Microorganisms and Nitrogen Sources

Transport and Utilization of Amino Acids, Peptides, Proteins, and Related Substrates

Edited by

J. W. PAYNE

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Department of Botany, University of Durham, Durham, UK

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Editor's Preface

The idea behind this book was simple and clear: to present an account of the 'transmembrane metabolism' of certain related nitrogen sources by various microorganisms. By integrating descriptions of the characteristics of their transport with accounts of the metabolic fate and utilization of the different substrates it was planned to emphasize and to explore the cellular coordination of these events. Reviews dealing with, for example, aspects of amino acid transport or utilization appear often in separate texts on these specific topics but rarely, if ever, are they placed together; the same may be said for an integrated coverage of these features in prokaryotic bacteria through to eukaryotic fungi and algae. In this book these various aspects are brought together. However, the book overall was also envisaged as one of general reference for the area and, to this end, other closely related topics such as peptide ionophores and antimicrobial agents are included. Each section was to be self-contained and occasionally, as a result some overlaps occurred during their preparation. In several instances these are preserved, partly to maintain the unity of the section, partly because the context of the discussions are different, and partly because they serve to emphasize the interrelatedness of the topics.

Certain aspects of nitrogen utilization have been intentionally excluded. Nitrogen fixation, for example, is not considered here, for it has been the subject of several other texts. So, although it is convenient for a book to have a short title such as 'Microorganisms and Nitrogen Sources', the full title gives a stricter indication of the material to be found here. Even amongst the topics selected the information currently available varies considerably, and several authors responded bravely to the request to combine fact and speculation by presenting an analysis of the questions that need to be asked rather than just the answers obtained. Some of these speculations may have borne fruit and others withered even before publication but they should remain a source for reference and for ideas for some time to come. In particularly active research areas the addenda go some way to bridge the gap that inevitably arises in the production of a large, multi-author volume such as this.

I am particularly grateful to the contributors for the enthusiastic way in which they greeted the idea for the book, for their willingness to give their time and expertise in writing the sections, and not least for their patience once these had been handed over to the editor. Mrs Ethne Ellis deserves especial thanks for so expertly typing the bulk of the material. Finally, I thank my wife, and Clare and Adrian, for their encouragement and tolerance; only on rare occasions did it seem that, whereas microorganisms and man might manage perfectly well on a diet of amino acids and peptides alone, women and children might need more varied fare.

Contributors

J. J. ANDERSON — *Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109, USA*

Y. ANRAKU — *Department of Biology, Faculty of Science, University of Hongo, Bunkyo-ku, Tokyo 113, Japan*

J. M. BECKER — *Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37916, USA*

I. R. BOOTH — *Department of Microbiology, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, UK*

C. M. BROWN — *Department of Biological Sciences, University of Dundee, Dundee DD1 4HU, UK (Present address: Department of Brewing and Biological Sciences, Heriot-Watt University, Chambers Street, Edinburgh EH1 1HX, UK)*

P. H. CLARKE — *Department of Biochemistry, University College, Gower Street, London WC1E 6BT, UK*

B. L. COHEN — *Department of Genetics, University of Glasgow, Glasgow G11 5JS, UK*

A. A. EDDY — *Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, UK*

M. DE FELICE — *International Institute of Genetics and Biophysics, Via G. Marconi 10 80125 Naples, Italy*

C. E. FURLONG — *Department of Genetics, SK-50, University of Washington, Seattle, Washington 98195, USA*

J. GUARDIOLA — *International Institute of Genetics and Biophysics, Via G. Marconi 10 80125 Naples, Italy*

W. A. HAMILTON — *Department of Microbiology, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, UK*

C. F. HIGGINS — *Department of Botany, University of Durham, South Road, Durham DH1 3LE, UK (Present address: Department of Biochemistry, University of California, Berkeley, California 94720, USA)*

C. L. HERMSDORF — *Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510, USA*

H. HOLZER — *Biochemisches Institut der Universität Freiburg im Breisgau und Institut für Toxikologie und Biochemie der Gesellschaft für Strahlenund, Umweltforschung D-7800, Freiburg im Breisgau, West Germany*

M. IACCARINO — *The International Institute of Genetics and Biophysics, Via G. Marconi 10 80125 Naples, Italy*

B. A. LAW — *National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, UK*

A. MEISTER — *Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, New York 10021, USA*

F. NAIDER — *Department of Chemistry, The College of Staten Island of The City University of New York, Staten Island, New York 10301, USA*

D. L. OXENDER — *Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109, USA*

J. W. PAYNE — *Department of Botany, University of Durham, South Road, Durham DH1 3LE, UK*

M. J. PINE — *Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York 14263, USA*

S. C. QUAY — *Department of Pathology, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114, USA*

C. RATLEDGE — *Department of Biochemistry, University of Hull, Hull HU6 7RX, UK*

P. S. RINGROSE — *Roche Products Ltd, P.O. Box 8, Welwyn Garden City, Hertfordshire AL7 3AY, UK (Present address: Chemotherapy Division, Sandoz Research Institute, Brunner Strasse 59, A-1235 Vienna, Austria)*

G. D. SCHELLENBERG — *Department of Medicine, Division of Medical Genetics, Center for Inherited Diseases, and Department of Genetics, SK50, Seattle, Washington 98195, USA*

S. SIMMONDS — *Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510, USA*

E. E. SNELL — *Department of Microbiology, University of Texas at Austin, Austin, Texas 78712, USA*

M. C. STEPHENSON — *Department of Biochemistry, University of Hull, Hull HU6 7RX, UK*

W. D. P. STEWART — *Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK*

J. M. TUFFNELL — *Department of Botany, University of Durham, South Road, Durham DH1 3LE, UK*

H. E. WADE — *Centre for Applied Microbiology and Research, Porton Down, Wiltshire SP4 0JG, UK*

D. H. WOLF — *Biochemisches Institut der Universität Freiburg im Breisgau und Institut für Toxicologie und Biochemie der Gesellschaft für Strahlenund, Umweltforschung D-7800, Freiburg im Breisgau, West Germany*

L. WOLFINBARGER, JR — *Department of Biological Sciences, Old Dominion University, Norfolk, Virginia 23508, USA*

Contents

Microorganisms and Nitrogen Sources
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Introduction

E. E. SNELL

Department of Microbiology, University of Texas at Austin, Austin, Texas 78712, USA

The late D. D. Woods (Woods, 1953), paraphrasing an unpublished lecture by Marjory Stephenson, differentiated five developmental levels, characterized by a decreasing complexity of interactions, in the study of microbial metabolism. These levels included the study of: (1) mixed populations in 'natural media'; (2) pure cultures in complex, undefined media; (3) pure cultures in defined media; (4) viable, non-growing cell suspensions of a single cell type with defined substrates; and finally (5) non-viable, fractionated cell extracts—down to the stage of pure enzymes—with defined substrates for the elucidation of individual steps in metabolism.

The reader will recognize in these overlapping investigational levels an encapsulated history of microbial biochemistry. Progression from level (1) to level (2) could occur only as pure culture techniques were evolved, including the development of appropriate crude culture media. The change from level (2) to level (3) depended upon the painfully slow but cumulatively tremendous increase in knowledge of microbial and animal nutrition that occurred mostly from the 1930s through the 1950s and resulted in identification of the vitamins, amino acids, many of the inorganic ions, and other factors required for growth. (For reviews reflecting our increasing understanding of this subject, see Woods, 1953; Williams, 1941; Peterson and Peterson, 1945; Knight, 1945; Guirard and Snell, 1963.) Such identification in turn stimulated development of synthetic procedures for these and related substances, and of microbiological, chromatographic, and other methods for their determination. It also made possible recognition of the first biochemical (nutritional) mutants of microorganisms. With the parallel development of isotopic methods and of biochemical supply houses that made these unlabelled and labelled metabolites generally available, the stage was set for the sophisticated studies at levels (3), (4), and (5) characteristic

of biochemistry today. These studies have resulted in a rather detailed understanding of intracellular pathways leading to and from most of the now-recognized components of living cells.

The same cannot yet be said for transport of amino acids, peptides, and proteins, which is the subject of this volume. Since, in the growing cell, transport must precede metabolism, a substantial if diminishing gap remains in our understanding of utilization of these substances for growth. Judging from recent reviews (Holden, 1962; Boos, 1974; Simoni and Postma, 1975; Wilson, 1978), the leading edge of experimentation here seems to lie principally at level (4) and the early stages of level (5). Particularly sketchy, in part because of the limited availability of pure substrates, has been our knowledge of peptide transport and utilization, and as an introduction to the many articles in this book that deal with various aspects of this topic, it may be useful to review briefly the experiments, mostly with growing cultures (level (3)) which led to the realization that peptides sometimes are far superior to their component amino acids as sources of amino acids for growth, that such peptides are nevertheless hydrolysed intracellularly prior to utilization, and that uptake systems exist for peptides that differ from those for amino acids.

Gale (1945) observed that partial hydrolysates of salmine were more effective than equimolar amounts of arginine in supplying the growth requirement of a Group D streptococcus that was auxotrophic for this amino acid. He attributed this superiority to the presence of arginine dihydrolase, which converts free arginine, but not its peptides, to the inactive ornithine. Since pure peptides were not available for testing, it could not be determined whether the active peptides of the partial hydrolysate were utilized directly, or were hydrolysed prior to use.

These points were later tested in a system more amenable to experimental analysis by Kihara *et al.* (1952). *Streptococcus faecalis* 8043 is auxotrophic for tyrosine; it also contains an active tyrosine decarboxylase when grown in the presence of vitamin B_6, but not when grown without this vitamin. In the absence of vitamin B_6, tyrosine peptides were equally (Leu-Tyr) or less (Gly-Tyr) active than free tyrosine in supporting growth and no tyramine was formed; when pyridoxamine was added to the growth medium, both peptides were more active than free tyrosine. Appropriate comparisons showed that the cell yield per unit of added tyrosine was greatly decreased by the presence of pyridoxamine, while that from tyrosine peptides decreased only slightly. Cell suspensions hydrolysed both peptides; the authors suggested that when peptides are being hydrolysed, tyrosine became available continously, at a relatively constant rate per cell, in concentrations sufficient for protein synthesis, but not sufficient to permit a large fraction of it to be destroyed by the decarboxylase before it could be utilized for growth (Kihara *et al.*, 1952).

In an analogous fashion, the presence of proline oxidase apparently accounts for the observed superiority of proline peptides to free proline in supporting growth of a mutant strain of *Escherichia coli* (Stone and Hoberman, 1953), a superiority, that, when first observed, had been ascribed to direct utilization of these peptides (Fruton and Simmons, 1948). Since numerous catabolic enzymes that destroy free amino acids are known, circumstances analogous to those described in the preceding examples could occur frequently and find their explanation in similar terms.

Discovery of a second set of circumstances under which peptides surpass free amino acids in growth-promoting activity was partly serendipitous. Although vitamin B_6 is normally required for growth of *Streptococcus faecalis*, we found that it was not required if sufficient alanine was added to a medium that contained each of the other amino acids (Snell and Guirard, 1943). To our surprise (since no D-amino acid had previously been implicated in growth processes), D-alanine proved more effective than L-alanine in supporting growth under these conditions (Snell, 1945). However, D-alanine did not 'replace' the growth requirement of *Lactobacillus casei* for vitamin B_6 under similar conditions (Snell, 1945). Reasoning from structural similarities, we speculated that D-alanine might serve as a biosynthetic precursor of vitamin B_6 by supplying the nitrogen at position 1, and the carbons at positions 2, 2′, and 3 (Snell and Guirard, 1943). Would *L. casei* also grow without vitamin B_6 if D-alanine and an unknown precursor for the other 'half' of the vitamin were supplied? We assayed for the missing 'precursor' and found indeed that *L. casei* grew well without vitamin B_6 if D-alanine and a partial hydrolysate of casein (vitamin B_6-free!) were supplied together in a medium that contained all of the L-amino acids (Snell, 1945).

In the meantime, however, our theory concerning D-alanine as a precursor of vitamin B_6 had collapsed—appropriate assays showed instead that vitamin B_6 was necessary for synthesis of D-alanine (Holden and Snell, 1949), which was present in all cells, however grown, and which we eventually identified as an essential component of the bacterial cell wall (Snell *et al.*, 1955)—and we were left to ponder the role of the partial casein hydrolysate in promoting the response of *L. casei* to D-alanine. We fractionated the hydrolysate until we obtained active fractions that contained chiefly alanyl and tyrosyl peptides of valine, isoleucine, and leucine (Kihara *et al.*, 1952). Tests then showed that synthetic dipeptides of L-alanine were fully active (Kihara and Snell, 1952). Each of the active peptides was hydrolysed by cell suspensions, and there was no evidence for destruction of L-alanine by catabolic processes.

To summarize the findings: (a) peptides of L-alanine are required for growth in the presence of D-alanine and the absence of vitamin B_6, (b) such peptides are hydrolysed by the cells, but (c) added D-alanine does not

induce a requirement for L-alanine peptides in the presence of vitamin B_6 where both D- and L-alanine are synthesized internally. The simplest explanation of these findings, which we adopted (Kihara and Snell, 1952), was that D-alanine interfered with *absorption* of free L-alanine, but not of L-alanine peptides from the external medium; no interference, however, occurred with utilization of intracellular L-alanine. Experimental evidence for the postulated interference of D-alanine with L-alanine absorption was also presented. The existence of specific absorption sites for amino acids that differed from those for peptides, and that could be separately inhibited without inhibiting intracellular processes, was implicit in this explanation of the findings.

These conclusions are notable because they antedate the subsequent demonstration by currently acceptable procedures of specific transport systems for amino acids in bacterial cells, (Cohen and Rickenberg, 1956; Cohen and Monod, 1957) and because they focussed attention on competition between external nutrients for common absorption sites as a possible explanation for other cases (many of which were known; e.g. Sprince and Woolley, 1944; Malin *et al.*, 1951) in which partial protein digests stimulated growth of bacteria in media that contained a full complement of free amino acids. In general terms, if peptides were absorbed at sites separate from amino acids, inhibition of amino acid absorption by an antagonistic amino acid should not interfere with absorption of peptides, which, since they entered the cell by independent pathways, could then be hydrolysed and thus supply the amino acid for protein synthesis even when absorption of external free amino acid was blocked. This possibility seemed especially attractive to us, since many instances of one amino acid antagonizing utilization of structurally related amino acids had been observed both in the early literature (e.g. Gladstone, 1939; Doudoroff, 1943) and during studies leading to the development of quantitative microbiological assays for amino acids (Snell and Guirard, 1943; Malin *et al.*, 1951; Brickson *et al.*, 1948). We studied a sufficient number of such instances to convince us that this set of circumstances occurred frequently and that the explanation was generally valid (Prescott *et al.*, 1953; Kihara and Snell, 1955, 1960*b*). We later demonstrated (Leach and Snell, 1960) the validity of these postulates by direct tests of the effects of D-alanine on uptake of [^{14}C]-L-alanine and its peptides by resting cells of *L. casei*.

Especially instructive in this connection were experiments with *Leuconostoc mesenteroides*, in which antagonists to two different essential amino acids, A and B, were added simultaneously to the growth medium (Kihara and Snell, 1960*b*). Growth then occurred on addition either of comparatively large amounts of the free amino acids A + B, of small amounts of a mixture of dipeptides, AC + BD, or of equally small amounts

of the dipeptide AB; all active peptides were hydrolysed by the cells. The activity of AB as a source of both A and B for growth under these conditions appears to preclude the necessity for mechanisms other than hydrolysis for the redistribution of these amino acid residues into other structures.

These observations supplied the background to our identification (Kihara and Snell 1960*a*) of 'strepogenin', an unidentified peptide growth factor required for rapid growth of *Lactobacillus casei* from small inocula in media based on acid hydrolysates of casein and vitamins. A considerable literature had accumulated on this subject; several different compounds, including peptides, showed some activity, but none of them was as effective as an enzymic protein digest in promoting growth. We adopted the working hypothesis that impediments to the utilization of several different amino acids, analogous to those discussed earlier, might be present in this medium. Correction of one such impediment by addition of a single limiting amino acid in excess or of smaller amounts of a peptide of that amino acid would not correct the overall deficiency: at best it could stimulate the growth rate to the point where availability of a second amino acid limited the growth rate. An enzymic protein digest would contain peptides that corrected all of the deficiencies; only in the unlikely event that one isolated a single peptide that contained each of the limiting amino acids in a structure that could be both rapidly absorbed by the cell and rapidly hydrolysed could one expect to duplicate the effects of an enzymic casein digest with a single peptide. Even then the peptide would account for only a small portion of the activity of the digest. Guided by this hypothesis, we showed that the availability of serine, cysteine, and glutamic acid limited growth in the strepogenin assay medium; simultaneous addition of large amounts of these three amino acids, or small amounts of a mixture of peptides that supplied them, greatly stimulated the growth rate; in their presence, addition of small amounts of other known growth factors (uracil, guanylic acid, spermine, fatty acids) gave rates of growth as great as those obtained with enzymic casein digests (Kihara and Snell, 1960*a*).

The foregoing discussion has given examples of three circumstances that result in increased activity of peptides as compared with their component amino acids for amino acid auxotrophs: (1) peptides may protect the amino acid from degradation, or (2) because peptides commonly enter the cell by routes other than those used by amino acids, they may bypass transport systems for free amino acids that are not fully operative because of (a) partial saturation of that system by a competing natural or synthetic amino acid antagonist or (b) loss of transport capability for other reasons (Peters *et al.*, 1953).

In none of the work described thus far has there been any indication that peptides serve any role other than as a source of limiting amino acids. On

the other hand, if one employs nutritional criteria, one should not be surprised if only nutritional answers are forthcoming. Nevertheless, considering the profusion of peptide hormones that operate in mammalian systems, one wonders if similar considerations may sometimes apply to their action. My colleagues indicate that far too little hormone is required to serve as a useful amino acid source in target cells. However, in these days of cascade amplification and differential rates of protein turnover, this objection is hardly compelling; the possibility at least needs study. It is clear in any case that transport phenomena, which control access to the cell of all types of compounds in its external environment, occupy a focal point in the utilization of nutrients. Volumes such as the present one play an important role in summarizing and systematizing our knowledge of such events and thus in directing future effort in the field.

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