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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

A Microbiological Method for the Determination of Sequences of Amino Acid Residues[1]

BY SIDNEY W. FOX, THOMAS L. HURST AND KENNETH F. ITSCHNER

A subtractive microbiological method for the determination of terminal amino acid residues in peptides is presented. Results are given for glycyl-DL-valine, L-prolyl-L-leucine, leucylphenylalanine, valylleucine, leucylvaline, L-valyl-D-valine, D-valyl-L-valine and valylglycylphenylalanine. By employing selective hydrolysis, the sequence of residues in the tripeptide could be assigned. The general procedure is uniquely applicable to quantitative determinations of terminal amino acid residues on a micro scale. When employed comparatively with paper-strip procedures, this method serves to indicate D-residues. Other advantages and recognized current limitations are discussed.

Elucidation of the structure of proteins and of peptides requires methods which will assign positions of amino acid residues in the chains. This is theoretically possible from determination of sequence in smaller peptides obtained on hydrolysis,[2] advances in this direction have been made.[3-5] Improvements in methodology are needed, as well as independent methods which will provide confirmation for those already available. In the determination of the structure of Gramicidin S,[3] for example, it was deemed desirable to synthesize the peptides indicated by a simpler method, and to compare the R_f values of the synthetic peptides with those of the isolated materials. Both of these procedures relied, however, on chromatography; an independent principle for a micro method is desirable.

The use of microbiological techniques was suggested earlier[2] and has now been applied to some synthetic peptides by proceeding subtractively as follows: (a) All residues are first determined on a complete hydrolysate by setting up tubes each of which is nutritionally complete except for one amino acid. (b) The terminal residue is blocked chemically, in a second sample. (c) Treated sample (b) is hydrolyzed, and the microbiologically recoverable amino acids are again evaluated. (d) The missing residue is the one which occupied the terminal position in the original peptide.

The microbiological procedure, in contrast to chromatography, is applicable for relatively convenient quantitative determinations, and this in turn for peptides with recurrent residues. In conjunction with chromatography, on the other hand, it is possible to locate D-residues readily, since the paper strip does not distinguish between L- and D-amino acids, whereas the microörganisms usually do. The occasional utilization of D-residues by bacteria (*cf.* ref. 6) may be avoided.

A number of blocking agents have been used in the present study in a comparative survey of the applicability of various reagents[2] in a microbiological approach. The dinitrophenyl residue[7] has been found to offer difficulties in quantitative applications, an observation which agrees with the report of Consden, Gordon, Martin and Synge.[3] Nitrous acid is not a practical reagent, since some of the resultant hydroxyacids can be utilized microanabolically.[8] Nitrous acid also reacts abnormally with a number of amino acids.[9] Phenyl isocyanate[10] and the thio analog[11] have proved to be useful and they are the type of reagent described here although others are, in principle, applicable. In chemical procedures these agents have also been used in a stepwise manner on peptides containing more than two residues, and this is a principal reason for studying the microbiological applicability of this kind of blocking group.

Model studies were performed on glycyl-DL-valine, L-prolyl-L-leucine, L-valyl-D-valine and D-valyl-L-valine, leucylphenylalanine, leucylvaline and valylleucine, and valylglycylphenylalanine (Table I).

The operating conditions first arrived at, and presented in the experimental section, proved adequate for conclusive results with all of the above dipeptides except glycyl-DL-valine. After treatment, recovery of 19% glycine indicated, however, sufficiently large quantitative loss in this residue that the correct sequence could be deduced from microbiological results even though the paper-strip picture was equivocal. The 19% recovery was smaller than a 41% value obtained when the isothiocyanate/peptide ratio was half of that given in the experimental section. When the blocking reaction was carried out with added alkali, the glycine recovery was eliminated entirely. The pertinent reaction conditions are being studied further; this one instance with glycine however suggests that a generally standard procedure may be described only after peptides of all amino acids in the free amino

(1) Journal Paper No. J-1891 of the Iowa Agricultural Experiment Station, project 863, supported in part by the Corn Industries Research Foundation and by a grant from the National Institutes of Health, Public Health Service.

(2) S. W. Fox, *Adv. Prot. Chem.*, **2**, 155 (1945).

(3) R. Consden, A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **41**, 596 (1947).

(4) F. Sanger, *ibid.*, **45**, 563 (1949).

(5) R. R. Porter, *ibid.*, **46**, 473 (1950).

(6) M. N. Camien and M. S. Dunn, *J. Biol. Chem.*, **184**, 283 (1950).

(7) E. Abderhalden and P. Blumberg, *Z. physiol. Chem.*, **65**, 318 (1910).

(8) D. M. Hegsted, *J. Biol. Chem.*, **167**, 741 (1945); B. A. Prescott, E. Borek, A. Brecher and H. Waelsch, *ibid.*, **181**, 273 (1949).

(9) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **41**, 590 (1947).

(10) E. Abderhalden and H. Brockmann, *Biochem. Z.*, **225**, 386 (1930).

(11) P. Edman, *Arch. Biochem.*, **22**, 475 (1949); P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

TABLE I

CONSTANTS OF PEPTIDES NOT FOUND IN EARLIER LITERATURE

Peptide	M.p., °C., uncor.	Neut. equiv. Calcd.	Neut. equiv. Found	Formol equiv. Calcd.	Formol equiv. Found	Nitrogen, % Calcd.	Nitrogen, % Found	Microcrystalline appearance
α-Bromoisovalerylleucine	148–150	294	291			4.76	4.66	
α-Bromoisocaproylvaline	127–130	294	293			4.76	4.68	
Valylleucine	274–275 dec.			230	232	12.16	11.9	Needles[a]
Leucylvaline	267–269 dec.			230	233	12.16	11.9	Hexagonal[b] platelets
α-Bromoisovalerylglycylphenylalanine	192 dec.	385	385			7.27	7.31	
Valylglycylphenylalanine				321	331	13.08	13.1	Needles[c]

[a] From water. [b] From aqueous alcohol, and from aqueous dioxane. [c] From water by addition of ethanol.

TABLE II

DATA FOR ASSIGNMENT OF SEQUENCE IN PEPTIDES SUBJECTED TO PHENYL ISOTHIOCYANATE AND HYDROLYTIC TREATMENTS

Peptide	Papergram recovery after isothiocyanate and hydrolysis	Microbiological recovery of hydrolyzed peptide, %	Microbiological recovery of treated and hydrolyzed peptide, %
Valylleucine[a]	Valine 0, leucine +	Valine 87, leucine 92	Valine 7, leucine 85
Leucylvaline[a]	Leucine 0, valine +	Leucine 92, valine 95	Leucine 26, valine 94
	.....	.....	Leucine 2,[b] valine 91[b]
Leucylphenylalanine[a]	Leucine 0, phenylalanine +	Leucine 95, phenylalanine 94	Leucine 10, phenylalanine 88
L-Prolyl-L-leucine	Proline 0, leucine +	Proline 93,[c] leucine 82[a]	Proline 7,[c] leucine 84[a]
Glycyl-DL-valine[d]	Glycine +, valine +	Glycine 88,[c] valine 92[a]	Glycine 19,[c] valine 89[a]
Glycyl-DL-valine[e]	Glycine 0, valine +	.....	Glycine 0,[c] valine 94[a]

[a] *L. arabinosus* for m.b. assay. [b] *S. faecalis* for m.b. assay. [c] *L. brevis* for m.b. assay. [d] Without added alkali in treatment. [e] With added alkali in treatment.

position have been tested. With proline, leucine, or valine in the amino position, coverage was obtained readily in the peptides tested (Table II).

The controls employed in the establishment of the sequence in L-prolyl-L-leucine revealed that a product earlier designated carefully only as prolyl-L-leucine[12] underwent resolution during recrystallization. This fact was confirmed in the present work by standard curves constructed from L-proline and from an hydrolyzate of prolylated DL-alanine. Further confirmation is found in the fact that the D-prolyl-D-leucine, similarly prepared, yielded no microbiologically recoverable L-proline on hydrolysis.

From the results on the valylvalines, as presented in Table III, it may be inferred that the simultaneous employment of paper-strip and microbiological assay provides a convenient alternative method to that employing D-amino acid oxidase[13] for identifying D-residues. Such a procedure can be helpful in residue isomer problems associated with antibiotics.[14]

TABLE III

DATA FOR ASSIGNMENT OF SEQUENCE IN TWO ENANTIOMORPHIC VALYLVALINES

Peptide	Papergram recovery after isothiocyanate and hydrolysis	Microbiological recovery of treated and hydrolyzed peptide, %
D-Valyl-L-valine[a]	Valine +	L-Valine 114
L-Valyl-D-valine[a]	Valine +	L-Valine 10

[a] *L. arabinosus* for m.b. assay.

In the tripeptide, valylglycylphenylalanine, it was found possible to assign all positions by successive subtraction (Table IV). This required a step involving selective hydrolysis of the phenyl isothiocyanated tripeptide into valine phenylthiohydantoin and a residue which could be recognized as glycylphenylalanine. The hydrolytic reagent was dioxane–HCl, used at 25° for six hours (warm methanol–HCl,[10] and nitromethane–HCl[11] have been used earlier). The entire procedure was operable without need for isolation of any intermediate.

TABLE IV

DATA FOR ASSIGNMENT OF SEQUENCE IN VALYLGLYCYLPHENYLALANINE

Treatment of tripeptide prior to assay	Valine recovery, %	Glycine recovery, %	Phenylalanine recovery, %
Hydrolysis[a]	103	99	99
Isothiocyanate, hydrolysis in aqueous HCl in autoclave[b]	2[a]	93[a]	100[a]
Isothiocyanate, cold hydrolysis in dioxane-HCl, followed by treatment[b] with hydrolysis in autoclave; (assay[c])	0	0	97

[a] *L. brevis* for m.b. assay. [b] As in experimental, with added alkali. [c] *L. arabinosus* for m.b. assay.

Another potential application of the microbiological method is as an index of purity of large peptides, and it is being so applied to ACTH preparations.

In the selection of bacteria for microbiological assignment of sequence, a coverage of all the amino acid requirements is necessary, and this can theoretically be achieved by use of two or more of those for which the requirements have been extensively catalogued.[15]

Experimental

Peptides.—Valylleucine was prepared from α-bromoisovaleryl bromide[16] and leucine by the Schotten–Baumann pro-

(12) M. Fling, F. N. Minard and S. W. Fox, THIS JOURNAL, **69**, 2466 (1947).

(13) T. S. G. Jones, *Biochem. J.*, **42**, lix (1948).

(14) J. W. Hinman, E. L. Caron and H. N. Christensen, THIS JOURNAL, **72**, 1620 (1950).

(15) M. S. Dunn, S. Shankman, M. N. Camien and H. Block, *J. Biol. Chem.*, **168**, 1 (1947).

(16) J. Volhard, *Ann.*, **242**, 161 (1887).

cedure. A precipitate was obtained upon acidification after amination of the intermediate for 5 days at 37° with 15 parts of concd. ammonium hydroxide in a stoppered flask. The leucylvaline and valylglycylphenylalanine were similarly prepared. Since none of these compounds were found in the literature, their constants and those of some intermediates are presented in Table I. Some of the results in Tables II and IV further confirm their identity. Four of the peptides are capable of existence as D–D, L–L racemate or D–L, L–D racemate (*cf.* refs. 14, 17). The proportion of unblocked L-residue is however, theoretically the same with either racemate. The valylvalines were a gift from Dr. J. W. Hinman of the Upjohn Laboratories; their preparation has been described.[14] These peptides were received as the hydrochlorides, for which theoretical neutral equivalents had been found in the Kalamazoo Laboratories. Since these substances are quite hygroscopic, samples were weighed by difference.

Papergrams.—The paper-strip work was carried out with use of details recommended by Gage, Douglass and Wender.[18] The solutions employed were the same as those used for microbiological determinations, except that it was necessary to concentrate the solutions in order to obtain visible color in the spots.

Culture.—*Lactobacillus arabinosus* 17-5 *and Streptococcus faecalis* R were organisms that were originally obtained from the American Type Culture Collection and had been maintained in this Laboratory for some time. Since each of these has been frequently used here in routine bioassay, and their observable cultural requirements have remained unchanged, their identity is relatively assured. *L. brevis* was obtained shortly before use, as ATTC 4510.

The cultural procedure was as described in an earlier paper[19] with, however, 400 mg. of norleucine, 400 mg. of glycine, 10 g. of succinic acid, 20 mg. of xanthine, and 500 mg. of urea added per l. of medium.

Procedure for Sequence Determination.—The following is typical of the runs on the five dipeptides in Table II and the two in Table III. Peptide to an amount of 1.0 mg. was dissolved in 1.0 ml. of water (in some cases aliquots of a larger amount of solution were employed). To this was added 1 ml. of dry pyridine containing 20 mg. of phenyl isothiocyanate (or of phenyl isocyanate) and the clear solution was placed at 37° for 3–4 hr. The solution was then evaporated in a vacuum desiccator over sulfuric acid, and the residue was hydrolyzed with 2 ml. of 6 *N* hydrochloric acid for 18 hr. in the autoclave at 15 lb. pressure.

Excess hydrolytic acid was removed by heating over steam and finally evaporation in a desiccator over sodium hydroxide flakes. The residue obtained was made up into solution, the *p*H adjusted to 6.8, and appropriate amounts of clear liquid taken for either paper analysis or for microbiological assay. Each recovery value used in the final calculations was an average of four points obtained at different assay levels.

The second run reported for glycyl-DL-valine involved the addition of a drop of aqueous brom thymol blue indicator solution to the reaction flask, with adjustment of *p*H to 7.2–7.4 at the start of reaction, and after two hours, by addition of 0.2 *N* sodium hydroxide solution.

The cold partial hydrolysis of the third treatment of Table IV employed 1 ml. of dioxane saturated with HCl per mg. of original tripeptide at 25° for 6 hr. After reaction, the dioxane–HCl was removed in a vacuum desiccator over sodium hydroxide.

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(17) E. Fischer and A. H. Koelker, *Ann.*, **354**, 39 (1907).

(18) T. B. Gage, C. D. Douglass and S. H. Wender, *J. Chem. Educ.*, **27**, 159 (1950).

(19) S. W. Fox, M. Fling and G. N. Bollenback, *J. Biol. Chem.*, **155**, 465 (1944).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE PURDUE RESEARCH FOUNDATION, PURDUE UNIVERSITY]

The Reaction of 1,3-Dichloro-2,4,6-trinitrobenzene with Amino Acids[1,2]

BY H. FEUER, G. B. BACHMAN AND J. P. KISPERSKY[3]

The reaction of 1,3-dichloro-2,4,6-trinitrobenzene with amino acids results in the formation of dibasic acids. The preparations of N,N′-bis-(carboxymethyl)-2,4,6-trinitrophenylenediamine (styphnylbisglycine) and N,N′-bis-(carboxyethyl)-2,4,6-trinitrophenylenediamine (styphnylbis-β-alanine) are described. Nitration of these acids leads to N,N′-dinitro substituted compounds which may be converted to the corresponding acid chlorides. These react readily with alcohols to give esters which also are obtained directly by nitrating the esters of styphnylbisglycine and styphnylbis-β-alanine.

1,3-Dichloro-2,4,6-trinitrobenzene (styphynyl chloride) has recently become readily available by a new synthesis which was developed in this Laboratory.[4] The reaction of styphnyl chloride (I) with amino acids has now been investigated (see Fig. 1). A literature search revealed that the only reference to this type of compound is reported by Hirayama[5] who prepared the trinitrophenyl derivatives of several amino acids from picryl chloride. His reaction conditions, a two-phase system (water–toluene) in the presence of sodium hydroxide at room temperature, have now been employed successfully for the preparation of the esters of two diamino dibasic acids from styphnyl chloride (Va,b), but have given negative results in the preparation of the corresponding free acids. When the reaction with the free amino acids was carried out at room temperacture (*ca.* 25°), 87% of the styphnyl chloride was recovered. At higher temperatures (*ca.* 90–95°) a very small yield of a dark red solid which still contained chlorine was obtained. A good yield of styphnylbisglycine, however, was obtained at 50° in the presence of aqueous ethanol and a sufficient quantity of sodium carbonate to form the disodium salt of the acid. The latter crystallized from the reaction mixture upon cooling. Styphnyl chloride was also condensed with β-alanine in a similar manner.

Nitration of the above acids and their esters to the N,N′-dinitro derivatives (IIIa,b and VIa,b) was successfully accomplished with a mixture of equal volumes of concd. sulfuric acid and fuming nitric acid. No good crystallizing medium could be found for the N,N′-dinitrostyphnylbisglycine. Attempts to recrystallize it from hot water caused hydrolysis of the side-chain with the formation of styphnic acid (1,3-dihydroxy-2,4,6-trinitrobenzene). This hydrolysis was not observed with N,N′-dini-

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(3) Aerojet Engineering Corporation, Azusa, California.

(4) H. B. Hass, H. Feuer and A. A. Harban, THIS JOURNAL, **72**, 2282 (1950).

(5) K. Hirayama, *Z. physiol. Chem.*, **59**, 290 (1909).