BIOCHEMICAL ASPECTS OF NUTRITION

The Proceedings of the First Congress of The Federation of Asian and Oceanian Biochemists

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PREFACE

This book records, in principle, the proceedings of the symposium on "Biochemical Aspects of Nutrition", which was held on October 10–12, 1977 at the Nagoya Kanko Hotel, Nagoya, as a part of the First Congress of the Federation of Asian and Oceanian Biochemists. However, it is not the simple proceedings, but resembles a monograph written by experts for the following reasons. First, the program committee for the symposium carefully selected speakers from all over the world so as to cover the main topics in this field. Second, the speakers were asked to prepare their papers in the form of reviews in miniature. Accordingly, the book is expected to be useful for biochemists in wider areas than the specialists. The editor's thanks are due to Drs. S. Fukui, N. Hosoya, A. Ichihara, Y. Imai, and A. Yoshida, the members of the program committee, for their efforts in preparing the program.

By the way, it should be mentioned that since the Federation of Asian and Oceanian Biochemists started in 1972, five years passed until the first congress was held. This period was necessary for the Federation to accumulate the energy to hold such an event. Accordingly, this book is a testimony to the cooperation of the members of the Federation. In this respect, encouragement received from the delegates of the member countries, especially Dr. A. W. Linnane, the president of the Federation, is highly appreciated. The efforts of the members of the organizing committee of this congress, Drs. O. Hayaishi, K. Imahori, B. Maruo, Y. Miura, D. Mizuno, and T. Murachi are also greatly appreciated.

Kunio YAGI
The Congress President
First Congress of the
Federation of Asian and
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CARBOHYDRATE

VITAMIN

UNSTABLE PROTEIN IN MAMMALIAN CELLS SYNTHESISED IN THE PRESENCE OF AMINO ACID ANALOGS OR UNDER CONDITIONS OF AMINO ACID IMBALANCE

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Labile proteins can be produced in *E. coli* strains which have a requirement for an amino acid by the substitution of a structural analog for the amino acid in the growth medium.[1] The relative stability of proteins fromed by this procedure will depend on the extent to which the analog is substituted and thus the degree of structural abnormality produced in the protein. In general, only those analogs which can charge tRNA will be incorporated and produce modified protein chains. Recently, we and others have shown that mammalian cells incubated in media lacking an essential amino acid are also capable of synthesising labile proteins in the presence of an analog to the omitted amino acid.[2,3] Although amino acid analogs are a convenient way of producing abnormal proteins, it is quite likely that such proteins are more normal events arising from translational errors in protein synthesis as well as from post-translational modifications. This concept is well established in bacteria, where mutants with reduced fidelity in protein synthesis have been isolated. The situation is less definite in higher organisms. However, one experimental finding consistent with translational errors in mammalian cells is the rapid degradation of approximately 10% of protein synthesised after a brief labelling period. This result has been found with several cell types and may be indicative of errors in translation followed by a "proof-reading" mechanism which detects and degrades the error proteins.[2,4,5] The experiments described in the following sections summarise our approach to an understanding of mechanisms responsible for the synthesis and breakdown of several types of abnormal proteins.

AMINO ACID SUBSTITUTIONS

We have tested a wide range of naturally-occurring and synthetic analogs for their ability to alter protein breakdown in mammalian cells. Our aims in these studies were to (a) determine the extent to which analogs modified the degradation rate, (b) look for tissue-specific effects, and (c) provide indirect evidence for the insertion of the analog into proteins. The experimental procedure used was incubation of reticulocytes in a complete medium containing ^{3}H-leucine, with various concentrations of the analog replacing the normal amino acid. After labelling for 30 min, the cells were washed, transferred to fresh medium and degradation of ^{3}H-protein was followed for 2 hr. A similar

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; L-DOPA, L-dihydroxyphenylalanine; TCA, trichloracetic acid.

TABLE I

EFFECTS OF AMINO ACID ANALOGS ON PROTEIN DEGRADATION AND PEPCK HEAT LABILITY

Labelled protein was synthesised in the presence of complete medium minus individual amino acids and with analogs added, usually at a concentration of 2mM. Those analogs which decreased protein labelling in comparison to controls with the normal amino acid omitted are indicated by superscript 1. Stimulation of protein synthesis is indicated by superscript 2. Although azetidine-2-carboxylic acid behaves as a proline analog in hepatoma cells, it competes with alanine and not proline in rat reticulocytes. Degradation of labelled protein was followed for 2 hr. Analogs which increased protein degradation above that occurring in controls are indicated by + (slight effect) or ++ (large effect). PEPCK heat lability was measured at 42° using cytosol fractions prepared from Reuber cells which had been incubated for 16 hr in the presence of analogs, usually at a concentration of 2 mM, with dibutyryl cyclic AMP, dexamethazone and theophylline added in order to induce the enzyme.[6] Small (+) or large (++) increases in lability are indicated.

	Effect on protein degradation in reticulocytes	*hepatoma*	*PEPCK lability*
Proline analogs			
L-Thioproline	$+^1$	0	0
4-Hydroxy-L-proline	0	0	0
L-Pipecolic acid	0	0	0
5-Hydroxy-L-pipecolic acid	$+^1$	0	0
3,4-Dehydro-L-proline	0	0	++
L-Azetidine-2-carboxylic acid	++	$++^1$	++
Tyrosine or phenylalanine analogs			
L-Mimosine	0	N.D.*	0
β-2-Thienyl-DL-alanine	0^2	0	+
DL-2-Fluorophenylalanine	0^2	+	+
DL-3-Fluorophenylalanine	0^2	+	++
DL-4-Aminophenylalanine	0	0	0
DL-α-Aminophenyl-acetic acid	0	0	0
2-Thiazolyl-DL-alanine	$++^2$	0	0
L-Dihydroxyphenylalanine	$+^1$	0	+
Arginine analogs			
L-Canavanine	0^2	++	++
L-Indospicine	0^2	+	++
L-Homoarginine	0	N.D.	0
Histidine analogs			
L-Histidinol	$+^1$	0^1	+
DL-1,2,4-Triazol-3-alanine	$+^2$	$+^2$	+
1-Methyl-L-histidine	0	N.D.	0
3-Methyl-L-histidine	0	N.D.	0

*N.D., not determined.

procedure was used for the Reuber H35 hepatoma cells in monolayer culture except for an increase in the labelling period to 4 hr. Since the specific activity of ^{3}H-leucine in newly-synthesised protein was also measured, we were able to tell whether the analog replaced the normal amino acid in protein synthesis.

Of the analogs tested in Table I with reticulocytes, thienylalanine, 2- and 3-fluoro-phenylalanine and thiazolylalanine increased protein synthesis above that found when tyrosine and phenylalanine were omitted from the medium; canavanine and indospicine

similarly increased labelling in arginine-deficient medium, and triazolalanine increased labelling in histidine-deficient medium. Only triazolalanine increased protein labelling in hepatoma cells.

Since PEPCK is present in hepatoma cells at a low basal activity and can be induced to a high activity by cyclic AMP, the heat lability of enzyme formed in the presence of an analog can be readily tested. Further, heat-labile enzyme would only be expected when analogs are actually incorporated into the enzyme. Thus, both this procedure and the stimulus of protein labelling by an analog provide indirect evidence for incorporation of the analog in place of the normal amino acid.

The amino acids which give rise to labile proteins or labile PEPCK are indicated in Table I. Several types of effect can be distinguished:

Type 1 analogs produce labile proteins after being incorporated. In this group we include all the analogs which affect PEPCK stability as well as those which increase protein labelling in either reticulocytes or hepatoma cells, *viz.*, dehydroproline, azetidine carboxylic acid, thienylalanine, 2- and 3-fluorophenylalanine, thiazolylalanine, L-DOPA, canavanine, indospicine, histidinol, and triazolalanine. Although most of these amino acids are known to be substrates of tRNA-acylating enzymes in bacterial or other systems, L-DOPA and histidinol are more difficult to explain. Some comments on histidinol are given in a later section. It should be noted that some analogs have large effects on reticulocytes but not in hepatoma cells (*e.g.*, thiazolylalanine), while several produce labile PEPCK or hepatoma protein but do not affect reticulocyte proteins.

Type 2 analogs produce labile reticulocyte or hepatoma protein without any evidence of incorporation. Analogs in this group do not affect PEPCK lability and usually inhibit protein labelling. Examples are thioproline and hydroxypipecolic acid. These analogs will be considered below in the section on amino acid omissions.

Type 3 analogs show evidence of incorporation into protein but do not produce labile proteins. These analogs presumably do not modify the structure of proteins in which they are incorporated to a sufficient extent to target the abnormal protein for rapid degradation. None of the analogs qualify for this category when data on both reticulocytes and hepatoma cells are considered, although thienylalanine and the fluorophenylalanines stimulate protein synthesis in reticulocytes without producing labile proteins.

Type 4 analogs show no effects on protein degradation or synthesis and must be considered as non-substrates for the tRNA acylation enzymes.

The most interesting analogs from a biochemical and nutritional standpoint are those type 1 analogs which are naturally occurring. Two such analogs, canavanine and indospicine (see Fig. 1) are present in legumes, plant species which are potentially useful foods. Indeed some legumes contain up to 5% of their dry weight as free canavanine.[7] Both arginine analogs are particularly toxic to liver cells because they can be present at very much higher concentration than arginine. This competitive situation arises because arginine is maintained at very low levels in liver by the high activity of arginase, while neither indospicine nor canavanine are good substrates for arginase. Feeding indospicine to rats leads to severe liver damage even when only small amounts are given.[8] The significance of this finding is also borne out by our heat lability measurements on hepatoma PEPCK. The enzyme has a heat inactivation rate up to 20 times more rapid than the normal enzyme, and even when indospicine is included in the culture medium at concentrations as low as 10 μM, a significant increase in lability is found (S. E. Knowles and F. J. Ballard, unpublished experiments). The lack of toxic effects found with canavanine or indospicine

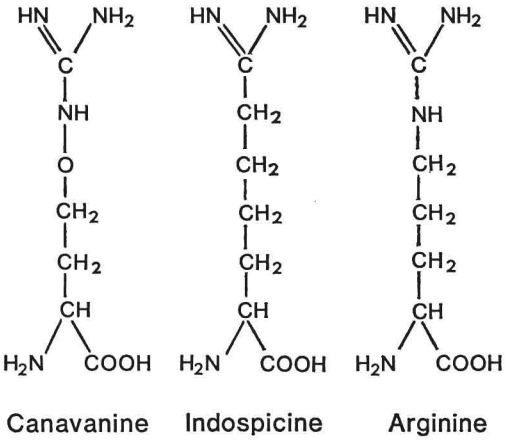

Fig. 1. Structures of the arginine analogs, canavanine and indospicine.

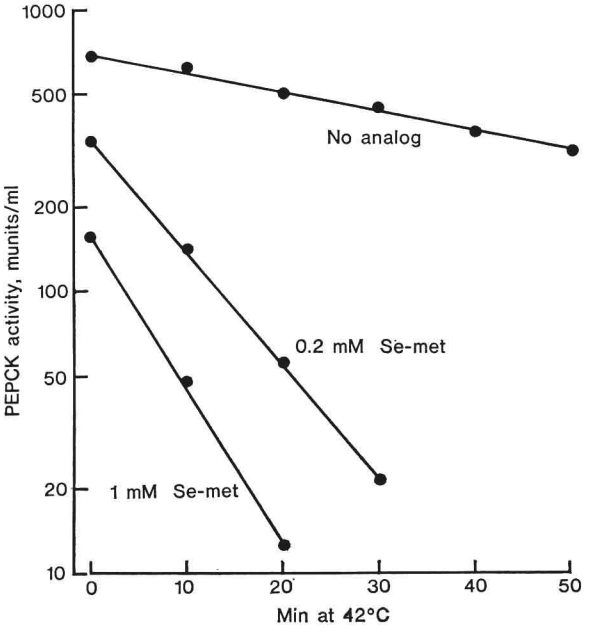

Fig. 2. Heat inactivation of normal and selenomethionyl-PEPCK in Reuber hepatoma cells. Enzyme was induced and the heat lability measured as described in the legend to Table I (see also 6). Se-met, selenomethionine.

in reticulocytes is presumably a result of the relatively high residual arginine concentrations in this cell type.

Canavanine is not incorporated into the protein of plant species which accumulate the analog, an adaptation resulting from a mutation in the tRNA synthetase in those species.[9] Indeed a similar mutation has been reported in the tRNA synthetase of bruchid beetles which live on seeds containing 13% canavanine by weight. In addition to their altered tRNA synthetase, the beetles have arginase together with an extraordinarily high activity of urease,[10] thus permitting the use of urea nitrogen. Clearly these adaptations offer a selective advantage to the insects.

Azetidine carboxylic acid, like canavanine, occurs as a free amino acid in substantial amounts. Again producer species such as lily-of-the-valley, cannot incorporate the analog in place of proline.[9] The analog leads to a large increase in haemoglobin degradation in reticulocytes, but surprisingly it behaves as an alanine analog rather than a proline analog (Table I). We have no evidence for the incorporation of azetidine carboxylic acid into reticulocyte protein in place of alanine, but the protein formed in its presence is extremely labile with up to 50% being degraded during a subsequent incubation of the cells. In the hepatoma cells azetidine carboxylic acid reacts as a proline analog and again produces large changes in protein lability.

Two analog amino acids not listed in Table I are the selenium derivatives selenocysteine and selenomethionine. These two amino acids are present at low concentrations in cells, are substrates for the cysteine-or methionine-specific tRNA synthetases respectively, and may be the means by which selenium is incorporated into proteins. Incorporation of both analogs occurs into reticulocyte protein and hepatoma protein to produce labile proteins. Thus PEPCK induced in the presence of either analog is extremely heat-labile, as illustrated for selenomethionine in Fig. 2. Yet selenomethionine does not change the degradation rate of total hepatoma protein even at concentrations which produce a 10-fold increase in the heat lability of PEPCK. Further, measurements of PEPCK degradation did not show that the heat-labile enzyme was selectively degraded by the intact cells. Presumably, therefore, the modifications to the enzyme structure resulting from selenomethionine incorporation do not lead to changes in conformation that can be detected by the proteolytic pathways of the cell.

NUTRITIONAL SIGNIFICANCE OF ANALOG AMINO ACIDS

Analog amino acids which either inhibit protein synthesis or can be incorporated into proteins might reduce the growth rate of organisms or lead to altered properties of enzymes and other cellular proteins. This could be a serious problem if the analog in question is present at high concentrations in feed and also if the analog is a good substrate or potent inhibitor of tRNA acylation. Incorporation into protein is explained since a tRNA molecule charged with an analog amino acid is not distinguished from correctly charged tRNA in subsequent reactions in protein synthesis. Accordingly any selection against the analog must occur at the tRNA acylation reaction, or at an earlier step in protein synthesis such as transport into the cell. The different types of antimetabolite actions by which analog amino acids can exert their effects have been reviewed recently.[9]

Even if a non-protein amino acid is a good substrate for tRNA acylation and is also present at high concentrations in the diet, deleterious effects on the animal may not be seen. Thus, unless some key enzymes or other proteins are modified to the extent that

their functions are altered, an increase in degradation rate will remove the abnormal protein and permit the organism to synthesise new protein molecules. However, even this response would be nutritionally important because the increased rate of protein turnover would require an increased energy expenditure. Although we have no useful information on the magnitude of any increase in nutritional requirements, it could become significant when substantial amounts of an analog are incorporated.

A more drastic effect of analog amino acids than an increase in energy requirements is the production of toxic symptoms. Mention has already been made of the selective advantage which arginine analogs have in mammalian liver. Thus ingestion of food containing canavanine or indospicine leads to liver damage because the arginine in liver is maintained at very low levels.[7,8] Indeed the toxicity of plant species which contain the analogs, for example *Dioclea megacarpa* or *Indigofera spicata*, is largely caused by their canavanine or indospicine content respectively.

IMBALANCES OF NORMAL AMINO ACIDS

It is essential for optimal rates of protein synthesis that all amino acids are present at concentrations sufficiently high to maintain each tRNA in the charged form. Thus drastic reduction in the concentration of a single amino acid will reduce protein synthesis by an amount dependent on both the degree to which the tRNA is present in the uncharged state and also the proportion of the amino acid in the protein being synthesised. For example, omission of histidine or valine from the medium in which reticulocytes are incubated produces a marked reduction in haemoglobin synthesis, while isoleucine or arginine omission has little if any effect (see Table II).

In order to provide controls in studies with analogs, we labelled protein with ^{3}H-leucine with single amino acids left out of the reticulocyte labelling medium, and subsequently measured degradation. As well as an inhibition of protein synthesis when some amino acids were omitted, the reduced amount of protein formed was unstable, often to the extent seen with amino acid analogs. Further, a negative correlation is seen between protein labelling and protein degradation (Table II). This surprising result could possibly be explained if haemoglobin synthesis was preferentially inhibited when single amino acids

TABLE II

EFFECTS OF SINGLE AMINO ACID OMISSIONS ON PROTEIN SYNTHESIS AND BREAKDOWN IN RETICULOCYTES

Protein was labelled in rat reticulocytes in the presence of a complete medium with amino acids added at concentrations equivalent to those occurring in rat plasma. Single amino acids were omitted. Labelling for 30 min in the presence of ^{3}H-leucine was followed by rapid washing of the cells. Degradation in complete medium was then measured over a 2-hr period.

Amino acid omitted	*Protein synthesis (% of control)*	*Protein degradation (% in 2 hr)*
None	100	6.6
Isoleucine	91	7.1
Arginine	80	7.1
Phenylalanine	68	8.8
Lysine	53	11.0
Valine	27	18.9
Histidine	21	24.2

TABLE III

CHANGES IN PROTEIN SYNTHESIS AND PROTEIN BREAKDOWN CAUSED BY INHIBITION OF PROTEIN SYNTHESIS

These experiments were carried out as indicated in the legend to Table II. Inhibitors were added only during the labelling period.

Inhibitor	*Protein synthesis (% of control)*	*Protein degradation (% in 2 hr)*
None	100	3.3
His omission	17	14.0
Histidinol 3mM, his omission	8	27.6
Cycloheximide, 30 μM	7	8.0
Cycloheximide, 3 μM	22	5.7
NaF, 30 mM	10	5.6

were left out of the labelling medium, leaving a labile protein or proteins. However, chromatography of the labelled protein on Sephadex columns or electrophoresis in the presence of sodium dodecyl sulphate gave good evidence for the labelling of haemoglobin or slightly shortened haemoglobin molecules. Accordingly we believe that omission of certain amino acid leads to a modified haemoglobin.

There does not seem to be any obvious mechanism how an abnormal protein could be produced simply by reducing the content of a single amino acid. Reduction in the content of charged $tRNA_{his}$ as compared to uncharged $tRNA_{his}$ might be expected to lower protein synthesis rates, but mischarging of the tRNA with other amino acids or substitutions of other charged tRNA species at positions coded for by histidine triplets seems unlikely. Yet the fraction of labile protein formed when histidine is omitted is quite substantial and is further increased when histidinol, an inhibitor of histidine charging to tRNA, is included in the labelling medium (Table III).

One possible explanation for the production of labile haemoglobin is that inhibition of protein synthesis by any inhibitor, not just by reduction of the proportion of tRNA in the charged form, will lead to an accelerated protein degradation. Inhibition of reticulocyte protein synthesis by cycloheximide or fluoride does indeed give rise to labile protein (Table III), but the increase in lability is well below that found when protein synthesis is inhibited by the reduction of tRNA charging produced by histidine omission or addition of histidinol. Since histidinol is not an amino acid, it cannot be incorporated in place of histidine, even if it could be charged by $tRNA_{his}$. However, any resultant uncharged $tRNA_{his}$ could bind to the ribosome at the "A" site in place of charged $tRNA_{his}$. Protein synthesis would then be stopped until the uncharged $tRNA_{his}$ was replaced by charged $tRNA_{his}$. However, although this would reduce the rate of protein synthesis, it is hard to envisage how a labile protein could be formed. Perhaps the growing peptide might "skip" the histidine coding triplet and give rise to a protein shortened by the absence of a histidine residue; but there is no evidence for a reaction of this type.

Incubation of histidinol with hepatoma cells in the presence of cyclic AMP results in the induction of a labile form of PEPCK. This finding is also hard to reconcile unless histidinol produces a modification to PEPCK such as a substitution of an amino acid for histidine or a histidine deletion, as suggested above. An alternate possibility is for histidinol to have an effect unrelated to protein synthesis, perhaps a stimulation of the breakdown of cellular proteins not restricted to proteins being synthesized during the labelling period.

Should this occur, it would be analogous to the stringent response in bacteria, a process which gives rise to a number of effects including the breakdown of cell protein. We have tested whether a "stringent"-type of response occurs in reticulocytes starved of histidine, by prelabelling reticulocyte protein before addition of histidinol. Under these conditions the pre-existing protein remains stable, since only that protein made in the presence of histidinol is degraded. Therefore the mechanism by which histidine starvation produces labile protein remains obscure.

REGULATION OF THE BREAKDOWN OF LABILE PROTEINS

Lysosomes are thought to be a site for the breakdown of intracellular proteins, because of their high content of proteolytic enzymes, and also by analogy with the known involvement of lysosomes in the catabolism of proteins pinocytosed into cells.[3] Since direct evidence for this hypothesis has been elusive, more indirect approaches have been attempted.

We have quantified degradation of canavanine-containing protein and normal protein in hepatoma cells by a double label technique.[2] Normal proteins were first labelled with ^{14}C-leucine, after which the medium was changed to an arginine-free, canavanine-containing medium with ^{3}H-leucine added. Following the second labelling period, the cells were thoroughly washed and placed in a medium containing 2mM leucine to restrict

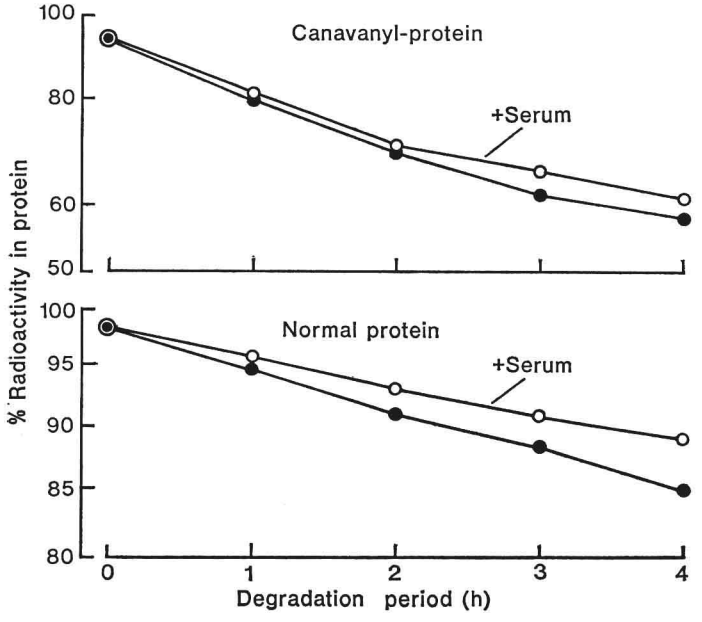

Fig. 3. Degradation of normal protein and canavanyl-protein in Reuber hepatoma cells. Normal and canavanyl-protein was labelled sequentially in hepatoma cells, after which the degradation of both protein types was followed in cells incubated in serum-free medium or medium with 10% fetal calf serum added.[2]