

PROTEIN RELEASE BY BARLEY ALEURONE
LAYERS AND METABOLISM OF PUROMYCIN
BY YEAST CELLS

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ULRICH MELCHER
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LAYERS AND METABOLISM OF FUROMYCIN
BY YEAST CELLS

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ABSTRACT

PROTEIN RELEASE BY BARLEY ALEURONE LAYERS AND METABOLISM OF PUROMYCIN BY YEAST CELLS

By

Ulrich Melcher

The initial amino acid polymerized during protein synthesis in eukaryotic organisms was investigated by studying N-terminal amino acids of proteins released by barley aleurone layers, and by studying the metabolism of puromycin by yeast cells.

Barley aleurone layers released large amounts of protein, including hydrolytic enzymes. Protein release was only partially dependent on added gibberellic acid (GA_3) and was reduced by pretreatment of tissue with CaCl_2 .

A procedure is described for the quantitative determination of protein N-terminal amino acids using 1-dimethylaminonaphthalene-5-sulfonyl (DNS) chloride, thin layer chromatography, and fluorescence spectrometry. The predominant N-terminal amino acid (determined by this

method) of proteins released in the presence of GA was glutamic acid, while in the absence of GA, it was leucine. The N-terminal glutamic acid arose from digestion by GA induced proteases.

Bromate inhibited amino acid release from aleurone layers. It was less effective on release of protein and amylase. Some of the reduction in amylase release was overcome by an exogenous supply of amino acids. Bromate was not effective in preventing the changes in N-terminal profile.

Yeast cells supplied with high concentrations of puromycin incorporated radioactive amino acids, formate, and acetate into puromycin derivatives. Formate and acetate were only incorporated into acyl aminoacyl puromycin, while amino acids were incorporated into this compound and aminoacyl puromycin.

These products were characterized by ethyl acetate extraction, thin layer chromatography, electrophoresis at pH 1.8, N-terminal analysis, and steam distillation. Acyl aminoacyl puromycin was further characterized by electrophoresis at pH 5.4, mild acid hydrolysis, and pronase digestion.

Many amino acids contributed radioactivity to the products. These were primarily aminoacyl puromycins and acyl aminoacyl puromycins, rather than their peptidyl analogues. Most of the ¹⁴C-acetate incorporate was

recovered as acetate after acid hydrolysis. Only 50% of the ^{14}C -formate incorporation was recovered as formate. Some of the ^{14}C -amino acid radioactivity was not incorporated as amino acids, formyl or acetyl groups.

Chloramphenicol inhibited incorporation of all precursors tested only when used as a saturated solution. Concentrations of cycloheximide and anisomycin which inhibited protein synthesis had no effect on formation of radioactive puromycin derivatives.

Radioactive methionine was incorporated into puromycin derivatives by yeast cells. The level of incorporation was no greater than that of other amino acids and the incorporation was not into N-formyl methionyl puromycin.

The possible relation of these puromycin derivatives to initiation of protein synthesis in yeast cells is considered.

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LIST OF ABBREVIATIONS

N-terminal	amino terminal
C-terminal	carboxy terminal
GA ₃	gibberellic acid
DNP	2,4-dinitrophenyl
PTH	3-phenyl-2-thiohydantoin
DNS	1-dimethylaminonaphthalene-5-sulfonyl
dansylate	react with DNS-Cl
dansylation	reaction with DNS-Cl
TLC	thin layer chromatography

GENERAL INTRODUCTION

Living cells make proteins by the stepwise addition of amino acids to the carboxyl terminus of a growing peptide chain (for reviews see Lengyel and Söll, 1969; Lengyel, 1969). More specifically, a subcellular particle (ribosome) and soluble proteins catalyze the transfer of a peptide from peptidyl tRNA to an aminoacyl tRNA yielding a free tRNA and a peptidyl tRNA longer by one amino acid residue. The correct aminoacyl tRNA is selected by the interaction of the tRNA with mRNA.

This process must be started and also must be ended. Initiation differs from elongation in at least three respects. First, initiation requires that mRNA, aminoacyl tRNA, ribosome, and protein factors be brought together. Elongation only requires the addition of one aminoacyl tRNA to a preexisting complex. Second, the initiation reaction is the transfer of an amino acid, rather than a peptide, from aminoacyl tRNA to another aminoacyl tRNA. Last, initiation requires the selection from the entire mRNA of one particular codon at which to start polymerization. Elongation merely requires moving from one codon to the next codon on the mRNA.

Study of initiation is important, since this step may control which proteins are synthesized. Evidence from bacteriophage systems indicates that control of translation does occur at initiation. The accumulation of phage coat protein inhibits the synthesis of the phage specific RNA polymerase directly by binding to a site on the phage RNA (Ward, et al., 1968). This binding prevents initiation of polymerase synthesis (Lodish, 1968). Initiation can also be prevented by the secondary structure of phage RNA (Lodish, 1970). In addition, the relative amounts of the proteins formed depend on the source of initiation factors (Eisenstadt and Brawerman, 1967; Hsu and Weiss, 1969; Dube and Rudland, 1970; Schedl et al., 1970; Szer and Brenowitz, 1970) or the source of 30s ribosomal subunits (Lodish, 1969a; Lodish, 1969b).

In higher organisms, initiation has been assayed mainly by measuring the attachment of ribosomes to mRNA. Using this assay, Fan and Penman (1970) concluded that the decline in protein synthesis during mitosis of Chinese hamster cells was due to a decline in the rate of initiation. Although many ribosomes from one tissue can attach to mRNA from another tissue or organism, there is a fraction of ribosomes which only attach to the homologous mRNA (Naora and Kodaira, 1970). Similar tissue specificity was noted by Heywood (1970) when he observed that muscle initiation factors were needed for the translation of

muscle mRNA on reticulocyte ribosomes. Initiation of protein synthesis within a cell can be controlled by physical separation of ribosomes from mRNA (Marcus and Feeley, 1965). Initiation could also be controlled by regulating the availability of the first aminoacyl tRNA (Arnstein and Rahaminoff, 1968).

In bacteria, protein synthesis begins with the formation of an initiation complex. This complex contains mRNA, one species of tRNA, the 30s ribosomal subunit, and initiation factors.

The only tRNA that can participate in the formation of this complex is N-formyl methionyl tRNA_f^{met} (Rudland, et al., 1969). It differs from the other species of methionine accepting tRNA, tRNA_m^{met}, in that it is bound to ribosomes in response to the codons AUG and GUG, while met tRNA_m^{met} is bound only with AUG (Marcker, et al., 1966; Ghosh, et al., 1967). Met tRNA_m^{met} can not be formylated by the transformylase that modifies met tRNA_f^{met} (Marcker, 1965). In in vitro protein synthesizing systems, the formyl methionine from this tRNA becomes the N-terminal amino acid of the newly synthesized protein chains (see int. al.: Adams and Capecchi, 1966; Clark and Marcker, 1966; Migita and Doi, 1970). Met tRNA_f^{met} does not incorporate methionine into internal positions of the peptide chain, while met tRNA_m^{met} donates methionine only internally (Marcker, et al., 1966).

Since met tRNA_f^{met} responds to codons AUG and GUG, one expects the mRNA to contain one of these codons at the initiation point. It has been shown in studies with synthetic polynucleotides that the codons AUG and GUG in the presence of F-met tRNA_f^{met} phase the mRNA (Thach, et al., 1966). Codons prior to AUG are not translated; and codons subsequent to it are translated in groups of three bases following the AUG triplet. That AUG is a natural initiation codon for bacterial protein synthesis has also been demonstrated. The sequence of several phage RNA initiation regions (Steitz, 1969; Gupta, et al., 1970) show that an AUG codon precedes the first codon that could be deduced from known protein sequences.

Enzyme activities exist in bacteria which modify the N-terminal of proteins. A deformylase removes the N-terminal formate group (Adams, 1968; Takeda and Webster, 1968). An aminopeptidase can then remove N-terminal methionine (Takeda and Webster, 1968; Matheson and Dick, 1970). The specificity of this peptidase can account for the observed N-terminal distribution of bacterial proteins (Waller, 1963; Horikoshi and Doi, 1967; Sarimo and Pine, 1969).

Chloroplast and mitochondrial protein synthesizing systems of higher organisms are believed to resemble the bacterial system closely (Roodyn and Wilkie, 1968). That the initiation reactions are similar is supported by

the isolation of N-formyl methionyl tRNA from mitochondria of HeLa cells (Galper and Darnell, 1969) of yeast and of rat liver (Smith and Marcker, 1968) and from bean chloroplasts (Burkard, et al., 1969). The production of N-formyl methionyl puromycin by Acetabularia chloroplasts (Bachmeyer, 1970) also supports this conclusion.

Three features of the bacterial mechanism of protein synthesis initiation are also found in eukaryotic cytoplasm. First, the cytoplasm of yeast (Yamane and Sueoka, 1963; Takeishi, et al., 1970; RajBhandary and Ghosh, 1969; Smith and Marcker, 1970), guinea pig liver (Caskey, et al., 1967 and 1968), rabbit liver (Bhaduri, et al., 1970; Petrissant, et al., 1970); mouse liver and mouse ascites tumour cells (Smith and Marcker, 1970); and wheat germ (Leis and Keller, 1970), contain at least two species of met tRNA (see, however, Brown and Novelli, 1968). One of these only can be aminoacylated by E. coli met tRNA synthetase (Yamane and Sueoka, 1963; Petrissant, et al., 1970). One of the met tRNA's can be formylated by E. coli transformylase (RajBhandary and Ghosh, 1969; Smith and Marcker, 1970; Caskey, et al., 1967; Bhaduri, et al., 1970; Leis and Keller, 1970). The formylated met tRNA can substitute for E. coli N-formyl met tRNA_f^{met} in bacterial cell free systems (Smith and Marcker, 1970; Marcker and Smith, 1969). In in vitro studies with synthetic polynucleotides and a eukaryotic cell free system,

the formylatable met tRNA incorporates methionine primarily into N-terminal positions, while the other species donates methionine into internal positions (Smith and Marcker, 1970).

Messenger RNA molecules from eukaryotes probably have an AUG codon near the site for the first amino acid. RHN's of plant viruses have been translated in bacterial cell free systems, yielding proteins closely resembling the in vivo viral products (Clark, et al., 1965; vanRavenswaay Claasen, et al., 1967). These systems depend on a formyl donor (Reineke, et al., 1968; Kolakofsky and Nakamoto, 1966) and some bacterial initiation factors (Brawerman, 1969; Wahba, et al., 1969). It has been shown for alfalfa mosaic virus (Hoogendam, et al., 1968) and tobacco mosaic virus (Schwartz, 1966) that the in vitro product has an N-formyl methionyl residue at the N-terminus. In contrast to these results, Laycock and Hunt (1969) could only demonstrate hemoglobin synthesis in a bacterial system programmed with rabbit hemoglobin mRNA when acetyl valyl tRNA was added to the system .

Second, revertants of iso-1-cytochrome c-less mutants of yeast sometimes have peptide chains longer by two residues at the N-terminal (Stewart, et al., 1969). The sequences of the revertant proteins are consistent with the hypothesis that an AUG codon immediately adjacent to the first amino acid codon was mutated in the original

lesion, and that the reversion consisted of mutation of the codon adjacent to mutated initiator to another AUG.

Third, the N-terminal incorporation of methionine into globin chains in a hemoglobin cell free system has been reported (Bhaduri, et al., 1970; Jackson and Hunter, 1970). In these cases, the information to add on an additional methionine must have been supplied in the mRNA as an AUG codon. Thus, as in bacteria, part of the initiation signal for eukaryotic protein synthesis is probably an AUG codon.

A third feature in common with bacterial initiation is the requirement for a number of initiation factors. Such factors have been isolated or their presence demonstrated in reticulocytes (Prichard, et al., 1970) muscle (Heywood, 1970) and wheat embryos (Marcus, et al., 1970). Although these factors function in initiation the details of their action may differ from those found for bacteria (Marcus, 1970a).

These three features of eukaryotic initiation (the involvement of a special met tRNA, the presence of an AUG initiator codon, and the involvement of initiation factors) are established. It is, however, not clear whether methionine, or another amino acid, possibly N-acetylated, is the first amino acid polymerized. Four approaches have been used to examine this problem.

The first approach is predicated on the assumption that an N-acylated amino acid is used as the first amino acid. If this is so, then there must be, in any tissue, an N-acyl aminoacyl tRNA and an enzyme to acylate aminoacyl tRNA. Neither N-formyl met tRNA (Marcker and Sanger, 1964; Clark and Marcker, 1966; Smith and Marcker, 1968) nor a met tRNA formylase (RajBhandary and Ghosh, 1969; Kolakofsky and Nakamoto, 1966) has been found in the cytoplasm of any eukaryote. Blocked aminoacyl tRNA's (Clark and Marcker, 1966; Mosteller, et al., 1968) and the corresponding formylases and acetylases (Mosteller, et al., 1968; Marchis-Mouren and Lipmann, 1965) have also been reported absent from eukaryotic cytoplasm.

In contrast, three laboratories have reported blocked aminoacyl tRNA's in eukaryotic systems. Rush and Starr (1970) have demonstrated the conversion of glutamyl tRNA into its cyclized form, pyrrolidone carboxyl tRNA, by cell free extracts. Kim (1969) claims to have shown that yeast cells make N-formyl seryl tRNA both in vivo and in vitro. Liew, et al. (1970) have shown the presence of N-acetyl seryl tRNA in regenerating rat liver and also demonstrated acetylation of seryl tRNA by cell free extracts.

Nascent polypeptide chains presumably have not been extensively modified at the N-terminal. Thus, analysis of their N-terminals provides a second approach to the

initiator amino acid. Analysis of nascent chains made by rabbit reticulocytes showed that most of the chains, if not all, had an unblocked N-terminal valine (Arnstein and Rahaminoff, 1968). Wilson and Dintzis (1969), however, reported that a small percentage of nascent hemoglobin chains have an additional residue masking the normal N-terminal valine. Jackson and Hunter (1970) have shown that some nascent globin chains have an additional methionine residue N-terminal. Nascent protamine chains also begin with free methionine (Wigle and Dixon, 1970). Similar experiments, with tumor cells synthesizing primarily one immunoglobulin, showed that nascent chains had more free N-terminal glutamyl residues than the finished product (Baglioni, 1970). The amino acid sequence of the isolated immunoglobulin begins with pyrrolidone carboxylic acid. Nascent chains (presumably of histones) from regenerating rat liver polysomes have N-terminal acetyl serine residues (Liew, et al., 1970). N-acetyl glycine has been identified as the N-terminus of nascent chains from polysomes of hen's oviduct minces, a tissue which synthesizes ovalbumin (Narita, et al., 1968).

The presence of acetyl residues at the N-terminus of a number of purified proteins led Pearlman and Bloch (1963) to propose that acetyl amino acids are involved in chain initiation in higher organisms. Some of these acetyl groups are added after polymerization (Marchis-Mouren

and Lipmann, 1965). In addition to N-acetyl proteins, the presence of one formyl protein has been reported (Kreil and Kreil-Kiss, 1967). N-terminal data on total cellular proteins, rather than purified individual proteins, of a number of eukaryotes are available (Sarimo and Pine, 1969). Methionine was not detected as an N-terminal. The transient N-terminal incorporation of methionine into complete protamine molecules has been reported (Wigle and Dixon, 1970).

The last approach to the question of which amino acid is N-terminal is the examination of which amino acids are incorporated as N-terminals in cell free systems. Synthetic polynucleotides are translated by a cell free system from ascites cells only when they contain AUG or GUG codons (Brown and Smith, 1970). When they are translated, methionine is incorporated into N-terminal positions. Incorporation of formyl methionine from ascites cell met tRNA formylated with E. coli transformylase was not observed. When a natural viral RNA was used in the same system, methionine was not incorporated N-terminally (Smith and Marcker, 1970). A cell free, hemoglobin synthesizing system has been reported (Arnstein and Rahaminoff, 1968) to incorporate α -hydroxy isovalerate rather than valine into the N-terminal of hemoglobin chains. These workers suggested that initiation required aminoacyl tRNA's lacking free α -amino groups. In

experiments with a similar system, Bhaduri, et al. (1970) have shown N-terminal incorporation of methionine.

In summary, methionyl tRNA, the codon AUG, and protein factors, are involved in initiation. The first amino acid polymerized under some conditions is methionine. Under other conditions it is either an unmodified amino acid or an acetylated amino acid. This study was undertaken to determine whether methionine, acyl amino acids, or other unmodified amino acids served as chain initiators for in vivo protein synthesis in barley aleurone and yeast.

PART I

N-TERMINAL AMINO ACIDS OF BARLEY

ALEURONE LAYER PROTEINS

INTRODUCTION

In order to study initiation of protein synthesis by examining N-terminal profiles of proteins, a suitable protein preparation is necessary. First, the preparation should contain more than one species of protein. Second, the protein must be free of post-synthesis modifications of the N-terminal region.

The barley aleurone layer (Varner and Ram Chandra, 1964; Varner et al., 1965; Chrispeels and Varner, 1967) was thought to conform to just such requirements. In response to a hormonal stimulus, amylase (Filner and Varner, 1967), protease (Jacobsen and Varner, 1967), and probably several other enzymes are synthesized de novo and secreted by the aleurone cells. It was thought that, since release followed soon after synthesis, post-polymerization modification would be at a minimum.

Closer examination of the system, however, showed that it was not suitable for studies of initiation by N-terminal analysis. Only some of the proteins found in the medium were newly synthesized enzymes. Altering the incubation procedure eliminated much of the contaminating

protein. However, such a partially purified protein preparation was still not suitable, because protease activity generated a non-random N-terminal profile. The partially purified protein preparation had an N-terminal profile which reflected the protease specificity. Therefore, it is likely that the newly synthesized proteins had been substantially altered after polymerization.

MATERIALS AND METHODS

Isolation and Incubation of Aleurone Layers

Barley (Hordeum vulgare) seeds, cultivar Himalaya, from 1964 and 1967 harvests were used. Seeds were imbibed, aleurone layers isolated and incubated, and media and extracts prepared according to the methods of Chrispeels and Varner (1967) with the following minor exceptions. A 5% solution of NaOCl was used in place of Chlorox. Half-seeds were stirred in 1% NaOCl with a magnetic stirring bar. When GA was omitted from incubations 0.20 ml of acetate buffer was added instead. Media after incubation were spun at 2,000 x g for 10 min (Jones and Varner, 1967) to remove debris. The supernatants were decanted and used as medium. In experiments where aleurone layers were transferred during incubation, the layers were removed aseptically, blotted dry on a sterile paper towel, and placed into another flask for further incubation.

Protein and Amino Acid Determinations

Protein was determined according to the method of Lowry, et al. (1951) as modified by Eggstein and Kreutz (1955). Results are expressed either as relative absorbance or as μg protein. In the latter case, suitable dilutions of a solution of two times recrystallized bovine serum albumin (calbiochem) were assayed concurrently with samples.

In cases where interfering substances, such as CaCl_2 , were present, protein was assayed by the Waddell method (1956). Protein values determined with this method agreed with values obtained by the Lowry procedure. The differences in absorbance between 215 and 225 nm were proportional to concentration over a 16 fold range of concentration. Acetate and nucleic acids, at the concentrations encountered, did not interfere.

The ninhydrin assay (Moore and Stein, 1946) was used to measure amino acid levels. Standard concentrations of glycine were assayed concurrently with all samples.

Enzyme Assays

Amylase activity was measured by the method of Chrispeels and Varner (1967). One unit of amylase activity is defined as the amount of enzyme required to reduce the absorbance of the starch iodine complex by 1.0 in 1

min. In some assays, 0.20 ml, instead of 1.0 ml, each of enzyme, starch, and iodine reagent were used. After reaction the solutions were diluted with 0.4 ml water. $A \Delta A_{620}$ of 5.0 in this assay was equivalent to 1.0 units.

Protease was assayed according to the method of Jacobsen and Varner (1967). The gliadin solution was made 10 mM in β -mercaptoethanol. The sulfhydryl reagent was omitted from the enzyme solution.

Ribonuclease was assayed by the method of Wilson (1963). The supernatant after uranyl acetate-perchloric acid precipitation of RNA was diluted 10 fold and the A_{260} determined. Minus enzyme blanks were subtracted from all samples.

Acid phosphatase was measured by a modification of the method of Burch (1957). Incubations contained 0.06 ml 0.10 M sodium acetate, pH 4.8, 0.24 ml diluted enzyme solution, and 0.50 ml 6 mM disodium p-nitrophenyl-phosphate. Incubations were for 30 min at 37°C, after which 0.20 ml of 0.1 N NaOH were added and the absorbance at 410 nm determined.

Hydrolysis of β -naphthyl acetate was used as a measure of esterase activity. To start the reaction, 0.40 ml of a saturated solution of β -naphthyl acetate was added to 0.10 ml of diluted enzyme solution. After 1.0 hr at room temperature, the reaction was stopped by immersion in ice. Color was developed by adding 0.10 ml

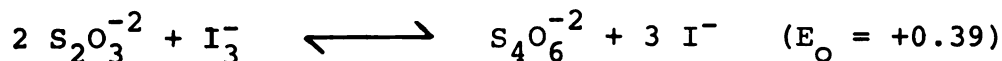
freshly prepared 0.2% fast green RR salt (Sigma), followed by 1.00 ml chloroform. Tubes were well mixed. After separation of layers, the absorbance of the lower organic phase at 534 nm was determined.

Bromate Determination

Bromate was determined using the coupled redox reaction:



($E_o = +1.29$). The reaction:



was used as indicator. Bromate was titrated in 125 ml erlenmeyer flasks containing in addition to the sample (100-300 nmol bromate), 5 ml 0.05 M KI, 1 ml 1 N HCl and a drop of starch solution. Reducing agent, 100 μM $\text{Na}_2\text{S}_2\text{O}_3$ was added from a 25 ml burette until the blue starch iodine color disappeared permanently. The reaction required 30 min for completion at room temperature. The reducing agent was standardized against known concentrations of KBrO_3 . The equivalence found was 15.86 nmol KBrO_3 per ml $\text{Na}_2\text{S}_2\text{O}_3$.

Bio-Gel P-100 Chromatography

Bio-Gel P-100 (Bio-Rad) was used as a medium for gel filtration. Five to ten g of the dry gel were allowed

to hydrate in 5×10^{-2} M sodium acetate, pH 4.8, for at least five hours with occasional stirring. A glass column (1 x 115 cm) was filled with buffer, the top stoppered with a tube leading to the imbibed gel in 0.5 to 1.0 l buffer. The gel was continuously stirred during packing of the column which occurred by siphon action.

Solid ammonium sulfate was slowly added to the medium from incubation of 60 aleurone layers, with stirring, to give a final concentration of 70% saturation in ammonium sulfate at 0°C. The resulting precipitate was collected by centrifugation at 18,000 x g for 15 min and then dissolved in 20% (w/w) sucrose. The sample in 20% sucrose was applied to the top of the gel column with a pasteur pipette. The column was developed with buffer without applied pressure. The flow rate was kept between 15 and 20 ml/hr. Fractions were collected every 10-12 min and were assayed for A_{280} , Folin positive material, refractive index, and enzyme activity.

The void volume was determined with Blue Dextran. The sucrose that was used to dissolve the samples served as an internal marker for the end of the column. Sucrose was determined by refractive index. The amino acid elution profile, determined by ninhydrin, coincided with the sucrose elution profile.

Preparation of "-GA Protein"

The "-GA protein" used in N-terminal studies was obtained by incubating 180 aleurone layers without GA for 24 hr. The media were collected, freed of debris by low speed centrifugation, and dialyzed five hours against two changes of distilled water. The sample was frozen and lyophilized. A yield of 38.34 mg lyophilized material was obtained. A sample was dissolved in water and analyzed spectrally. An absorption maximum was found at 278 nm, with a broad shoulder at 320 nm. The extinction of a 1 mg/ml solution was 0.710 A/cm at 278 nm. The ratio, A_{280}/A_{260} was 1.24.

Reaction of Protein with 1-Fluoro-2,4-dinitrobenzene and Isolation and Separation of DNP Amino Acids

For dinitrophenylation (Sanger, 1945), 5.08 mg of "-GA protein" were dissolved in 0.2 ml 0.1 M NaHCO_3 . The dissolved protein was reacted with 0.2 ml 5% fluoro-dinitrobenzene in ethanol by shaking 4 hours in the dark at room temperature. The solution was acidified with HCl and the precipitated protein spun down. After extraction of the precipitate with ether, water, acetone, and again with ether, the residue was dried over P_2O_5 in vacuo. The dried precipitate weighed 3.35 mg. It was hydrolyzed with 0.4 ml 5.7 N HCl in vacuo at 105°C for 20 hours.

The hydrolysate was diluted with 2.0 ml water and extracted three times with ether. The ether soluble DNP amino acids were separated by two dimensional ascending thin layer chromatography on silica gel H using the solvents: benzene: pyridine: acetic acid:: 80: 20: 2 (v: v: v) and CHCl_3 : CH_3OH : acetic acid:: 95: 5: 1 (v: v: v) of Brenner, et al. (1961a). The plates were chromatographed twice in the first solvent, with 10 min drying in between runs. After the second chromatography, they were dried 2 hours at 70°C and turned at right angles for chromatography in the second solvent. Yellow spots corresponding to DNP-amino acids were marked, scraped off and eluted with 1 N NH_4OH in 50% ethanol. The spectra of the eluates were taken.

Edman Degradation of Protein
and Isolation and Separation
of PTH Amino Acids

For Edman degradation (Edman, 1950: Eriksson and Sjöquist, 1960), 12.66 mg of "-GA protein" were dissolved in 1.0 ml water. Phenyl isothiocyanate was added as a 5% solution in pyridine. After overnight reaction in the dark, 2.0 ml water were added and the reaction mixture extracted four times with 3 ml benzene. After bringing the aqueous phase to dryness with a Rotary Evapo-Mix (Buchler Instruments) glacial acetic acid and 1 N HCl were added in proportions that dissolved the residue.

After 2 hr at 40°C, the acid was removed under vacuum and the remaining solution brought to 2.0 ml with water saturated with ethyl acetate: methyl ethyl ketone:: 2: 1 (v: v). Phenylthiohydantoins of amino acids were obtained by four extractions with water saturated ethyl acetate: methyl ethyl ketone:: 2: 1 (v: v). They were chromatographed on thin layers of silica gel H according to the method of Smith and Murray (1968), using solvents 7 and 8 of Brenner, et al. (1961b), and solvents C and A of Cherbuliez, et al. (1963). Spots were identified, eluted, and examined spectrally by the method of Smith and Murray (1968).

Thin Layer Chromatography of DNS Amino Acids

DNS amino acids were separated and identified by chromatography on thin layers of silica gel H. Layers, 250 μ thick, were made by spreading a slurry of 30 g silica gel H (E. Merck, Darmstadt) in 80 ml water on 20 x 20 cm glass plates. The layers were used after 24 hr. Samples were applied in a spot 2 cm from each edge. Standard DNS amino acids were applied at opposite ends from the sample spot, also 2 cm from the edge. Samples and standards were applied with a microliter syringe or with a disposable microcapillary. The following solvents were used: 1. benzene: pyridine: acetic acid:: 80: 20: 2 (v: v: v) (Morse and Horecker, 1966).

Thiophene free benzene and 99.5% glacial acetic acid were used without further purification. Pyridine was purified by 2 days of refluxing with barium oxide, followed by distillation. 2. chloroform: ethanol: ammonia:: 20: 29: 1 (v: v: v). These reagents did not require purification. Ninety-five % ethanol and 28% (w/w) aqueous ammonia were used.

DNS amino acids were obtained from Calbiochem. DNS homoserine, DNS methionine sulfone, and DNS p-methoxyphenylalanine were prepared by reaction of the amino acid with DNS-Cl under the conditions described below. p-Methoxyphenylalanine was synthesized according to the method of Behr and Clarke (1932) in 38% yield. The crystals melted between 190 and 191°C.

Chromatograms were developed in solvent 1 until the solvent front was about 5 cm from the top. They were then dried for 2 hr in a circulating air oven at 70-80°C. The plates were turned at right angles, placed in the solvent 2 tank, equilibrated, and chromatographed until the solvent front was about 5 cm from the top. The plates were immediately sprayed with 2-propanol: triethanolamine:: 8: 2 (v: v) (Seiler and Wiechmann, 1966). Yellow fluorescent spots were detected with an ultraviolet Mineralight.

The separations were reproducible if the silica gel layer had been equilibrated with the vapor phase of the solvent before immersion in it. For this purpose, one

side of the chromatography tank was lined with chromatography paper. The plates were placed in the tank on a metal support. After 10 to 15 minutes equilibration, they were lowered into the solvent. Although fresh solvent, mixed in the tank, was always used, the tank was only emptied of old solvent just prior to introduction of fresh solvent.

DNS amino acids were chromatographed singly and in mixtures. Migration values (Table 1) were computed relative to DNS isoleucine = 1.00 in solvent 1, and relative to DNS leucine = 0.75 in solvent 2. Migration values were more reproducible when expressed in this way than when expressed relative to the solvent front.

This two dimensional system allowed unambiguous identification of most DNS amino acids. DNS glutamine, DNS asparagine, and di-DNS histidine were not tested. These compounds, as well as di-DNS cystine, are not expected in acid hydrolysates. DNS cysteic acid and DNS arginine are obscured by the presence of large amounts of O-DNS tyrosine and ϵ -DNS lysine.

Quantitative Determination of DNS Amino Acids

DNS amino acids were eluted from silica gel H chromatograms with methanol. The gel containing the fluorescent spot was scraped together with spatulas and transferred to a conical test tube. Then 2.0 ml redistilled

Table 1. R Values of DNS Amino Acids in Two Dimensional TLC.

Compound	Solvent 1	Solvent 2
DNS Asp	0.05	0.01
DNS Glu	0.17	0.03
DNS Ser	0.11	0.45
DNS Thr	0.15	0.57
DNS Gly	0.31	0.61
DNS Ala	0.56	0.74
DNS Val	0.88	0.82
DNS Leu	0.96	0.75
DNS Ile	1.00	0.84
DNS Pro	0.85	0.43
DNS Hypo	0.22	0.26
DNS Try	0.35	0.66
DNS Phe	0.68	0.77
N,O-diDNS Try	0.45	0.92
O-DNS Try	0.00	0.25
N,N-diDNS Lys	0.45	0.95
ϵ -DNS Lys	0.00	0.17
DNS Met	0.57	0.81
DNS MetO ₂	0.00	0.62
(DNS Cys) ₂	0.00	0.14
DNS Arg	0.00	0.00
DNS His	0.00	0.41
DNS p-methoxy Phe	0.69	0.76
DNS-OH	0.00	0.95
DNS-NH ₂	1.09	0.62

R values for solvent 1 are expressed relative to DNS-Ile = 1.00; for solvent 2 relative to DNS Leu = 0.75.

DNS hser	0.21	0.58
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methanol was added to make a suspension. After several minutes standing, the gel was spun down in a table top centrifuge. The supernatant was used as DNS amino acid solution.

DNS amino acids were assayed with an Aminco-Bowman spectrofluorometer. The excitation beam was passed through slits of 3, 2 and 3 mm, while the emitted light was passed through slits of 3, 2, 1, and 4 mm. The DNS amino acid solution was excited at 340 nm and the emission spectrum recorded. The relative transmission at the peak of the emission spectrum was taken as a measure of the concentration of DNS amino acid. N-DNS amino acids had emission maxima at 515 nm. The emission maxima of O-DNS tyrosine and DNS-OH were 495 and 467 nm, respectively.

Methanol was chosen as eluting agent because the quantum yield of fluorescence, although lower than in other solvents such as butanol and dioxane (Chen, 1967), was high enough to allow direct quantitation. Butanol was effective in eluting some DNS amino acids, such as DNS isoleucine but was ineffective on others, such as DNS glutamic acid. Dioxane was a poor eluter of most DNS amino acids.

Elution efficiencies of representative DNS amino acids were obtained by comparing the fluorescence of equivalent samples--one diluted in methanol, the other spotted on silica gel H and eluted. The resulting

efficiencies are tabulated in Table 2. By visual observation with ultraviolet light, it was determined that other DNS amino acids were eluted with similar efficiencies.

Table 2. Elution Efficiencies of DNS Amino Acids from Silica Gel H.

DNS Amino Acid	% eluted
DNS isoleucine	114
DNS glycine	82
DNS glutamic acid	80
DNS serine	98
DNS phenylalanine	95
ϵ -DNS lysine	88

Equal aliquots (1-3 nmol) of DNS amino acid solutions were spotted on a thin layer of silica gel H or were added to 2.0 ml methanol. Gel containing DNS amino acids were scraped off and eluted with 2.0 ml methanol.

The concentration dependence of fluorescence emission by DNS amino acids was studied. The response of the detector was found to vary linearly with concentration of DNS amino acid over a 2,000 fold range of concentrations. At concentrations greater than 10 μ M the response became less than linear, probably due to self absorption. With pure solutions of DNS amino acids the limit of detection was 5 nM, whereas eluates from

thin layer chromatograms had a higher background which raised the limit of detection to 25 nM.

The spectrofluorometer was calibrated with a solution of DNS glycine of known concentration. The conversion factor converting instrument response into concentration was applied to all DNS amino acids. Gros and Labouesse (1969) have reported on the quantum yield of fluorescence for the DNS amino acids. The quantum yields of most DNS amino acids varied by less than 10%. DNS proline, DNS tryptophan, and O-DNS tyrosine were reported to have 85, 80, and 10% of the quantum yield of DNS glycine, respectively.

Reaction of Amino Acids,
Peptides, and Proteins
with DNS-Cl

Amino acids, peptides, and proteins were derivatized with DNS-Cl under similar conditions. A typical reaction mixture contained: sample of 20 μ l 0.1 M NaHCO_3 , 30 μ l acetone, and 2 to 3 μ l 20 mg/ml DNS-Cl (Calbiochem). In reaction with proteins a larger total volume was often used. In this case, all the reagents, including DNS-Cl, were proportionately increased. Reaction tubes were closed from the atmosphere to prevent extensive loss of acetone. They were placed in the dark and allowed to react at room temperature, generally overnight.

DNS amino acids were purified from the reaction mixture either by use of an ion exchange column as described by Gray and Hartley (1963) or by direct thin layer chromatography. DNS peptides were hydrolysed by addition of 1 ml 6 N HCl directly to the reaction mixture. The tubes were sealed and hydrolysed 10-18 hr at 105°C.

Protein reaction mixtures were transferred to dialysis tubes and dialysed in the dark at 4°C against (three changes of) 2 l distilled water over a 24 hr period. The dialyzed DNS protein was transferred to hydrolysis tubes, concentrated, and hydrolyzed with 1 ml 6 N HCl at 105°C for 10 to 20 hr. After removal of HCl, the hydrolyzate was ready for thin layer chromatography. Prior purification by ion exchange (Gray and Hartley, 1963) was not found to be necessary and sometimes resulted in losses of yellow fluorescent material.

In order to determine what concentrations of DNS-Cl and amino acid were necessary for complete reaction, the relative rates of DNS serine and DNS-OH formation were determined. The ratio of the two rates, k_s/k_h , was determined by analysis of end products after complete reaction of DNS-Cl. Under conditions where the concentration of serine and of water do not change significantly, this ratio is given by:

$$k_s/k_h = \frac{(\text{DNS ser}) (\text{H}_2\text{O})}{(\text{DNS-OH}) (\text{Serine})} .$$

Equal volumes of stock solutions of serine in 0.1 M NaHCO_3 and of DNS-Cl in acetone were added to tubes containing different volumes of a 1:1 mixture of 0.1 M NaHCO_3 and acetone. After reaction, the mixtures were spotted on a thin layer of silica gel H and chromatographed in solvent 2. The fluorescent spots corresponding to DNS serine and DNS-OH were eluted with methanol and the concentration of products determined with a spectrofluorometer. Table 3 shows that the ratio k_s/k_h is not constant at the higher concentrations of serine used. At these concentrations, the serine concentration changes significantly during reaction. As the concentration of serine was lowered by dilution, the value of the ratio becomes constant at 2.3×10^5 . This value then can be used to calculate how much DNS-Cl must be added for a given concentration of amino acid in order to produce complete reaction.

Gros and Labouesse (1969) have determined the rates of formation of DNS-OH and DNS glycine. From their data a ratio of rates k_g/k_h can be calculated to be 1.3×10^5 . Gray (1967) has determined the second order rate constants for formation of all DNS amino acids. From his data a ratio of k_s/k_h of 1.1×10^5 can be calculated. The slight discrepancies may arise from a difference in the reaction conditions, such as temperature or acetone concentration.

Table 3. Relative Rates of DNS Serine and DNS-OH Formation.

Initial serine concentration (mM)	DNS serine formed (nmol)	DNS-OH formed (nmol)	k_s/k_h
5.00	60.3	18.8	1.66×10^5
2.50	49.2	29.3	1.74 "
1.67	43.8	36.4	1.87 "
1.25	47.0	46.6	2.10 "
1.00	40.2	47.3	2.21 "
0.72	30.6	47.1	2.37 "
0.56	25.5	50.3	2.37 "
0.45	22.8	57.1	2.29 "

Each reaction mixture contained 10 μ l 10.0 mM L-serine in 0.1 M NaHCO_3 and 10 μ l 20 mg/ml DNS-CL in acetone. A volume of 1:1 acetone: 0.1 M NaHCO_3 was added to give the indicated initial serine concentration. k_s/k_h was calculated as described in the text; (water) was assumed = 26 M.

Recovery of DNS Amino Acids
after Hydrolysis of
DNS Protein

A DNS protein preparation from barley aleurone layers ("-GA protein") was examined for losses during hydrolysis. Samples were examined at 12, 18, 24, and 36 hours of hydrolysis by thin layer chromatography followed by elution and quantitation. The response of the spectrofluorometer was converted to nmol DNS glycine equivalents. Table 4 shows that losses of all DNS amino acids occurred

Table 4. Effect of Length of Hydrolysis on Recovery of DNS Amino Acids.

DNS Amino Acid	nmoles DNS amino acid recovered after hydrolysis			
	12 hr.	18 hr.	24 hr.	36 hr.
Isoleucine	0.62	0.55	0.59	0.41
Leucine	2.00	2.25	1.71	1.17
Valine	0.82	0.87	0.57	0.54
Phenylalanine	0.45	0.35	0.27	0.29
Alanine	0.57	0.47	0.36	0.37
Glycine	0.42	0.27	0.15	0.25
Threonine	0.59	0.42	0.25	0.27
Serine	0.90	0.55	0.38	0.25
Aspartic acid	1.12	0.62	0.62	0.54
Glutamic acid	1.55	0.82	0.61	0.45
Unknown	18.6	17.6	15.3	9.2
ϵ -Lysine	125	129	149	125
O-Tyrosine	9.2	10.6	9.5	8.3

2.88 mg of "-GA protein" was reacted with DNS-Cl. An aliquot of 4/5 was removed for this study. The dialysed dansylated protein was concentrated and hydrolysed in vacuo at 105°C. with 2.0 ml 6 N HCl. At the indicated times aliquots were removed for analysis.

as hydrolysis progressed. The destruction of DNS serine, DNS aspartic acid and DNS glutamic acid appeared to be more extensive than destruction of other DNS amino acids,

which had similar loss rates. On the other hand, increases in the amounts of DNS leucine, DNS valine, ϵ -DNS lysine and O-DNS tyrosine occurred between 12 and 18 hours. These amino acids must have been released from the protein more slowly than others.

Since this experiment was done, Gray (1967) and Gros and Labouesse (1969) have published more detailed experiments on this point. They also find that DNS leucine and DNS valine require longer periods of hydrolysis for complete release. Gros and Labouesse (1969), however, report that ϵ -DNS lysine and O-DNS tyrosine were released from protein in 6 hours. Furthermore, the rapid destruction of DNS amino acids reported by them is not supported by the present results nor by the data of Gray (1967).

Losses occurred at two other stages in the analysis of DNS proteins. Losses during elution from the thin layer chromatograms were mentioned above. There was, in addition, a loss of fluorescence during chromatography in solvent 1. The loss was reproducible between 45 and 50%. Losses were greater if impure pyridine was used, or if the two hour oven drying before chromatography in the second dimension was omitted. No losses occurred during chromatography in solvent 2.

The complete method was tested on insulin and on porcine pancreatic α -amylase. The former gave 0.9 moles

glycine and 0.7 moles phenylalanine per mole protein [compared to the expected 1.0 moles of each per mole, (Sanger, 1945)]. The latter yielded 0.9 moles glycine, 0.9 moles phenylalanine and 0.8 moles alanine per mole of protein, in agreement with the results of McGeachin and Brown (1965).¹ These values were corrected for chromatographic but not for hydrolytic losses.

¹A similar enzyme preparation to that used by McGeachin and Brown was used here. It has since been reported (Rowe, et al., 1968) that such an enzyme preparation is contaminated with inactive peptides. These peptides are responsible for the phenylalanine and glycine observed.

RESULTS

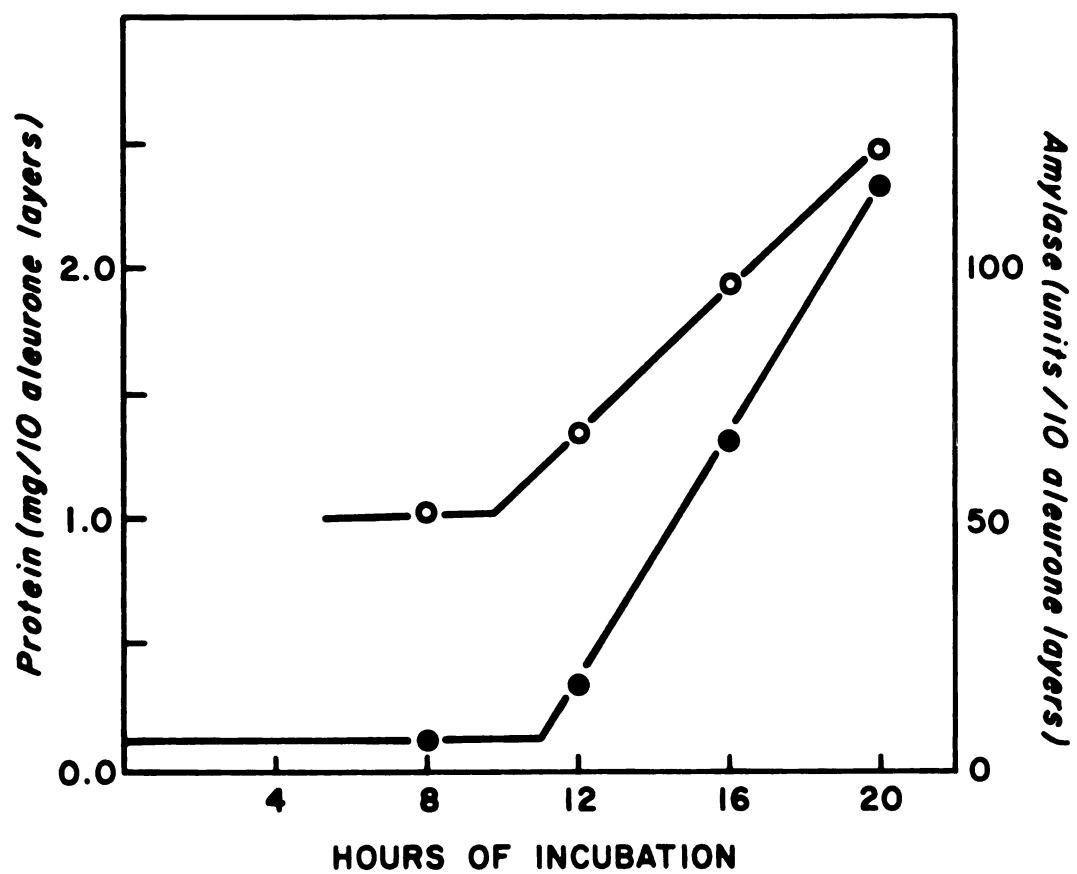
Release of Non-Hydrolase Proteins from Aleurone Layers

The importance of analyzing only newly synthesized proteins was outlined above. In the barley aleurone layer system, this means that the hormone induced enzymes must be the sole proteins released. Experiments were designed to determine whether this was the case.

The time courses of release into the medium of several enzyme activities are known. Amylase appears in the medium after an initial lag of ca. 8 hours (Chrispeels and Varner, 1967). Protease increases coincide with amylase (Jacobsen and Varner, 1967). Ribonuclease activity is released later than amylase activity (Chrispeels and Varner, 1967). Phosphatase increases slightly precede amylase release (Pollard and Singh, 1968). Thus, a period of 6 to 10 hours seems to be required before hormone induced enzymes appear in the medium.

Therefore, aleurone layers were incubated for varying lengths of time with GA. At the end of incubation, the media were assayed for amylase and protein (Figure 1). Amylase activity began to accumulate in the medium after an initial lag of 11 hours. Protein in the

Figure 1. Release of Protein and Amylase by Barley Aleurone Layers. Aleurone layers were incubated in +GA medium for the indicated times. Clarified media were assayed for amylase (closed circles) and Lowry protein (open circles).



medium increased during amylase accumulation. However, 42% of the protein was present before the onset of amylase release.

That the hormone induced enzymes were not the sole proteins released was further demonstrated by gel filtration. Aleurone layers were incubated for 12 hours with GA, transferred to fresh medium, and incubated for another 12 hours. Proteins in the 12 to 24 hour medium were precipitated with ammonium sulfate and analyzed by filtration on Bio-Gel P-100. Fractions were assayed for amylase activity and protein (Figure 2). Most of the protein eluted was of low molecular weight. As will be shown later (Figure 3) most hydrolytic enzymes eluted before this material.

Enrichment for Amylase by CaCl_2 Treatment

Incubation with calcium chloride prior to treatment with GA increased the specific activity of amylase released. Aleurone layers were washed once or twice with 1.0 M CaCl_2 or buffer and then incubated for 24 hours with GA. Amylase and protein were measured in the clarified media. Table 5 shows that one wash with CaCl_2 increased the level of amylase recovered more than three fold. Two washes with CaCl_2 were less effective on enzyme activity, but reduced the level of protein by more than 50%. As a result, treatment with CaCl_2 increased

Figure 2. Bio-Gel P-100 Filtration of Proteins Released by Barley Aleurone Layers 12-24 Hours after Addition of GA.

Sixty aleurone layers from 1964 harvest barley seeds were incubated 12 hr in +GA medium, removed, transferred to fresh +GA medium, and incubated a further 12 hr. Medium from the last incubation was collected and analyzed by gel filtration. Fractions were assayed for amylase (solid line), Lowry protein (dashed line) and refractive index (dotted line).

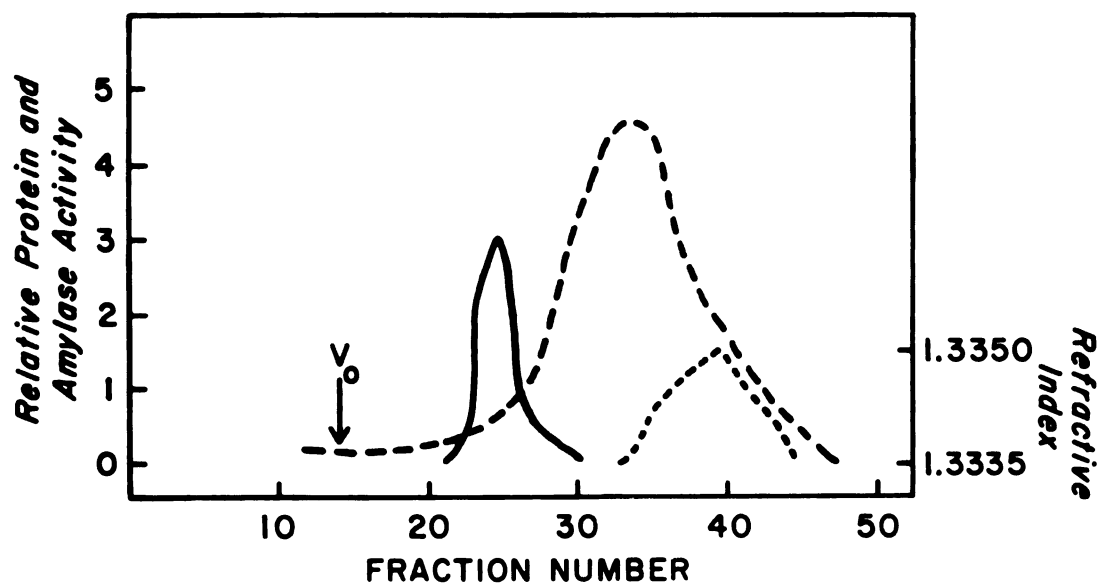


Table 5. Effect of Calcium Chloride on Release of Amylase and Protein during Subsequent Incubation of Aleurone Layers

Washes		Amylase (units/ 10 aleurone layers)	Protein (mg/ 10 aleurone layers)	Specific Activity (units/mg)
1st	2nd			
none	none	24	4.85	4.9
buffer	buffer	21	3.09	6.8
CaCl ₂	buffer	29	1.98	14.6
CaCl ₂	none	85	3.63	23.4
CaCl ₂	CaCl ₂	59	2.12	27.9

Aleurone layers from 1964 barley seeds were incubated 2 hr/wash in buffer (5×10^{-3} M sodium acetate, pH 4.8) or CaCl₂ (1.0 M CaCl₂ in the same buffer). They were then transferred to +GA medium and incubated 24 hr. Protein was determined by the Waddell method.

the specific enzyme activity, the largest increase (more than five fold) being observed with two washes.

Although the calcium chloride wash procedure was an improvement over the original method, contaminating protein was still present. This was shown by two experiments. Aleurone layers were incubated with or without GA for 1.5 or 24 hours. The incubation media were collected and assayed for protein. The appearance of protein in the incubation medium (Table 6) did not require GA, although it was enhanced by the hormone. Incubation

Table 6. Release of Protein by Aleurone Layers in Absence of Presence of GA.

Time of Incubation	Protein (mg/10 aleurone layers)	
	-GA	+GA
1.5 hr	0.480	0.510
24 hr	0.665	2.455

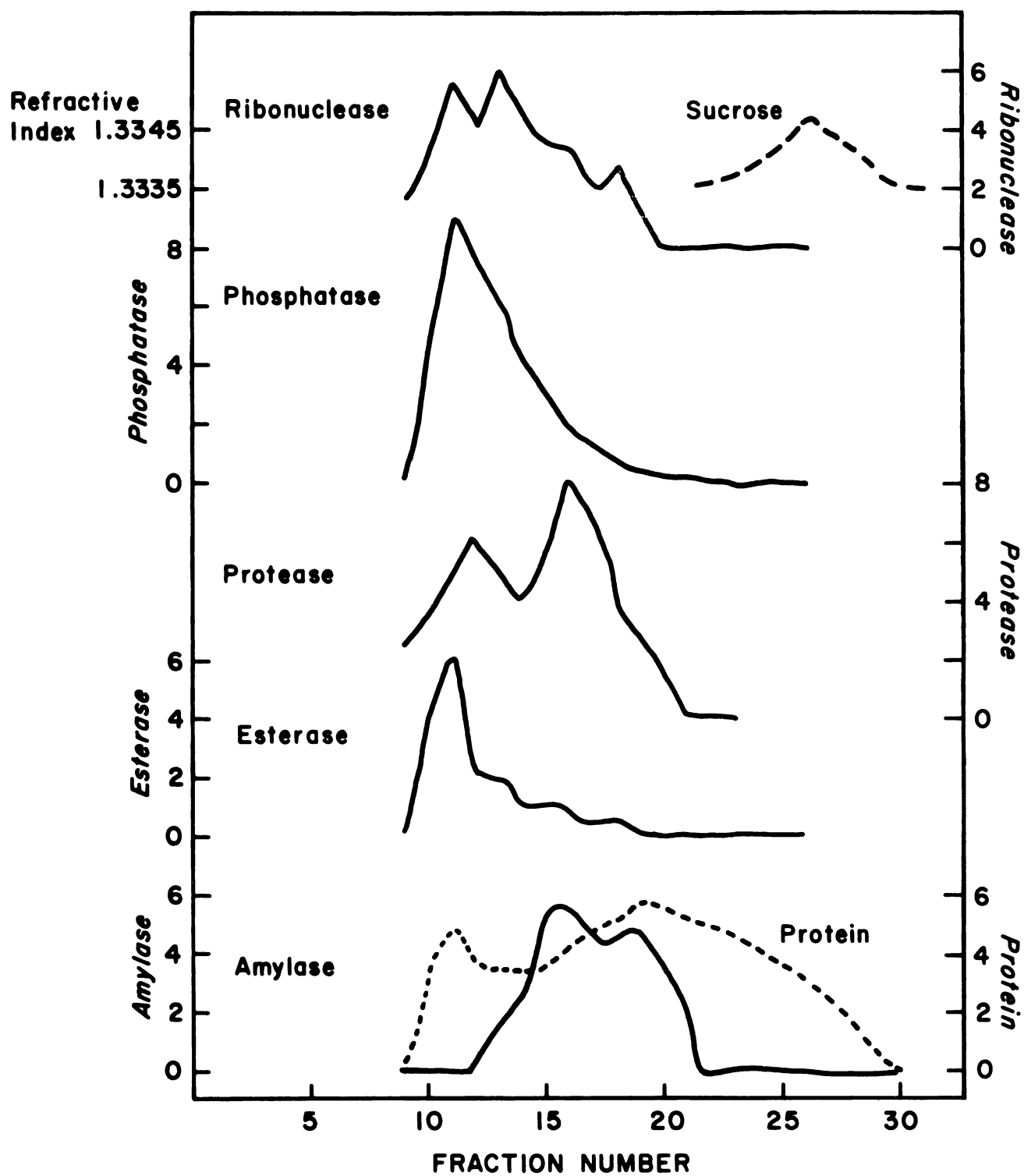
1964 barley aleurone layers were washed twice for two hr with 1.0 M CaCl_2 in 5×10^{-3} M sodium acetate, pH 4.8. Incubations were for the indicated times with or without GA. Protein was determined on the clarified media by the Waddell method.

of aleurone layers without hormone beyond 1.5 hours only slightly increased the level of protein.

The proteins released by aleurone layers after washing twice with calcium chloride were chromatographed on Bio-Gel P-100. Fractions were assayed for protein and for various hydrolytic enzyme activities. Several of the hydrolytic activities examined chromatographed in multiple peaks (Figure 3). The position of these peaks was reproducible, although their relative heights were not. A reduction, although not complete, of low molecular weight protein compared with the results in Figure 2 was obtained. This area was devoid of any of the enzyme activities tested. Thus, although the calcium chloride elution procedure gave a more satisfactory protein preparation, the preparation was still not free from non-GA induced, non-hydrolase protein.

Figure 3. Filtration on Bio-Gel P-100 of Enzymes Released by Barley Aleurone Layers.

Sixty aleurone layers from 1964 barley seeds were washed twice for 2 hr with 1.0 M CaCl_2 in 5×10^{-3} M sodium acetate, pH 4.8. They were then incubated for 24 hr in +GA medium. The medium was collected and analyzed by gel filtration. Fractions were assayed for relative enzyme activities (solid lines), Lowry protein (dotted line), and refractive index (dashed line).



Enrichment for Amylase by
Mid-Course Transfer

The increase in protein production between 1.5 and 24 hours in the absence of hormone (Table 6) was small. Therefore, the possibility of eliminating such protein by transfer of aleurone layers during incubation with GA was examined.

Transfer of aleurone layers at both 8 and 16 hours of incubation resulted in a lower recovery of protein (Table 7, column 1). This reduction in protein was accompanied by a decrease in amylase activity (Table 8, lines 1 and 2). The reduction in protein and amylase was not due to the transfer procedure (Table 7, column 4 and Table 8, lines 3 and 4).

This "transfer inhibition" was still notable when the 16 hour transfer was omitted (Table 8, lines 5 and 6). On the other hand, no reduction of amylase activity occurred on omission of the 8 hour transfer (Table 8, lines 7 and 8). More than half of the enzyme activity was produced during the last 8 hour period. In the same period, protein release was small (Table 7, column 3). As a result, the average specific amylase activity produced during these last 8 hours was 61 units/mg. The highest specific activity observed in these experiments was 90 units/mg, corresponding to 18.5% of the total protein as amylase.

Table 7. Effect of Aleurone Layer Transfer on Protein Release.

Release during	Protein (mg/10 aleurone layers)			
	Transfers at:			8, 16 (to same) hrs.
	8, 16 hrs.	8 hrs.	16 hrs.	
0-8 hrs.	0.52	0.54	) 1.17	--
8-16 hrs.	0.32	) 1.39		--
16-24 hrs.	0.34		0.62	--
0-24 hrs.	1.18	1.94	1.79	1.76
Control, no transfer	1.73	2.02	2.04	2.05

1964 barley aleurone layers were washed twice for two hrs in 1.0 M CaCl_2 in 5×10^{-3} M sodium acetate, pH 4.8. They were then placed in GA incubation medium and incubated 24 hrs. At 8 and/or 16 hrs of incubation, layers were removed, blotted on sterile paper towels, and placed into the same flask or into a flask with fresh GA incubation medium. Protein was measured on the clarified media by the Waddell method. Each value is the average of at least four incubations. Values may not add up due to rounding off.

Table 8. Effect of Aleurone Layer Transfer on Amylase Production.

	Amylase		
	Units/ 10 aleurone layers		
	Medium	Extract	Total
No transfer	57	20	78
Transfer, 8 and 16 hrs.	11	28	38
No transfer	62	18	80
Transfer to same flask, 8 and 16 hrs.	50	24	74
No transfer	58	17	75
Transfer, 8 hrs.	31	21	52
No transfer	67	20	86
Transfer, 16 hrs.	59	26	85

Experimental conditions were as described in Table 7.
Medium amylase values represent the total of all flasks.

The above experiments were done using barley seeds obtained from the 1964 harvest. A more recent harvest, 1967, when examined by the same procedures, gave higher amylase values (Table 9). In this case, however, prior washing with calcium chloride decreased the yield of amylase as well as that of protein.

Table 9. Effect of Calcium Chloride on Production of Amylase and Protein by 1967 Barley Aleurone Layers.

Washes	GA	Amylase (units/ 10 aleurone layers)			Protein
		Medium	Extract	Total	(mg/ 10 aleurone layers
-	+	126	22	148	11.34
-	-	1.7	1.3	3.0	4.22
+	+	39	17	56	2.66
+	-	0.9	1.3	2.2	0.58

Experimental details as in Table 6, except 1967 barley seeds were used and were incubated for 24 hr.

N-Terminal Profiles of Released Proteins

The above results show that the N-terminal amino acids of newly synthesized enzymes in the barley aleurone layer system were not directly approachable. The approach taken here was to first determine the N-terminal profile of proteins in the medium without purification. The effect of purification procedures on the N-terminal profiles was then examined.

Aleurone layers isolated from either 1964 or 1967 harvest barley seeds were incubated for 24 hours either with or without GA. The layers were not washed with calcium chloride prior to incubation. Media from incubation

were analyzed for N-terminals by the DNS-Cl technique. The results are expressed as percent of total fluorescence due to N-terminal amino acids (i.e., all DNS amino acids with the exception of ϵ -DNS lysine and O-DNS tyrosine). These results were not corrected for hydrolytic losses, elution efficiencies, or relative quantum yields.

N-terminal profiles of 1964 and 1967 barley proteins (Table 10) were similar. Non-random distributions were found both with and without GA. In the presence of GA, the major N-terminal amino acid was glutamic acid (and/or glutamine). It accounted for over half of the total fluorescence. In the absence of GA, a different fluorescent spot was encountered which similarly accounted for more than half of the fluorescence. It had R_f values in the two dimensional thin layer system similar, but not identical, to DNS methionine sulfone.

Characterization of Unknown N-Terminal Amino Acid

That the unknown was not DNS methionine sulfone was demonstrated by rechromatography in benzene: pyridine: acetic acid:: 60: 13: 27 (v: v: v). The migration of DNS methionine sulfone relative to DNS isoleucine was 0.62, while the unknown moved with an $R_{DNS\ Ile}$ of 0.49. The unknown was not one of the common DNS amino acids which failed to separate because of overloading. The unknown spot was eluted and rechromatographed in the same two

Table 10. N-Terminal Profiles of Proteins Released from 1964 and 1967 Barley Aleurone Layers with or without GA.

DNS amino acids	% Total N-terminal fluorescence			
	-GA		+GA	
	1964	1967	1964	1967
Isoleucine	6.4	4.5	3.3	8.3
Leucine			2.9	
Valine			1.5	
Proline	1.3	0.6	0.6	2.0
Phenylalanine	3.6	2.2	0.9	3.5
Alanine			1.8	
Lysine, Tyrosine	1.0	n.d.	2.0	n.d.
Glycine	7.9	3.2	5.1	2.6
Threonine			3.8	4.3
Serine			3.1	3.7
Unknown	61.8	86.4	5.9	13.7
Glutamic acid	12.5	3.3	71.7	61.9

Aleurone layers isolated from 1964 or 1967 harvest barley seeds were incubated 24 hr with or without GA. Media were analyzed for N-terminals. n.d. = less than 1%.

dimensional system. No alteration in migration was observed. The unknown spot was further chromatographed in solvent B of Seiler and Wiechmann (1964) where it migrated more slowly than DNS-OH. Seiler and Wiechmann reported

higher R_f values for all DNS amino acids with the exception of DNS arginine and DNS cysteic acid. The unknown was significantly separated from the exceptions in the original two dimensional system.

The unknown was a DNS amino acid associated with the N-terminal of the proteins. It had fluorescence excitation and emission spectra identical to known N-DNS amino acids. It was adsorbed onto an anion exchange resin (AG 2 X-8) at neutral pH, indicating the possible presence of a carboxyl group. It associated with protein during dialysis and during ammonium sulfate precipitation. Finally, calculations of an average molecular weight of the protein preparation (from total N-terminal fluorescence of a known weight of protein) gave a value of 54,000 g/mol when the unknown was included. This average molecular weight was consistent with the results of gel filtration.

The "-GA protein" was analyzed by the fluorodinitrobenzene technique as described in methods. From 5.08 mg of protein, a yield of 3.35 mg of DNP protein was obtained. The DNP amino acid spots found on thin layer chromatography are listed in Table 11 with their tentative identification and the spectrally determined absorbance associated with them. Rechromatography of the DNP leucine spot in solvent 1 with authentic DNP leucine, DNP valine, and DNP isoleucine established its identity as DNP leucine.

Table 11. DNP Amino Acids Obtained from "-GA Protein."

DNP amino acids	$R_{\text{DNP Leu}}$		
	solvent 1	solvent 2	$A_{\lambda\text{max}}$
Serine	16	7	0.0570
Threonine	29	14	0.0500
Glycine	27	33	0.0122
Alanine	50	57	0.0346
Phenylalanine	57	74	0.0152
Valine	90	93	0.0418
Leucine	100	100	0.287
Proline	73	93	0.0090
Lysine, Tyrosine	23	61	0.0105

It accounted for 56% of the recovered absorbance. Assuming no hydrolytic losses and an average extinction coefficient for DNP amino acids of 18×10^3 (Fraenkel-Conrat, et al., 1955), an average molecular weight of 5.9×10^4 g/mol was calculated.

For Edman degradation 12.66 mg of -GA protein were dissolved in 1.0 ml water and reacted with 2 ml 5% phenylisothiocyanate in pyridine. The phenylthiohydantoins obtained by the procedure described in methods were chromatographed two dimensionally on a thin layer of silica gel HF using solvents 7 and 8 of Brenner, et al.

(1961b). Seven ultraviolet absorbing spots which on elution gave spectra characteristic of phenylthiohydantoins were obtained. Rechromatography of this spot in solvents C and A of Cherbuliez, et al. (1963), confirmed that the spot contained the hydrophobic phenylthiohydantoins. The phenylthiohydantoin of leucine predominated.

If no losses and an extinction coefficient of 15×10^3 (Smith and Murray, 1968) are assumed, then an average molecular weight of 4.2×10^4 g/mole can be calculated. The average molecular weights calculated from DNP amino acids, and from phenylthiohydantoins, were similar to those calculated from DNS amino acids, assuming that the unknown spot was an N-terminal amino acid. Therefore, the unknown must be a derivative of DNS leucine, possibly a dipeptide. The stability of DNS dipeptides with leucine, isoleucine or valine N-terminal has been noted before (Gray, 1967; Gros and Labouesse, 1969).

Distribution of N-Terminal Amino Acids in Gel Filtration

The results of dinitrophenylation and Edman degradation thus confirmed the conclusion drawn from Table 10 that two amino acids (leucine, -GA, and glutamic acid, +GA) predominate as N-terminals of proteins released by barley aleurone layers. I proceeded to investigate whether the N-terminal glutamic acid was associated with

newly synthesized enzymes. Gel filtration of medium proteins (Figure 3) resulted in a separation into enzymatic proteins and non-enzymatic low molecular weight material. The fractions from a gel filtration similar to that in Figure 3 were divided into four groups and analyzed for N-terminal profiles by the DNS-Cl procedure. Table 12 shows that DNS glutamic acid, although present in all fractions, accounted for a much greater part of the total fluorescence in fractions containing low molecular weight non-enzymatic material. The unknown DNS amino acid was found predominantly in the highest molecular weight fractions. The proportion of other DNS amino acids either did not change or decreased with decrease in molecular weight.

Effect of Transfer on N-Terminal Profiles

Table 7 indicated that the enzymes released between 16 and 24 hours of incubation with GA were relatively free of contamination. Aleurone layers, washed twice with calcium chloride, were incubated for 16 hours with GA and then transferred to fresh medium for another 8 hours. Both the 0-16 hour and the 16-24 hour media were analyzed for N-terminals by the DNS-Cl technique. Table 13 shows that DNS glutamic acid was the main N-terminal amino acid during the period of hydrolase production. It, however, accounted for a lesser percentage

Table 12. N-Terminal Profiles of P-100 Fractions of Released Aleurone Proteins

DNS amino acids	% Total N-terminal Fluorescence			
	Fraction nos.			
	10-13	14-17	18-21	21-25
Isoleucine	6.2	7.1	6.9	) 11
Leucine	7.5	6.6	6.5	
Valine	6.2	4.1	3.6	3
Proline	5.6	4.1	3.8	3
Phenylalanine	4.6	5.6	3.8	) 9
Alanine	5.2	7.1	4.2	
Tyrosine	3.3	2.0	n.d.	n.d.
Glycine	8.8	9.2	4.8	) 11
Threonine	5.2	4.1	3.8	
Serine	4.9	5.1	4.0	
Aspartic acid	4.6	9.7	5.5	n.d.
Glutamic acid	11.8	29.6	48.8	60
Unknown	24.2	5.6	4.2	6

Media from 60 aleurone layers from 1964 barley seeds were treated as described in Figure 3. The indicated fractions were pooled and analyzed for N-terminals. n.d. = less than 2%.

Table 13. N-Terminal Profiles of Early and Late Released Aleurone Proteins.

DNS amino acid	% Total N-terminal fluorescence	
	0-16 hr	16-24 hr
Isoleucine	4.2	24.4
Leucine	4.7	
Valine	3.9	
Proline	2.9	6.0
Phenylalanine	3.4	5.0
Alanine	4.4	5.9
Glycine	7.1	7.9
Threonine	4.1	3.9
Serine	4.2	3.4
Glutamic acid	17.0	31.6
Aspartic acid	5.6	5.7
Unknown	37.2	9.6
Lysine, Tyrosine	2.3	n.d.

30 aleurone layers from 1964 barley seeds were washed twice with 1.0 M CaCl_2 in 5×10^{-3} M sodium acetate, pH 4.8. They were incubated for 16 hr with GA, removed, blotted dry, and placed in fresh +GA medium for a further 8 hr. The resulting media were clarified and analyzed for N-terminals. n.d. = less than 2.0%.

of the total than it did in an unpurified preparation (Table 10). The 0-16 hour incubation had appreciable quantities of glutamic acid and unknown as N-terminals.

Protease Action on N-Terminal Profile

The above experiments showed that the glutamic acid N-terminal released in the presence of GA appeared during the period of hydrolase synthesis, but was mainly associated with low molecular weight, non-enzymatic material. The possibility that the glutamic acid N-terminal was due to proteolysis was examined.

Aleurone layers were incubated with or without GA for 24 hours. One-half of the -GA medium was removed for N-terminal analysis by DNS-Cl. To the other half, 0.1 ml of +GA medium was added. The addition of this much protein by itself does not significantly affect the N-terminal profile of the mixture. After a further incubation for 24 hours, this mixed medium was analyzed for N-terminals by the same procedure. Table 14 shows that the addition of +GA medium, presumably possessing proteolytic activities, altered the N-terminal profile. The unknown N-terminal almost completely disappeared, while the N-terminal percentage of glutamic acid rose markedly. At the same time, the total fluorescence of N-terminal amino acids only rose 53%. The profile of the

hydrolyzed protein was similar but not identical to the profile of +GA proteins (Table 10).

Table 14. Effect of +GA Medium on the N-Terminal Profile of -GA Proteins.

DNS amino acids	% total N-terminal fluorescence	
	-GA	-GA and +GA
Isoleucine, Leucine, Valine	4.4	20.5
Proline	0.8	2.8
Phenylalanine, Alanine, Methionine	1.3	15.0
Lysine, Tyrosine	n.d.	2.6
Glycine, Threonine, Serine	1.8	15.4
Aspartic, Glutamic acids	2.8	36.9
Unknown	88.9	6.8

10 aleurone layers from 1967 barley half seeds were incubated for 24 hr. with or without GA. 1.0 ml of -GA medium was removed for N-terminal analysis. 0.9 ml of the same medium was further incubated for 24 hrs. with 0.1 ml of +GA medium. N-terminals were analyzed. n.d. = less than 0.5%.

Use of Bromate as a Protease Inhibitor

Since bromate is a known inhibitor of barley proteases (Macey and Stowell, 1961), experiments were conducted to determine if it could inhibit proteolysis without affecting production of amylase activity. Aleurone

layers were incubated with GA and with or without different concentrations of KBrO_3 . After 24 hours media and extracts were prepared and assayed for bromate, amino acids and protein (Table 15) and for amylase (Table 16). After incubation of bromate with aleurone layers, there was a loss of 0.2 μ equivalents bromate. Thus, although some of the bromate was reduced, most could be recovered when concentrations of 1 mM and greater were used. The amount of bromate found in the extracts was consistent with complete equilibration between the medium and the layers. Bromate was effective in reducing proteolysis. The level of amino acids in the medium was reduced by more than 90% by 1 mM KBrO_3 ; and by more than 99% by 10 mM KBrO_3 . The release of protein into the medium was inhibited 50% by 1 mM bromate.

Bromate inhibited the production of amylase. The inhibition, however, was less than the inhibition of amino acid release at the same bromate concentrations. A similar result was obtained by other workers (MacLeod and Millar, 1962; Jacobsen and Varner, 1967). It was thought that the effect of bromate on amylase might be due to a limitation of amino acid supply for amylase synthesis. The addition of amino acids, although consistently effective in raising amylase production at higher bromate concentrations, did not bring amylase production up to the

Table 15. Effect of Bromate on Amino Acids and Protein in and out of Barley Aleurone Layers.

	Amino Acids		Protein		Bromate	
	ueq glycine		mg		ueq	
	10 aleurone layers		10 aleurone layers		10 aleurone layers	
	Medium	Extract	Medium	Extract	Medium	Extract
GA	23.8	1.3	4.56	1.86	0.025	0.000
GA, 1 mM KBrO ₃	1.6	0.8	2.30	1.55	1.75	0.058
GA, 10 mM KBrO ₃	0.2	0.6	1.55	1.42	19.0	0.725

Aleurone layers were incubated in 2.0 ml solution containing 10^{-6} M GA and KBrO₃ in the indicated concentrations. After 24 hr of incubation media and extracts were prepared. Amino acids were assayed by the ninhydrin reaction, using glycine as standard. Protein was assayed as Folin positive material. Bromate was determined by titration against standardized Na₂S₂O₃ to a starch-iodine end point.

Table 16. Effect of Bromate and Amino Acids on Amylase Production by Aleurone Layers.

	Amylase (units/10 aleurone layers)					
	without amino acids			with amino acids		
	Medium	Extract	Total	Medium	Extract	Total
GA alone	39	15	53	33	22	55
GA, 1 mM KBrO ₃	26	17	43	12	26	38
GA, 3 mM KBrO ₃	12	13	25	--	--	--
GA, 5 mM KBrO ₃	4	9	13	10	20	30
GA, 5 mM KBrO ₃ imbibed	2	4	6	5	9	14
GA, 10 mM KBrO ₃	1	3	4	3	8	11
-GA	2	2	4	2	2	5

1964 barley half seeds were imbibed for three days on sand moistened with sterile distilled water or sterile 5 mM KBrO₃. Aleurone layers were isolated and incubated in 2.0 ml sterile solution containing 10⁻⁶ M GA, KBrO₃ in the concentrations indicated with or without the addition of 20 mg neutralized casein hydrolyzate powder. After 24 hr of incubation, media and extracts were prepared and assayed for amylase activity.

level obtained without bromate. In addition, the presence of exogenous amino acids slightly inhibited amylase release.

Proteins released in the presence of 1 mM KBrO_3 and GA were analyzed for N-terminals. Of the total N-terminal fluorescence, 30% was due to DNS glutamic acid. Thus, the inhibition of amino acid release, and presumably proteolysis, at concentrations of bromate which did not affect amylase production, was not enough to prevent changes of the N-terminal profile.

DISCUSSION

The first amino acid polymerized during protein synthesis in barley aleurone layers cannot be deduced from N-terminal profiles of released aleurone protein for two reasons. First, proteolytic activities substantially alter the N-terminal profile of proteins. Second, not only GA induced hydrolases, but also other proteins are released by aleurone layers. The release of protein may be physiologically important and is discussed below.

During incubation with GA, aleurone layers released a large amount of protein (0.25 to 0.50 mg per aleurone layer). A large part of this protein release was not dependent on GA. Protein release was not temporally correlated with release of amylase. Second, only a small portion of the released protein was GA induced hydrolase. Release of hydrolases could be experimentally separated from release of protein by CaCl_2 washing and by mid-course transfer of aleurone layers. These experimental procedures resulted in a ten fold increase in amylase specific activity. This means that only 10% of the protein released when layers were not prewashed or transferred was hydrolase protein. Third, protein release was observed on washing with CaCl_2 or on incubation

without GA. Although the proteins released in the presence or absence of GA had different N-terminal profiles, that one profile could be converted to the other by GA induced enzymes indicates that the two groups of proteins were similar. Some of the protein release seen in the presence of GA was, therefore, not GA induced. A similar partial GA independence of protein release from deembryonated cereal seeds has been reported by several workers (Varner, et al., 1965; Ingle and Hageman, 1965; Paleg, 1961).

During normal germination of barley seeds, the growing embryo utilizes sugars and amino acids released from the endosperm and aleurone layer. The present experiments allow one to distinguish two processes involved in the conversion of aleurone layer protein into amino acids. The first process is release of protein from aleurone cells. As mentioned above, this does not require GA. The release is partially dependent on proteolysis during incubation, since inhibition of proteolysis by 1 mM bromate reduced the amount of protein released by 50%. Since proteases are present in the ungerminated grain (Mikola and Enari, 1970; Jones, 1968), proteolysis during inhibition may be responsible for the remaining release observed. The second stage in making amino acids available is the degradation of protein to amino acids. This process is dependent on GA induced proteases. These

proteases must have a different specificity than the proteases already present. A number of proteolytic activities in germinating barley grain, including an aminopeptidase, (TenHooen, 1968; Bhatt, 1969; Prentice, et al., 1969; Mikola and Enari, 1970) have been described. It is not clear which of these are responsible for the changes in N-terminal profile observed here.

Factors other than GA may regulate amylase production. The "transfer inhibition" of amylase production (Table 3 and 4) suggests the release of a factor whose continuous presence for more than eight hours is necessary for maximal amylase production. The difference between 1964 and 1967 harvest barley seeds may be due to a factor in the dry seed, necessary for amylase production, which is slowly denatured on storage. After removal of the factor by CaCl_2 , a new, active factor takes its place. The difference in harvests may also be due to a different sensitivity to damage of cells by 1.0 M CaCl_2 . None of these possibilities has been further explored.

Since amylase is synthesized de novo from amino acids derived from hydrolysis of reserve protein (Filner and Varner, 1967), amylase production should depend on the availability of amino acids. Incubation of layers with an inhibitor of proteolysis, made the production of amylase partially dependent on exogenous amino acids. This confirms the prediction and indicates that one source

of amino acids for enzyme synthesis could be exogenous amino acids. Amylase production was partially dependent on exogenous amino acids at concentrations of bromate which only lowered the endogenous pool by one half. It is thus possible that during germination, protein is released by the aleurone cell, and then degraded by proteolytic enzymes. The resulting amino acids may then re-enter the aleurone cell and be used for further enzyme synthesis. A dependence on amylase production on exogenous amino acids has also been demonstrated in wild oats (Naylor, 1965).

PART II

METABOLISM OF PUROMYCIN BY YEAST CELLS

INTRODUCTION

Puromycin is an analogue of the aminoacyl adenosine end of aminoacyl tRNA (Yarmolinsky and DeLaHaba, 1959). It releases nascent chains (Morris and Schweet, 1961) from peptidyl tRNA in polysomes. The reaction is catalyzed on the large ribosomal subunit by the peptidyl transferase center which normally catalyzes peptide bond formation (Maden and Monro, 1968). The product of the reaction is peptidyl puromycin (Nathans, 1964). Upon further incubation with the antibiotic, the polyribosome structures engaged in protein synthesis break down (Colombo, et al., 1965; Lin and Key, 1967). This breakdown requires energy (Marks, et al., 1963) whereas the release of peptidyl puromycin occurs at 0° without energy. Breakdown is not complete--a new steady state level of polyribosomes is established (Williamson and Schweet, 1964; Villa-Trevino, et al., 1964). The presence of puromycin causes a more rapid equilibration of subunits with the free subunit pool (Kaempfer and Meselson, 1969). After the release of nascent chains and during subsequent incubation with puromycin, initiation of new peptide bond formation takes place at

other than the normal initiation sites (Williamson and Schweet, 1965). These reinitiated chains are also released with puromycin. The products are oligopeptidyl puromycins (Smith, et al., 1965).

These observations lead me to the following interpretation of the mechanism of action of puromycin on polyribosomes. The release of the nascent chains leaves a ribosome-messenger-tRNA complex. After an energy dependent translocation, the tRNA-OH is released. The incoming aminoacyl tRNA may or may not be bound firmly enough to be able to form a peptide bond with the new aminoacyl tRNA in the acceptor site. If it is, peptide bond formation occurs and short peptidyl puromycin chains are synthesized. If the aminoacyl tRNA is not bound firmly enough, the whole ribosome messenger complex dissociates. The resulting ribosomes (subunits?) are available for chain initiation.

Another model has been proposed by Kaempfer and Meselson (1969). In their model, upon release of tRNA-OH the 50s ribosomal subunit dissociates from the message, leaving the 30s subunit free to travel along the mRNA. Periodic reassociation with the larger subunit causes reinitiation. The smaller subunit finally dissociates when it reaches the termination signal. In this model, one would expect equal synthesis of all sequences within a cistron. In my model, preferential synthesis of N-terminal sequences is predicted. Nathans (1965) has shown that in the presence

of puromycin, more N-terminal sequences are synthesized than C-terminal ones. It is thus likely that in the presence of sufficiently high concentrations of puromycin only puromycin derivatives of the initial amino acid will be formed.

In this study use has been made of the antibiotic, puromycin, to stop polymerization after the first amino acid is in position at the peptidyl transferase center. This approach has been used before to demonstrate formation of N-formyl methionyl puromycin by bacteria and blue green algae (Bachmeyer and Kreil, 1968) and Acetabularia (Bachmeyer, 1970). It has also been used to show N-acetyl glycyl puromycin formation in hen's oviduct minces (Narita, et al., 1969). Despite its use in these studies, the rationale for its use has not been clearly outlined.

I used yeast cells for this investigation for three reasons. First, yeast cells synthesize a variety of proteins, rather than one particular protein. Second, yeast is a single-celled organism, so that reagents and radioactive precursors should have equal access to all cells. Third the N-terminal amino acid profile of the combined yeast cellular proteins are known (Sarimo and Pine, 1969).

METHODS

Preincubation of Yeast Cells

The preincubation solution consisted of 5.0 ml sterile 0.2 M citrate (Na), pH 4.8; 5.0 ml sterile 0.5 M glucose; 0.2 ml 2 mg/ml chloramphenicol (omitted in some experiments); and 2.0 gm yeast. Yeast was added last and was suspended by shaking about 5 min at room temperature. In some experiments, 5.0 ml glucose was replaced with 4.9 ml sterile water and 0.1 ml absolute ethanol. In these cases, yeast was preincubated for 0.5 hr. at room temperature

Incubation of Yeast Cells

All incubations were in sterile 10 x 75 mm vials. All components of the incubation solution (puro-mycin, radioactive precursors, cycloheximide, etc.) except chloramphenicol were added to the vials as aqueous solutions. The volume was made up to 35 μ l by the addition of sterile water. Lastly, 15 μ l of the preincubated yeast suspension was added. The vials were immediately placed in a 37°C water bath to start the incubation.

When high concentrations of chloramphenicol were used, the desired amount of an aqueous solution of the inhibitor was pipetted into a vial. The vial was stoppered with cotton, frozen, and then lyophilized. In experiments requiring dead yeast, the preincubated yeast suspension was placed in a boiling water bath for 5 min prior to addition of 15 μ l to the incubation mixture.

Ethyl Acetate Extraction

At the end of incubation, the vials were put in ice and immediately diluted with 1.0 ml water. The diluted solution was transferred with a Pasteur pipet to a large test tube. An additional 1.0 ml water was added to the vial and also transferred. Routinely, 0.4 ml 0.1 N NaOH was added. This aqueous solution was extracted four times with 2 to 3 ml of ethyl acetate (Leder and Bursztyn, 1966). The combination of alkaline pH and ethyl acetate was sufficient to release most of the cell contents. This was determined by comparing the amount of ninhydrin positive material released under these conditions with that released by boiling the yeast suspension for 5 min (Table 17). The combined

Table 17. Release of Ninhydrin Positive Material from Yeast Cells
by Ethyl Acetate.

	<u>nmol glycine equiv.</u>		% release	
	mg yeast		-EtOAc	+EtOAc
water	28.4	38.8	20.6	27.7
0.05 N HCl	21.7	132.4	15.5	94.9
0.05 N NaOH	24.5	125.2	17.5	89.7
5 min boiling	139.4	--	100	

Seventy μ l of a 0.2 gm/ml suspension of yeast in 0.2 M citrate (Na), pH 4.8, was added to 2.0 ml of water, HCl or NaOH. Then 2.0 ml of water (-EtOAc) or 2.0 ml of ethyl acetate (+EtOAc) were added. After thorough mixing, tubes were centrifuged 10 min. at 1,400 x g. 0.5 ml of the aqueous phase supernatant was assayed with ninhydrin reagent. The A_{570} of the sample was compared with that of 30 μ l of 0.010 M glycine in water, HCl, or NaOH to obtain nmol glycine equivalents. The ethyl acetate phase contained negligible ninhydrin reactive material.

ethyl acetate extracts were brought to dryness with an air stream.

Chromatography of Puromycin Derivatives

The concentrated ethyl acetate phases were purified by chromatography on thin layers of silica gel HF₂₅₄. Layers, 250 or 500 μ thick, were made by spreading a slurry of 30 g silica gel in 80 ml water on 5 x 20 cm glass plates. The plates were used between 12 and 24 hr after spreading.

Samples were dissolved in 50 μ l eluting agent [ethyl acetate: methanol: water:: 1: 1: 1(v: v: v)]. This solution was spotted with a disposable capillary tube as a streak 2 cm from the edge of the plate. To ensure complete transfer two further additions of 20 to 30 μ l eluting agent to the tube were also spotted. The plates were immersed directly in the solvent, chloroform: 95% ethanol: aq ammonia:: 40: 58: 2(v: v: v), without pre-equilibration. Chromatograms were developed until the solvent front was about 3 cm from the top edge (about 75 min). The solvent front was marked. Puromycin was identified as an ultra-violet absorbing band with an ultraviolet Mineralight.

The position of radioactive components was determined on a Packard radiochromatogram scanner.

Recovery of Puromycin
Derivatives from
Silica Gel

The area of the chromatogram which contained the puromycin derivatives was scraped off with a spatula into small test tubes. The gel was suspended in 2.0 ml eluting agent, and the suspension allowed to equilibrate about 10 min. The gel was spun down in a table top centrifuge and the supernatant decanted. This procedure was repeated once with 2.0 or 3.0 ml eluting agent. The combined supernatants were concentrated to dryness on a Rotary Evapo-Mix. The elution efficiency of this method was better than 90%.

Electrophoresis of
Puromycin Derivatives

The gel eluates were electrophoresed according to the procedure of Bachmeyer and Kreil (1968) with slight modifications. Samples were dissolved in 30 μ l eluting agent. They were spotted with disposable capillary tubes at the centers of 12 x 1 in. paper strips (Schleicher & Schuell 2043A). The sample tubes were rinsed with two

additions of 30 μ l eluting agent. These rinses were also spotted. The strips were electrophoresed in a Durrum Cell (Beckman Instruments) at 350 V for 2.5 hr. The buffer was 1.0 M acetic acid adjusted to pH 1.8 with 90% formic acid. After electrophoresis, the strips were dried at room temperature. Ultraviolet absorbing bands were located with an ultraviolet Mineralight. The position of radioactive components was determined with a Packard radiochromatogram scanner. Radioactive bands were cut out and placed in scintillation vials for radioactivity counting or into test tubes for elution and further analysis.

Incorporation into macromolecules

Incorporation of radioactive precursors into cold trichloroacetic acid precipitable material was used as a measure of incorporation into macromolecules. Aqueous phases, after ethyl acetate extraction, were freed of remaining ethyl acetate by several days storage at 4°C. When free of ethyl acetate, 2.0 ml of 30% (w/w) trichloroacetic acid containing 0.1% (w/w) casein hydrolyzate was added to each tube. The tubes were thoroughly mixed. Precipitates which formed after 12 to 24 hr. at 4°C were collected on Millipore filters. The filters were rinsed 5 times with 5 ml cold 15% (w/w) trichloroacetic acid containing 0.1% (w/w) casein hydrolysate. The filters

were placed in scintillation vials and freed of water and trichloroacetic acid in a 70°C oven.

In experiments where puromycin derivatives were not extracted with ethyl acetate, the diluted incubation mixtures were transferred at 0°C to tubes containing several drops t-butyl alcohol. The tubes were well mixed prior to addition of trichloroacetic acid as above.

Radioactivity Determination

Radioactivity was determined by liquid scintillation spectrometry. As scintillation fluid, 5 or 10 ml Bray's solution (Bray, 1960) was used.

Steam Distillation

A 2 l flask heated by an electrical hot plate was used as a steam generator. The flask was fitted with a two-holed rubber stopper. A glass tube through one hole was used as a safety valve. A glass tube through the other hole led into the distillation apparatus. This apparatus consisted of: two 75° three way connecting tubes (tubes 1 and 3), one 105° two way connecting tube with suction and extended delivery tubes (tube 2), a 200 ml round bottom flask (distillation flask), a 50 ml round bottom flask (alkaline trap flask), and a condenser. All joints beyond the steam generator were ground glass.

The steam delivery tube was led through tube 1 to the bottom of the distillation flask. The side arm of tube 1 was connected to tube 2, the delivery tube of which was led through tube 3 to the bottom of the alkaline trap flask. The side arm of tube 3 was connected to the condenser. The suction tube outlet on tube 2 was closed off with a rubber pipet bulb.

The sample, 1 ml 6 N H_3PO_4 , and 9 ml water were put in the distillation flask. The alkaline trap flask contained 10 ml 0.01 N NaOH. Distillation was begun by closing the safety valve of the steam generator. During steam distillation, the volume in both flasks was maintained by intermittent heating with a Fisher burner. Distillation was stopped when 120 ml water were collected from the condenser.

For determination of radioactivity distilled into the alkaline trap, the contents of the trap were transferred to a scintillation vial. The liquid was brought to dryness in a 70°C oven. Radioactivity was determined in a liquid scintillation spectrometer, using a window which eliminated sodium hydroxide caused spurious counts.

Mild Acid Hydrolysis

Eluates from electropherograms or from thin layer chromatograms were divided into equal portions. The eluates were brought to dryness in conical tubes. To the chilled

tubes, 50 μ l 1 N HCl was added, and the tubes were covered with Parafilm. Hydrolysis was begun by putting the tubes in a 37°C water bath. Hydrolysis was stopped by spotting the solution on paper electrophoresis strips, removing HCl and water with a hair dryer. A rinse of the conical tube with 30 μ l eluting agent was also spotted. Non-hydrolyzed aliquots were spotted on paper electrophoresis strips as described above. The use of HCl in spotting controls was avoided, since appreciable hydrolysis occurred during spotting. Both methods of spotting gave greater than 90% transfer of radioactivity from the tube to the strip.

Amino Acid Thin Layer Chromatography

Amino acids were separated by two dimensional chromatography on thin layers of silica gel H using N-butanol: glacial acetic acid: water:: 4: 1: 1(v: v: v) in the first dimension and phenol: water:: 75: 25(w: w) in the second (Brenner and Niederwieser, 1960). The sample in 0.05 N HCl was spotted two cm from each edge and then chromatographed twice in the first solvent (with 10 min intermediate drying). After drying for 30 min, the plate was turned at right angles and chromatographed in the second solvent. After removal of phenol, amino acids were detected with ninhydrin (Brenner and Niederwieser, 1960). The resulting separation was intermediate between those

reported by Opienska-Blauth, et al. (1964) and that reported by Pataki (1965). With peptide hydrolyzates, 20 μ l 0.1% casein hydrolyzate was added to the hydrolyzed samples before evaporation of HCl.

MATERIALS

One pound cakes of Red Star brand pressed baker's yeast were obtained from the University's food stores. The yeast cakes were stored at 4°C, never for more than a week, prior to use. Anabaena cylindrica was a gift from Dr. C. P. Wolk.

Puromycin dihydrochloride was obtained from Nutritional Biochemical Co. in vials. Sterile water was added to the vial to make an 0.30 M solution. An equivalent of NaOH was added, bringing the pH, as determined with pH paper, to 7. More sterile water was added to bring the final concentration to 0.25 M. Concentrations were checked by spectral determinations on a suitable diluted aliquot, assuming an extinction coefficient of 17,700 at 274.5 nm. The solution was stored in a freezer between uses. Cycloheximide was obtained from Nutritional Biochemical Co., chloramphenicol from California Biochemicals. Anisomycin was a gift from Dr. N. Belcher (Pfizer and Co.). It was dissolved in sterile water with the addition of one equivalent of HCl.

Distilled water was used after passage through cation exchange and mixed bed exchange columns. Methanol and ethyl acetate were redistilled prior to use.

N-formyl methionyl puromycin (methoxy-³H) and ¹⁴C-glycyl glycyl puromycin, synthesized by the method of Leder and Bursztyn (1966), were a gift from Mr. Warren Evins. N-formyl glycine was synthesized by the method of Sheehan and Yang (1958) in 58% yield. The crystals melted between 147.5 and 149.0°C, two degrees below the melting point reported by Fischer and Warburg (1905).

RESULTS

Inhibition of Amino Acid Incorporation by Puromycin

Puromycin derivatives of initiating amino acids should only be formed when protein synthesis is highly inhibited by the antibiotic (Bachmeyer and Kreil, 1968). Thus, the puromycin concentration dependence of radioactive amino acid incorporation into trichloroacetic acid precipitable material was examined. The inhibition of incorporation by puromycin is plotted in Figure 4. Concentrations of 50 mM and greater resulted in essentially complete inhibition.

In the above experiment, the pH of the incubation solution may have been altered by the addition of concentrated non-buffered solutions of puromycin. However, incorporation was independent of pH in the range 4.0 to 6.0 as shown in Table 18. Thus, the inhibition of incorporation shown in Figure 4 was not due to changes in pH resulting from the addition of large amounts of puromycin.

Table 18. Effect of pH on Incorporation of ^{14}C -Alanine into Macromolecules by Yeast Cells.

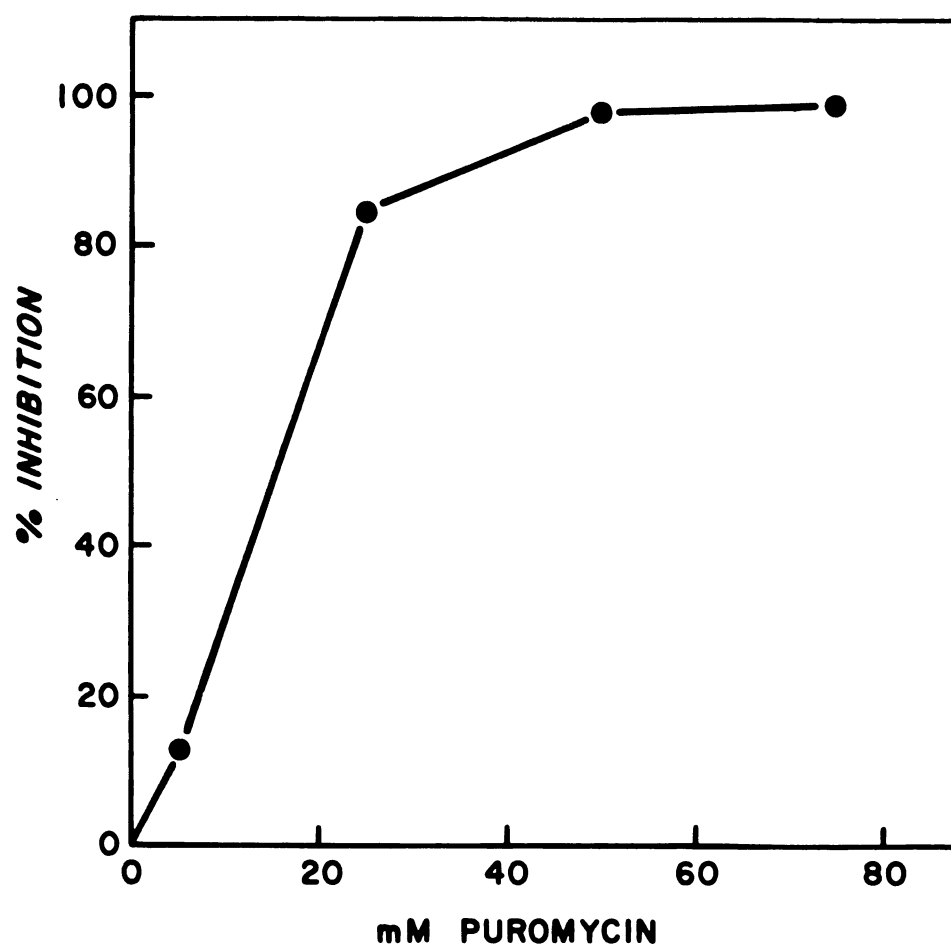
pH	cpm
4.00	6,745.8
4.70	6,868.0
5.45	6,574.4
6.00	6,948.4

Each incubation mixture contained 0.05 μCi L-alanine/ ^{14}C (U.L., sp.act. = 0.10 mCi/0.073 mg). Yeast (4.0 mg) was preincubated in a mixture of 5.0 ml 0.5 M glucose and 5.0 ml 0.10 M citric acid, made to the indicated pH with NaOH. Fifteen μl of preincubated yeast suspension was incubated with label in a total volume of 50 μl for 30 min, before dilution and precipitation.

At high puromycin concentrations there was a low, but significant, level of incorporation of radioactive amino acids into trichloroacetic acid precipitable material. This incorporation continued for at least three hours. However, when protein synthesis was not inhibited, incorporation (Table 19) ceased after 60 minutes of incubation. When radioactive amino acids were supplied for 30 minute periods, rather than throughout incubation, incorporation

Figure 4. Inhibition by Puromycin of ^{14}C -Amino Acid Incorporation into Macromolecules by Yeast Cells.

Points are the average of 3 experiments. All incubations were for 3 hr. NaCl was added to give a final concentration of 75 mM in all incubations. Radioactive labels supplied and minus puromycin control incorporations were: Expt. 1: 2.0 μCi ^{14}C -amino acid mixture (sp.act.= 1 mCi/mg, Volk), control incorporation = 694,403 cpm; Expt. 2: 0.5 μCi reconstituted ^{14}C -protein hydrolyzate (sp.act.= 1 mCi/mg, Schwarz), control incorporation 220,532 cpm; Expt. 3: as in Expt. 2, control incorporation 199,531 cpm.



could be measured beyond 60 minutes. Amino acids supplied at the start of incubation were .97% utilized in 60 minutes. Because of this difference in utilization of amino acids with and without puromycin, the inhibition by puromycin was actually greater than shown in Figure 4.

Table 19. Effect of Labeling Time on ^{14}C -Serine Incorporation Into Macromolecules by Yeast Cells.

Time of Labeling (min)	Incorporation (cpm)	Time of Labeling (min)	Incorporation (cpm)
0-30	67,834	0-30	67,834
0-60	83,975	30-60	43,748
0-90	86,345	60-90	34,295
0-120	86,128	90-120	28,656
no incubation	289		

Yeast cells were incubated under standard conditions at 37°C. At the indicated times, label (0.1 μCi of U.L.

L-serine- ^{14}C , sp.act. 0.05 mCi/0.0445 mg) was added. Incorporation was stopped by placing the vials in ice, followed by treatment with t-BuOH.

Isolation of Puromycin Derivatives

Puromycin and its derivatives were extractable from basic aqueous solutions with ethyl acetate (Leder and Bursztyn, 1966). With most radioactive precursors used, the ethyl acetate phase contained radioactive components

that interfered with the determination of puromycin derivatives by direct electrophoresis. For this reason, the extracts were first purified by thin layer chromatography.

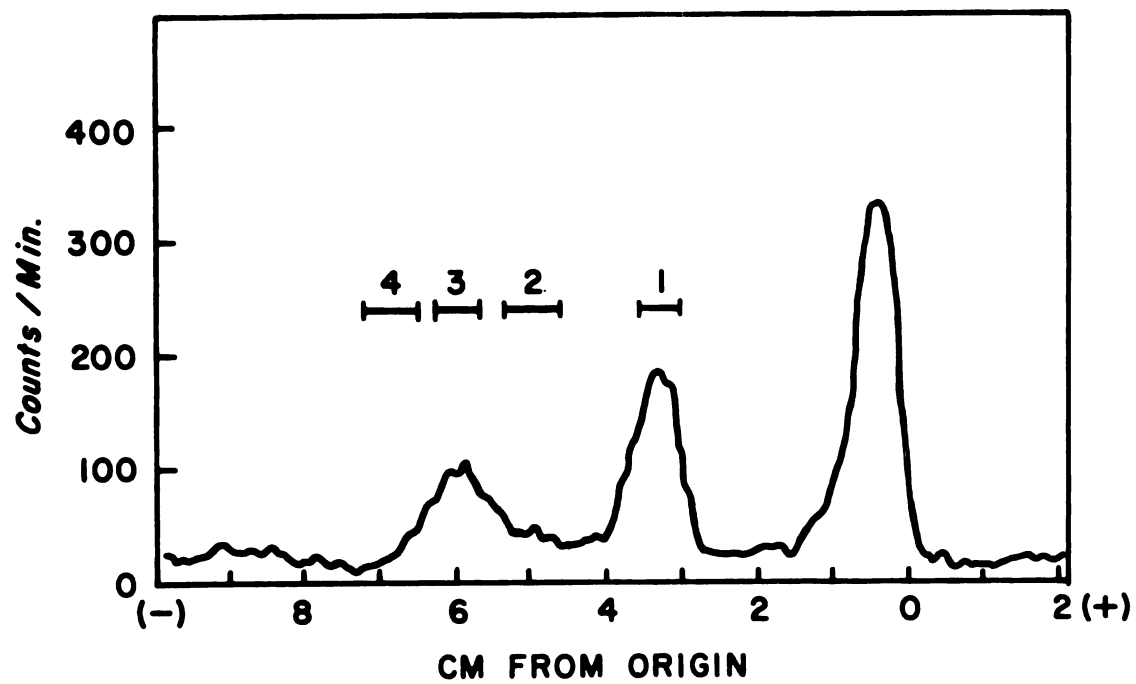
The mobilities of puromycin, N-formyl methionyl puromycin, and dipeptidyl puromycin on silica gel HF relative to the solvent front were: 0.65, 0.78, 0.75, respectively. Both the blocked and the free aminoacyl puromycin compounds had greater R_f values than puromycin itself did. Most interfering radioactive substances, including amino acids, had lower R_f values than puromycin did.

Bachmeyer and Kreil (1968) used electrophoresis at pH 1.8 to separate N-formyl methionyl puromycin from methionyl puromycin, puromycin, and methionine. I also used paper electrophoresis at pH 1.8 here. The separation obtained (Figure 5) agreed well with their report. The order of test substances after electrophoresis, was, from origin to cathode: N-formyl methionyl puromycin, amino acids, dipeptidyl puromycin, puromycin.

Also shown in Figure 5 is the distribution, determined with a radiochromatogram scanner, of radioactivity from ^{14}C -amino acids along the electropherogram. One peak of radioactivity coincided with N-formyl methionyl puromycin, and a second peak coincided with dipeptidyl puromycin. The large peak near the origin was always observed. Its

Figure 5. Electrophoresis of Amino Acids, Puromycin, and Puromycin Derivatives at pH 1.8.

Yeast cells were incubated with puromycin and 1.0 μCi ^{14}C -amino acid mixture (sp.act.= 1 mCi/mg) for 6 hr. This electropherogram was scanned for radioactivity with a Packard Radiochromatogram Scanner, strip speed 0.5 cm/min; time constant = 30 sec; slit 5 mm; 1,000 cpm full scale. Migration of standard substances was determined in separate experiments. N-formyl methionyl puromycin (1) and dipeptidyl puromycin (3) were determined by radiochromatogram scanner. Puromycin (4) was determined by UV absorbance. Amino acids (2)--methionine and glutamic acid--were determined by ninhydrin.



formation was not dependent on puromycin, and it contained no spectrally detectable puromycin.

Although most of the puromycin applied to electropherograms had the mobility indicated in Figure 5, a small fraction coelectrophoresed with acyl aminoacyl puromycin. That this was due to disproportionation of puromycin during electrophoresis, rather than to a contaminant of the puromycin, was shown by eluting the low mobility band and subjecting it to electrophoresis a second time. Most of the ultraviolet absorbance now moved with high mobility. Reelectrophoresis of material in the original puromycin band again produced a disproportionation into the two migration positions. The cause of this disproportionation is unknown. It made quantitation of puromycin derivative formation, either by ultraviolet spectroscopy or by the use of radioactive puromycin, difficult. On the other hand, it provided an internal marker for the position of acyl aminoacyl puromycin.

Incorporation of Radioactive Amino Acids into Puromycin Derivatives

When yeast cells were supplied with puromycin and a mixture of radioactive amino acids, they formed radioactively labeled puromycin derivatives (Figure 5). In order to demonstrate that puromycin had reacted with a radioactive component in a biologically catalyzed reaction,

the requirements for this reaction were examined. The results (Table 20) show that incorporation of radioactivity into both puromycin derivatives required the integrity of the yeast, the presence of puromycin, and a period of incubation. Inhibition of protein synthesis by cycloheximide, instead of puromycin, did not produce incorporation of radioactivity into these electrophoretic bands.

Table 20. Incorporation of ^{14}C -Amino Acids into Puromycin Derivatives by Yeast Cells.

Treatment	Incubation Time	cpm Incorporated into	
		Band 1	Band 2
75 mM puromycin	3 hr	1,058	718
75 mM puromycin, boiled yeast	3 hr	72	104
75 mM puromycin	0 hr	96	70
75 mM NaCl	3 hr	56	32
75 mM NaCl, 0.34 mM cycloheximide	3 hr	49	82

Each incubation mixture contained 1.0 μCi ^{14}C -amino acid mixture (sp.act.= 1 mCi/mg). Areas of the electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and eluted with 2.0 ml of eluting agent. An aliquot of 0.2 ml of the eluate was counted by liquid scintillation spectrometry. Background cpm have been subtracted.

In order to establish optimum conditions for the formation of labeled puromycin compounds, the time course of their formation was examined. Figure 6 shows that

incorporation into both bands occurred throughout three hours of incubation, although not at a sustained linear rate. A three hour incubation period was used in most later experiments.

As mentioned above, formation of puromycin derivatives of initiating amino acids was only expected at high concentrations of puromycin. Therefore, the concentration dependence of incorporation of radioactive amino acids into puromycin compounds was determined. Figure 7 shows that the optimum concentration for formation was 50 mM puromycin. Increasing the puromycin concentration from 0 to 50 mM resulted in an almost linear increase in incorporation into both derivatives. Further increases in concentration reduced incorporation into puromycin compounds. A concentration of 50 mM was thus used in most later experiments.

Nature of ^{14}C -Amino Acids Incorporated into Puromycin Derivatives

Whether the incorporation of radioactivity from a mixture of uniformly labeled ^{14}C -amino acids (all protein amino acids except methionine, cysteine, glutamine, asparagine, and tryptophan) was due to the incorporation of one amino acid or to incorporation of many amino acids was determined by four methods. First, the radioactively labeled puromycin derivatives were hydrolyzed with 5.7 HCl. The hydrolysis products were reacted with DNS-Cl and

Figure 6. Time Dependence of ^{14}C -Amino Acid
Incorporation into Puromycin
Derivatives by Yeast Cells.

Each incubation mixture contained 0.5 μCi of reconstituted ^{14}C -protein hydrolyzate (sp.act. = 1 mCi/mg). Puromycin concentration was 50 mM. Areas of the electropherograms corresponding to acyl aminoacyl puromycin (closed circles) and aminoacyl puromycin (open circles) were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm and minus puromycin control cpm (30-50) have been subtracted.

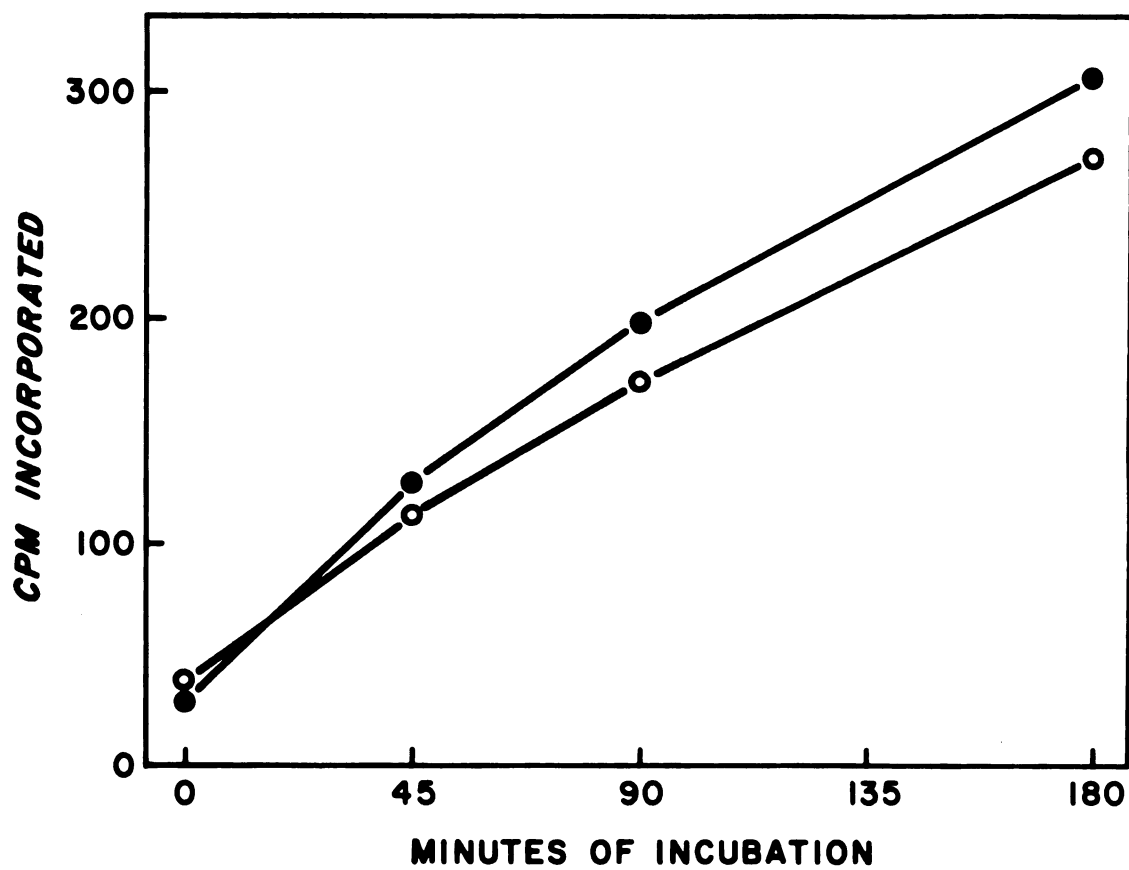
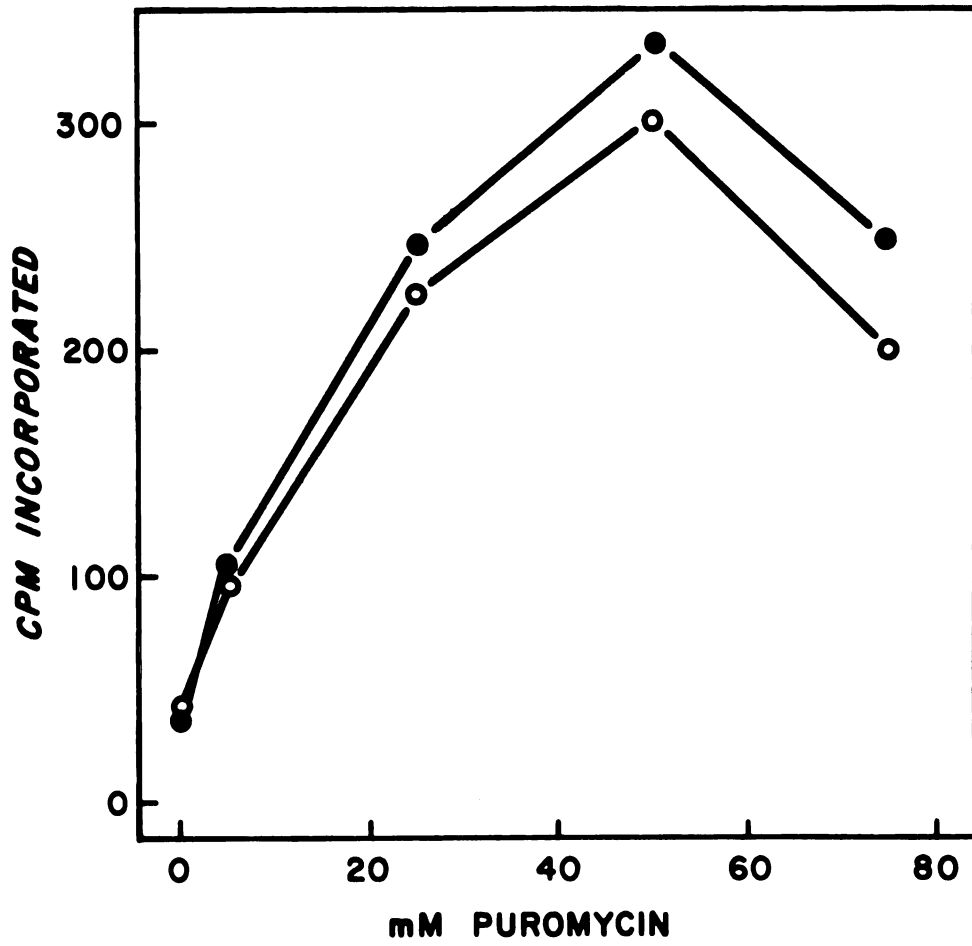


Figure 7. Puromycin Concentration Dependence of ^{14}C -Amino Acid Incorporation into Puromycin Derivatives by Yeast Cells.

Each incubation mixture contained 0.5 μCi reconstituted ^{14}C -protein hydrolyzate (sp.act. = 1 mCi/mg). All incubations were for 3 hr at 37°C . NaCl was added to all incubations to give a final concentration of 75 mM. Areas of the electropherograms corresponding to acyl aminoacyl puromycin (closed circles) and aminoacyl puromycin (open circles) were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted.



separated by two dimensional thin layer chromatography. Fluorescent spots corresponding to DNS amino acids were located and their content of radioactivity determined. Table 21 shows that the radioactivity was distributed among all of the DNS amino acids. Some amino acids, particularly the aliphatic ones, had more radioactivity than others. Whether this was due to actual differences in incorporation or merely to differences in the specific activity of the respective amino acid pools in the yeast cell was not determined.

Although DNS-Cl is known to react effectively with amino acids under the conditions used, the possibility that the results in Table 21 reflected some peculiarity of the DNS-Cl reaction was considered. Aminoacyl puromycin was hydrolyzed with 5.7 N HCl as above. The hydrolysis products were separated directly by two dimensional amino acid chromatography. Amino acid spots were located with ninhydrin and their content of radioactivity determined. Recovery of radioactivity was less than with the dansyl procedure by 10-20%. This is probably due to loss of the carboxyl group as CO_2 during the ninhydrin reaction. Table 22 shows that radioactivity was indeed distributed among most amino acids. The aliphatic amino acids, isoleucine, leucine, and valine were most highly labeled, while the basic amino acids, lysine, arginine, and histidine, contributed little label. Incorporation of

Table 21. Distribution of Radioactivity Among DNS Amino Acids from ^{14}C -Amino Acid Labeled Puromycin Derivatives.

DNS Amino acid	cpm in DNS amino acids from:	
	Band 1	Band 2
Isoleucine	32.2	64.0
Leucine	36.1	75.7
Valine	31.3	36.3
Proline	1.4	24.4
Phenylalanine	24.8	
Alanine	6.3	114.1
Lysine, Tyrosine	20.7	27.0
Glycine, Tryptophan	21.5	102.8
Serine, Threonine	17.1	19.5
Glutamic acid	42.3	15.5
Aspartic acid	22.0	--

Puromycin derivatives were obtained by incubating yeast cells with 2.0 μCi ^{14}C -amino acid mixture (sp.act. = 1 mCi/mg) and 75 mM puromycin for 3 hr. Areas of electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and eluted with 2.0 ml eluting agent. After concentration and *in vacuo* hydrolysis with 1 ml 5.7 N HCl at 105°C for 12 hr, hydrolyzates were concentrated to dryness several times and reacted with 2 μl 20 mg/ml DNS-Cl in 30 μl acetone and 20 μl 0.1 M NaHCO_3 . Fluorescent spots from the two dimensional thin layer chromatography which followed were scraped off and radioactivity determined. Background cpm have been subtracted. Standard counting error = ± 5 cpm.

Table 22. Distribution of Radioactivity Among
Amino Acids in ^{14}C -Aminoacyl Puromycin.

Amino Acids	cpm
Lysine, Histidine, Arginine	1.8
Proline	26.7
Glycine, Serine	20.1
Aspartic, Glutamic Acids	62.7
Threonine	34.2
Alanine	41.3
Isoleucine, Leucine Valine, Tyrosine	188.8
Phenylalanine, Methionine	124.8

Yeast was incubated with 75 mM puromycin and 1.0 μCi ^{14}C -amino acid mixture (sp.act. = 1 mCi/mg), for varying time periods. Electrophoretic bands were pooled and an aliquot removed for this analysis. The aliquot was concentrated to dryness and hydrolyzed with 1 ml 5.7 N HCl for 12 hr at 37°C. After hydrolysis, 20 μl 0.2% casein hydrolyzate was added and the solution brought to dryness. Amino acids were chromatographed on silica gel H. Spots were located with ninhydrin and their content of radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted. Standard counting error = ± 10 cpm.

basic amino acids into band 2 was not expected since lysyl, arginyl or histidyl puromycin should have a charge of +3 at pH 1.8. It is not known whether acyl aminoacyl puromycin containing basic amino acids (+2) comigrate with aminoacyl puromycin.

If many amino acids contributed label to puromycin derivatives, it should be possible to incorporate radioactivity from several individual ^{14}C -amino acids. Incorporation, with and without puromycin, of radioactively labeled alanine, serine, isoleucine, arginine and glycine into the electrophoretic bands was examined. Table 23 shows that puromycin stimulated appearance of radioactivity in these areas when labeling was with alanine, serine, isoleucine and glycine. In agreement with the results obtained with a mixture of ^{14}C -amino acids (Table 22), best incorporation was obtained with isoleucine, an aliphatic amino acid, while incorporation of arginine, a basic amino acid, was not significant.

The incorporation of radioactive methionine was examined in greater detail, since a critical role for methionine in eukaryotic chain initiation has been proposed (Smith and Marker, 1970). Yeast were grown on ethanol, as well as glucose, in order to stimulate mitochondrial protein synthesis. Table 24 shows that ethanol grown yeast were as effective as glucose grown yeast in incorporating ^3H -methionine (methyl- ^3H) into

Table 23. Incorporation of Individual ^{14}C -Amino Acids into Puromycin Derivatives by Yeast Cells.

^{14}C -Amino Acid	Puromycin	cpm Incorporated into	
		Band 1	Band 2
Alanine	+	53	107
	-	0	44
Serine	+	67	154
	-	14	27
Isoleucine	+	193	726
	-	77	246
Arginine	+	72	125
	-	53	110
Glycine	+	265	357
	-	27	6

Amounts of radioactivity used were: U.L. ^{14}C -L-alanine, 1.0 μCi (sp.act. = 135 mCi/mmol); U.L. ^{14}C -L-serine, 1.0 μCi (sp.act. = 0.05 mCi/0.0445 mg); ^{14}C -L-isoleucine, 1.0 μCi (sp.act. = 240 mCi/mmol); ^{14}C -L-arginine, 1.0 μCi (sp.act. = 240 mCi/mmol); 2- ^{14}C -glycine, 5.0 μCi (sp.act. = 2.5 mCi/mmol). Puromycin concentration for the first four amino acids was 75 mM, for glycine it was 50 mM. Alanine and serine incubations were for 70 min., the remainder were incubated for 3 hours. Incubations without puromycin contained an equivalent amount of NaCl. Areas of electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted.

Table 24. Incorporation of ^3H -Methionine into Puromycin Derivatives by Yeast Cells

Treatment	Incubation Time	cpm Incorporated			
		by glucose-grown yeast	Band 1	Band 2	by ethanol-grown yeast
50 mM puromycin	3 hr	300.5	180.9	313.6	197.4
50 mM NaCl	3 hr	29.8	58.2	27.0	52.0
50 mM puromycin	0 hr	81.4	101.3	--	--
50 mM puromycin, boiled yeast	3 hr	79.4	83.2	--	--

Each incubation mixture contained 5.0 μCi L-methionine-methyl- ^3H (sp.act. = 1.0 mCi/2.2 mg). Areas of the electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted.

puromycin derivatives. The absence of a large stimulation when growth was on ethanol indicates that much of the incorporation observed was not mitochondrial. Furthermore, the level of incorporation observed was similar to that of other amino acids (Table 23). Thus puromycin derivatives of methionine were no more predominant than puromycin derivatives of other amino acids. A high background was noted in the zero time and boiled yeast controls (Table 24). This may be due to slight bacterial contamination of reagents or vials.

Lastly, if no one amino acid contributed all the label from a ^{14}C -amino acid mixture, then addition of high concentrations of several ^{12}C -amino acids should result in only small inhibition of labeling of puromycin derivatives. Yeast cells were incubated with puromycin and a mixture of ^{14}C -amino acids. Unlabeled amino acids in groups of four were added to give a final concentration of 0.2 mg/ml of each amino acid. Incorporation into puromycin derivatives was assayed and is shown in Table 25. None of the three groups of four amino acids was able to completely inhibit incorporation. Most effective was the group phenylalanine, tyrosine, valine, and leucine, while the least effective group was that containing two basic amino acids, histidine, and lysine. This result was consistent with the distribution of radioactivity among amino acids (Tables 21 and 22) and with the incorporation of individual ^{14}C -amino acids

(Table 23). These experiments demonstrated that there was no one single predominant radioactive amino acid in the puromycin derivatives.

Table 25. Inhibition of ^{14}C -Amino Acid Incorporation into Yeast Puromycin Derivatives by Excess ^{12}C -Amino Acids

^{12}C -amino acids added	cpm Incorporated into	
	Band 1	Band 2
none	346	377
threonine, arginine, isoleucine, aspartic acid	259	321
proline, histidine, glutamic acid, lysine	296	365
phenylalanine, tyrosine, leucine, valine	237	268

Yeast was incubated with 1.0 μCi ^{14}C -amino acid mixture, 75 mM puromycin and with or without added amino acids, for 3 hr. Final concentration of each added amino acid was 0.2 mg/ml. All amino acids added were L-amino acids, except D, L-histidine hydrochloride monohydrate and L-lysine hydrochloride. Radioactivity was determined by measuring the area under peaks of a radiochromatogram scan. Band 1 = acyl aminoacyl puromycin; Band 2 = aminoacyl puromycin.

N-Terminal Analysis of ^{14}C -Puromycin Derivatives

Although the radioactivity analyzed above as "acyl aminoacyl puromycin" and "aminoacyl puromycin" was obtained from areas of the electropherograms where these compounds

should have migrated, further proof that they were indeed acyl aminoacyl puromycin and aminoacyl puromycin was desired. The main difference between the two compounds is that the α amino group of the amino acid is blocked with an acyl group in the former, but is free in the latter. That the radioactivity that coelectrophoresed with N-formyl methionyl puromycin did not have a free amino group is supported by three observations.

DNS-Cl reacts with free amino groups. Therefore, it should react with aminoacyl puromycin and not with acyl aminoacyl puromycin. It should be possible to isolate radioactive DNS amino acids from dansylated "aminoacyl puromycin" after hydrolysis. Dansylation and hydrolysis of "acyl aminoacyl puromycin" should not yield radioactive DNS amino acids.

"Acyl aminoacyl puromycin" and "aminoacyl puromycin" were labeled with ^{14}C -amino acids. Each group of compounds was then divided in half. One half was reacted with DNS-Cl, hydrolyzed and chromatographed. The other half was hydrolyzed before reaction with DNS-Cl and chromatography. The radioactivity found in the dansyl amino acids is shown in Table 26. Only a small percentage of the radioactive amino acids in acyl aminoacyl puromycin was found to be N-terminal. On the other hand most of the radioactivity in aminoacyl puromycin was N-terminal. This supports the identification of the band as aminoacyl

puromycin and also indicates that mainly aminoacyl puromycin, rather than peptidyl puromycin were present in the preparation.

Table 26. N-Terminal Analysis of ^{14}C -Amino Acid Labeled Puromycin Derivatives from Yeast Cells

	cpm in DNS Amino Acids after		
	1. Dansylation 2. Hydrolysis	1. Hydrolysis 2. Dansylation	% of cpm N-terminal
Acyl aminoacyl puromycin	7.2	226.4	3
Aminoacyl puromycin	263.4	301.4	87

Yeast cells were incubated with 2.0 μCi ^{14}C -amino acid mixture (sp.act. = 1 mCi/mg) and 50 mM puromycin for 3 hr. Electrophoretic bands were eluted with 2.0 ml eluting agent and divided into two equal portions. One half was treated as described in Table 21. The other half was treated similarly except that reaction with DNS-Cl preceded hydrolysis. Results represent the total in DNS amino acids. Standard counting error = 8.5 cpm.

Characterization of Acyl
Aminoacyl Puromycin Labeled
with ^{14}C -Amino Acids

The above results are also consistent with the "acyl aminoacyl puromycin" being an oligopeptidyl puromycin. That this was not the case was demonstrated by re-electrophoresis of dansylated and non-dansylated acyl aminoacyl puromycin at pH 5. The pK of the dimethylamino groups of puromycin (Smith, et al., 1965) and DNS amino

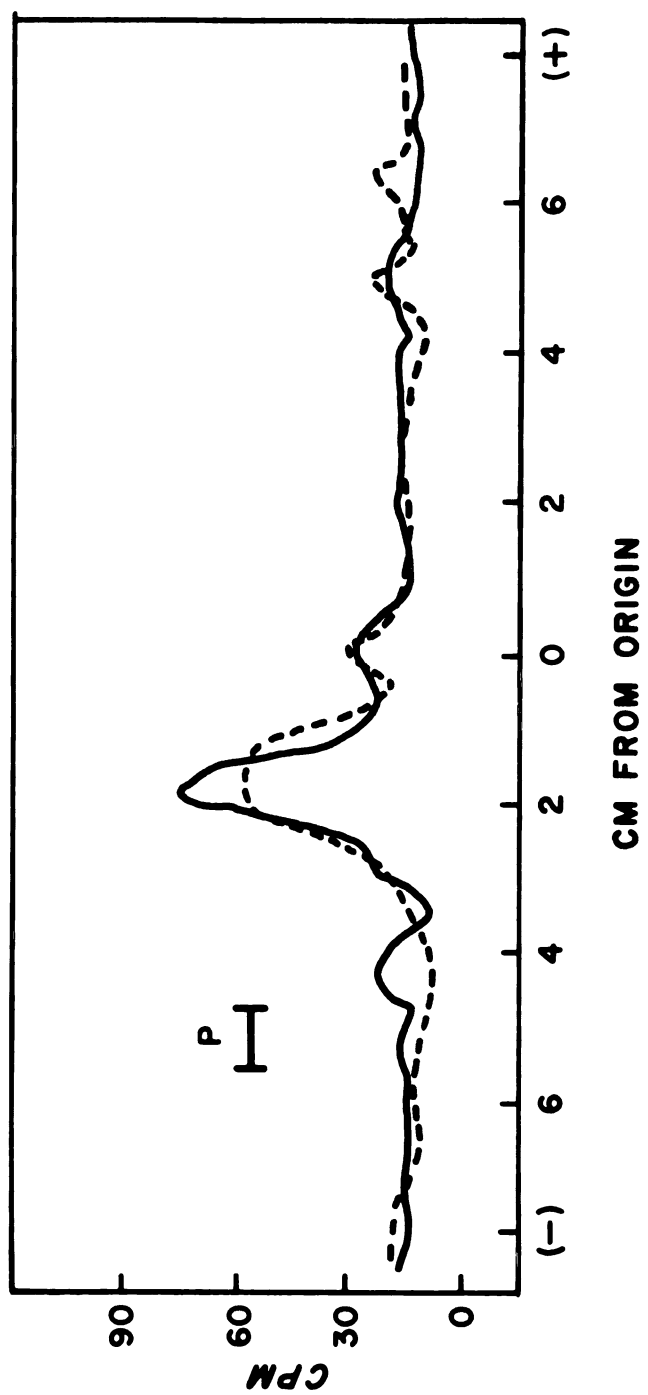
acids (Gray and Hartley, 1963) are between 3 and 4. The pK values of amino groups whose carboxyl is in peptide linkage range around 7. Therefore, at pH 5, acyl aminoacyl puromycin should be uncharged, while oligopeptidyl puromycin should have a charge of +1. At pH 5, the DNS group should have no net charge. Substitution of an α amino group with DNS-Cl should substitute a group with a pK of 3 to 4 for a group with a pK of 7. At pH 5, this should result in the net loss of one positive charge.

"Acyl aminoacyl puromycin" was labeled with ^{14}C -amino acids and divided in half. One half was reacted with DNS-Cl. The other half was treated identically except DNS-Cl was omitted from the reaction mixture. Both halves were electrophoresed at pH 5.4. The radioactivity scans of the electropherograms are presented in Figure 8. As predicted, acyl aminoacyl puromycin did not move much from the origin, indicating that it had no net charge. After reaction with DNS-Cl, the mobility of acyl aminoacyl puromycin was unchanged. Therefore, it did not have an amino group that reacted with DNS-Cl.

Digestion with pronase supported the identity of acyl aminoacyl puromycin. Pronase, a proteolytic enzyme from Streptomyces griseus (Nomoto and Narahashi, 1959), hydrolyzes amino acid--puromycin bonds (Bachmeyer and Kreil, 1968; Nathans, 1964), but not acetyl--amino acid

Figure 8. Electrophoresis at pH 5.4 of Yeast ^{14}C -Acyl Aminoacyl Puromycin Before and After Reaction with DNS-Cl.

Yeast cells were incubated with 2.0 μCi ^{14}C -amino acid mixture (sp.act. = 1 mCi/mg) for 3 hr. The electrophoretic band corresponding to acyl aminoacyl puromycin was eluted with 4.0 ml eluting agent and divided in two. Each half was brought to dryness and redissolved in 20 μl 0.1 N NaHCO_3 and 30 μl acetone. To one sample, 2 μl 20 mg/ml DNS-Cl was added. After overnight reaction, the samples were spotted on paper strips and electrophoresed in 2% pyridine, 1% acetic acid, pH 5.4, at 350 V for 2.5 hr. Radiochromatogram scanning conditions were: 0.5 cm/min, 30 sec time constant, and 5.0 mm slit width.



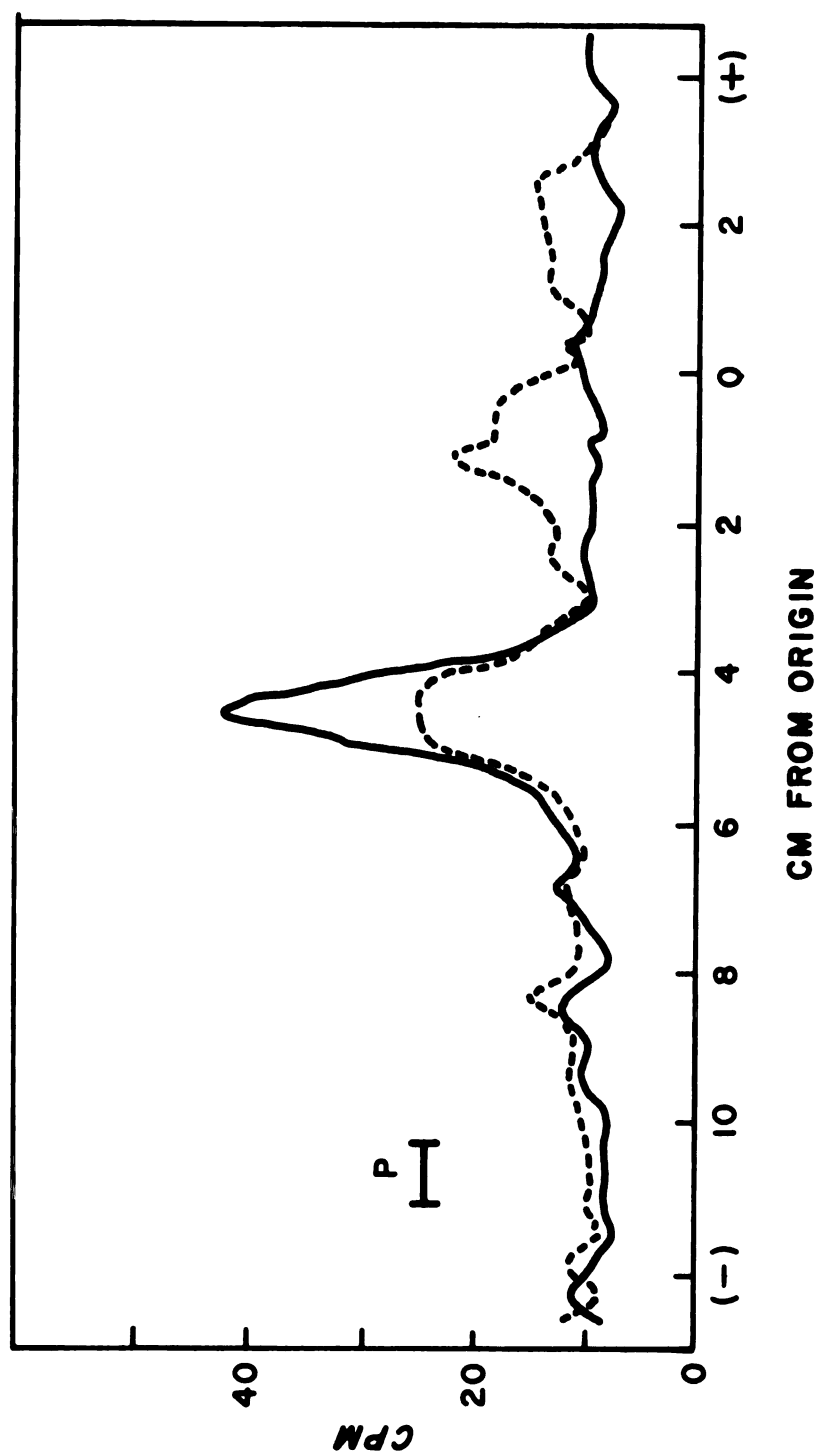
bonds (Nomoto, et al., 1960). Thus, acyl aminoacyl puromycin should yield acyl amino acids and puromycin after pronase digestion.

"Acyl aminoacyl puromycin" was labeled with ^{14}C -amino acids. One half of the sample was treated with pronase in sodium phosphate, pH 7.4, the other half with buffer only. After a period of digestion, the solutions were electrophoresed. The results (Figure 9) show that pronase treatment converted radioactivity in the acyl aminoacyl puromycin region to radioactive bands near the origin. Very little radioactivity was recovered in the amino acid area of the electropherogram. The bands near the origin were probably N-acyl amino acids and N-acyl aminoacyl p-methoxy phenylalanine, since these should be uncharged at pH 1.8. The latter arises from hydrolysis of the peptide bond in the puromycin moiety (Nathans, 1964).

The production of radioactive acyl amino acids by pronase supports the contention that the radioactivity is incorporated into acyl amino acyl puromycin. The absence of appreciable radioactivity in amino acid products indicates that most of the radioactivity incorporated was linked directly to the acyl group. Thus, like aminoacyl puromycin, acyl aminoacyl puromycin had primarily one amino acid residue per puromycin moiety.

Figure 9. Electrophoresis at pH 1.8 of Yeast ^{14}C -Acyl Aminoacyl Puromycin Before and After Pronase Digestion.

Yeast cells were incubated with 2.0 μCi reconstituted ^{14}C -protein hydrolyzate (sp.act. = 1 mCi/mg) and 50 mM puromycin for 3 hr. Acyl aminoacyl puromycin was eluted from the electropherogram with 4.0 ml eluting agent, and divided into two equal portions. To one half, 0.05 ml 0.033 M sodium phosphate, pH 7.4, was added (solid line). To the other half (dotted line), 0.05 ml of a 1 mg/ml solution of pronase (45,000 P.U.K./mg, Calbiochem) in the same buffer was added. Both samples were covered with Parafilm and incubated 20 hr at 37°C. Samples were spotted on paper strips for electrophoresis for 3.0 hr at 350 V. Radioactivity scan conditions were: 0.2 cm/min, 100 sec time constant, 10 mm slit width.



Of the radioactivity incorporated into acyl aminoacyl puromycin from a ^{14}C -amino acid mixture, only 30-50% could be recovered as radioactive DNS amino acids. The chromatograms of dansyl amino acids were examined for other radioactive compounds. One spot, accounting for most of the remaining radioactivity was found. The $R_{\text{DNS Ile}}$ values were: in solvent 1, 1.11; and in solvent 2, 0.34. Acyl aminoacyl puromycin was purified with the latter solvent as a band with $R_{\text{DNS Ile}}$ of 1.0. No fluorescence was associated with the spot, nor did dansylation affect its mobility. This radioactive compound could only be isolated from acyl aminoacyl puromycin and not from aminoacyl puromycin.

Incorporation of Radioactive
Formate and Acetate into
Puromycin Derivatives

The incorporation of radioactive formate and acetate into puromycin derivatives was examined. Tables 27 (formate) and 28 (acetate) show that both precursors were incorporated into acyl aminoacyl puromycin in a reaction that required the integrity of the yeast, the presence of puromycin and a period of incubation. Only a slight amount of radioactivity was incorporated into aminoacyl puromycin. Almost all the radioactivity must therefore have been incorporated into the acyl group of acyl aminoacyl puromycin, rather than into the amino acid moiety. In these

Table 27. Incorporation of ^{14}C -Formate into Puromycin Derivatives by Yeast Cells

Treatment	Incubation Time	cpm Incorporated into Band 1	cpm Incorporated into Band 2
50 mM puromycin	3 hr	658.1	64.6
50 mM puromycin, boiled yeast	3 hr	61.2	26.8
50 mM puromycin	0 hr	0.0	17.2
50 mM NaCl	3 hr	2.1	2.8

Each incubation mixture contained 5.0 μCi ^{14}C -sodium formate (sp.act. = 4.0 mCi/mmol). Areas of electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted.

Tabel 28. Incorporation of ^{14}C -Acetate into Puromycin Derivatives by Yeast Cells

Treatment	Incubation Time	cpm Incorporated into Band 1	cpm Incorporated into Band 2
50 mM puromycin	3 hr	274.1	29.2
50 mM puromycin, boiled yeast	3 hr	33.7	17.2
50 mM puromycin	0 hr	15.7	1.9
50 mM NaCl	3 hr	5.7	7.8

Each incubation mixture contained 0.5 μCi sodium acetate-2- ^{14}C (sp.act. = 0.59 mCi/mmol). Other experimental conditions as described in Table 27.

experiments, ten times as much radioactivity of formate of ten fold higher specific activity, with respect to acetate, was added. In subsequent experiments, a higher ^{14}C -acetate specific radioactivity was occasionally used. Both specific activities gave the same degree of incorporation.

The time dependence of labeling of acyl aminoacyl puromycin with ^{14}C -formate and ^{14}C -acetate was examined. Figure 10 shows that formate incorporation occurs at an almost linear rate throughout three hours of incubation. The time course was similar to that of ^{14}C -amino acid incorporation into puromycin derivatives (Figure 6). Acetate incorporation leveled off during the second and third hour of incubation. No increase in incorporation could be detected beyond three hours of incubation. This was probably due to rapid metabolism of acetate by yeast cells.

The puromycin concentration dependence of radioactive formate and acetate incorporation was also examined. The same optimum concentration was found (Figure 10) as was found for amino acid incorporation into puromycin derivatives (Figure 7). Concentrations higher than 50 mM also resulted in decreased incorporation. Radioactive acetate and formate incorporation differed from amino acid incorporation into puromycin derivatives in that concentration less than 50 mM were much less effective in promoting incorporation.

Figure 10. Time Dependence of ^{14}C -Formate and ^{14}C -Acetate Incorporation into Acyl Aminoacyl Puromycin by Yeast Cells.

Each incubation mixture contained 2.0 μCi ^{14}C -sodium formate, sp.act. = 4.0 mCi/mmol (closed circles); or 0.5 μCi 2- ^{14}C -sodium acetate, sp.act. = 50 μCi /7.1 mg (open circles). Puromycin concentration was 50 mM. Areas of the electropherograms corresponding to acyl aminoacyl puromycin were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted.

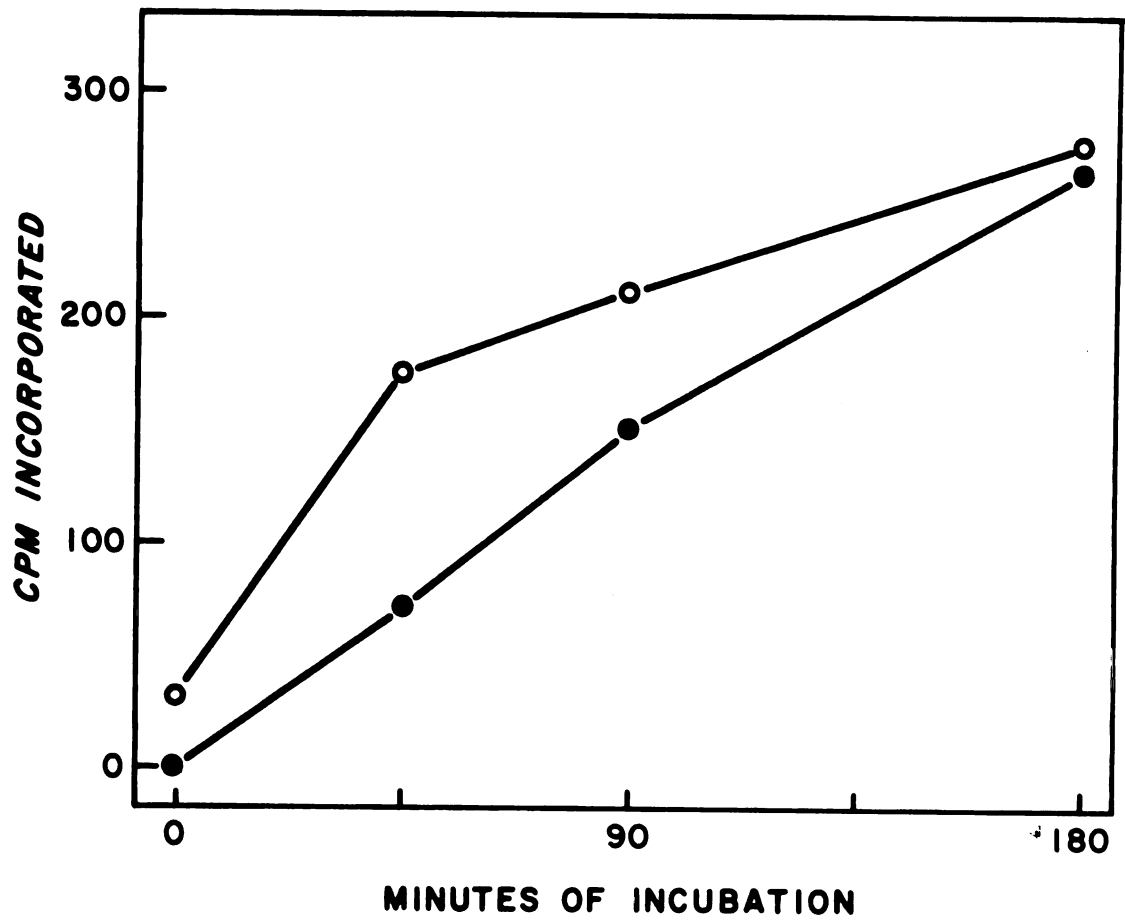
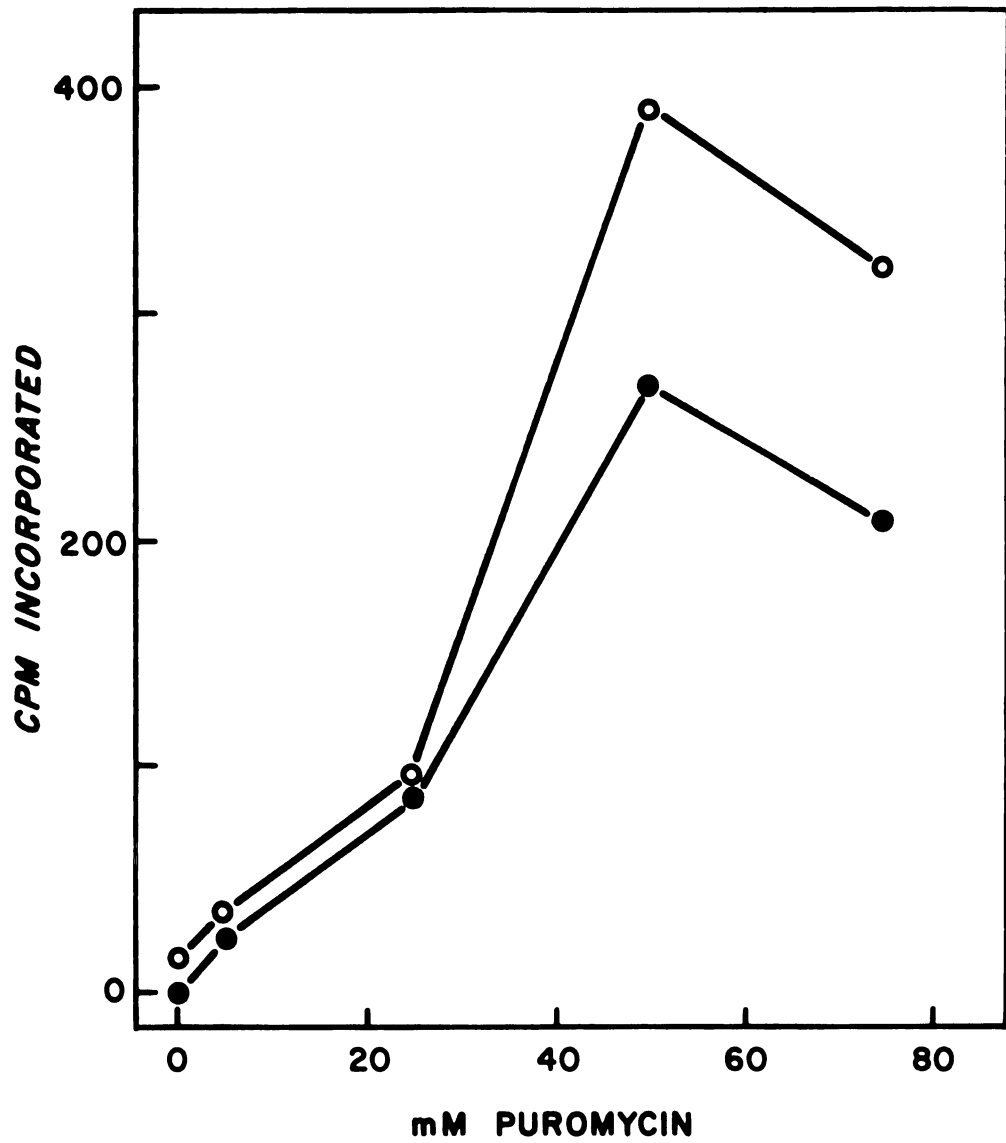


Figure 11. Puromycin Concentration Dependence of ^{14}C -Formate and ^{14}C -Acetate Incorporation into Acyl Aminoacyl Puromycin by Yeast Cells.

Each incubation mixture contained 2.0 μCi ^{14}C -sodium formate, sp.act. = 4.0 mCi/mmol (closed circles) or 0.5 μCi 2- ^{14}C -sodium acetate, sp.act. = 50 $\mu\text{Ci}/7.1\text{ mg}$ (open circles). All incubations were for 3 hr at 37°C . NaCl was added to all incubations to give a final concentration of 75 mM. Areas of the electropherograms corresponding to acyl aminoacyl puromycin were cut out and radioactivity determined by liquid scintillation spectrometry. Background cpm have been subtracted.



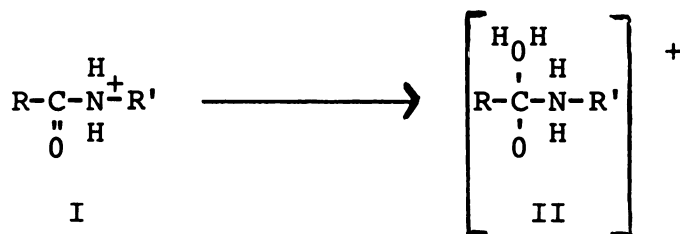
Mild Acid Hydrolysis of
¹⁴C-formate and -Acetate
Labeled Acyl Aminoacyl
Puromycin

Several methods were used to determine whether the radioactivity incorporated from formate and acetate was present as formyl and acetyl groups. The lability of the label to mild acid hydrolysis was studied. Cleavage of the formyl group from formyl peptides with 0.2 N HCl in methanol at room temperature (Sheehan and Young, 1958) and at 37°C (Bretscher and Marcker, 1966) has been reported. I hoped that stronger acid conditions would produce hydrolysis of both acyl groups. The conditions chosen (1 N HCl, 37°C) should not produce appreciable hydrolysis of peptide bonds (Sanger, 1952).

The effectiveness of these conditions was tested by studying the hydrolysis of N-acetyl glycine and N-formyl glycine. The hydrolysis of both compounds in 1 N HCl was followed by the production of groups that could react with picryl sulfate (Satake, et al., 1960). Both hydrolyses followed pseudo first order kinetics at 37°C. The rate constant for N-formyl glycine was $7.5 \times 10^{-5} \text{ sec}^{-1}$, and that for N-acetyl glycine was $1.5 \times 10^{-6} \text{ sec}^{-1}$. The hydrolysis of formyl groups from formyl methionyl puromycin under less strong conditions (Bachmeyer and Kreil, 1968) is complete in two hours, much shorter than would be required for complete hydrolysis for formyl glycine.

The effect of 1 N HCl at 37°C on labeled puromycin derivatives was examined. Acyl aminoacyl puromycins were labeled with ^{14}C -formate and with ^{14}C -acetate. Two specific radioactivity levels of acetate were used. The puromycin derivatives were divided into equal portions and hydrolyzed for different lengths of time with 50 μl 1 N HCl at 37°C. Figure 12 shows that a rapid hydrolysis of label from all three radioactive preparations occurred during the first hour of hydrolysis. Thereafter, no detectable hydrolysis took place. This behavior would be expected from a mixture of two components, one rapidly hydrolyzed and the other not hydrolyzed under the conditions used. The rate of hydrolysis of the rapid component was at least ten times the rate of hydrolysis of N-formyl glycine.

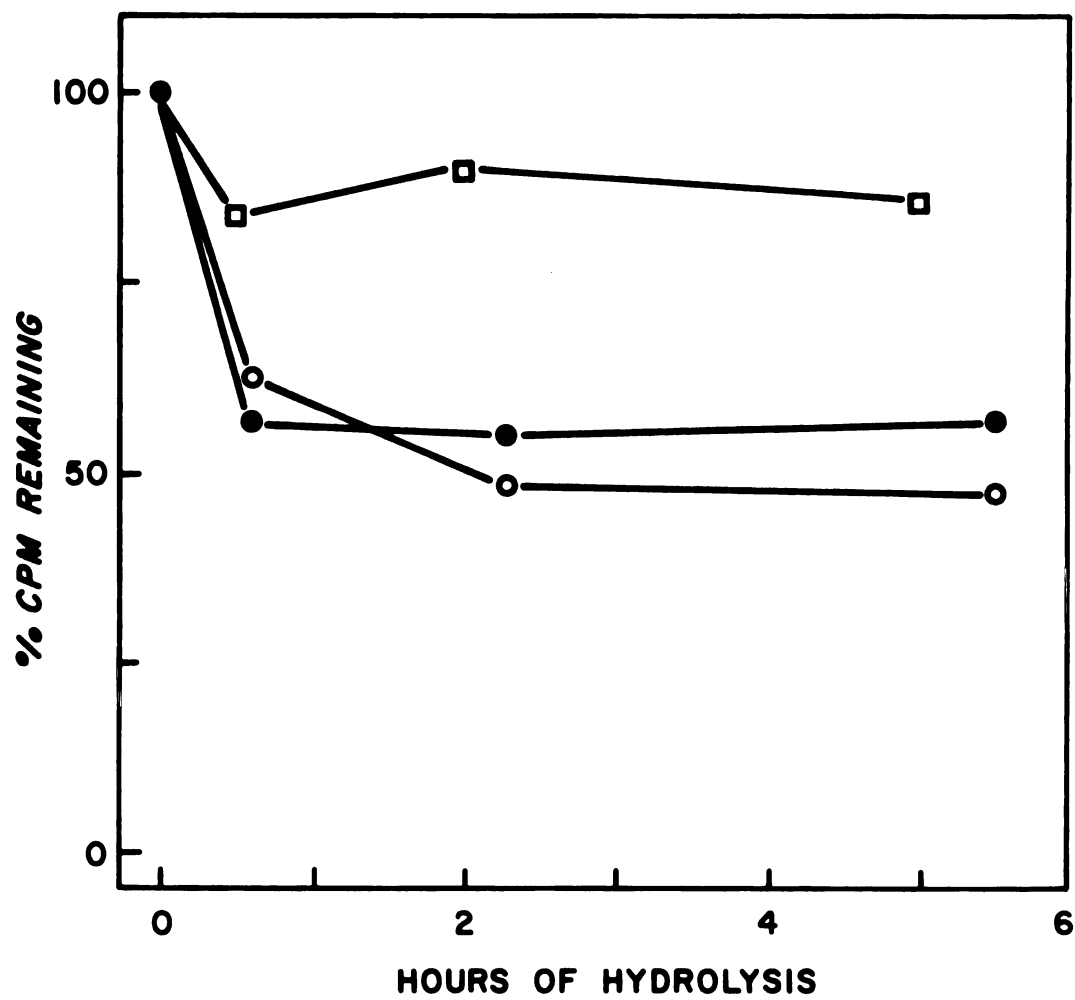
The explanation of the observed differences in hydrolysis rates lies in the mechanism of acid catalyzed hydrolysis of amides. The rate limiting step is probably (Gould, 1959).



Under conditions where the acid base equilibrium between I and its conjugate base strongly favors the acid, the effect of varying R and R' can be predicted. Electron donating R groups should reduce the reaction rate by

Figure 12. Mild Acid Hydrolysis of Radioactivity in Yeast Acyl Aminoacyl Puromycins.

Yeast cells were incubated with either 1.0 μCi sodium acetate-2- ^{14}C (sp.act. = 0.25 mCi/0.398 mg) (closed circles); 1.0 μCi sodium acetate-2- ^{14}C (sp.act. = 0.5 mCi/mmol) (open squares); or 5.0 μCi ^{14}C -sodium formate (sp.act. = 4.0 mCi/mmol) (open circles), and 50 mM puromycin for 3 hr. Eluates from thin layer chromatograms were divided into four equal portions and subjected to mild acid hydrolysis for the indicated times. Areas of electropherograms corresponding to acyl aminoacyl puromycin were cut out and counted by liquid scintillation spectrometry. 100% values were 249.8, 222.9, 215.4 cpm respectively.



reducing the partial positive charge on the carbonyl carbon in I. Lowering of the pK_a of the nitrogen by R' groups should increase the reaction rate.

Formyl glycyL puromycin differs from formyl glycine in that the pK_a of the nitrogen is lower in the former, thus increasing the rate of hydrolysis of formyl glycyL puromycin. Acetyl glycyL puromycin should also be hydrolyzed more rapidly than acetyl glycine, but in this case, the increase in rate may not be enough to produce detectable hydrolysis under the conditions used.

It can be, therefore, concluded from Figure 12 that only about 50% of the radioactive formate incorporated was present as formyl groups. On the other hand, acyl aminoacyl puromycin labeled with high specific activity acetate had 50% of its label present as formyl groups. When lower specific activities of acetate were used the labeling of formate groups decreased. Whether none of the ^{14}C -acetate incorporated was present as acetyl groups or whether acetyl aminoacyl puromycins were not appreciably hydrolyzed under these conditions could not be determined from these data.

Steam Distillation

The presence of radioactive acetate in histones has been determined by steam distillation (Allfrey, et al., 1964). Histones were hydrolyzed with 6N H_3PO_4 for

20 hours at 110°C and the products steam distilled. Steam distillation can also be used for formyl groups. In this case, hydrolysis before steam distillation should not be necessary since the conditions during distillation (100°C, 0.6N H₃PO₄, 30 min.) should be sufficient to produce complete hydrolysis.

An equation to describe steam distillation of water soluble acids was derived from three assumptions. First, it was assumed that the equation:

$$-d m_a = \frac{m'_a}{v'} d v'$$

(m_a is the amount, in moles, of acid in the liquid phase of the distillation flask; m'_a is the amount of acid in the vapor phase; v' is the volume of the vapor phase) describes the system. It was also assumed that the system obeys Raoult's law and that the concentrations of acid were so small that their contribution to the sum of mole fractions and the sum of vapor pressures was negligible. From these assumptions the following equation was derived:

$$-\ln \frac{(m_a)_f}{(m_a)_i} = \frac{P_a^O}{P_h^O} \times \frac{v_f}{v}$$

(i and f refer to initial and final states; P_a^O and P_h^O are the vapor pressure of the acid and water, respectively, at the boiling point of the mixture; v is the volume of

liquid in the distillation flask; v_f is the volume collected from the condenser).

The above equation predicts that a straight line should result from a plot of the natural logarithm of the fraction of acetic acid that was not steam distilled against the ratio of volume collected to volume in the distillation flask. The slope of the line should be the ratio of the vapor pressures. This was tested by steam distilling 3.0 ml of 0.01 M acetic acid for various lengths of time. The initial amount of acetic acid present, $(m_a)_i$, was determined by measuring the decrease in volume of 0.01 M potassium hydrogen phthalate required to titrate the alkaline trap to a phenolphthalein end point, that occurred on the addition of 3.0 ml 0.01 M acetic acid. The quantity of acetic acid steam distilled was determined similarly. The difference between $(m_a)_i$ and the amount steam distilled is $(m_a)_f$, the amount of acetic acid not distilled. The resulting points agreed well with the theoretical line assuming $P_a^O = 417$ and $P_h^O = 760$.

As a further control, the recovery of radioactivity from ^{14}C -sodium acetate after steam distillation was examined. A mean value for percent recovery of 1.0 nCi of radioactivity during 11 steam distillations was 94.8 with a mean deviation from the mean of 7.1%. The addition or omission of carrier acetic acid did not result in any reproducible difference in recovery.

Acyl aminoacyl puromycin was labeled with ^{14}C -amino acids, formate or a high or low specific activity acetate. Aminoacyl puromycin was labeled with ^{14}C -amino acids. An aliquot of each sample was taken for radioactivity determination. The remainder of the sample was divided in half. One half was subjected to hydrolysis with 6 N H_3PO_4 at 105°C for 12 hr, and then steam distilled. The other half was steam distilled without hydrolysis. Table 29 shows the radioactivity that was steam distillable with and without prior hydrolysis.

Aminoacyl puromycin labeled with ^{14}C -amino acids had negligible steam distillable radioactivity either before or after hydrolysis. This result is consistent with the lack of an acyl group in this compound.

Some steam distillable radioactivity was produced from acyl aminoacyl puromycin labeled with ^{14}C -amino acids by acid hydrolysis. Evidently, some of the radioactive amino acids had been metabolized to acetate.

Low specific activity ^{14}C -acetate derived radioactivity had the characteristics expected of acetyl aminoacyl puromycin. It was not steam distillable before hydrolysis, but was almost completely distilled after hydrolysis. This result is consistent with the stability of 80-90% of the label to mild acid hydrolysis (Figure 12).

The situation was different with high specific activity ^{14}C -acetate derived radioactivity. About 40% of

Table 29. Steam Distillation of Radioactivity in Yeast Puromycin Derivatives.

Radioactive label	Total cpm	Steam Distilled cpm	Steam Distilled %	Steam Distilled After Hydrolysis cpm	Steam Distilled After Hydrolysis %
¹⁴ C-amino acids (Band 2)	413	24	6	21	5
(Band 1)	437	18	4	69	16
¹⁴ C-acetate (Expt. 1)	206	10	5	201	97
(Expt. 2)	357	133	37	149	42
¹⁴ C-formate	500	206	41	231	46

Puromycin derivatives were labeled with 1.0 μ Ci of ¹⁴C-amino acid mixture (sp.act. = 1.0 mCi/mole), 1.0 μ Ci of 2-¹⁴C-sodium acetate (sp.act. = 50 μ Ci/7.1 mg, expt. 1), (sp.act. = 0.25 mCi/0.398 mg, expt. 2) or 5.0 μ Ci ¹⁴C-sodium formate (sp.act. = 4.0 mCi/mole). Acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were prepared as described in methods. Radioactive bands were eluted from electropherograms and divided into three, one aliquot was counted directly in a liquid scintillation counter, another was steam distilled, and the last was concentrated, hydrolyzed with 6 N H₃PO₄ at 105°C for 12 hr before steam distillation.

the label was steam distilled without prior hydrolysis, while strong acid hydrolysis produced no further increase in steam distillable radioactivity. This result is consistent with the data of Figure 12 in that 44% of the radioactivity was rapidly hydrolyzed under the mild conditions. This result also indicates that little or none of the radioactivity is actually present in the form of acetyl groups.

Radioactivity from ^{14}C -formate was 40-50% steam distillable with or without prior hydrolysis. This result is again consistent with the results of mild acid hydrolysis, and is probably due to the presence of formyl groups. The remainder of the radioactivity could not have been present as acetyl groups.

Effects of Protein Synthesis Inhibitors

The formation of puromycin derivatives by yeast cells is probably catalyzed by the protein synthetic apparatus. The concentration dependence of formation of puromycin derivatives (Figure 7) is similar to the puromycin concentration dependence of inhibition of protein synthesis (Figure 4). Further proof that puromycin derivatives were formed by the protein synthetic machinery of the yeast cell was sought.

The use of other inhibitors of protein synthesis was attempted. The formation of a peptide bond between an amino acid and puromycin is probably catalyzed by the same ribosome enzyme systems that catalyze protein peptide bond formation (Maden and Monro, 1968). Therefore, inhibitors of protein synthesis that inhibit peptide bond formation would be effective in inhibiting formation of puromycin derivatives.

Chloramphenicol

Chloramphenicol has been used to distinguish chloroplast (Aaronson, et al., 1967; Smillie, et al., 1967) and mitochondrial (Clark-Walker and Linnane, 1966) protein synthesis from cytoplasmic synthesis. By binding to ribosomes from these organelles (Anderson and Smillie, 1966), chloramphenicol inhibits the peptide bond forming reaction (Monro and Vazquez, 1970). It does not affect in vitro amino acid incorporation by yeast (So and Davies, 1963) nor does it bind to yeast ribosomes (Vazquez, 1964). The antibiotic does not inhibit peptide bond formation by eukaryotic cytoplasmic ribosomes (Neth, et al., 1970).

Yeast was incubated with puromycin, radioactive precursors, and chloramphenicol. Incorporation into puromycin derivatives was assayed. At chloramphenicol concentrations of 1 mg/ml and below, no effect was noted. Only in saturated solutions did any decreases occur

(Table 30). At this concentration, formate and acetate incorporation were both inhibited by about 50%. Incorporation from ^{14}C -amino acids into acyl aminoacyl puromycin was reduced 30%, while incorporation into aminoacyl puromycin was unaffected. The effect of chloramphenicol on ^3H -methionine incorporation was also examined, since mitochondria would be expected to make N-formyl methionyl puromycin. When yeast were grown on either glucose or ethanol, incorporation into aminoacyl puromycin was stimulated by the inhibitor. No reduction of incorporation into acyl amino acyl puromycin was observed with glucose grown yeast, while a 50% inhibition was obtained with ethanol grown yeast. The inhibitions observed with glucose grown yeast were not due to inhibition of respiration. Chloramphenicol, either alone, or in combination with cycloheximide, did not affect oxygen uptake over a three hour period of incubation.

That concentrations of chloramphenicol of 1 mg/ml did not produce any significant inhibition of incorporation into puromycin derivatives indicates that none of the observed incorporation was due to mitochondrial protein synthesis. This chloramphenicol concentration is sufficient to inhibit in vivo yeast mitochondrial protein synthesis completely (Clark-Walker and Linnane, 1966). That some inhibition was observed at the higher chloramphenicol concentration could, however, mean that these derivatives

Table 30. Effect of Chloramphenicol on Incorporation of Radioactivity into Puromycin Derivatives by Yeast Cells.

Radioactivity Source	Control cpm	+Chloramphenicol cpm	% control
I. Band 1			
^{14}C -formate	743.4	353.8	47.6
^{14}C -acetate	321.2	166.7	51.9
^{14}C -amino acids	401.7	285.4	71.1
^3H -methionine glucose	264.3	245.1	91.8
ethanol	301.7	140.2	46.6
II. Band 2			
^{14}C -amino acids	417.4	441.5	105.6
^3H -methionine glucose	111.2	175.6	158
ethanol	80.5	94.2	117

Yeast cells were incubated with 50 mM puromycin and label: 5.0 μCi ^{14}C -sodium formate (sp.act. = 4.0 mCi/mmol), 0.5 μCi sodium acetate-2- ^{14}C (sp.act. = 50 $\mu\text{Ci}/7.1$ mg), 0.5 μCi ^{14}C -amino acid mixture (sp.act. = 1.0 mCi/mmol), 5.0 μCi ^3H -methyl-L-methionine (sp.act. = 1.0 mCi/2.2 mg). All incubations were for 3 hr. To the indicated samples, 300 μg chloramphenicol were added. Areas of electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and counted by liquid scintillation spectrometry. Background cpm have been subtracted.

were formed by mitochondria. This is unlikely since the only product expected from mitochondria is N-formyl methionyl puromycin. High concentrations of chloramphenicol are known to have effects on eukaryotic protein synthesis (Borsook, et al., 1957; Allen and Schweet, 1962). The antibiotic may inhibit some step in initiation in these systems (Weisberger and Wolfe, 1964; Godchaux and Herbert, 1966).

These results do not eliminate the possibility that under the particular conditions used, high concentrations of chloramphenicol were needed to inhibit a mitochondrial puromycin reaction. A different test for the effectiveness of chloramphenicol on the in vivo puromycin reaction had to be devised. A blue green alga, Anabaena cylindrica, was therefore used to determine if chloramphenicol could inhibit N-formyl methionyl puromycin formation.

Algae were incubated with puromycin, ^3H -methionine, and ^{14}C -formate, and with or without 0.6 mg/ml chloramphenicol. Table 31 shows that both formate and methionine radioactivity was incorporated into acyl aminoacyl puromycin. Incorporation could not be obtained in the absence of puromycin. The incorporation was not further characterized, but was assumed to be N-formyl methionyl puromycin (Bachmeyer and Kreil, 1968). Radioactivity from methionine was incorporated into aminoacyl puromycin to the same extent as into acyl aminoacyl puromycin. Some incorporation of formate into aminoacyl puromycin was also observed, but

Table 31. Incorporation of Radioactivity into Puromycin Derivatives by Anabaena cylindrica.

Radioactivity Source	Puro-mycin	Chloram-phenicol	Band 1 cpm	Band 2
^{14}C -formate	+	-	4,771.2	140.5
"	+	+	2,012.0	153.0
"	-	+	68.8	41.1
^3H -methionine	+	-	724.9	736.2
"	+	+	467.0	475.6
"	-	+	18.7	19.8
^{14}C -amino acids	+	-	255.6	122.3
"	-	-	32.1	49.1

Filaments were allowed to settle from a 14 day old culture of the alga. Four-fifths of the supernatant medium were decanted, and the cells suspended in the remaining medium to a density of $10.0 A_{660}/\text{ml}$. Conditions of incubation were identical to those used with yeast, the algae suspension being used as yeast suspension, except the algae were incubated at 23°C for 12 hr under a fluorescent light with constant shaking. Amounts and descriptions of radioactive precursors were as in Table 30. Indicated incubations contained 50 mM puromycin and/or 0.6 mg/ml chloramphenicol. Areas of electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and counted by liquid scintillation spectrometry. Background cpm have been subtracted.

the level was less than 5% of the incorporation into N-formyl methionyl puromycin. Chloramphenicol inhibited formate incorporation into N-formyl methionyl puromycin by 57%, and methionine incorporation into the same compound by 35%. A similar inhibition of methionine incorporation into aminoacyl puromycin occurred, while chloramphenicol did not affect formate incorporation into this band. Significant incorporation of radioactivity into both compounds was also obtained from ^{14}C -amino acids. Whether this incorporation was due to conversion of radioactive amino acids into methionine or formate was not determined.

These experiments offer further proof that the methods used were effective in isolating puromycin derivatives formed in vivo. They also show that concentrations of chloramphenicol below 1 mg/ml do inhibit the in vivo puromycin reaction in susceptible organisms.

Cycloheximide

Cycloheximide inhibits in vitro amino acid incorporation by yeast ribosomes (Rao and Grollman, 1967) while having no effect on mitochondrial (Clark-Walker and Linnane, 1966) or chloroplast (Smillie, et al., 1967) protein synthesis. Yeast was incubated with puromycin and radioactive precursors with or without cycloheximide. A concentration of cycloheximide was used that was known by experiment to give greater than 95% inhibition of

incorporation of radioactive amino acids into trichloroacetic acid precipitable material. Incorporation into puromycin derivatives was assayed. Radioactivity incorporation into acyl aminoacyl puromycin from all precursors tested (formate, acetate, and amino acids) was not affected by cycloheximide (Table 32). Amino acid incorporation into aminoacyl puromycin was slightly inhibited by cycloheximide.

Cycloheximide inhibits translocation of peptidyl tRNA from the acceptor site to the donor site on the ribosome (McKeehan and Hardesty, 1969; Baliga, et al., 1970). In bacterial systems, this step is catalyzed by a different enzyme during initiation than during elongation (Kolakofsky, et al., 1969; Swan, et al., 1969). The possible action of cycloheximide at two sites has been raised (Godchaux, et al., 1967). It is, however, unlikely that cycloheximide inhibits initiation (Stanners, 1966; MacKintosh and Bell, 1969; Marcus, 1970; Huang and Baltimore, 1970; Fan and Penman, 1970; McCormick and Penman, 1969; see however Colombo et al., 1965, and Vesco and Colombo, 1970) nor peptide bond formation with puromycin (McKeehan and Hardesty, 1969; Neth, et al., 1970). The lack of effect of cycloheximide on the observed incorporations is in accord with these reports.

Table 32. Effect of Cycloheximide on Incorporation of Radioactivity into Puromycin Derivatives by Yeast Cells.

Radioactivity Source	Control cpm	+Cycloheximide cpm	% control
I. Band 1			
^{14}C -formate	610.1	660.5	108.2
^{14}C -acetate	321.2	336.4	104.7
^{14}C -amino acids	401.7	400.4	99.7
II. Band 2			
^{14}C -amino acids	417.4	387.3	92.8

Experimental details were as in Table 30. The indicated incubations contained 0.34 mM cycloheximide. Band 1 is acyl aminoacyl puromycin and Band 2 is aminoacyl puromycin.

Anisomycin

Since cycloheximide only indirectly inhibits peptide bond formation, a more direct inhibitor was tried. Anisomycin is also specific for 80s ribosomal protein synthesis (Grollman, 1967). It is effective in preventing the fragment reaction between acyl aminoacyl hexanucleotide and puromycin (Neth, et al., 1970). The fragment reaction is analogous to the postulated initiation reaction investigated here.

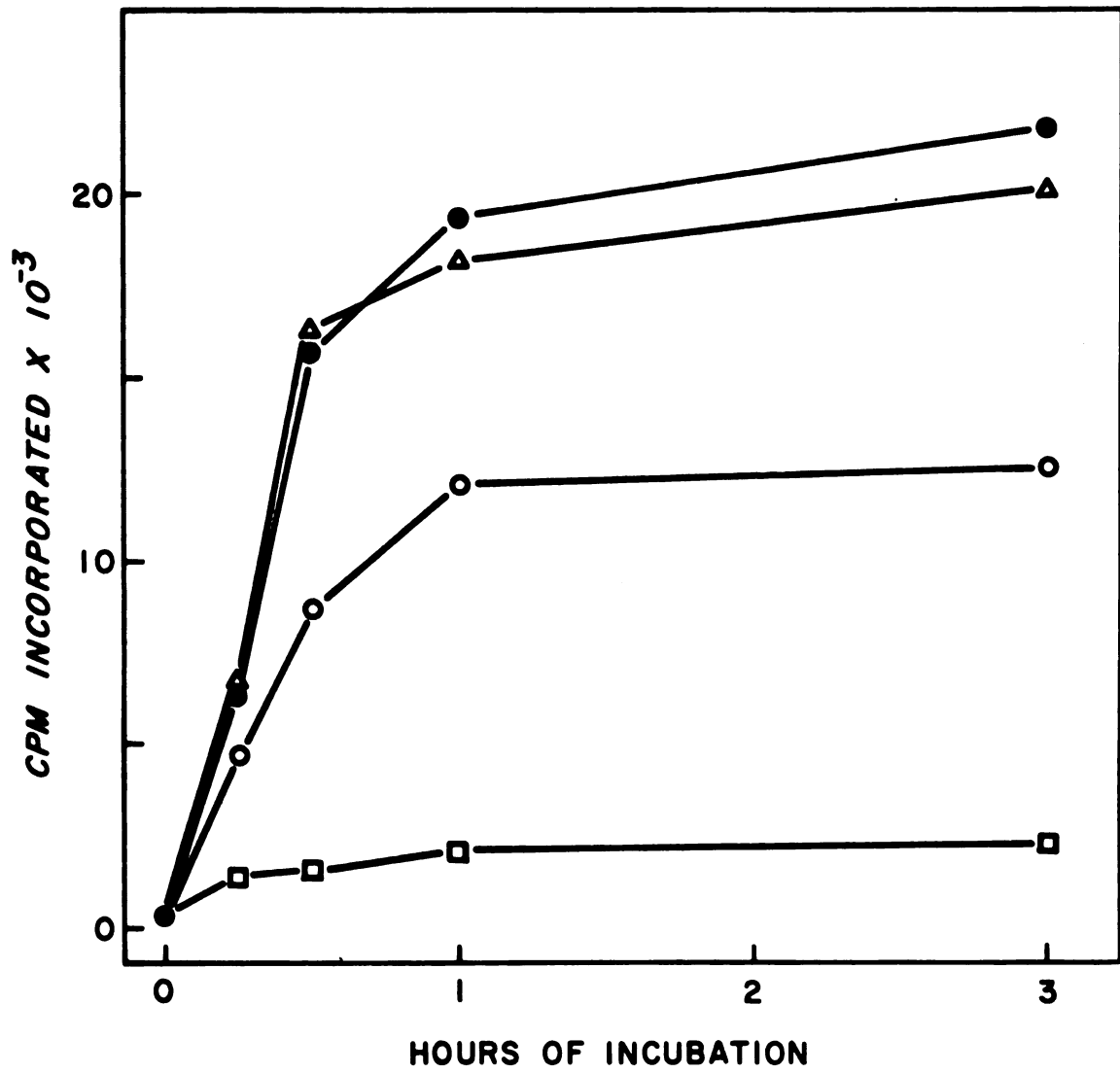
The effect of anisomycin on protein synthesis in yeast cells was examined first in order to establish an

optimum concentration for use in studies with puromycin. Incorporation of ^{14}C -amino acids into trichloroacetic acid insoluble material as a function of time and of anisomycin concentration was followed. The results (Figure 13) show that anisomycin did inhibit amino acid incorporation. Higher concentrations of the antibiotic were necessary to produce the same degree of inhibition reported by Grollman (1967) for Saccharomyces fragilis.

The effect of 1 mM anisomycin on incorporation of radioactive precursors into puromycin derivatives was tested (Table 33). Incorporation into acyl aminoacyl puromycin from any of the precursors examined (formate, acetate and amino acids) was not significantly inhibited by anisomycin. No significant inhibition was noted of ^{14}C -amino acid incorporation into aminoacyl puromycin. Although this concentration of anisomycin was sufficient to inhibit peptide bond formation (Figure 13), higher concentrations may be needed to compete with the high concentration of puromycin. At 2 and 20 mM anisomycin inhibitions of ^{14}C -amino acid incorporation into both compounds were noted. The degree of inhibition varied from experiment to experiment, was never more than 25%, and was not consistently concentration dependent. ^{14}C -acetate incorporation was inhibited 40% by 20 mM anisomycin, while formate incorporation was unaffected. The conclusion drawn from these results is that anisomycin,

Figure 13. Effect of Anisomycin on ^{14}C -Amino Acid
Incorporation into Macromolecules by
Yeast Cells.

Each incubation mixture contained 0.05 μCi reconstituted ^{14}C -protein hydrolyzate (sp.act. = 1 mCi/mg) and one of the following concentrations of anisomycin: 1.0 mM (squares); 0.10 mM (open circles); 0.010 mM (triangles); or no anisomycin (closed circles).



at concentrations effective in inhibiting amino acid incorporation into trichloroacetic acid precipitable material, had little or no effect on formation of radioactively labeled puromycin derivatives. The ineffectiveness of anisomycin will be considered in the discussion.

Table 33. Effect of Anisomycin on Incorporation of Radioactivity into Puromycin Derivatives by Yeast Cells.

Radioactivity Source	Anisomycin (mM)	Control cpm	+Anisomycin cpm	% control
I. Band 1				
¹⁴ C-formate	1	610.1	662.3	108.4
"	20	653.2	652.0	99.9
¹⁴ C-acetate	1	319.4	361.0	112.9
"	20	820.8	478.4	58.3
¹⁴ C-amino acids	1	380.4	367.3	96.6
"	2	380.4	317.6	83.3
"	20	364.2	312.3	85.8
II. Band 2				
¹⁴ C-amino acids	1	364.6	348.2	95.6
"	2	364.6	328.4	90.1
"	20	316.5	264.7	83.7

Experimental details were as in Table 30, except that 0.5 μ Ci sodium acetate-2-¹⁴C (sp.act. = 0.5 mCi/mmol) was used at 1 mM anisomycin. 1.0 μ Ci of the same label were used at 20 mM anisomycin. Band 1 = acyl aminoacyl puromycin. Band 2 = aminoacyl puromycin.

Mild Acid Hydrolysis of
Acyl Aminoacyl Puromycin

The results in Tables 24 and 30 imply that ^3H -methionine was not incorporated into N-formyl methionyl puromycin. Whether this was the case could be tested more directly by mild acid hydrolysis. N-formyl methionyl puromycin should be completely converted to methionyl puromycin after acid treatment.

Yeast cells grown on either glucose or ethanol were supplied with puromycin and ^3H -methionine. After incubation, acyl aminoacyl puromycins were isolated as an electrophoretic band. The eluate from this band was divided in two, and one sample hydrolyzed for one hour. Both samples were re-electrophoresed. Bands corresponding to acyl aminoacyl puromycin and aminoacyl puromycin were counted for radioactivity. In these experiments, slight (never more than 10%) depurination (Nathans, 1964), detected as cpm not moving from the origin, occurred during acid treatment. The results (Table 34) are corrected for this depurination. Similar results were obtained whether glucose or ethanol was used as a carbon source. The slight loss of radioactivity from Band 1 did not appear in Band 2. Possibly, some of the ^3H -methionine (methyl- ^3H) was converted to ^3H -formate. No methionyl puromycin was formed indicating that none of the ^3H -methionine incorporation was into N-formyl methionyl puromycin.

Table 34. Effect of Mild Acid Treatment on Variouslly Labeled Acyl Aminoacyl Puromycins.

Treatment	Hydro- lysis	Band 1	cpm	Band 2
1. ^3H -methionine				
glucose	-	241.6		19.9
glucose	+	218.2		21.3
ethanol	-	193.2		22.7
ethanol	+	179.0		14.8
2. ^{14}C -amino acids				
none	-	450.8		37.8
none	+	421.0		47.1
3. ^{14}C -formate				
none	-	653.2		--
none	+	305.2		--
chloramp.	-	299.2		--
chloramp.	+	174.2		--

Yeast cells were preincubated on glucose (1,2,3) or ethanol (1); yeast cells were incubated with 50 mM puromycin the indicated label and with or without the addition of 300 μg chloramphenicol for 3 hr. Labels used were: 5.0 μCi ^3H -methyl-L-methionine (sp.act. = 1.0 mCi/2.2 mg) (1); 1.0 μCi ^{14}C -amino acid mixture (sp.act. = 1.0 mCi/mg) (2); 5.0 μCi ^{14}C -sodium formate (sp.act. = 4.0 mCi/mmol) (3). Eluates from thin layer chromatograms (3) or electropherograms (1, 2) were divided into two equal portions. One half was treated with mild acid for 1 hr, the other spotted directly on paper. Areas of electropherograms corresponding to acyl aminoacyl puromycin (Band 1) and aminoacyl puromycin (Band 2) were cut out and counted by liquid scintillation spectrometry. Background cpm have been subtracted.

The same technique can be used to test whether any incorporation of ^{14}C -amino acids into puromycin derivatives was into N-formyl aminoacyl puromycin. Although a slight conversion of cpm from acyl aminoacyl puromycin to aminoacyl puromycin occurred (Table 34, section 2), the amount of this conversion was within experimental error, and may not be significant. At least 90% of the radioactivity from ^{14}C -amino acids in acyl aminoacyl puromycin was not present as formyl aminoacyl puromycin.

Since ^{14}C -formate was incorporated into acyl aminoacyl puromycin in at least two forms (Table 29 and Figure 12), and since chloramphenicol inhibited its incorporation 53% (Table 30), chloramphenicol inhibition of one form and not the other was possible. This possibility was also tested using mild acid hydrolysis. In agreement with the results of Table 30, chloramphenicol inhibited ^{14}C -formate incorporation 54% (Table 34, section 3). Of the remaining incorporation 42% was released by mild acid, while 54% release was obtained from the control. Thus, chloramphenicol inhibited incorporation into formyl and non-labile components to about the same extent.

Independent Formation of Puromycin Derivatives

Three possible relationships between the two types of puromycin derivatives were considered. First, one could be produced first and then be converted to the other.

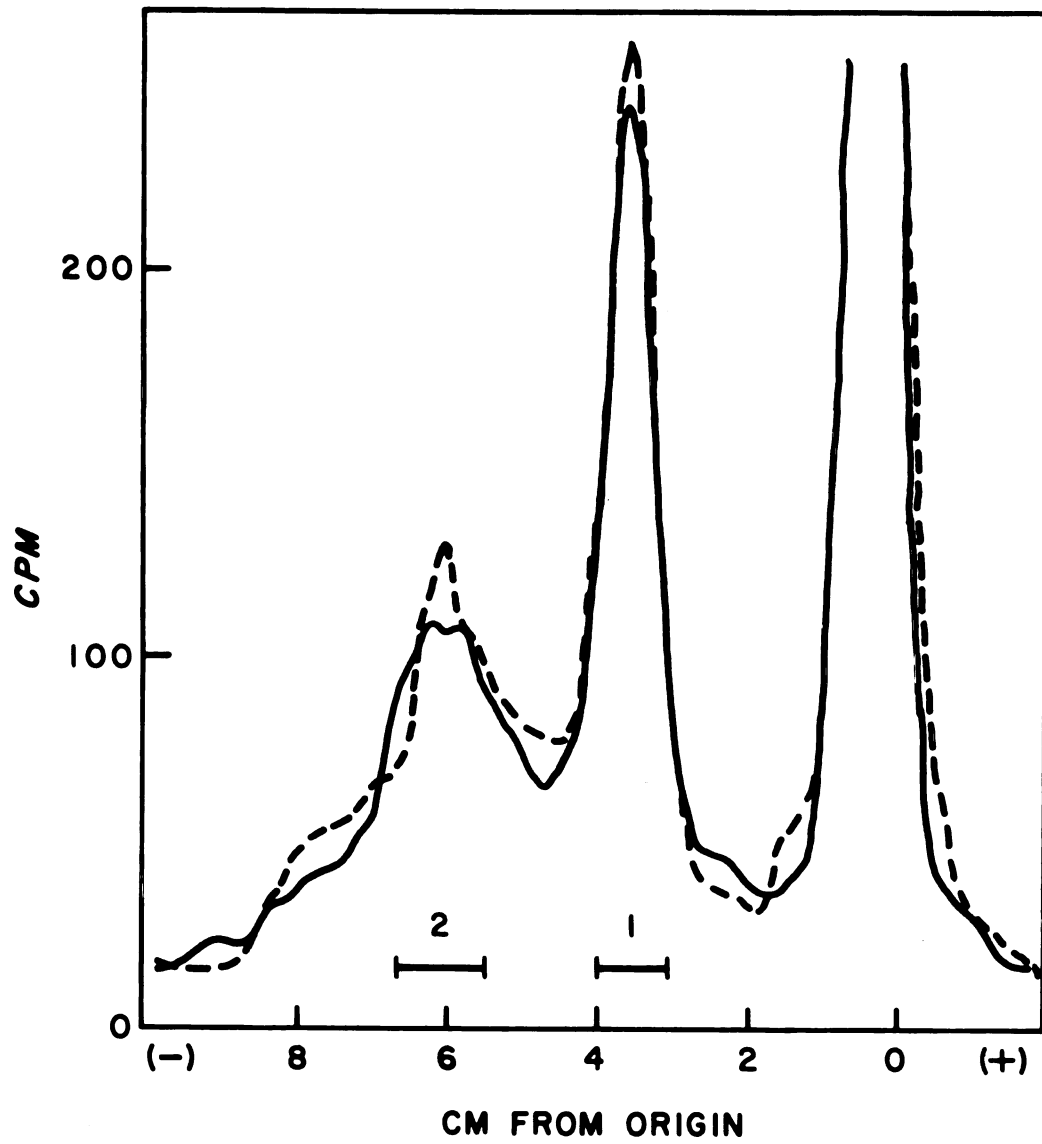
Second, they could be freely interconverted in an equilibrium process. Last, the derivatives could be formed independently of one another. The first possibility was ruled out by two experiments. In studying the time course of ^{14}C -amino acid incorporation into puromycin derivatives (Figure 6) it was noted that both derivatives were formed with similar kinetics. This is not the relationship expected for a precursor product situation.

The possibility was further tested by a chase experiment. Three samples were incubated with yeast, puromycin and ^{14}C -amino acids for three hours. At this time, one sample was removed for analysis of puromycin derivatives. To another, a concentrated casein hydrolyzate solution was added, while an equal volume of water was added to the last sample. After an additional hour of incubation, the samples were analyzed for puromycin derivatives. Figure 14 shows that the chase was effective in that only small increases in incorporation occurred during the chase period. In the control (water during fourth hour), which is omitted for clarity, marked increases in incorporation occurred. Comparison of the peaks in Figure 14 shows that there was no detectable conversion of acyl aminoacyl puromycin to aminoacyl puromycin or vice versa.

The above experiments do not rule out the possibility that most of the radioactive puromycin derivatives

Figure 14. Effect of ^{12}C -Amino Acid Chase on Labeling of Yeast Puromycin Derivatives.

Yeast cells were incubated with 50 mM puromycin and 2.0 $\mu\text{Ci } ^{14}\text{C}$ -amino acid mixture (sp.act. = 1 mCi/mg) for 3 hr. One sample was analyzed for radioactive puromycin derivatives (solid line). A second sample received 10 μl 10 mg/ml casein hydrolyzate neutralized power and was incubated another hr before analysis (dashed line). Radioactivity scanning conditions were: 0.5 cm/min, 7.5 mM slit width, 30 sec time constant. In a third sample (not shown) which received 10 μl water during the fourth hour, the radioactivity peak heights were 310 cpm (Band 1, acyl aminoacyl puromycin) and 190 cpm (Band 2, aminoacyl puromycin).



observed did not have access to the site where the conversion occurs. For instance, if the hypothetical conversion enzyme is localized on the ribosome in such a position that it removes the acyl group as soon as the acyl aminoacyl puromycin is formed, then conversion should not be detected in the above chase experiment. The experiment also does not rule out the possibility that there is an equilibrium between the two forms. Acetylation of an initiator aminoacyl tRNA has been shown to require acetyl coenzyme A (Liew, et al., 1970). This makes an equilibrium unlikely because the deacylation reaction probably proceeds with the release of a large free energy. One is left then with the possibility that a conversion takes place immediately after synthesis or that the two compounds are indeed produced independently of one another.

DISCUSSION

The behavior of the compounds isolated as "Band 1" to ethyl acetate extraction, chromatography, electrophoresis at pH 1.8, electrophoresis at pH 5.4, N-terminal analysis, steam distillation, mild acid hydrolysis, and pronase digestion supports their identification as N-acyl aminoacyl puromycin. The data do not eliminate the possibility that some, but not all, of the compounds were N-acyl puromycin. The compounds isolated as "Band 2" were less well characterized. Their behavior to ethyl acetate extraction, chromatography, electrophoresis at pH 1.8, and steam distillation suggest their identification as aminoacyl puromycin. N-terminal analysis supports this identification, but does not rule out the presence of other components.

Several acyl groups were present in N-acyl aminoacyl puromycin. The presence of N-formyl aminoacyl puromycin was demonstrated by mild acid hydrolysis and steam distillation. Although present, formyl groups did not constitute a significant part of the acyl groups in N-acyl aminoacyl puromycin labeled with radioactive amino acids or methionine. The predominant acyl group was probably the acetyl group. One nanomole (calculated from

the specific radioactivity of supplied ^{14}C -acetate, assuming no dilution) N-acetyl aminoacyl puromycin was formed during a three hour incubation with 50 mM puromycin. Under the same conditions, 0.3 nanomole (calculated from the ^{14}C -amino acid specific radioactivity and the amount of ninhydrin positive material in yeast cells) N-acyl aminoacyl puromycin were formed. The discrepancy could be due to an increase in amino acid pool size consequent to inhibition of amino acid utilization through protein synthesis. N-acyl aminoacyl puromycin contained a constituent in addition to formyl and acetyl groups, amino acids, and puromycin. This constituent was labeled by a ^{14}C -amino acid mixture, was not an amino acid, and possibly could be another acyl group.

Aminoacyl puromycins and acyl aminoacyl puromycins may have been formed by the protein synthetic machinery of yeast cells. The puromycin concentration dependences for formation of the derivatives and for inhibition of amino acid incorporation were similar. It is well known that the inhibition of protein synthesis is due to the formation of peptide bonds between the growing chain and puromycin. If protein synthesis was inhibited, then puromycin derivatives should have been formed. The demonstration of N-formyl methionyl puromycin formation by Anabaena cylindrica, a prokaryote, shows that the methods used were effective in isolating puromycin derivatives.

The methods were designed not to separate derivatives that varied in the amino acid portion of the molecule. At least some of the isolated products must have been made by the protein synthetic apparatus.

Other known actions of puromycin can not account for the formation of puromycin derivatives. Puromycin inhibits the leucyl, phenylalanyl tRNA--protein transferase from E.coli, but is inactive against the corresponding arginyl tRNA enzyme from higher organisms (Leibowitz and Soffer, 1970). It inhibits cyclic nucleotide phosphodiesterase (Appleman and Kemp, 1966), but how this action could give rise to the observed compounds is unclear. On the other hand, that puromycin derivatives could be labeled with a ^{14}C -amino acid mixture by Anabaena cylindrica indicates that formation of puromycin derivatives through mechanisms other than protein synthesis may occur.

If puromycin derivatives are formed on ribosomes, are they made by the cytoplasmic or the mitochondrial protein synthetic apparatus? The fraction of total protein synthesis in yeast cells that is due to mitochondria is very low (Schwegen and Kaudewitz, 1970). Since puromycin acts on both systems similarly, only a small fraction of the derivatives could have been formed by the mitochondria. Mitochondria, when supplied with puromycin, should make N-formyl methionyl puromycin. The formation of other puromycin derivatives is possible in view of the ability

of Anabaena cylindrica to form derivatives labeled with ^{14}C -amino acid mixture. None of the observed methionine or amino acid incorporation by yeast cells was into N-formyl methionyl puromycin. Moderate concentrations of chloramphenicol (effective in reducing N-formyl methionyl puromycin formation by Anabaena cylindrica) did not inhibit incorporation of any of the radioactive precursors tested. For these reasons, the yeast puromycin derivatives studied may have been made by the cytoplasmic protein synthetic machinery.

No support for the above conclusion was obtained by the use of inhibitors of cytoplasmic protein synthesis. The translocation step in protein synthesis elongation is catalyzed by an enzyme, and is inhibited by cycloheximide (McKeehan and Hardesty, 1969; Baliga, et al., 1970). It is possible that, as occurs in bacteria (Kolakofsky, et al., 1969; Swan, et al., 1969), a different enzyme is involved in the initial translocation. If this is the case, then cycloheximide should not inhibit peptide bond formation between puromycin and the initial amino acid. Thus, although experiments with cycloheximide do not support puromycin derivative formation by cytoplasmic ribosomes, they do not contradict this hypothesis.

The results with anisomycin, however, do contradict the hypothesis. Since anisomycin inhibits peptide bond formation between acyl aminoacyl hexanucleotide and

puromycin by yeast ribosomes (Neth, et al., 1970), it should have inhibited the formation of the puromycin derivatives studied here. The detailed mechanism of anisomycin action has not been elucidated. It is possible that it binds to the ribosome so as to impair binding of acyl aminoacyl hexanucleotide and of non-initiating aminoacyl tRNA's, while not affecting the binding of initiator aminoacyl tRNA's. It is, however, also possible that the formation of labeled puromycin derivatives was not catalyzed by cytoplasmic or mitochondrial ribosomes. If this is the case, then puromycin must prevent continuing protein synthesis by an unknown mechanism not involving peptide bond formation with nascent chains.

Assuming for the moment that the puromycin derivatives examined were formed by cytoplasmic ribosomes, they must contain the initial amino acid polymerized. First, at high concentrations of puromycin, only puromycin derivatives of the initial amino acid should be formed (see INTRODUCTION). The results with Anabaena cylindrica confirm that the principal, although not the only, derivative formed is that of the initiator amino acid (in this organism, N-formyl methionine). Second, N-acyl aminoacyl puromycins, by virtue of their structure, can not arise from the reaction of internal amino acid residues with puromycin. Third, the observation that high concentrations of chloramphenicol inhibit formation of some puromycin

derivatives agrees with reports that, at high concentrations, chloramphenicol can inhibit initiation of eukaryotic protein synthesis (Weisberger and Wolfe, 1964). Last, internal re-initiation in the presence of puromycin results uniquely in the formation of di- and oligo- peptidyl puromycin (Smith, et al., 1965). In the experiments reported here, aminoacyl puromycin was the predominant product.

If the puromycin derivatives reflect initiation of protein synthesis, then some conclusions about amino acids involved in initiation of yeast protein synthesis can be drawn. No one single amino acid is the first amino acid polymerized for every protein chain. This conclusion agrees with the literature since different amino acids have been implicated as initiators in the different systems studied: glycine in ovalbumin synthesis (Narita, et al., 1968 and 1969); serine in histone synthesis (Liew, et al., 1970); glutamine in immunoglobulin synthesis (Baglioni, 1970); and valine in hemoglobin synthesis (Arnstein and Rahaminoff, 1968). Each of these systems is making predominantly one protein and therefore only one N-terminal amino acid is expected.

A blocked amino group is not required in initiation. Free amino acids, acetyl amino acids, and some formyl amino acids participate in initiation of yeast protein synthesis. This again agrees with the literature. In some systems acetyl amino acids seem to function as

initiators (Liew, et al., 1970; Narita, et al., 1968 and 1969). In others, free amino acids are required (Baglioni, 1970). Even formylated amino acids have been suggested as initial amino acids (Kim, 1968; Kreil and Kreil-Kiss, 1967).

The only studies with which these results are not in accord are those that suggest that methionine is polymerized into the N-terminal of all proteins and then later removed to reveal the true terminal amino acid. The level of methionine incorporation observed was no greater than that of any other amino acid studied. As mentioned above, met tRNA and the codon AUG play a role in initiation (see GENERAL INTRODUCTION). The current study provides support for the hypothesis proposed by Smith and Marcker (1970): " . . . Met tRNA_F* could select the reading frame only and not be incorporated into proteins."

Bhaduri, et al. (1970) and Jackson and Hunter (1970) have shown N-terminal incorporation of methionine in the hemoglobin system. However, Takeishi, et al. (1970) were not able to find any incorporation of methionine from met tRNA_F* in the same system. Wigle and Dixon (1970) have shown that methionine is transiently incorporated into the N-terminus of protamine.

Since methionine is incorporated in vivo into N-terminal positions in at least two systems (Jackson and Hunter, 1970; Wigle and Dixon, 1970), and since no evidence that puromycin derivatives were formed by the yeast protein

synthetic apparatus was found in this study, it is possible that the derivatives were formed by some other pathway. In this case, the puromycin derivatives that should have been formed through protein synthesis must be present in quantities small compared to the non-ribosomally formed compounds. Although this did not seem to be the case with Anabaena cylindrica, it may be the case with yeast. Alternatively, if ribosomal puromycin derivatives were more than a few amino acids long, they would not have been extracted with ethyl acetate, and thus ignored in this study. Also possible is rapid enzymatic hydrolysis of ribosomal aminoacyl puromycins, although such hydrolysis does not occur in reticulocytes (Jackson and Hunter, 1970).

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