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Bacterial utilization and sequence determination of peptides

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BACTERIAL UTILIZATION AND SEQUENCE DETERMINATION OF PEPTIDES

by

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INTRODUCTION

The known importance of proteins and peptides in the life processes makes the elucidation of the structure of these compounds a worthwhile objective. The most difficult part of the problem is the determination of the sequence of the amino acid residues of which proteins and peptides consist. This thesis is partly devoted to the search for techniques of sufficient simplicity and sensitivity to aid in this determination.

Results obtained in the investigation of the utilization by Lactobacillus arabinosus of the four stereoisomeric leucylleucines[1] indicated that it might be possible to employ the enzyme systems of this microorganism in vivo to determine amino acid residue sequence in peptides. Investigation of this possibility yielded results which, in turn, stimulated interest in the specificities exhibited by bacteria in their utilization of amino acid derivatives. A part of this thesis records the study of bacterial utilization of these derivatives.

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[1] S. W. Fox, Y. Kobayashi, S. Melvin, and F. N. Minard, J. Am. Chem. Soc., 70, 2404 (1948).

HISTORICAL

Determination of Amino Acid Sequence in Peptides

The extensive literature on the subject of sequence determination in peptides has been reviewed by Fox[1] in 1945. Since that time some of the older methods have been further developed and several methods which are new in principle or application have been reported.

Methylation

Abderhalden and Sickel[2] methylated leucylproline by means of diazomethane, forming N-dimethylleucylproline methyl ester. It was not necessary to hydrolyze the compound for identification. Zimmerman[3] and Zimmerman, McPhail and Canzanelli[4] used trimethylation by methyl

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[1] S. W. Fox, Advances in Protein Chemistry, 2, 155 (1945).

[2] E. Abderhalden and H. Sickel, Z. physiol. Chem., 159, 163 (1926).

[3] W. Zimmermann, Z. physiol. Chem., 231, 19 (1935).

[4] W. Zimmermann, M. K. McPhail, and A. Canzanelli, Z. physiol. Chem., 231, 25 (1935).

sulfate to detect glycine residues when present on the amino end of peptides. If glycine occupied the terminal position, betaine hydrochloride could be detected after hydrolysis by reason of its insolubility in absolute ethanol. A method so obviously limited is, however, of slight value, and an improved procedure was introduced by Bowman and Stroud[1], and Bowman[2]. These workers found that peptides were converted to their dimethyl derivatives by reductive condensation with formaldehyde, palladized charcoal being used as a catalyst. After acid hydrolysis the dimethyl derivative of the amino acid which bore the terminal amino group on the original peptide could be extracted by boiling ethanol and identified. This method is still limited to the determination of a single residue per molecule, however.

The reaction has been applied in a subtractive way by Ingram[3] in developing a method applicable to very small amounts of peptide. The reaction is run in a centrifuge tube on five micromoles of peptide. The catalyst is

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[1]R. E. Bowman and H. H. Stroud, J. Chem. Soc., 1950, 1342.

[2]R. E. Bowman, J. Chem. Soc. 1950, 1349.

[3]V. M. Ingram, Nature, 166, 1038 (1950).

removed after reaction by centrifugation and excess formaldehyde by evaporation. The treated peptide is then hydrolyzed and the hydrolysate chromatographed on paper. Since a dimethylated amino acid does not produce a color with ninhydrin the terminal residue may be detected by its absence from the chromatogram after treatment. Any free amino groups other than the terminal one would also be dimethylated, but the amino acid bearing such a group would still produce a spot, though at a different position. The method is able to detect only the terminal amino acid of a peptide.

Aryl isocyanates and isothiocyanates

Bergmann, Kann and Miekeley[1] employed phenylisocyanate to form the phenylureido derivative of a peptide. Subsequent acid hydrolysis split the peptide bonds, forming the phenylureido derivative of the terminal amino acid. This compound underwent cyclization under the conditions of the hydrolysis, yielding the corresponding phenylhydantoin, which could be easily separated and identified. These reactions may be represented as follows:

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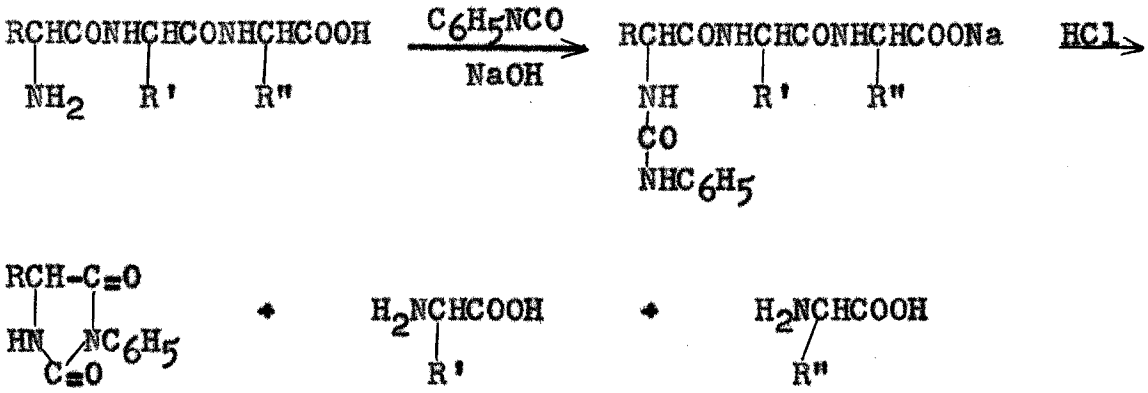
[1] M. Bergmann, E. Kann, and A. Miekeley, Ann., 458, 56 (1927).



Using methanolic hydrochloric acid at 60-65° for one-half hour, Abderhalden and Brockmann[1] were able to split phenylureido peptides at the peptide bond adjacent to the phenylcarbamyl group without marked hydrolysis of the other peptide bonds. The other product of the hydrolysis was the original peptide less its terminal residue, and this shortened peptide could be subjected to a repetition of the treatment with phenylisocyanate and a second hydrolysis to determine a second residue. Thus a stepwise degradation of the peptide was achieved. The authors found that the peptide linkages beyond the one adjacent to the phenylcarbamyl group were also hydrolyzed to a slight extent, however, and this fact would prevent use of the method for determination of large peptides.

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[1] E. Abderhalden and H. Brockmann, Biochem. Z., 225, 386, (1930).

Edman[1,2] substituted phenyl isothiocyanate for phenyl isocyanate as the marking reagent and found that the derivatives of peptides obtained by its use were cleaved swiftly and at room temperature by dry hydrogen chloride in nitromethane. Cleavage took place exclusively at the peptide bond adjacent to the phenylthiocarbamyl group. Hydrolysis of the thiohydantoin to obtain the corresponding amino acid followed by paper chromatography served to identify it. The method in this form constitutes a practical means of stepwise degradation and characterization of a peptide, but requires about ten milligrams of the peptide per residue. Edman[3] could not obtain the phenyl thiohydantoins of cystine, serine and theonine in crystalline form. He also reports that the formation of the hydantoins is attended by racemization.

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[1] P. Edman, Arch. Biochem., 22, 475 (1949).

[2] P. Edman, Acta Chem. Scand., 4, 283 (1950).

[3] P. Edman, Acta Chem. Scand., 4, 277 (1950).

Nitrophenyl reagents

Abderhalden and Blumberg[1] showed that 2,4-dinitrophenyl chloride gave derivatives with amino acids which could be readily characterized. Abderhalden and Stix[2] attempted to use this reagent to mark the terminal amino acids of peptides but were not able to fractionate the mixtures obtained upon hydrolysis. Barger and Tutin[3] used 2,4,5-trinitrotoluene in the characterization of carnosine and Keil[4] employed the same reagent for anserine. Quastel and coworkers[5] used 2,3,4-trinitrotoluene in partially elucidating the structure of glutathione. In the case of each of these reagents the labilizing influence of the two nitro groups, ortho and para respectively to a third substituent

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[1]E. Abderhalden and P. Blumberg, Z. physiol. Chem., 65, 318 (1910).

[2]E. Abderhalden and W. Stix, Z. physiol. Chem., 129, 143 (1923).

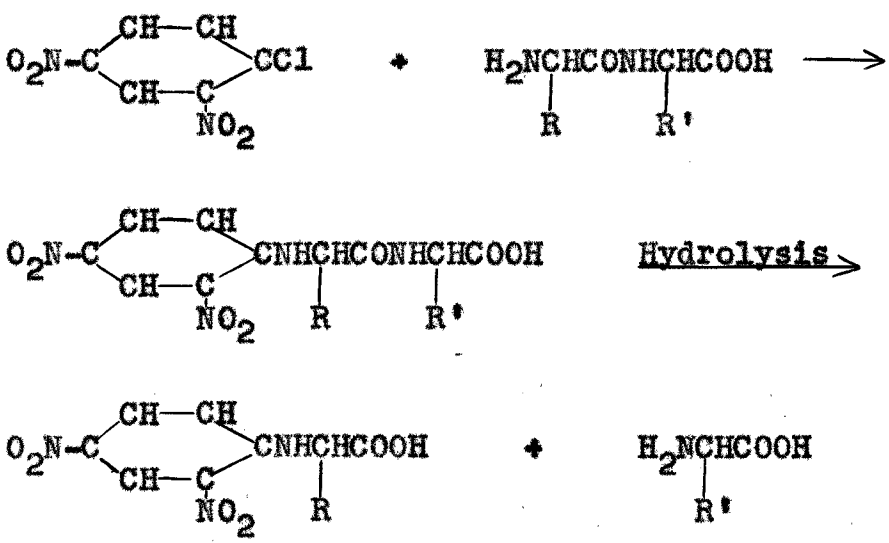
[3]G. Barger and F. Tutin, Biochem. J., 12, 402 (1918).

[4]W. Keil, Z. physiol. Chem., 187, 1 (1930).

[5]J. H. Quastel, C. P. Stewart, and H. E. Tunnicliffe, Biochem. J., 17, 586 (1923).

on the benzene ring, facilitated the removal of the substituent and condensation of the dinitrophenyl group with the free amino group of the peptide. The bond so formed was found to be more resistant to hydrolysis than the peptide linkage, so that the terminal amino acid was marked for identification.

The action of the dinitrophenyl reagents in general may be typified by that of 2,4-dinitrophenyl chloride:



Peptides couple with 2,4-dinitrofluorobenzene in the cold. Using this reagent and introducing partition chromatography as a means of separating mixtures of 2,4-dinitrophenyl peptides resulting from partial hydrolysis of the

corresponding derivative of insulin, Sanger[1,2,3] obtained evidence leading to the conclusion that insulin is composed of two pairs of peptide chains connected by cross-linkages. Two of the four chains begin with the sequence glycyl-isoleucyl-valyl-glutamyl-glutamic acid and the other two begin with the sequence phenylalanyl-valyl-aspartyl-glutamic acid. Somewhere within each of the latter pair of chains is the group threonyl-prolyl-lysyl-alanine. This work probably represents the farthest advance to date toward the elucidation of the structure of a protein, but the procedure must be rather tedious and the sample required rather large.

In their brilliant investigation of the structure of gramicidin S, Consden, Gordon, Martin and Synge[4] introduced the use of paper chromatography. After partial hydrolysis of the antibiotic, four dipeptides and two tripeptides were separated on paper. The structure of each dipeptide was then determined in two steps. In the first step, the

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[1]F. Sanger, Biochem. J., 39, 507 (1945).

[2]F. Sanger, Nature, 162, 491 (1949).

[3]F. Sanger, Biochem. J., 45, 563 (1949).

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

peptide was hydrolyzed completely and its amino acids identified by chromatography. In the second step the peptide was deaminated by nitrosyl chloride, then hydrolyzed and chromatographed. The acid which carried the free amino group in the peptide failed to produce a color when treated with ninhydrin. Only the terminal amino acid of the tripeptides could be determined by this method, but the order of the other two acids could be arrived at from a study of the dipeptides considered as degradation products of the tripeptides. The results were checked by comparison with synthetic peptides. In this manner Gramicidin S was demonstrated to contain amino acid residues in the order -valyl-ornithyl-leucyl-phenylalanyl-prolyl-. Other evidence indicates that it is a cyclic compound and contains the above sequence twice repeated.

This method was been applied to the study of the structure of wool[1] and a large number of dipeptides from wool hydrolysates have been identified. Large proteins are probably too complex to be completely characterized by this method, however.

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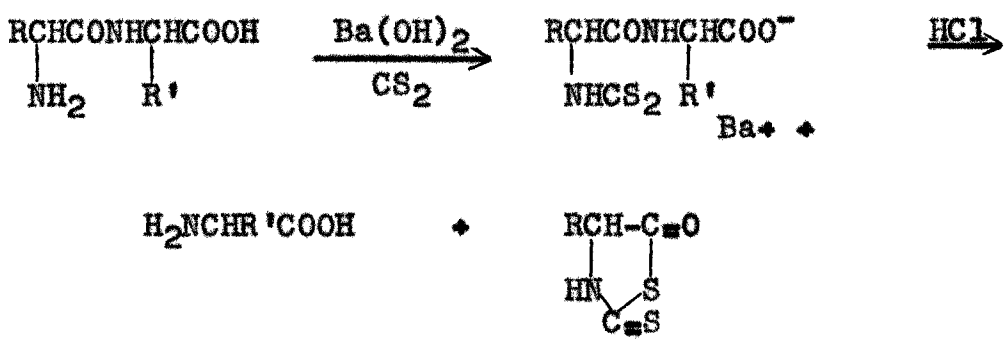
[1]F. Sanger, "Partition Chromatography". Biochemical Society Symposia No. 3, p. 27. Cambridge, England, Cambridge University Press. 1950.

Carbon disulfide

Levy[1] reported the use of carbon disulfide in a new method for peptide degradation. The N-dithiocarboxy derivative formed by this reagent is split on treatment with dilute mineral acid, the products being the original peptide, less its terminal residue from the amino end, and the 2-thiothiazolid-5-one corresponding to the residue lost. This method is therefore adapted to stepwise degradation, since the shortened peptide may be subjected to a repetition of the treatment. The degradation of a peptide by one residue is illustrated below:



Methyl ethyl xanthate

Another recently developed procedure is that reported by Khorana.[2] Peptides are reacted with methyl ethyl

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[1]A. L. Levy, J. Chem. Soc., 1950, 404.

[2]H. G. Khorana, Chemistry & Industry, 1951, 129.

xanthate to form the N-thiocarbethoxy derivatives which can then be split exclusively at the peptide linkage adjacent to the thiocarbethoxy group by dry hydrogen chloride in nitromethane. The thiazolid-2, 5-dione corresponding to the terminal amino acid is produced and may be identified. The shortened peptide may be subjected to repeated applications of the same procedure to characterize it more completely. This method also is too recent to have been thoroughly tested.

Enzymic methods

Efforts to use enzymes which split off terminal amino acids for peptide sequence studies have been hampered by the difficulty of obtaining pure enzyme preparations.[1] The possibility of resynthesis, with formation of sequences not originally present in the peptide, also exists. However, Grassman, Dyckerhoff and Eibeler[2] showed that glycine is terminal in glutathione by use of carboxypeptidase and Waldschmidt-Leitz and coworkers[3] used protaminase to

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[1]S. W. Fox, Advances in Protein Chemistry, 2, 155 (1945).

[2]W. Grassmann, H. Dyckerhoff, and H. Eibeler, Z. physiol. Chem., 189, 112 (1930).

[3]E. Waldschmidt-Leitz, F. Ziegler, A. Schaffner, and L. Weil, Z. physiol. Chem., 197, 219 (1931).

demonstrate that both clupein and salmine contain a terminal arginine.

Lens[1] attempted to determine more than one terminal amino acid residue in insulin by means of carboxypeptidase. The course of digestion was followed by van Slyke amino nitrogen analysis, and samples digested for different periods of time were subjected to ultrafiltration and the filtrate chromatographed. When analysis showed that 2.6 amino groups had been liberated per mole of insulin these groups were found to belong to alanine. When 3.16 groups had been liberated there appeared on the chromatogram faint spots representing glycine, valine, the leucines, tyrosine and the monoaminodicarboxylic acids.

Fromageot and coworkers[2] found that two of the peptide chains of insulin have glycine residues at their carboxyl ends and that the remaining two have terminal alanine residues. Their work thus agrees with the report of Lens. The method used for determining the terminal residues consisted in reducing the free carboxyl groups of insulin by means of lithium aluminum hydride, then

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[1] J. Lens, Biochim. et Biophys. Acta, 3, 367 (1949).

[2] C. Fromageot, M. Jutisz, D. Meyer, and L. Penasse, Biochem. et Biophys. Acta, 6, 283 (1950).

hydrolyzing and identifying the aminoalcohol produced by paper chromatography. The method was made quantitative by treating the aminoalcohol with periodic acid and determining the aldehyde formed.

Bacterial Utilization of Amino Acid Derivatives

During the past decade the nutritional requirements of certain lactic acid bacteria have been sufficiently well defined in terms of known compounds to permit the use of these organisms in assays for vitamins and amino acids[1,2]. The principle of such assays consists in comparing the growth effect produced by the sample with that produced by the pure substance to be determined.

With the increasing use of lactic acid-forming bacteria in amino acid assays has come an interest in the specificity of the growth response of the assay organisms. Peptides are obviously among the compounds found in biological

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[1]S. Shankman, M. N. Camien, H. Block, R. B. Merrifield, and M. S. Dunn, J. Biol. Chem., 168, 23 (1947).

[2]M. S. Dunn, S. Shankman, M. N. Camien, and H. Block, J. Biol. Chem., 168, 1 (1947).

preparations which are most likely to interfere in determinations of amino acids. Reports of experiments on utilization of peptides began to appear almost as soon as assay methods were developed, but at first consisted chiefly of scattered observations.

Kuiken and others[1] reported that the L-leucine content of DL-leucylglycine was used to the extent of 80 per cent, while that of glycyl-L-leucine was used from 80 per cent to 100 per cent by Lactobacillus arabinosus. Schweigert and Snell[2] reported the testing of four peptides composed of leucine and glycine residues for ability to replace leucine for L. arabinosus. All four peptides were utilized to a considerable degree although the reported results for each compound varied over a rather wide range. The least available was DL-leucyl-glycylglycine, utilized 30-60 per cent.

Lewis and Olcott[3] found that L-glutamyl-L-glutamic acid was only slightly available (10-24 per cent) as a

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[1]K. A. Kuiken, W. H. Norman, C. M. Lyman, F. Hale, and L. Blotter, J. Biol. Chem., 151, 615 (1943).

[2]B. S. Schweigert and E. E. Snell, Nutrition Abstracts & Revs., 16, 497 (1947).

[3]J. C. Lewis and H. S. Olcott, J. Biol. Chem., 157, 265 (1945).

replacement for free glutamic acid for L. arabinosus, while L-glutamyl-L-tyrosine was practically unavailable. On the other hand, the glutamic acid content of glutathione was readily available (69-94 per cent).

Agren[1] was led to investigate the utilization of amino acids involved in peptide linkages by observation of the differences in the conditions used by various investigators for hydrolysis of proteins prior to amino acid assay, and especially by the work of Lugg[2] who showed that some of these methods did not produce complete hydrolysis. Agren first used three lactic acid bacteria, Streptococcus faecalis (ATCC No. 9790), Lactobacillus delbrueckii, and Lactobacillus casei, studying their growth responses to peptides containing leucine or valine combined in various arrangements with glycine and alanine. The medium of McMahan and Snell[3] was used for L. casei while that of Stokes and others[4] was used for the other two micro-

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[1]G. Agren, Acta Physiol. Scand., 13, 347 (1947).

[2]J. W. H. Lugg, Biochem. J., 40, 88 (1946).

[3]J. R. McMahan and E. E. Snell, J. Biol. Chem., 152, 83 (1944).

[4]J. L. Stokes, M. Gunness, I. M. Dwyer, and M. C. Caswell, J. Biol. Chem., 160, 35 (1945).

organisms. The configuration of the leucine and valine residues was not definitely stated, but in a later publication[1] it was implied that they were racemic.

Agren's results showed that the ability of a given organism to use the valine or leucine content of the peptides tested depended both on the position of the valine or leucine in the peptide and on the nature of the other residues present. Moreover, the microorganisms differed in their ability to use the same peptide. The presence of alanine, and to a lesser extent glycine, on the carboxyl end of a peptide made utilization of the valine or leucine content more difficult.

The author suggested that further development of his investigations might yield a method for the determination of amino acid residue sequence in peptides, but he did not enlarge upon this statement, and it is not clear what sort of procedure he had in mind.

Krehl and Fruton[2] studied the growth response of L. arabinosus and Strep. faecalis to leucine peptides containing glycine and tyrosine residues. Both bacteria were

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[1] G. Agren, Acta Chem. Scand., 2, 611 (1948).

[2] W. A. Krehl and J. S. Fruton, J. Biol. Chem., 173, 479 (1948).

able to use the L-leucine content of all the peptides, although the percentage utilization varied with the microorganism and with the peptide. In the case of glycylleucine and of leucylglycine, a comparison could be made of the results obtained by Krehl and Fruton and those of Agren.[1] The former found that 70 to 80 per cent of the L-leucine content of these peptides was available to Strep. faecalis while Agren obtained complete utilization with this organism. In his second publication on the subject, Agren[2] suggested that the difference might be due to the use of different media, and he accordingly altered his medium to coincide with that of Henderson and Snell[3] in order to afford a better basis for comparison of results. He also extended his investigations to include a total of ten lactic acid bacteria. Four dipeptides and four tripeptides containing leucine in various combinations with glycine and alanine were tested.

The results confirmed those previously obtained by the author in showing that the utilization of leucine in

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[1] G. Agren, Acta Physiol. Scand., 13, 347 (1947).

[2] G. Agren, Acta Chem. Scand., 2, 611 (1948).

[3] L. M. Henderson and E. E. Snell, J. Biol. Chem., 172, 15 (1948).

leucine peptides depends on the position of the leucine and on the nature of the surrounding residues. The results obtained with the new medium did not differ from those obtained on the old medium in the cases of L. casei and Strep. faecalis. In the case of L. delbrueckii LD5, however, complete utilization of the leucine peptides occurred when the medium was that of Henderson and Snell while the L-leucine of DL-leucylglycine was only partially used and the L-leucine of glycyl-DL-leucylglycine was not used at all on the medium of Stokes and others. Agren suggested that the higher magnesium and manganese content of the medium of Henderson and Snell may result in the activation of bacterial peptidases necessary for utilization.

Fox, Kobayashi, Melvin and Minard[1] assayed the four stereoisomeric leucylleucines for growth-promoting activity toward L. arabinosus and obtained results which are in agreement with those of Agren in assigning importance to the order in which amino acids are incorporated in peptides. All the L-leucine content of D-leucyl-L-leucine and L-leucyl-L-leucine was utilized, and D-leucyl-D-leucine gave no growth response at all, as predicted. However, L-leucyl-D-leucine, surprisingly, also showed no activity. Thus

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[1]S. W. Fox, Y. Koyabashi, S. Melvin, and F. N. Minard, J. Am. Chem. Soc., 70, 2404 (1948).

only when the residue on the carboxyl terminus of the peptide was of the L-configuration was any part of the peptide available.

There have been numerous instances of better growth response by microorganism to a given amino acid supplied as a part of a peptide than to the free acid itself in equimolar quantity. Woolley[1] found that DL-serylglycyl-glutamic acid had "strepogenin activity" of a low order for Strep. lactis. Later experiments showed that glycyl-serylglutamic acid, alanylglycylglutamic acid, and glycylalanylglutamic acid also possessed such activity.[2] Riesen and others[3] reported that L. lactis grew twenty times as well when glutathione rather than free cystine was used as a source of that amino acid. Lactobacillus pentosus also utilized glutathione better than cystine.

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[1]D. W. Woolley, J. Biol. Chem., 166, 783 (1946).

[2]D. W. Woolley, J. Biol. Chem., 172, 71 (1948).

[3]W. H. Riesen, H. H. Spengler, A. R. Robblee, L. V. Hankes, and C. A. Elvehjem, J. Biol. Chem., 171, 731 (1947).

Simmonds and Fruton[1] discovered a mutant strain of E. coli which required a source of proline in the medium but which utilized proline peptides much better than free proline. Strains requiring phenylalanine and leucine, however, did not respond better to peptides containing these amino acids than to the acids themselves.[2] The same workers[3,4] isolated a microorganism called by them Strain SF which grew more rapidly in a medium containing leucylglycine than in a medium containing a mixture of leucine and glycine as the source of nitrogen.

Malin, Camien and Dunn[5] tested a number of glycine derivatives for availability as sources of glycine for five lactobacilli. Seven peptides were among the compounds tested and the results which pertain to them are reproduced in Table I.

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[1] S. Simmonds and J. S. Fruton, J. Biol. Chem., 174, 705 (1948).

[2] S. Simmonds and J. S. Fruton, J. Biol. Chem., 180, 635 (1949).

[3] S. Simmonds and J. S. Fruton, Science, 109, 561 (1949).

[4] S. Simmonds and J. S. Fruton, Science, 111, 329 (1950).

[5] R. B. Malin, M. N. Camien, and M. S. Dunn, Arch. Biochem. Biophys., 32, 106 (1951).

Table I

Per Cent Utilization of Glycine in Peptides[a]

Peptide	Microorganism				
	Leuc. citrovorum	Leuc. mesenteroides	L. brevis	L. pentoaceticus	S. faecalis R
	76 hrs.	76 hrs.	96 hrs.	96 hrs.	96 hrs.
Glycylglycine	119	100	216	132	85
Glycyl-L-leucine	158	98	296	233	81
Glycyl-L-tyrosine	124	104	514	278	148
DL-alanylglycine	56	73	171	144	43
DL-leucylglycine	59	50	171	188	44
L-leucylglycylglycine	122	83	128	114	89
Glutathione	6	13	10	11	5

[a]R. B. Malin, M. N. Camien, and M. S. Dunn, Arch. Biochem. Biophys., 32, 106 (1951).

This table shows that all the dipeptides and one of the tripeptides exhibited greater activity for one or more of the lactobacilli than could be accounted for on the basis of glycine content. The variation of activity with concentration and incubation time was studied and activity was found to decrease as either of these two factors increased. The concentration used in obtaining the data

for the table above was that required to give half maximal growth, in each case.

Several reports have appeared to the effect that partial hydrolysates of various proteins are more active in promoting growth than a mixture of their constituent amino acids.[1,2] This enhanced activity may be due to better utilization of amino acids incorporated in peptides.

Three possible explanations of the stimulatory effect of proline peptides have been advanced by Simmonds and Fruton.[3] They may presumably be extended to cases of the same effect when produced by other peptides. The first explanation, and the one which has received most attention from other workers, is that some peptides are incorporated in the protein molecule intact. But in the case of the proline peptides, all those tested were more efficient in promoting the growth of a prolineless E. coli mutant than was free proline. It is hard to understand how every position requiring a proline residue in every protein of a

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[1] H. Sprince and D. W. Woolley, J. Am. Chem. Soc., 67, 1734 (1945).

[2] M. Klungsor, R. J. Sirny, and C. A. Elvehjem, J. Biol. Chem., 189, 557 (1951).

[3] S. Simmonds and J. S. Fruton, J. Biol. Chem., 180, 635 (1949).

bacterial cell could be adequately filled by proline bound in an unvarying sequence to other amino acids. It is even more difficult to visualize the same positions filled in a second experiment by proline bound in a second, different, invariant sequence. These objections, however, do not rule out hydrolysis of a part of the peptide supplied, the remainder being used intact and accounting for the stimulation.

The second possibility suggested was that free proline may be converted to products not used for growth, whereas proline peptides are not so converted. But if the peptides are hydrolyzed before use, then their proline content becomes free proline and should be subject to the same sort of diversion as if it had been supplied in the free form originally. Splitting of the peptide might occur, however, after attachment of the proline at the site of its use or simultaneously to its attachment, and this is in fact the third alternative, which Simmonds and Fruton call transpeptidation. It is understood, of course, that other explanations of peptide stimulation of growth are not excluded.

Virtanen and Nurmikko[1] have reported an experiment designed to elucidate the mechanism of utilization of peptides by Leuc. mesenteroides. The same workers[2] had tested four dipeptides involving glycine residues and found that Leuc. mesenteroides could use the glycine content of each almost completely. To investigate the mechanism of utilization, three of these peptides, DL-alanylglycine, DL-leucylglycine, and glycylglycine were incubated with saline suspensions of the microorganism. Samples were withdrawn at intervals and chromatographed on paper. At first only the spot corresponding to the intact peptide appeared, but with longer incubation the spots characteristics of the constituent amino acids of the peptide appeared and the spot of the peptide grew fainter. In the case of glycylglycine the peptide spot disappeared completely. The conclusion that hydrolysis had occurred in the case of each of the three peptides was checked by reacting samples of the incubated solutions with ninhydrin after removal of the bacteria by centrifugation. Since the peptides and their amino acids produce colors of varying intensity with

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[1] A. I. Virtanen and V. Nurmikko, Acta Chem. Scand., 5, 681 (1951).

[2] V. Nurmikko and A. I. Virtanen, Acta Chem. Scand., 5, 97 (1951).

ninhydrin, breakdown of the peptides could be detected. The results supported the conclusion that hydrolysis had occurred in the case of each peptide.

The replacement of amino acids in the nutrition of lactic acid bacteria by their acyl derivatives has been studied because of interest in the specificity of the growth response of the organisms.[1] In addition, since it seems unlikely that the acylamino acids are incorporated intact in protein molecules, those which are used must be split by enzymes. It should be possible, therefore, to obtain information about the enzyme systems of bacteria by studying the utilization of acylamino acids and acylpeptides.

More than one type of enzyme may be involved in the hydrolysis of acylated amino acids. One possibility is the carboxypeptidases. Bergmann and associates[2] showed that the carboxypeptidase from the pancreas will attack chloroacetyl-L-tyrosine and it has since been shown to be active against many other acylamino acids. Another possibility is hippuricase, an enzyme or mixture of enzymes

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[1] C. H. Eades, Jr., J. Biol. Chem., 187, 147 (1950).

[2] M. Bergmann, L. Zervas, and H. Schleich, Z. physiol. Chem., 212, 72 (1934).

first discovered in animal kidney by Schmiedeberg.[1] Hippuricase preparations split hippuric acid and the N-acyl derivatives of other amino acids and have been prepared from certain bacteria. It has been suggested that in hippuricase one may be dealing with a number of closely related enzymes which might be called "aminoacylases".[2]

Hegsted[3] investigated the response of L. arabinosus to the acetyl derivatives of L-valine, DL-leucine, and DL-isoleucine. Of these, only acetyl-DL-leucine was utilized at all. Utilization varied from 30 to 60 per cent in different tests, based on the leucine content of both configurations. Hegsted also tested the four stereoisomeric benzoylvalylvalines and benzoyl-L-valine and found no activity for any of these compounds.

Eades[4] tested the acetyl and chloroacetyl derivatives of racemic leucine, phenylalanine, valine, and tryptophan

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[1]O. Schmiedeberg, Arch. exptl. Pathol. Pharmakol., 14, 379 (1881).

[2]F. Leuthardt, Hippuricase (Histozyme). In J. B. Sumner and K. Myrback, eds. The Enzymes. p. 951. New York, N. Y., Academic Press. 1951.

[3]D. M. Hegsted, J. Biol. Chem., 157, 741 (1945).

[4]C. H. Eades, Jr., J. Biol. Chem., 187, 147 (1950).

for utilization by L. arabinosus, L. casei, and Leuc. mesenteroides. Acetyl-DL-leucine was active only for L. arabinosus and acetyl-DL-tryptophan only for L. casei. The activities of the chloroacetylamino acids paralleled those of the corresponding acetylamino acids exactly. The acetyl-dehydro derivatives produced no growth response in any case.

Eades' work establishes the fact that striking variations exist in the abilities of microorganisms to use acylamino acids. The responses obtained here seem to be more clean-cut than those typically obtained with peptides, complete activity or no response at all being obtained. No cases of stimulation were observed. Leuc. mesenteroides proved to be the most fastidious of the bacteria tested, utilizing none of the acylamino acids.

Although both Hegsted and Eades found that acetyl-DL-leucine could replace leucine with high efficiency in the nutrition of L. arabinosus, Krehl and Fruton[1] found only six per cent activity for acetyl-L-leucine. Since it hardly seems probable that the DL form would be more readily available than the L-form, the divergent results obtained must be the result of some difference in the conditions of the test. The critical factor is difficult to determine, but

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[1] W. A. Krehl and J. S. Fruton, J. Biol. Chem., 173, 479 (1948).

it is clear that a different medium was used in each test. No obvious deficiencies appear in the medium of Krehl and Fruton, however. Perhaps the most likely explanation is that some genetic difference existed in the L. arabinosus strains used for the three experiments.

Among the glycine derivatives tested by Malin, Camien and Dunn[1] were hippuric acid and hippurylglycine. None of the five lactobacilli used in this experiment were able to utilize the latter compound with greater than five per cent efficiency. Hippuric acid gave growth responses ranging from 40 per cent for Leuc. citrovorum to 141 per cent for L. pentoaceticus. The apparent stimulation of the latter organism resembles the effect, previously discussed, of some peptides. An explanation involving splitting off of the benzoyl group is implied, since the hippuryl radical is not known to occur in biologically important materials. The failure of the five bacteria to utilize hippurylglycine is surprising in view of the fact that both hippuric acid and glycylglycine are readily utilized by them.

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[1]R. B. Malin, M. N. Camien and M. S. Dunn, Arch. Biochem. Biophys., 32, 106 (1951).

Nurmikko and Virtanen[1] also reported utilization of hippuric acid and failure to utilize hippurylglycine by Leuc. mesenteroides. They were, however, unable to demonstrate hydrolysis of hippuric acid by the microorganism by the procedure which they used to show hydrolysis of peptides.[2] They suggested that the breakdown of hippuric acid may occur inside the bacterial cell.

A few other acylamino acids have been tested. Stokes and coworkers[3] reported that the benzoyl derivatives of DL-lysine, DL-threonine, and DL-tryptophan are unavailable to Strep. faecalis (9790), while formyl-L-valine showed only barely detectable activity. Lewis and Olcott[4] reported that the p-aminobenzoyl and p-nitrobenzoyl derivatives of L-glutamic acid are not utilized by L. arabinosus.

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[1]V. Nurmikko and A. I. Virtanen, Acta Chem. Scand., 5, 97 (1951).

[2]A. I. Virtanen and V. Nurmikko, Acta Chem. Scand., 5, 681 (1951).

[3]J. L. Stokes, M. Gunness, I. M. Dwyer, and M. C. Caswell, J. Biol. Chem., 160, 35 (1945).

[4]J. C. Lewis and H. S. Olcott, J. Biol. Chem., 157, 265 (1945).

EXPERIMENTAL

Preparation of Compounds

Benzoyl-DL-isoleucine and benzoyl-DL-alloisoleucine

A procedure which is essentially that of Ingersoll and Babcock[1] was used in benzoylating DL-isoleucine. Ten grams (0.077 moles) of DL-isoleucine (Merck, Lot 41258) were dissolved in 75 ml. of 1 N NaOH and 11 gm. (0.078 moles) of benzoyl chloride (General Chemical) and 150 ml. 1 N NaOH were added in portions with cooling and stirring during about one hour. The solution was then acidified with HCl until no more precipitation occurred and stored overnight in the refrigerator. The precipitate was collected by filtration and boiled for ten minutes with carbon tetrachloride, then filtered and recrystallized from 50 per cent aqueous ethanol. Ten grams of product which melted at 136-137° were obtained (59 per cent yield). A second recrystallization did not change the melting point. Since

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[1] A. W. Ingersoll and S. H. Babcock, Org. Syntheses, coll. 2, 328 (1943).

Bouveault and Locquin[1] reported a melting point of 118° for benzoyl-DL-isoleucine it was necessary to check the identity of the product obtained. For this purpose samples of acetyl-DL-isoleucine and acetyl-DL-alloisoleucine[2] were obtained from Dr. Jesse Greenstein of the National Cancer Institute. A small sample of the acetyl-DL-isoleucine was hydrolyzed in 2 N HNl during two hours. The solution was evaporated to dryness and the dry residue was benzoylated by a procedure similar to that described for the Merck isoleucine. The product was extracted with warm carbon tetrachloride and recrystallized from aqueous alcohol. It melted at 138-139° and a mixed melting point taken with the product from benzoylation of the Merck isoleucine also melted at 138-139°. The material had a neutral equivalent of 235. (Calculated for $C_{13}H_{17}O_3N$: neutral equivalent, 236).

A sample of acetyl-DL-alloisoleucine treated in the same manner as the acetyl-DL-isoleucine gave a product melting at 118-119°. It was concluded that the product obtained from Merck isoleucine was benzoyl-DL-isoleucine

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[1]L. Bouveault and R. Locquin, Compt. rend., 141, 115 (1905).

[2]J. P. Greenstein, L. Levintow, C. G. Baker, and J. White, J. Biol. Chem., 188, 647 (1951).

and that the product obtained by Bouveault and Locquin was actually benzoyl-DL-alloisoleucine.

DL-valylglycyl-DL-phenylalanine

Eleven grams (0.05 moles) of glycyl-DL-phenylalanine (laboratory preparation) were dissolved in 50 ml. (0.05 moles) of 1 N NaOH. An additional 100 ml. of 1 N NaOH and 10 gm. (0.05 moles) of α-bromoisovaleryl chloride (laboratory preparation) were added in portions with stirring and cooling during 50 minutes. Upon acidification of the reaction mixture a solid precipitate was formed which was collected by filtration and dissolved in 1400 ml. of boiling ethanol. Upon cooling there was formed a precipitate which was collected by filtration and which weighed 4 gm. when air-dried. A sample decomposed at 206°. This material will be designated as Precipitate A. It was not subsequently used, or further identified.

To the filtrate from Precipitate A were added 1400 ml. of water which produced a second precipitation. The material obtained weighed 7.5 gm. and a sample decomposed at 192°. It will be designated as Precipitate B. Precipitates A and B were believed to be the two racemates possible for DL- α-bromoisovalerylglycyl-DL-phenylalanine or mixtures of the two racemates in different proportions. In the case of Precipitate B the analysis provided evidence

that it was some form of the bromo compound. The yield of Precipitate B amounted to 39 per cent based on the total glycylphenylalanine used.

Anal. Calc'd for $C_{16}H_{21}O_4N_2Br$: N, 7.3; Neut. equiv., 385.

Found for Precipitate B: N, 7.3[a]; Neut. equiv., 385.

[a]Determination run by Mr. A. MacMillan.

Seven grams (0.018 moles) of ∝-bromoisovalerylglycylphenylalanine (Precipitate B) were sealed with 60 ml. (3.0 moles) of 15 N ammonium hydroxide in a bottle and placed in an incubator at 37 for 15 days. The long period of amination was deemed necessary since it was known that some ∝-bromoisovaleryl compounds are difficult to aminate.[1] The amination mixture was evaporated under reduced pressure to a viscous oil. The oil was dissolved in 5 ml. of absolute alcohol, 200 ml. of ether were added and the solution stored in the refrigerator. The material reprecipitated as an oil which gradually solidified. The solid was

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[1]E. Abderhalden and V. Vlassopoulos, Fermentforschung, 10, 365 (1928-29).

filtered off and extracted several times with 90 per cent ethanol in an attempt to free it of bromide ion. Finally the solid was dissolved in 75 ml. of water and silver oxide added to precipitate the bromide. Excess silver was removed by means of hydrogen sulfide. The solution was concentrated under reduced pressure until precipitation began, then 100 ml. ethanol were added. After cooling, the precipitate was collected by filtration and dried at 100°. The yield was 0.8 gm. (14 per cent based on the α-bromoisovalerylglycylphenylalanine).

Anal. Calc'd for $C_{16}H_{23}O_4N_3$: N, 13.1;
Neut. equiv., 321.
Found, N, 13.1[a]; Neut. equiv., 331.

[a]Determination run by Mr. A. MacMillan.

Benzoyl-DL-leucylglycyl-DL-phenylalanine

DL-Leucylglycyl-DL-phenylalanine to the amount of 0.83 gm. was dissolved in 2.5 ml. of 1 N NaOH and 0.4 ml. of benzoyl chloride and 5.0 ml. of 1 N NaOH were added in alternate portions with cooling and shaking. The solution was poured into 5 ml. of 6 N HCl and cooled in the refrigerator. The precipitate which formed was filtered off and boiled in carbon tetrachloride for five minutes to

remove benzoic acid, then collected by filtration and recrystallized from aqueous ethanol. The yield was 0.4 gm. (36 per cent based on the tripeptide). A sample melted at 185-187°.

Anal. Calc'd for $C_{24}H_{29}O_5N_3$: N, 9.6;
Neut. equiv., 439.
Found: N, 9.4; Neut. equiv., 440.

Hippuryl-DL-phenylalanine

Four gm. (0.018 moles) of glycyl-DL-phenylalanine (laboratory preparation) were dissolved in 18 ml. of 1 N NaOH and 2.6 gm. (0.018 moles) of benzoyl chloride (General Chemical) and 36 ml. of 1 N NaOH were added in portions with cooling and stirring. After addition was complete the solution was acidified with HCl and cooled in the refrigerator. The precipitate was filtered off and recrystallized from aqueous alcohol. The yield of hippuryl-DL-phenylalanine was 4.9 gm. (83 per cent based on glycyl-DL-phenylalanine). A sample melted at 175°. Curtius and Muller[1] reported a melting point of 172°.

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[1] T. Curtius and E. Muller, J. prakt. Chem. N. S., 70, 223 (1904).

Compounds synthesized by procedures previously reported

Many of the compounds used in the experiments to be described have already been reported in the literature. When no major changes were introduced in the methods of synthesis used, these compounds are listed in Table II, together with a reference to a report of the synthesis of each. The benzoylamino acids were identified by melting point and the peptides by elementary analysis, supplemented in some cases by determination of the neutral equivalent, and by microbiological analysis.

The valylvalines were a gift from Dr. J. W. Hinman of the Upjohn Laboratories; their preparation has been described.[1] These peptides were received as the hydrochlorides, for which theoretical neutral equivalents had been found in the Kalamazoo Laboratories.

Benzoyldipeptides

A series of benzoylated dipeptides which were used in some of the experiments to be described were synthesized by Louis A. Carpino. The same general procedure was employed for the synthesis of all these compounds and may be outlined as follows.

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[1] J. W. Hinman, E. L. Caron, and H. N. Christensen, J. Am. Chem. Soc., 72, 1620 (1950).

Table II

Compounds Synthesized by Methods Previously Reported

Compound	Reference
Benzoyl-DL-alanine[a]	E. Fischer, Ber., 32, 2451 (1899).
Dibenzoyl-L-cystine	A. Schoberl, Ber., 76B, 964 (1943).
Benzoyl-L-glutamic acid[b]	E. Fischer, Ber., 32, 2451 (1899).
Benzoyl-DL-leucine[a]	E. Fischer, Ber., 33, 2370 (1900).
∝-Benzoyl-DL-lysine[c]	E. Fischer, Untersuchungen uber Aminosauren, Polypeptide, und Proteine, I., p. 233. Berlin, Verlag von Julius Springer. (1906)
Benzoyl-DL-methionine[a]	E. M. Hill and W. Robson, Biochem. J., 30, 248 (1936).
Benzoyl-DL-phenylalanine[a]	E. Fischer and A. Mouneyrat, Ber., 33, 2383 (1900).
Benzoyl-DL-threonine	H. D. West and H. E. Carter, J. Biol. Chem., 119, 109 (1937).
Benzoyl-DL-tryptophan[c]	J. Elks, D. F. Elliott, and B. A. Hems, J. Chem. Soc., 1944, 629. C. P. Berg, W. C. Rose, and C. S. Marvel, J. Biol. Chem., 85, 207 (1929).
N-Benzoyl-L-tyrosine[a]	S. W. Fox and C. W. Pettinga, Arch. Biochem., 25, 13 (1950).
Benzoyl-DL-valine[b]	M. D. Slimmer, Ber., 35, 400 (1902).

[a]Obtained from Dr. C. W. Pettinga.
[b]Obtained from Dr. Harry Wax.
[c]Obtained from Mrs. Jacquetta Strifert Halverson.

Table II (Cont'd)

Compound	Reference
DL-valyl-DL-leucine[a] and DL-leucyl-DL-valine[a]	S. W. Fox, T. L. Hurst, and K. F. Itschner, J. Am. Chem. Soc., 73, 3573 (1951).
DL-leucyl-DL-phenylalanine[b]	H. Leuchs and U. Suzuki, Ber., 37, 3306 (1904).
Glycyl-DL-leucine	E. Abderhalden, E. Rindtorff, and A. Schmitz, Fermentforschung, 10, 213 (1928-29).
Glycyl-DL-valine	Ibid.
DL-leucylglycyl-DL-phenylalanine	H. Leuchs and U. Suzuki, Ber., 37, 3306 (1904).
L-prolyl-L-leucine[c] and D-prolyl-D-leucine[c]	M. Fling, F. N. Minard, and S. W. Fox, J. Am. Chem. Soc., 69, 2466 (1947).
Benzoyl-DL-leucylglycine[d]	E. Fischer and A. Brunner, Ann., 340, 123 (1905).
Benzoyl-DL-valyl-DL-[d] valine B	J. W. Hinman, E. L. Caron, and H. N. Christensen, J. Am. Chem. Soc., 72, 1620 (1950).
Benzoylglycyl-DL-valine[d]	E. Abderhalden, E. Rindtorff and A. Schmitz, Fermentforschung, 10, 213 (1928-29).

[a]Obtained from Mr. T. L. Hurst.

[b]Obtained from Mr. Y. Kobayashi.

[c]Obtained from Dr. S. W. Fox.

[d]Obtained from Mr. L. A. Carpino.

The appropriate ∝-bromoacid bromides were synthesized by the Hell-Volhard-Zelinsky reaction. Chloroacetyl chloride was purchased from Eastman Kodak. One of the acid bromides, or chloroacetyl chloride, was coupled with an amino acid (racemic form) by the Schotten-Baumann reaction to form the ∝-haloacylamino acid. In every case where the compound possessed two asymmetric carbon atoms two racemic forms were possible for it. These were the L-L, D-D and the L-D, D-L racemates.[1] It was sometimes possible to separate these racemates by taking advantage of solubility differences, and when such a separation was accomplished, as indicated by a difference in physical properties, the two forms were arbitrarily designated as Form A and Form B.

The next step was the amination of the ∝-haloacylamino acids in a large excess of 15 N ammonium hydroxide, usually at 37° for three days, but sometimes at higher temperature. If separated, the A and B forms were aminated. The amination products were dipeptides which were benzoylated through the use of benzoyl chloride in a second Schotten-Baumann reaction. The A and B designations were carried through to the final products when applicable.

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[1] E. Fischer and A. H. Koelker, Ann., 354, 39 (1907).

Three of the benzoylated dipeptides have been previously reported and these are included in Table II. References to ten others have not been found, however, and these compounds are listed in Table III with analytical data pertaining to them.

Table III

Analytical Data on New Benzoyldipeptides

Benzoyl derivative of	Calculated		Found	
	Per cent nitrogen	Neut. equiv.	Per cent nitrogen	Neut. equiv.[a]
DL-Leucyl-DL-leucine A	8.04	348	7.90	352
DL-Leucyl-DL-valine A	8.37	334	8.22	341
DL-Leucyl-DL-valine B	8.37	334	8.19	343
DL-Leucyl-DL-alanine A	9.14	306	9.06	309
DL-Alanyl-DL-leucine	9.14	306	9.19	310
DL-Valylglycine	10.06	278	10.18	278
DL-Alanyl-DL-valine	9.58	292	9.25	299
DL-Valyl-DL-alanine A	9.58	292	9.58	295
DL-Valyl-DL-valine A	8.74	320	9.12	
Glycyl-DL-leucine	9.58	292	9.43	292

[a]Determinations by Louis A. Carpino.

Bacteriological Procedure

The organisms used were Lactobacillus arabinosus 17-5, ATCC No. 8014, Streptococcus faecalis R, ATCC No. 8043, and Lactobacillus brevis, ATCC No. 8287. Stock cultures were carried on 2 per cent yeast extract agar deeps[1] by monthly transfers. Loop transfers were made from the stock cultures to tubes containing 5 ml. of yeast extract medium[2] which were incubated for 18 hours at 37°. These 18-hour cultures were centrifuged, the broth was decanted, and the organisms were washed twice with physiological saline solution and resuspended in 20 ml. saline solution. One drop of this saline suspension was used to inoculate each tube.

The basal medium used for L. arabinosus was essentially that of Kuiken and others[3]. The composition of the complete, double-strength medium is given in Table IV. The medium used for Strep. faecalis was essentially that of Horn, Jones,

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[1] J. R. McMahan and E. E. Snell, J. Biol. Chem., 152, 83 (1944).

[2] J. R. McMahan and E. E. Snell, J. Biol. Chem., 152, 83 (1944).

[3] K. A. Kuiken, W. H. Norman, C. M. Lyman, F. Hale, and L. Blotter, J. Biol. Chem., 151, 615 (1943).

Table IV

Complete Medium for Lactobacillus arabinosus[a]

Glucose	40	gm.	$FeSO_4 \cdot 7H_2O$	20	mgm.
Sodium acetate (anhydrous)	14.4	gm.	$MnSO_4 \cdot 4H_2O$	20	mgm.
			L-Arginine HCl	400	mgm.
Adenine sulfate	10	mgm.	DL-Alanine	400	mgm.
Guanine HCl	10	mgm.	DL-Aspartic acid	800	mgm.
Uracil	10	mgm.	DL-Glutamic acid.H_2O	800	mgm.
Thiamin chloride	200	γ	L-Histidine HCl	400	mgm.
Pyridoxine HCl	200	γ	DL-Lysine	800	mgm.
Calcium pantothenate	200	γ	DL-Phenylalanine	400	mgm.
			L-Proline	400	mgm.
Biotin	0.8	γ	DL-Serine	400	mgm.
Riboflavin	400	γ	DL-Tryptophan	800	mgm.
Nicotinic acid	800	γ	DL-Methionine	400	mgm.
p-Aminobenzoic acid	1.0	γ	L-Tyrosine	200	mgm.
			DL-Leucine	400	mgm.
Folic acid	2.0	γ	DL-Valine	400	mgm.
K_2HPO_4	1.0	gm.	DL-Isoleucine	400	mgm.
KH_2PO_4	1.0	gm.	L-Cystine	400	mgm.
$MgSO_4 \cdot 7H_2O$	400	mgm.			
NaCl	20	mgm.			

Adjust to pH 6.8 and dilute with water to 1 liter.

[a]A slight modification of the medium given in Kuiken, et al., J. Biol. Chem., 151, 615 (1944).

Table V

Complete Medium for *Streptococcus faecalis*[a]

Glucose	40	gm.	DL-Alanine	400 mgm.
Sodium acetate (anhydrous)	15	gm.	L-Arginine HCl	400 mgm.
			DL-Aspartic acid	800 mgm.
			L-Cystine	400 mgm.
Adenine sulfate	10	mgm.	DL-Glutamic acid.H_2O	940 mgm.
Guanine HCl	10	mgm.	Glycine	400 mgm.
Uracil	10	mgm.	L-Histidine HCl.H_2O	400 mgm.
Thiamin chloride	2.0	mgm.	DL-Isoleucine	400 mgm.
Pyradoxamine HCl	0.4	mgm.	DL-Leucine	400 mgm.
Calcium pantothenate	0.4	mgm.	DL-Lysine HCl	300 mgm.
Riboflavin	0.4	mgm.	DL-Methionine	400 mgm.
Nicotinic acid	0.8	mgm.	DL-Norleucine	400 mgm.
p-Aminobenzoic acid	0.4	mgm.	DL-Phenylalanine	400 mgm.
Biotin	10	γ	L-Proline	400 mgm.
Folic acid	2.0	γ	DL-Serine	400 mgm.
K_2HOP_4	1.0	gm.	DL-Threonine	400 mgm.
KH_2PO_4	1.0	gm.	DL-Tryptophan	300 mgm.
$MgSO_4.7H_2O$	400	mgm.	L-Tyrosine	400 mgm.
$MnSO_4.4H_2O$	20	mgm.	DL-Valine	400 mgm.
NaCl	20	mgm.		
$FeSO_4.7H_2O$	20	mgm.		

Adjust to pH 6.8 and dilute with water to 1 liter.

[a]A modification of the medium given in Horn, Jones, and Blum, U. S. Dept. Agr. Misc. Pub., 696, 1 (1950).

and Blum.[1] Its composition is given in Table V. The medium used for L. brevis was the same as that used for Strep. faecalis except that 30 gm. of L-arabinose and 5 gm. of glucose per liter of medium were used in place of 40 gm. of glucose, and the sodium acetate level was increased to 40 gm. per liter.

The ingredients of the media were made up in several stock solutions which were combined as needed. Four solutions contained, respectively, all the vitamins, all the purines, the phosphates, and the remaining minerals, each in hundredfold concentration. The amino acids were made up singly in hundredfold concentration except that in some cases those acids considered non-essential were combined in a single stock solution, of fortyfold concentration in each acid. It was necessary to dissolve cystine in acid solution and to dissolve tyrosine and tryptophan in basic solution. The other acids were dissolved in distilled water. The stock solutions were stored in the refrigerator.

In order to make up medium for a run, the proper stock solutions were combined, carbohydrates and sodium acetate were added, and the pH adjusted to 6.8 ± 0.1 pH unit. Five

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[1] M. J. Horn, D. B. Jones, and A. E. Blum, U. S. Dept. Agr. Misc. Pub., 696, 1 (1950).

ml. of this double-strength medium was added to each tube and the final volume made up to 10 ml. by addition of water or of the solution to be tested. The pH was again adjusted if the test solution was not neutral. This adjustment was made by use of a Beckman pH meter equipped with a nine-inch electrode. After plugging with cotton the tubes were autoclaved at 15 pounds pressure for 15 minutes, then cooled and inoculated with one drop of saline suspension of the appropriate microorganism.

The tubes were incubated at 37°. Incubation lasted for 70-72 hours except where noted otherwise. Growth was estimated by titration with 0.1 N or 0.05 N sodium hydroxide or by reading turbidities on a Coleman Universal Spectrophotometer at 575 millimicrons.

In qualitative tests of the ability of a compound to replace an amino acid for one of the microorganisms, duplicate tubes were set up containing the basal medium with the omission of the acid in question. To these tubes was added the substance to be tested. At the same time duplicate tubes containing the medium lacking any source of the acid and duplicate tubes containing complete medium were prepared as controls.

In tests for inhibition, the substance tested was added to duplicate tubes containing the complete medium. Tubes

containing the complete medium without additions served as controls.

In quantitative assays for utilization of an amino acid derivative, the derivative was added to medium from which the acid had been omitted, as in the qualitative tests. In addition, graded amounts of the amino acid were added in duplicate to a series of tubes containing the medium complete except for this amino acid. From the growth response obtained in the latter tubes a standard curve could be constructed, turbidity or lactic acid production being plotted against concentration of the amino acid being assayed for. By comparing growth response in the tubes containing the derivative with the standard curve the percentage utilization of the derivative could be calculated. Each derivative was tested at several different levels.

Chromatographic Procedure

The procedure of Gage, Douglass, and Wender[1] was used. The solvent mixture consisted of the upper layer formed when

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

400 ml. n-butanol, 100 ml. glacial acetic acid, and 500 ml. of water were mixed. The chromatographic chamber was an empty reagent jar of suitable size, closed at the top only by a watchglass. Strips of Whatman No. 4 filter paper one inch wide and 15 inches long were ordinarily used. A pencil line drawn across the strip one inch from the lower end served as the starting line.

In making a chromatographic run, the solution to be tested was spotted on the center of the starting line by means of a capillary pipette. The diameter of the spot was kept as near one-half centimeter as possible. When the spot was dry, the strip was suspended in the chamber by means of a rubber band around the neck of the jar or by fastening the top of the strip with cellulose tape. In order to keep the strip from twisting, a short piece of glass rod was inserted through two parallel slits cut below the starting line in the long dimension of the strip. The strip was allowed to dip into the solvent mixture approximately one-half inch. Solutions of pure amino acids were run as controls simultaneously with the solutions tested.

When the solvent front had risen to the desired height in the paper, the strips were removed, the location of the solvent front was marked, and the strips were allowed to dry. When dry, the strips were sprayed with a 0.1 per cent

solution of ninhydrin (Eastman Kodak) in n-butanol and then heated in an oven at 80° for five to ten minutes. The amino acids and peptides appeared as colored spots.

Each spot was encircled by pencil and the center of density marked. The R_f value for each spot was calculated by the equation:

$$R_f = \frac{\text{Distance traveled by amino acid}}{\text{Distance traveled by solvent front}}$$

each distance being measured from the starting line. Identification was made by comparison of R_f's of unknown substances with those of known substances chromatographed simultaneously since the R_f value of a given compound varied in successive runs through the influence of a number of factors.[1]

Formation of butyl acetate in the solvent mixture upon standing necessitated frequent preparation of fresh solvent.

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

Procedure and Results in Amino Acid Sequence Studies

A review of the literature on the subject shows that there is no lack of chemical reagents suitable for use in determining amino acid residue sequence in peptides. The chief difficulty encountered in the exploitation of such reagents lies in the lack of techniques of sufficient simplicity and reliability for practical use.

Since peptides or proteins are difficult to prepare and to purify, a procedure capable of use on a micro scale is often essential. Methods allowing the determination of only the terminal residue of a peptide have value for determining the molecular weight and details of protein structure, but the development of procedures for the determination of a number of residues in succession is highly desirable if the complete structure of a peptide is to be elucidated.

Preliminary experiments were performed to test the feasibility of combining the use of certain blocking reagents with microbiological assays in a subtractive method for determining the order of amino acid residues in peptides. The first step in each experiment consisted of hydrolysis of a sample of the peptide and quantitative determination of its amino acid content by microbiological

assay. Next, a second sample of the peptide was blocked chemically, the treated sample was hydrolyzed and the hydrolysate again assayed. If the blocking reaction was complete, and if the blocking group could not be split off in the hydrolysis or the blocked amino acid utilized by the bacteria, then the residue occupying the terminal position on the original peptide should not show up in the second assay. If a selective method of hydrolysis could be employed in the second step so as to split off only the blocked, terminal residue, then the method could be extended to determine several residues in successive steps.

The first blocking reagent tested was nitrosyl bromide. One hundred milligrams of DL-leucylglycyl-DL-phenylalanine was dissolved in 20 ml. of water containing 0.35 ml. of 48 per cent hydrobromic acid. The solution was cooled in an ice bath and 1 ml. of bromine (General Chemical) was added, then nitric oxide passed into the solution for half an hour. Excess bromine was removed by aeration for 45 minutes. The solution was evaporated to dryness in a vacuum desiccator over sodium hydroxide. A sample of the treated peptide was hydrolyzed in 3 N hydrochloric acid in a sealed tube at 110° for two hours. The hydrolysate was assayed for leucine and phenylalanine, using L. arabinosus. A sample of the untreated peptide was similarly hydrolyzed and assayed, and

samples of the treated and untreated peptide, unhydrolyzed, were included in the determination.

The results obtained in this experiment are shown in Table VI. Percentages for each amino acid are based on the total L-form as 100 per cent.

Table VI

Effect of Nitrosyl Bromide Treatment on Recovery of Amino Acids in Leucylglycylphenylalanine

Treatment of peptide	Per cent recovery	
	Leucine	Phenylalanine
Nitrosyl bromide for 30 min., unhydrolyzed	16	6
Nitrosyl bromide for 30 min., hydrolyzed	8	6
Untreated peptide, unhydrolyzed	47	55-70
Untreated peptide, hydrolyzed	79	80

The results show not only a marked reduction in the availability of the leucine residue, but also a similar and unexpected reduction of the availability of the phenylalanine after treatment. The effect on phenylalanine may indicate attack by the reagent on the phenyl ring. Hydrolysis did not affect the utilization of the leucine in the blocked peptide.

The results for phenylalanine in the untreated, unhydrolyzed tripeptide varied considerably for the different levels at which the peptide was tested. A later determination showed utilization ranging from 45 to 53 per cent for four levels.

Since the nitrosyl bromide treatment appeared to produce side effects which would prevent its use for the purpose intended, this line of experimentation was discontinued.

The reagent of Abderhalden and Blumberg[1], 2,4-dinitrochlorobenzene was next employed as a blocking agent in an experiment similar to the one just described. The peptides treated were DL-leucylglycyl-DL-phenylalanine, DL-valyl-DL-leucine, and DL-leucyl-DL-valine. DL-leucine was also run for purposes of comparison.

The following is typical of the procedure used. Thirty-three mgm. of leucylglycylphenylalanine and 168 mgm. of sodium bicarbonate were dissolved in 2 ml. of water. Forty mgm. of 2,4-dinitrochlorobenzene in 5 ml. ethanol were added and the mixture refluxed for two hours. The solvent was evaporated and 7 ml. of water were added to the residue which partly dissolved. Three extractions by 15-ml. portions

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[1]E. Abderhalden and P. Blumberg, Z. physiol. Chem., 65, 318 (1910).

of ether removed all undissolved material. Aliquots of the aqueous solution were hydrolyzed in 3 N or 6 N hydrochloric acid for varying periods of time at 110° in sealed tubes. The hydrolysates were assayed with L. arabinosus.

Under the conditions of the experiment, 2,4-dinitrochlorobenzene (DNCB) failed to block effectively the utilization by L. arabinosus of the terminal residues of treated peptides, even when the molar ratio of reagent to peptide was increased beyond that specified in the procedure above. In Table VII are recorded the results obtained when treated peptides, hydrolyzed and unhydrolyzed, were assayed. All hydrolyses were done in sealed tubes at 110°, but the duration of the hydrolysis varied, as did the normality of the hydrochloric acid used.

Peptides treated with DNCB and hydrolyzed gave lower recoveries of the terminal amino acids than did the hydrolyzed, untreated peptides (see Table VI for hydrolyzed, untreated leucylglycylphenylalanine). This indicates that some blocking was achieved. The blocking was so incomplete, however, that the reagent, used under these conditions, was of no value as a means of determining sequence, and no further experiments with it were attempted. Free leucine was almost completely unavailable to L. arabinosus after treatment with DNCB.

Table VII

Effect of Treatment with 2,4-Dinitrochlorobenzene on Recovery of Amino Acids of Peptides

Material tested	Molar ratio DNCB to peptide	Hydrolysis conditions	Per cent utilization of amino acids
Leucylglycylphenylalanine, treated and hydrolyzed	2/1	3 N HCl 2 hours	Leucine, 51 Phenylalanine, 97
Leucylglycylphenylalanine, treated and unhydrolyzed	2/1	-------	Leucine, 33 Phenylalanine, 48
DL-Leucine, treated and unhydrolyzed	2/1	-------	Leucine, 9
Leucylglycylphenylalanine, treated and hydrolyzed	10/1	6 N HCl 2 hours	Leucine, 66 Phenylalanine, 198*
Leucylglycylphenylalanine, treated and hydrolyzed	10/1	6 N HCl 36 hours	Leucine, 63 Phenylalanine, 180*
Leucylglycylphenylalanine, treated and unhydrolyzed	10/1	--------	Leucine, 42 Phenylalanine, 62*
DL-Leucine, treated and unhydrolyzed	10/1	--------	Leucine, 6
Valylleucine, treated and hydrolyzed	10/1	6 N HCl 2 hours	Leucine, 76 Valine, 39

Table VII (Cont'd)

Material tested	Molar ratio DNCB to peptide	Hydrolysis conditions	Per cent utilization of amino acids
Valylleucine, treated and unhydrolyzed	10/1	-------	Leucine, 50 Valine, 0
Valylleucine, not treated, but hydrolyzed		6 N HCl 2 hours	Leucine, 79 Valine, 63

*The solution of phenylalanine used in tubes for the standard curve was found to contain bacterial growth.

When the treated peptides were hydrolyzed, assays for the terminal residue gave higher results than were obtained with samples treated but not hydrolyzed. This possibly indicated removal of the 2,4-dinitrophenyl group, but the effect could also be attributed to hydrolysis of that portion of the peptide sample which had failed to couple with the reagent.

DL-Leucyl-DL-valine and DL-valyl-DL-leucine were treated with carbon disulfide in alkaline medium according to the method of Levy.[1] Twenty mgm. of each peptide were separately treated with 0.2 ml. of 1 N potassium hydroxide and 0.1 ml. of carbon disulfide in small test tubes. The tubes were shaken for three hours at room temperature by fastening them to an electric sander. At the end of the period of shaking, the contents of each tube had developed a yellow color.

One drop of concentrated hydrochloric acid was added to each tube after cooling. The acidification resulted in the formation of a gum in each case. The gums did not dissolve when the contents of the tubes were diluted prior to microbiological assay with L. arabinosus.

The results obtained from the attempt to use carbon disulfide as the blocking reagent in a method for studying

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[1] A. L. Levy, J. Chem. Soc., 1950, 404.

peptide sequence appear in Table VIII. Samples of the untreated peptides, hydrolyzed and unhydrolyzed, were included in the assay.

Table VIII

Availability of Amino Acids of Peptides Treated with Carbon Disulfide

Material tested	Per cent recovery	
	Leucine	Valine
Leucylvaline treated with CS_2	11	25
Leucylvaline hydrolyzed eight hours in 6 N HCl at 120°	83	74
Leucylvaline, unhydrolyzed and untreated	36	75
Valylleucine treated with CS_2	11	0
Valylleucine hydrolyzed eight hours in 6 N HCl at 120°	90	73
Valylleucine, unhydrolyzed and untreated	55	0

The results indicate that both residues of both dipeptides were unavailable to L. arabinosus after carbon disulfide treatment. The most striking part of the data, however, concerned the inability of L. arabinosus to utilize the valine content of racemic valylleucine even though the leucine content of this peptide was available to the extent

of 55 per cent. Referring to Table VII it may be seen that valylleucine treated with 2,4-dinitrochlorobenzene gave a similar recovery. This seemed at the time to be due to coverage of the valine residue by the reagent.

Paper chromatography was used in conjunction with the blocking technique of Edman[1] to determine the order of amino acids in several synthetic dipeptides in a subtractive manner. The following procedure was found to yield satisfactory results in most cases.

To 1.0 mgm. of the dipeptide dissolved in 1.0 ml. of water was added 1.0 ml. of pyridine containing from 10 to 40 mgm. of phenylisothiocyanate. The solution was allowed to stand at 37° for four hours, then evaporated in a vacuum desiccator over sulfuric acid. The residue was hydrolyzed in 2.0 ml. of 6 N hydrochloric acid for 6, 12, or 18 hours in an autoclave at 15 lbs. pressure. The acid was evaporated in a vacuum desiccator over sodium hydroxide and the hydrolysate taken up in water at suitable dilution for paper chromatography.

It was found desirable to alter the procedure at the point of the phenylisothiocyanate treatment in order to

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[1] P. Edman, Acta Chem. Scand., 4, 283 (1950).

secure more complete blocking of the terminal residue. Glycine was found to be especially hard to block. The change consisted in adding alkali during incubation of the peptide with phenylisothiocyanate so as to keep the pH of the solution at a value between 7 and 8. Bromthymol blue was used as an indicator.

Table IX contains data obtained through use of the above method on a number of synthetic dipeptides.

When the untreated dipeptides were hydrolyzed and the hydrolysates chromatographed two spots always appeared in the chromatogram except in the case of the dipeptides composed of two like residues. By comparison with the chromatograms of the amino acid standards these spots could be recognized as being characteristic of the component amino acids of the peptides. Identification was made through R_f values, but in the case of leucine and phenylalanine, which have R_f values which differ only slightly, color was an aid to identification since the phenylalanine spot is much more blue than that of leucine.

When the dipeptides were treated with phenylisothiocyanate, then hydrolyzed and the hydrolysates chromatographed, one of the two spots always disappeared, and the remaining spot was observed to be that characteristic of the amino acid on the carboxyl end of the peptide. It was

Table IX

Data for Assignment of Sequence in Dipeptides Subjected to Phenylisothiocyanate and Hydrolytic Treatment

Material chromatographed	Spots on chromatograms		
	Treated, hydrolyzed peptide	Untreated hydrolyzed peptide	Amino acid standard
	R_f	R_f	R_f
DL-Valyl-DL-leucine	0.61	0.64; 0.45	
DL-Leucyl-DL-valine	0.44	0.45; 0.65	
DL-Leucyl-DL-phenylalanine	0.58	0.56; 0.65	
Glycyl-DL-leucine	0.65	0.66; 0.19	
L-Prolyl-L-leucine	0.63	0.65; 0.35	
D-Prolyl-D-leucine	0.62	0.66; 0.32	
Glycyl-DL-valine	0.45	0.45; 0.17	
D-Valyl-L-valine	0.48	0.48	
L-Valyl-D-valine	0.44	0.46	
DL-Leucine			0.66
DL-Valine			0.48
DL-Phenylalanine			0.60
L-Proline			0.32
Glycine			0.20

therefore possible to determine the order of the two residues in the peptide, except in the case of the valylvalines, where the two spots coincided in spite of the fact that the two residues possessed different configurations.

It has already been mentioned that terminal glycine was only partially blocked in earlier experiments, and a faint spot having the R_f value of glycine appeared on the chromatograms of glycyl peptides in such cases.

Complete determination of the sequence of amino acids in a tripeptide, DL-valylglycyl-DL-phenylalanine, involved the use of a method for selective hydrolysis after the first blocking reaction to split only the peptide bond nearest the phenylthiocarbamyl group. Edman[1] achieved this effect by hydrolyzing with nitromethane saturated with dry HCl. In these experiments dioxane was substituted for nitromethane with good results.[2] The sequence determination was accomplished in a series of steps.

In the first step, 5 mgm. of tripeptide were hydrolyzed with 5 ml. of 6 N hydrochloric acid for 16 hours in

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[1]P. Edman, Acta Chem. Scand., 4, 283 (1950).

[2]T. L. Hurst, A Quantitative Method for Determining Sequences of Amino Acid Residues. Unpublished M.S. Thesis. Ames, Iowa. Iowa State College Library. 1951.

an autoclave at 15 lbs. pressure. The acid was evaporated partially on a steam bath and the evaporation was completed in a vacuum desiccator. The residue was dissolved in the appropriate amount of water and chromatographed.

In the second step, 5 mgm. of the tripeptide were dissolved in 5 ml. of water. To this solution were added 5 ml. of pyridine containing 0.1 ml. phenylisothiocyanate and 0.1 mgm. bromthymol blue. Sodium hydroxide, 0.1 N, was added until a blue-green color was produced, and the pH was readjusted hourly during four hours incubation at 37°. After incubation, the solution was evaporated to dryness in a vacuum desiccator over sulfuric acid. Hydrolysis and chromatography were performed as in the first step.

Finally, another sample of 5 mgm. of the tripeptide was treated as in the second step up to the hydrolysis. The residue from the phenylisothiocyanate treatment in this case was hydrolyzed by adding 5 ml. of dry dioxane saturated with dry HCl and placing the mixture in a desiccator over calcium sulfate. Hydrolysis was continued for six hours at room temperature and was followed by evaporation to dryness over sodium hydroxide in a vacuum desiccator. To the residue were added 4 ml. of water and 5 ml. of pyridine containing 0.1 ml. of phenylisothiocyanate and the determination was completed as in the second step.

Figure 1 illustrates the extension of the phenylisothiocyanate method to the assignment of sequence in a tripeptide, in this case, valylglycylphenylalanine. Strips 1, 2, and 3 are chromatograms of the hydrolysates obtained from the first, second, and third steps, respectively, of the procedure outlined. Strips 4, 5, and 6 are, respectively, the chromatograms of standard solutions of DL-valine, glycine, and DL-phenylalanine. Spots representing all three of the amino acids appear in the first chromatogram. In the second hydrolysate valine was not present in the free form and so produced no characteristic spot on the chromatogram. In the third chromatogram both valine and glycine failed to appear. The sequence of amino acids in the tripeptide was thus shown to be valine-glycine-phenylalanine.

Availability of Amino Acid Derivatives in Bacterial Nutrition

Results obtained in some of the experiments on amino acid sequence in which microbiological assays were used stimulated interest in the availability of certain derivatives of amino acids in bacterial nutrition. Table X shows

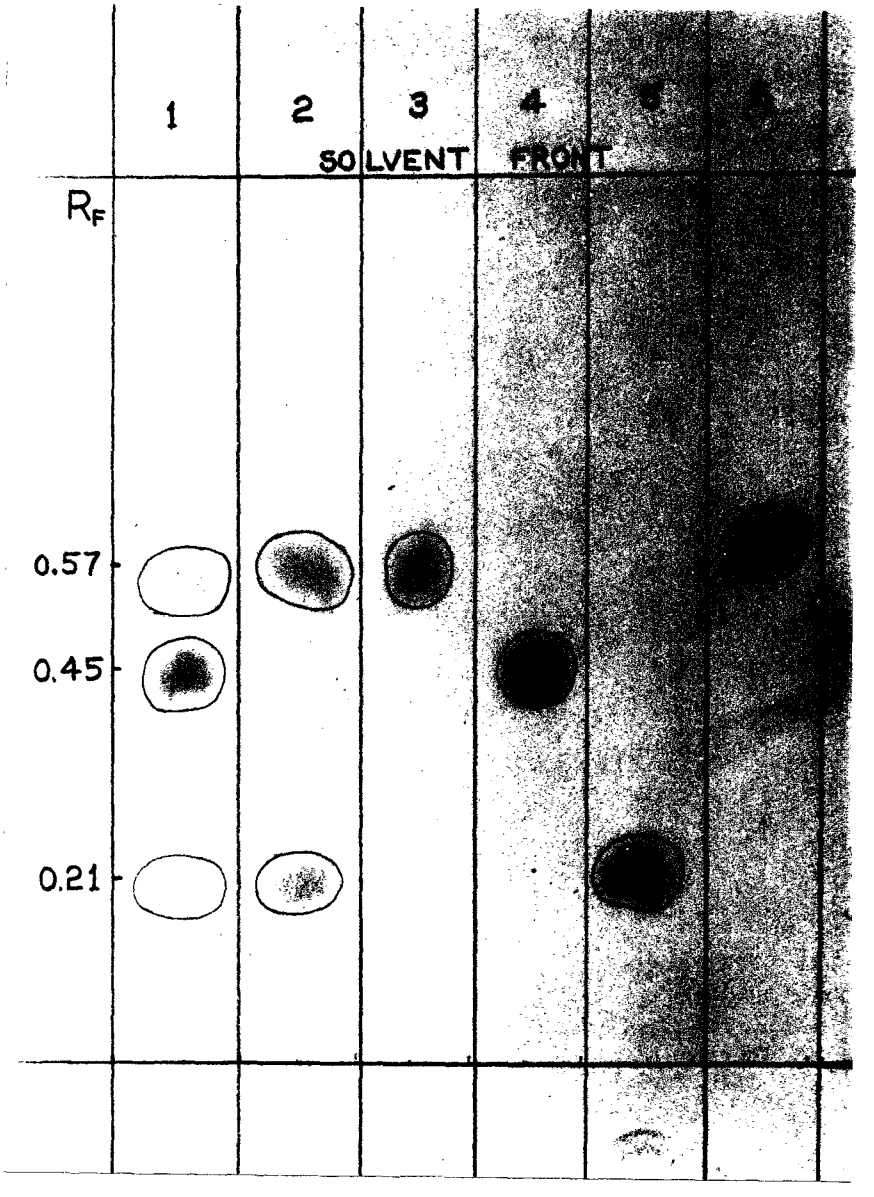

Figure 1. Chromatograms Illustrating Sequence Determination in Valylglycylphenylalanine

1. First step hydrolysate.
2. Second step hydrolysate.
3. Third step hydrolysate.
4. DL-Valine.
5. Glycine.
6. DL-Phenylalanine.

the utilization by L. arabinosus of the amino acid content of three peptides. These data have already been reported as a part of the experiments on amino acid sequence, but were not heretofore presented in a single table.

Table X

Peptides as Sources of Amino Acids in L. arabinosus Nutrition

Peptide assayed	Assayed for	Per cent acid used
DL-Leucylglycyl-DL-phenylalanine	Leucine	47
	Phenylalanine	55-70
DL-Leucyl-DL-valine	Leucine	35
	Valine	75
DL-Valyl-DL-leucine	Leucine	55
	Valine	0

None of the peptides stimulated growth beyond the amount which could be accounted for on the basis of amino acid content, and none were utilized fully. The case of valylleucine which could be used in place of leucine but not in place of valine has been previously referred to.

The use of peptides in the study of bacterial nutrition is complicated by the presence of more than one reactive group and of more than one amino acid residue,

each residue being of possible biological importance. The stimulatory effects produced by certain peptides which are not attributable to amino acid content alone have already been discussed.

Amino acids blocked at the amino or carboxyl group lend themselves more readily to the systematic study of the specificities involved in the nutrition of microorganisms because of the removal of some of these complicating factors. Only one residue has to be dealt with. The blocking group need not introduce an additional asymmetric carbon atom. If the derivative is utilized by the microorganism, the fact that one of the reactive groups is blocked makes it possible to gain a clearer idea of the type of enzyme or enzymes responsible.

Table XI shows the results of qualitative tests for utilization of the benzoyl derivatives of the nine amino acids essential for growth of L. arabinosus according to Kuiken and coworkers.[1] Definite utilization of benzoyl-DL-leucine, questionable utilization of dibenzoyl-L-cystine, and failure of the derivatives of the seven other acids to support growth are demonstrated.

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[1] K. A. Kuiken, W. H. Norman, C. M. Lyman, F. Hale, and L. Blotter, J. Biol. Chem., 151, 615 (1943).

Table XI

Utilization of Benzoyl Amino Acids by L. arabinosus

Amino acid absent from medium	Added per 10 ml. medium		Volume 0.1 N NaOH
			ml.
Glutamic acid	1.0 mg.	benzoyl-L-glutamic acid	1.50
	Nothing		1.45
Tryptophan	2.0 mg.	benzoyl-DL-tryptophan	2.90
	Nothing		2.90
Threonine	2.0 mg.	benzoyl-DL-threonine	2.00
	Nothing		1.70
Valine	2.0 mg.	benzoyl-DL-valine	1.45
	Nothing		1.47
Leucine	2.0 mg.	benzoyl-DL-leucine	13.53
	Nothing		2.07
Isoleucine	2.0 mg.	benzoyl-DL-isoleucine	3.66
	Nothing		3.55
Lysine	2.0 mg.	α-benzoyl-DL-lysine	3.87
	Nothing		3.91
Phenylalanine	2.0 mg.	benzoyl-DL-phenylalanine	2.40
	Nothing		2.26
Cystine	2.0 mg.	N, N'-dibenzoyl-L-cystine (sodium salt)	7.65
	Nothing		3.85
None	Nothing		13.80

Since the utilization tests of the benzoylamino acids had been run at rather high levels, the possibility existed that failure to support growth in the case of seven of these compounds was due to inhibition rather than simple unavailability. Accordingly, the seven benzoylamino acids were tested for inhibition at the same level previously used to test for utilization. The results of this experiment are presented in Table XII. They show no indication of inhibition by any of the compounds tested except in the case of

Table XII

Test of Benzoylamino Acids for Inhibitory Action Against L. arabinosus

Added to 10 ml. complete medium	Ml. 0.1 N NaOH
1.0 mg. benzoyl-L-glutamic acid	14.85
2.0 mg. benzoyl-DL-tryptophan	13.23
2.0 mg. benzoyl-DL-threonine	14.97
2.0 mg. benzoyl-DL-valine	15.03
2.0 mg. benzoyl-DL-isoleucine	14.65
2.0 mg. α -benzoyl-DL-lysine	15.00
2.0 mg. benzoyl-DL-phenylalanine	14.56
Nothing added	15.15

benzoyl-DL-tryptophan. Slightly less growth occurred in the presence of this compound than in its absence. The difference was slight, and at first was thought to have arisen from some variation accidentally introduced in setting up the tubes. However, a check run in quadruplicate confirmed the result obtained in the initial run.

It was of interest to determine to what extent benzoyl-DL-leucine and dibenzoyl-L-cystine were used by L. arabinosus. It was found that the L-leucine of benzoyl-DL-leucine was available to the organism almost as completely as the free acid (Table XII). On the other hand, dibenzoyl-L-cystine was available only to a slight extent. It was suspected that the latter compound might be contaminated by the free amino acid, but a sample of 100 mgm. gave a negative ninhydrin reaction.

Table XIII

Percentage Utilization of Benzoyl-DL-leucine and Dibenzoyl-L-cystine by Lactobacillus arabinosus

Compound	Per cent utilization of amino acid content
Benzoyl-DL-leucine	89
Dibenzoyl-L-cystine	0-5

It was desired to know whether the specificity exhibited by L. arabinosus in regard to utilization of benzoyl-amino acids was shared by other lactic acid bacteria. The benzoyl derivatives of ten of the twelve amino acids essential to Strep. faecalis R according to Dunn and others[1] were tested qualitatively for availability to that microorganism. It was found that benzoyl-DL-methionine could replace methionine for Strep. faecalis but that benzoyl-DL-leucine was completely inactive when substituted for leucine in the medium (Table XII). Cystine is not required for Strep. faecalis. It was also found that under the conditions of the experiment tyrosine was not essential to the strain of Strep. faecalis used, since the organism grew very well in the absence of tyrosine. When inhibition tests were run, none of the derivatives showed inhibition.

The benzoyl derivatives of twelve of the amino acids essential for L. brevis[2] were tested for utilization. Of these, only two, dibenzoyl-L-cystine and hippuric acid,

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

[2]M. S. Dunn, S. Shankman, M. N. Camien, and H. Block, J. Biol. Chem., 168, 1 (1947).

could replace the parent compounds. These results appear in Table XIV. Inhibition tests showed no inhibition by any of the benzoylamino acids against L. brevis. The finding that L. brevis can use hippuric acid to replace glycine is in agreement with the results obtained by Malin, Camien and Dunn.[1]

Since it had been demonstrated that L. arabinosus could utilize, and therefore probably could split, benzoylleucine and none of the other benzoylamino acids tested, experiments were performed with the object of determining whether the presence of a leucine residue having a free carboxyl group was necessary for the utilization of a peptide blocked at the amino end. A number of benzoylated peptides were tested for utilization qualitatively in two separate experiments. In the first experiment were tested the benzoylated peptides listed in Table XVI.

The results of the first run were in agreement with the hypothesis that benzoylated peptides having a leucine residue on the carboxyl end could be utilized by L. arabinosus. In a second experiment a number of benzoylated peptides containing glycine, leucine, valine and alanine were tested for availability of the leucine and valine residues to L.

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[1]R. B. Malin, M. N. Camien, and M. S. Dunn, Arch. Biochem. Biophys., 32, 106 (1951).

Table XIV

Utilization of Benzoylamino Acids by Strep. faecalis

Amino acid absent from medium	Added per 10 ml. medium	Volume 0.05 N NaOH
		ml.
Glutamic acid	0.5 mg. benzoyl-L-glutamic acid	1.60
	Nothing	1.63
Valine	0.5 mg. benzoyl-DL-valine	2.41
	Nothing	3.05
Isoleucine	0.5 mg. benzoyl-DL-isoleucine	4.15
	Nothing	3.89
Leucine	0.5 mg. benzoyl-DL-leucine	1.80
	Nothing	1.75
Methionine	0.5 mg. benzoyl-DL-methionine	10.40
	Nothing	4.83
Tryptophan	0.5 mg. benzoyl-DL-tryptophan	2.33
	Nothing	2.30
Threonine	0.5 mg. benzoyl-DL-threonine	2.45
	Nothing	2.20
Tyrosine	0.5 mg. N-benzoyl-L-tyrosine	8.18
	Nothing	9.68
Lysine	0.5 mg. α-benzoyl-DL-lysine	2.30
	Nothing	2.43
Alanine	0.5 mg. benzoyl-DL-alanine	3.10
	Nothing	3.13
None	Nothing	12.27

Table XV

Utilization of Benzoylamino Acids by *L. brevis*

Amino acid absent from medium	Added per 10 ml. medium		Volume 0.10 N NaOH
			ml.
Glutamic acid	0.5 mg.	benzoyl-L-glutamic acid	1.30
	Nothing		1.15
Isoleucine	0.5 mg.	benzoyl-DL-isoleucine	1.43
	Nothing		1.45
Leucine	0.5 mg.	benzoyl-DL-leucine	1.33
	Nothing		1.53
Methionine	0.5 mg.	benzoyl-DL-methionine	2.90
	Nothing		2.75
Valine	0.5 mg.	benzoyl-DL-valine	1.35
	Nothing		1.38
Tryptophan	0.5 mg.	benzoyl-DL-tryptophan	2.25
	Nothing		2.80
Cystine	0.5 mg.	dibenzoyl-L-cystine	2.75
	Nothing		1.25
Threonine	0.5 mg.	benzoyl-DL-threonine	1.48
	Nothing		1.40
Phenylalanine	0.5 mg.	benzoyl-DL-phenylalanine	2.00
	Nothing		1.95
Tyrosine	0.5 mg.	N-benzoyl-L-tyrosine	2.45
	Nothing		2.35
Lysine	0.5 mg.	α-benzoyl-DL-lysine	1.03
	Nothing		1.20
Glycine	0.5 mg.	benzoylglycine	12.78
	Nothing		1.65
Nothing	Nothing		21.90

Table XVI

Utilization of the Benzoyl Derivatives of Three Peptides by L. arabinosus

Amino acid absent from medium	Added per 10 ml. medium	Volume 0.1 N NaOH
		ml.
Leucine	0.6 mgm. benzoylglycyl-DL-leucine	11.55
	0.8 mgm. benzoyl-DL-leucyl-glycyl-DL-phenylalanine	1.48
	Nothing	1.50
Phenylalanine	0.6 mgm. benzoylglycyl-DL-phenylalanine	1.55
	0.8 mgm. benzoyl-DL-leucyl-glycyl-DL-phenylalanine	1.48
	Nothing	1.38
None	Nothing	16.28

arabinosus (Table XVII). None of the compounds could replace valine, while only benzoyl-DL-alanyl-DL-leucine having leucine at the carboxyl terminus, could replace leucine to a significant extent for L. arabinosus. The other benzoylated leucine peptides supported a very slight amount of growth if any at all. In the case of benzoyl-DL-leucyl-DL-leucine A this result did not fit the hypothesis.

The results obtained with the two racemic valylvalines agree with those obtained by Hegsted[1] when he tested the four stereoisomeric benzoylvalylvalines.

The two benzoylpeptides which were utilized by L. arabinosus were assayed for leucine activity. The results are shown in Table XVIII.

Table XVII

Availability of Leucine and Valine Content of Benzoylated Peptides for L. arabinosus

Amino acid absent from medium	Added per 10 ml. medium	Volume 0.1 N NaOH
		ml.
Leucine	Nothing	2.00
	0.6 mgm. benzoyl-DL-leucyl-DL-leucine A	3.70
	0.6 mgm. benzoyl-DL-leucyl-DL-valine A	1.87

Table XVII (Cont'd)

Amino acid absent from medium	Added per 10 ml. medium	Volume 0.1 N NaOH
		ml.
	0.6 mgm. benzoyl-DL-leucyl-DL-valine B	1.80
	0.6 mgm. benzoyl-DL-leucyl-DL-alanine	2.25
	0.6 mgm. benzoyl-DL-alanyl-DL-leucine	10.18
	0.6 mgm. benzoyl-DL-leucyl-glycine	2.05
Valine	Nothing	1.25
	0.6 mgm. benzoylglycyl-DL-valine	1.15
	0.6 mgm. benzoyl-DL-leucyl-DL-valine A	1.18
	0.6 mgm. benzoyl-DL-leucyl-DL-valine B	1.32
	0.6 mgm. benzoyl-DL-valylglycine	1.28
	0.6 mgm. benzoyl-DL-alanyl-DL-valine	1.25
	0.6 mgm. benzoyl-DL-valyl-DL-alanine A	1.40
	0.6 mgm. benzoyl-DL-valyl-DL-valine A	1.35
	0.6 mgm. benzoyl-DL-valyl-DL-valine B	1.35
	Complete medium	16.85

Table XVIII

Utilization of Benzoyl-DL-alanyl-DL-Leucine and Benzoylglycyl-DL-Leucine by L. arabinosus

Compound	Per cent utilization of leucine content
Benzoylglycyl-DL-leucine	96
Benzoyl-DL-alanyl-DL-leucine	97

DISCUSSION

The method described herein for determination of amino acid sequence in peptides differs from the method as developed by Edman[1] chiefly in the manner in which paper chromatography is used to identify amino acids. In the method of Edman the phenylthiohydantoin is extracted and hydrolyzed, and the amino acid in the hydrolysate identified by chromatogram. The procedure described here avoids extractions which are tedious and demand the use of larger samples.

The phenylisothiocyanate technique was tested on a number of synthetic peptides with success. In addition to the use of paper chromatography, microbiological assays were employed[2] to identify the amino acids present in hydrolysates of the treated and untreated peptides. The results obtained by the two methods were in agreement but the fact that the microbiological method is a quantitative one made

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[1] P. Edman, Acta Chem. Scand., 4, 283 (1950).

[2] T. L. Hurst, A Quantitative Method for Determining Sequences of Amino Acid Residues. Unpublished M.S. Thesis. Ames, Iowa. Iowa State College Library. 1951.

it possible to assign sequence by that method even in cases of only partial blocking of the terminal amino acid residue. Although the chromatographic technique may be roughly quantitative if the operator is sufficiently skilled, the microbiological method has the advantage on this point.

If the peptide investigated should contain one or more residues of the D-configuration the presence of such residues would probably not be detected by use of microbiological assays, since the D-forms of amino acids ordinarily do not promote growth of bacteria. On the other hand, the two optical forms of an amino acid may be expected to have the same R_f value, and in that case the chromatographic method would detect the presence of an amino acid of the D-form but not its configuration. The use of the two methods in conjunction should determine both the presence and configuration of an amino acid.

If more than one residue of the same amino acid were present in the peptide, blocking of one of these residues would not be detected by the chromatographic method unless the operator was skillful enough to employ the technique in a semi-quantitative way.

The chromatographic procedure has the advantage of simplicity relative to the microbiological method, which requires a standard curve for each amino acid determined,

and possibly the use of more than one microorganism. Chromatograms can be run in a matter of hours for small peptides, whereas microbiological tests usually require three days for incubation alone.

The results obtained when the availability of the benzoylamino acids for L. arabinosus was tested paralleled those obtained by other workers for the acetyl and chloroacetyl derivatives, wherever these derivatives had been tested[1,2,3], with one exception. The exception was the low utilization of acetyl-L-leucine reported by Krehl and Fruton.

The fact that benzoyl-DL-leucine was the only one of these derivatives available in a high degree for L. arabinosus was striking, since benzoylvaline and benzoylisoleucine are quite similar in structure to benzoylleucine. It has been shown that valine peptides are particularly resistant to hydrolysis by acid and alkali and this resistance is attributed to steric hindrance by

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[1] D. M. Hegsted, J. Biol. Chem., 157, 741 (1945).

[2] C. H. Eades, Jr., J. Biol. Chem., 187, 147 (1950).

[3] W. A. Krehl and J. S. Fruton, J. Biol. Chem., 173, 479 (1948).

the methyl groups.[1] It is also known that benzoylvaline forms the anilide with difficulty when catalyzed by papain, in contrast to the behavior of benzoylleucine, and this effect is also attributed to steric hindrance.[2] In the case of leucine, the methyl groups are farther away from the α-carbon by the space of a methylene group, and the steric effects are accordingly lessened.

These considerations may account for the fact that benzoylvaline and benzoylisoleucine are unavailable to the microorganism but cannot be applied to most of the compounds tested. The utilization of two of three benzoylated peptides having leucine at the carboxyl terminus and the failure of other benzoylated peptides to promote growth indicates a preference for leucine at the carboxyl terminus. The failure of benzoyl-DL-leucyl-DL-leucine A to support any considerable growth is not easily explained. The behavior of benzoyl-DL-leucyl-DL-leucine A is similar to the nonavailability of hippurylglycine reported by Malin, Camien, and Dunn[3], who found hippuric acid and glycylglycine utilized by their test organisms in contrast.

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[1]R. L. M. Synge, Biochem. J., 39, 351 (1945).

[2]S. W. Fox and M. Winitz, unpublished experiments.

[3]R. B. Malin, M. N. Camien, and M. S. Dunn, Arch. Biochem. Biophys., 32, 106 (1951).

The utilization of the leucine of DL-valyl-DL-leucine and the failure of L. arabinosus to use the valine of this compound has been noted. These facts can be explained if two assumptions can be made. The first assumption is that the leucine of any form of valylleucine must have the L-configuration if either residue of this dipeptide is to be utilized. This assumption is based on the discovery of a similar requirement for availability of leucylleucines by Fox and others.[1] The second assumption is that the DL-valyl-DL-leucine was inadvertently resolved at some time of its preparation or purification into the D-D, L-L and the D-L, L-D racemates, only the latter being recovered. If these two assumptions are granted, then the behavior of L. arabinosus in relation to valylleucine becomes understandable. In the case of the D-L isomer, the leucine is available but the valine is not, being of the D-configuration. The L-D isomer cannot be used because the configuration of the leucine residue blocks utilization of any part of the molecule.

Except for dibenzoyl-L-cystine which was available in some degree for both L. arabinosus and L. brevis, each

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[1] S. W. Fox, Y. Kobayashi, S. Melvin, and F. N. Minard, J. Am. Chem. Soc., 70, 2404 (1948).

microorganism exhibited the ability to debenzoylate a different benzoylamino acid. This indicates that the respective enzyme systems of the three bacterial strains differ considerably, even in such a restricted area of metabolism.

CONCLUSIONS

1. Paper chromatography can be used in conjunction with chemical blocking to determine the terminal amino acid residue of a peptide through a subtractive procedure.

2. The same method may be extended so as to determine amino acid residue sequence in higher peptides.

3. Paper chromatography and microbiological techniques used in conjunction on hydrolysates of treated peptides allow the determination of configuration as well as position of amino acid residues in peptides.

4. Marked differences exist among strains of lactic acid bacteria with regard to ability to debenzoylate benzoylamino acids.

5. When the amino end of a peptide is blocked by the benzoyl group, a leucine residue at the carboxyl terminus favors utilization by the strain of L. arabinosus employed.

6. The ability to debenzoylate benzoylamino acids appears to be strictly limited for a given strain of bacteria.

SUMMARY

Preliminary experiments were performed to investigate the possibility of combining chemical blocking with microbiological assays in a method for determining terminal amino acids in peptides.

A subtractive chromatographic procedure for determination of terminal amino acid residues in peptides is presented. Results are given for a total of nine dipeptides and one tripeptide. By employing selective cleavage of the treated tripeptide with dioxane-HCl the sequence of residues could be determined.

The benzoyl derivatives of nine amino acids essential for L. arabinosus, of twelve amino acids essential for L. brevis, and of ten amino acids essential for Strep. faecalis were tested for utilization by these microorganisms. It was found that, under the conditions of the experiment, L. arabinosus could utilize only benzoyl-DL-leucine with efficiency, whereas activity so slight as to be questionable was noted for dibenzoyl-L-leucine with efficiency, whereas activity so slight as to be questionable was noted for dibenzoyl-L-cystine. Only benzoyl-DL-methionine was available for Strep. faecalis.

L. brevis used hippuric acid efficiently, while results for dibenzoyl-L-cystine were equivocal for this organism also. It should be noted, however, that only one strain of each species of bacteria was used, and the results obtained may apply only to these strains.

Twelve benzoylated dipeptides and one benzoylated tripeptide containing leucine, phenylalanine and valine residues were tested for availability to L. arabinosus. Only two, hippuryl-DL-leucine and benzoyl-DL-alanyl-DL-leucine were utilized, the microorganism seeming to show a requirement for leucine at the carboxyl end of the compound used. One other such compound, however, benzoyl-DL-leucyl-DL-leucine A, gave little or no growth response.

Three peptides were assayed for availability to L. arabinosus. One of these, DL-valyl-DL-leucine, was found to support growth in a leucine-free medium but not in a valine-free medium. A possible explanation is presented.

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