0	2.7	2.8
Valine	6.1	± .1	5.7	6.0

The values for the fifteen amino acids in untreated lysozyme are taken from either five or six analyses in each case, and the standard deviations computed are also presented in Table IV. The individual assays were conducted on samples of lysozyme admixed with 1% by weight of either chymotrypsin, trypsin, papain or pepsin. The negligible contribution of the enzyme to the analytical figures is established by the small standard deviation in each case.

Discussion

The results indicate that the thiohydantoin from leucine can be relatively directly utilized by *L. arabinosus*. This is similar to the utilizability of benzoylleucine by the same organism,[35] and to the direct utilizability of the phenylthiohydantoin.[39] The data in Table I indicate that about the same extent of utilization is recorded after hot acid treatment such as typically used for complete hydrolysis of protein.

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The experiments which provided the data in Table II more closely simulated the conditions employed in carboxoid treatment of peptides. It should be emphasized that these results cannot be used to interpret directly experiments with peptides; they do appear however to have relative value. It is significant that after the treatment with excess of reagents virtually no leucine was recovered. This suggests that such treatment destroys the thiohydantoin other than by a mechanism involving mere conversion to amino acid. Most of the amino acids exhibit a degree of destruction which suggests a workable basis for a quantitative carboxoid method. L-Aspartic acid, L-glutamic acid, L-lysine and L-proline display recoveries which, if parallel to those in peptides, would need at least to be recognized as large imperfections in quantitative studies. The essential lack of effect on aspartic acid is explainable by the known difficulty in preparing a thiohydantoin from this amino acid.[40] Glutamic acid and proline underwent loss of utilizability of the L form of about one-half. This is probably due to racemization by the reagents. Such behavior can however be utilized. Whenever loss in terminal glutamic acid or proline after such treatment is significant, the decrement can be corrected by being multiplied by two. The basic amino acids showed smaller recoveries in the range of 12–33%.

The incompletely understood side-reactions in the carboxoid hydrolysis undoubtedly contribute to the generally low recoveries which have been recorded.[15,18,19,21] These reactions, insofar as they lead to destruction of carboxoid residues, serve the desired purpose in the subtractive technique described here. This fortunate circumstance has been pointed out also for the aminoid method.[7]

The specificities in utilization of amino acid derivatives by bacteria[35] suggest that other bacteria might be successfully used in those cases in which a given bacterium would be found to utilize at all a terminal residue carried through a carboxoid treatment.

Table III provides a number of controlled comparisons of treatment. The recovery of the aminoid component of dipeptides has been generally lower than for tripeptides (treatments 1, 1a, 2 and 3). This was also observed in many experiments not included in Table III. This difference in behavior in fact led to the use of tests of evaluation directly on yet larger peptides such as lysozyme. Although there is no certain explanation for this phenomenon, it seems probable to these authors that dipeptides might be particularly prone to form diketopiperazines in the presence of warm acetic anhydride.[41]

Treatments 4, 5 and 6 employed added ammonia, hydrogen peroxide or both. These reagents were used inasmuch as recovery and other experiments with lysozyme and soybean meal indicated more satisfactory results than with synthetic peptides alone. It was accordingly postulated that oxidizing and/or ammoniacal potential in the presence of protein improved the carboxoid reaction. Treatments 4, 5 and 6 on leu-gly-phe indicate that the combined treatments gave the most quantitative results. Under these conditions (No. 6) recovery of the glycine from glycylphenylalanine was close to complete.

When studied on either leucylvaline or the benzoyl derivative, treatments 7 and 8 reveal the interesting fact that the aminoid reagent, phenyl isothiocyanate, can be used carboxoidwise. This use is not practical, however, as judged by the greater decrement with ammonium thiocyanate in each case.

In Table IV it may be seen that the only integral decrement is that for leucine; other amino acid residues are evidently unharmed. It is of special interest that the N-lysine exhibits no loss. The integral decrement for leucine serves to validate the method in that leucine alone has been previously shown to be C-terminal[42] in a qualitative study. The present work indicates that this leucine is of the L configuration. In addition, after 240 hr. of chymotryptic hydrolysis the total amount of carboxoid opening for twelve amino acids was the same, 15.8 per molecule, as for aminoid opening, 15.6 per molecule.[43]

When rounded to the nearest integral number, the compositional figures of lysozyme in Table IV do not differ from the nearest whole numbers in an analysis entirely performed by another worker in this Laboratory.[7]

In general, the results with synthetic dipeptides and tripeptides are more quantitative for the aminoid subtractive method than for the carboxoid subtractive method. In the application to large peptides the two methods may be quantitatively equivalent except that the carboxoid reaction may not proceed normally with residues of aspartic acid, glutamic acid or proline.

Ames, Iowa

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