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Final Report



# DEVELOPMENT AND USE OF ANUCLEATED BACTERIAL CELLS TO ASSAY THE IN VIVO ACTIVITY OF POLLUTANTS

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April 1981 - July 1985

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There were 2 objectives in this research project: development of an in vivo assay for mistranslation-inducing activity of pollutants and characterization of amino acid substitutions. The first objective proved to be the more difficult. The T7 0.3 gene product (0.3 protein) was purified by a modification of the published procedure (2), and used to raise rabbit antibody to this protein. A radioimmune precipitation assay was developed which could be used to estimate increased misincorporation of cysteine into 0.3 protein. A simple assay

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involving only counting of the radioimmune precipitate (RIP) was not achieved because we were not able to obtain the 0.3 protein free of contaminating proteins in the RIP. However, increased cysteine misincorporation could be estimated by RIP-polyacrylamide gel electrophoresis (PAGE) combined with scanning densitometry. Efforts to obtain only the 0.3 protein in the RIP involved production of a monoclonal antibody to 0.3 protein, the use of protein A Sepharose or a second immunoglobulin instead of S. aureus to precipitate the immune complex and preincubation of the cell lysate with these 3 reagents to remove the protein which adhered to them. None of these techniques could remove all the trace extraneous proteins from the RIP. But even trace contaminating proteins cannot be tolerated in a simple assay for mistranslation based on counting of the immune precipitate only.

The 2nd objective proved more fruitful. We have been able to successfully identify the cysteine substitution sites in the N-terminal 42 positions of 0.3 protein. Cleavage of 0.3 protein with trypsin to identify cysteine for arginine substitutions showed that the major sites of cysteine misincorporation were at residues other than arginine. Sequencing of [$^{35}$S]cysteine-labeled 0.3 protein showed that the most frequent substitution was at residue 15 (tyrosine) and other substitutions were at positions 9 (asparagine), 12 (aspartate), 41 (alanine) and 42 (aspartate). Mistakes at these positions were unexpected and show that context greatly affects misincorporation and that misreading of 2 bases in the triplet at a time occurs relatively frequently. We have recently successfully purified an internal 0.3 peptide by RP-HPLC and preparative gel electrophoresis and can now obtain sequencing data on this portion of the 0.3 protein. We have also begun to compare mistakes made in 0.3 protein synthesized under "normal" conditions with the protein synthesized in the presence of the error-inducing aminoglycoside antibiotic, streptomycin. Preliminary results indicate that, contrary to what has been generally believed, streptomycin does induce novel types of errors (cysteine for valine (GUU), phenylalanine (UUC) and glutamate (GAA). The misreading errors seen without the drug were also apparent when streptomycin was added. Thus, streptomycin apparently can induce misreading which requires codon-anticodon mispairing at all 3 bases, and errors which require C to be misread as G in the internal position of the codon -mistakes not usually seen during <u>in vitro</u> mistranslation experiments.

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# PROGRESS REPORT

Grant #AFOSR 81-0087

Title: Development and use of anucleate bacterial cells to assay the *in vivo* activity of pollutans

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Report for research undertaken between April 1, 1981 - August 1, 1982

Progress Report, April 1, 1981 - August 1, 1982

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- B. Detection of $^{35}$S-cysteine incorporation into T7 polypeptides
- C. Purification of 0.3 protein
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- C. Determination of the normal mistranslation level *in vivo*
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  2. Determination of experimental conditions for calculations of mistranslation levels
  3. Demonstration that $^{35}$S is not transferred from cysteine to methionine

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I. Report for research undertaken between April 1, 1981 - February 1, 1982

A. Abstract

Experiments were undertaken to determine if misincorporation of $^{35}$S-cysteine into non-cysteine containing T7 proteins could be quantitated by autoradiography. It was found that a minimum of $10^7$ radioactive sulphur atoms were required to sufficiently expose X-ray film so that scanning densitometry could be used for quantitation. Calculations based on this determination indicated that the expected misincorporation of $^{35}$S-cysteine into T7 proteins in infected minicells would be insufficient to permit autoradiography to be used as the assay for misincorporation. It was therefore decided to develop a more sensitive assay based on immunoprecipitation of specific T7 proteins synthesized in infected minicells. A modification of a published procedure to isolate T7 0·3 gene product has been developed and we are now in the position to raise antibody to this purified protein.

Experiments have begun to determine if it is feasible to construct a recombinant bacteriophage containing a large (1000-3000 base-pairs) stretch of homopolymer i.e. poly dA-dT or poly dG-dC. The intent is to develop a probe for detection of mistranscription *in vivo*. These experiments have so far been unsuccessful. It appears that although monopolymers can be constructed *in vitro* they are not stable *in vivo*.

B. Detection of $^{35}$S-cysteine incorporation into T7 polypeptides

Anucleate bacterial cells (minicells) were infected with T7 and allowed to incorporate $^{35}$S-methionine or $^{35}$S-cysteine. The radioactively labeled polypeptides, synthesized in infected minicells, were separated by electrophoresis and the resulting gels used to expose X-ray film using the technique of fluorography. As previously observed three T7 proteins (products of genes 0·3, 9 and 16) were not labeled by incorporation of $^{35}$S-cysteine, i.e. they do not contain cysteine residues. Sample volumes were increased to increase the amounts of radioactive proteins on the gels. We were still unable to detect $^{35}$S-cysteine incorporation into 0·3, 9 or 16 proteins at sample sizes so large that gel resolution was impaired. Faint exposures of the X-ray film were obtained using $^{35}$S-cysteine incorporation into 0·3 protein by exposing the gel to the film for 8-12 weeks. This is not, however, a practical laboratory procedure.

Figure 1 shows the results of exposing known amounts of $^{35}$S-cysteine to X-ray film. It can be calculated that a minimum of $10^7$ $^{35}$S-atoms are required to produce an exposure suitable for quantitation by scanning densitometry in two days. Assuming a random amino-acid misincorporation rate of 1 per 10,000 peptide bonds (1) and a polypeptide length of 116 amino-acids (0.3 protein (2)) then one

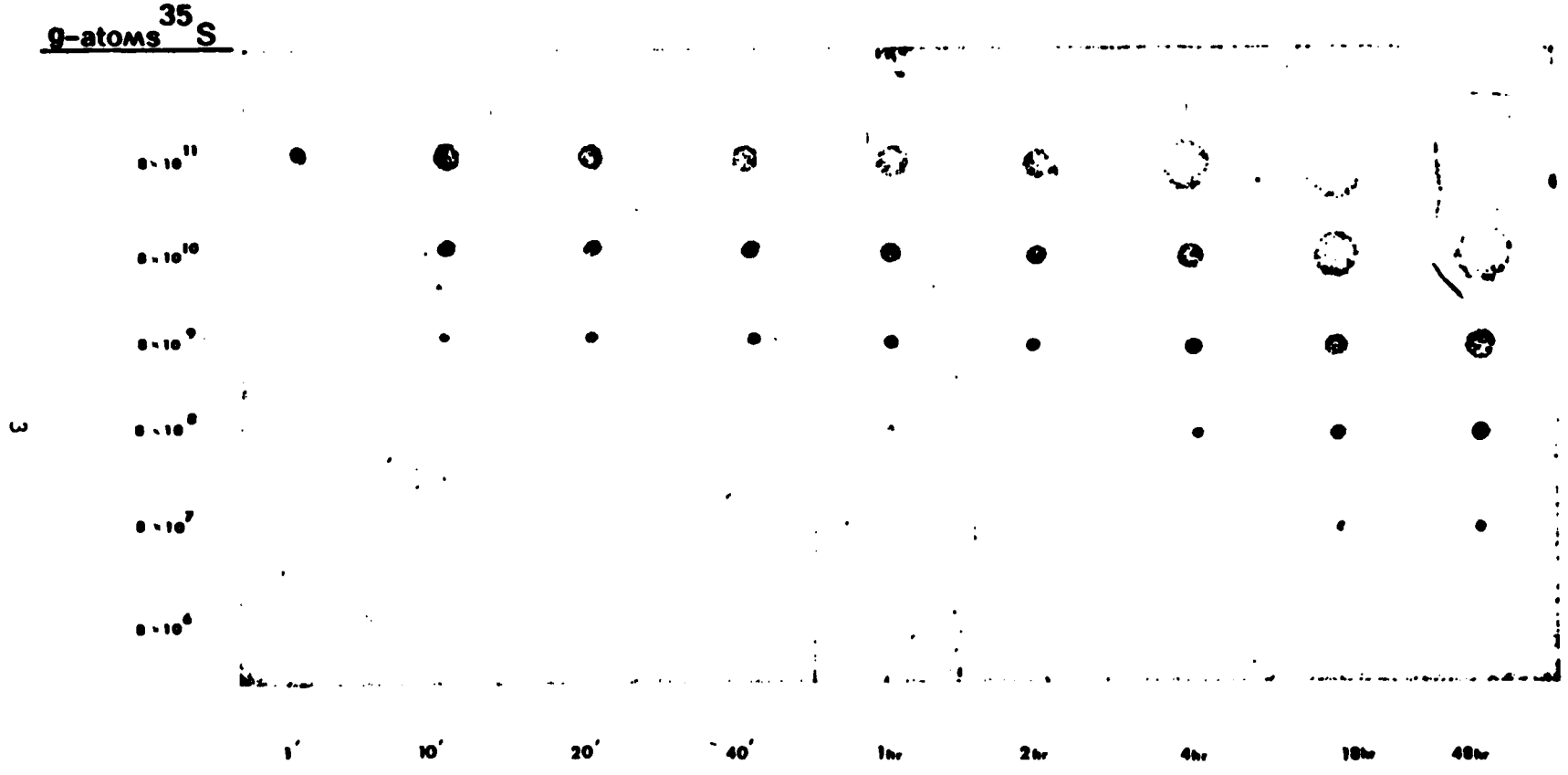



Figure 1. Known amounts of $^{35}S$-cysteine were impregnated into a dryed polyacrylamide gel prepared for fluorography. The gel was exposed to X-ray film for time periods indicated below each series of spots. The number of atoms of $^{35}S$ in each sample are given at the side of the figure.

$^{35}$S-cysteine should be misincorporated into approximately one 0·3 molecule per 2000 molecules synthesized. (This assumes that misincorporation of cysteine occurs at 1/20th the rate of total amino-acid misincorporation.)

If one 0·3 molecule is labeled per 2000 molecules synthesized and $10^7$ $^{35}$S-atoms are required for detection then a minimum of $2x10^{10}$ 0·3 molecules must be loaded onto the gel for there to be a reasonable probability that the misincorporation of $^{35}$S-cysteine will be detected by our autoradiography procedures. Our routine procedures employ $1x10^9$ minicells per sample and therefore each T7 infected minicell would have to synthesize 20 molecules of 0·3 protein to obtain the $2x10^{10}$ molecules per sample needed for minimum detection of $^{35}$S-cysteine misincorporation. Synthesis of 20 molecules of 0·3 protein per infected minicell should occur; however, our experimental results indicate that we do not routinely observe $^{35}$S-cysteine labeling of 0·3 protein. We must, therefore, assume that one or more of the quantitative assumptions used above in making calculations is incorrect. Attempts to stimulate $^{35}$S-cysteine incorporation by addition of streptomycin (known to increase mistranslation by a factor of 2 to 3) to give detectable incorporation were not successful. The conclusion of these studies was that quantitation of $^{35}$S-cysteine incorporation by direct autoradiography of extracts of T7 infected minicells is not sufficiently sensitive to develop as a routine procedure.

C. Purification of 0·3 protein

Based on our calculations that quantitation of $^{35}$S-cysteine misincorporation into 0·3 protein could not be successfully obtained by scanning densitometry we decided to purify the 0·3 protein so that antibody could be raised against the protein and used to specifically precipitate 0·3 protein from T7 infected minicells. The misincorporation of $^{35}$S-cysteine could then be quantitated by scintillation counting. In addition, antibody against 0·3 protein could be used to precipitate 0·3 protein synthesized in T7 infected cells, as well as in minicells, increasing the potential alternative systems in which 0·3 protein could be used to assay errors in protein synthesis.

A procedure for purification of 0·3 protein has been published (2) and we initially followed the procedure precisely as described. In essence the purification depends on the ability of 0·3 protein to bind to DEAE even in the presence of 0·3M $NH_4Cl$ plus the unusual property that 0·3 protein is soluble in ethanol. Our experiments confirmed that 0·3 can be separated from most proteins in an extract of T7 infected cells by binding to DEAE at 0·3M $NH_4Cl$ and elution at 0·7M $NH_4Cl$. Our problems arose in our attempts to repeat the published procedures to dissolve 0·3 protein in ethanol following TCA precipitation. A very time consuming series of experiments were undertaken varying salt concentration, pH, sequence of additions, etc. to obtain pure 0·3 protein as determined by SDS-gel electrophoresis using silver nitrate staining. We now have resolved these problems and have 0·3 protein in sufficient amounts to begin vaccination procedures for antibody production. Figure 2 is a flow chart of our procedure for isolating 0·3 protein from T7 infected cells.

Figure 2. Purification of T7 0·3 protein. Modification of the procedure described by Mark and Studier (1981).

Extract of T7 H3; lam193;LG3 infected E. coli cells

↓

DNase digestion. Bring to 0·3M $NH_4Cl$

↓

Slow speed centrifugation
High speed centrifugation

↓

Pass supernatant through DEAE column in 0·3M $NH_4Cl$. Most proteins do not bind.

↓

Elute with 0·3M → 1M $NH_4Cl$ gradient

↓

Test fractions for 0·3 protein by running 10–20% polyacrylamide gel and $AgNO_3$ staining

↓

Add 10% TCA to 0·3 containing fractions
Collect precipitate
Dissolve in Tris buffer + 0·3M $NH_4Cl$

↓

Add 4 volumes ethanol; pH2
Discard precipitate

↓

Add 8 volumes ethanol

↓

Discard supernatant
Dissolve pellet (pure 0·3) in 0·1M Tris buffer

D. Construction of a lambda based recombinant

Only a limited number of experiments have been undertaken on this part of the project as Dr. Alice Desmyter, who was hired to carry out these experiments, did not arrive in Columbus until December 1981. The problem of recruiting trained personnel for recombinant DNA studies was the basis for the no-cost extension granted to the project. Our experiments to date have been designed to determine the procedural conditions to synthesize long (1000-3000) base-pair homoduplexes using DNA polymerase I in the absence of template DNA and using terminal transferase. Based on the results of incorporation of radiochemically labeled precursors and gel electrophoresis of products it does appear that molecules of the appropriate length can be synthesized in vitro. We have not, so far, been able to clone these molecules into lambda vectors. The preliminary experiments already completed indicate that homopolymers may not be stable in vivo. A similar conclusion has been reached by Prof. R.D. Wells (University of Wisconsin; Personal communication 1981).

Report for research undertaken between Feb. 1, 1982 - Aug. 1, 1982

## A. Abstract

The T7 0.3 gene product (0.3 protein) was purified by a modification of the published procedure (2), and used to raise antibody to this protein. A radioimmune precipitation (RIP) assay was developed which could be used to estimate the increased misincorporation of cysteine into 0.3 protein. Parameters of the RIP assay were varied to make the RIP-polyacrylamide gel electrophoresis (RIP-PAGE) assay specific for the 0.3 protein. A single protein band was, however, never achieved although increased misincorporation of cysteine into the 0.3 protein can now be estimated by RIP-PAGE combined with scanning densitometry.

Experiments to determine the normal level of mistranslation *in vivo* are currently in progress. All necessary preliminary experiments for these assays have been completed. We have devised a labeling medium, determined the minimum saturating levels of cysteine and developed satisfactory methods to purify small amounts of labeled 0.3 protein, to demonstrate nonconversion of cysteine to methionine and to reliably determine the specific activity of purified, labeled 0.3 protein.

## B. Detection of $^{35}$S-cysteine incorporation into 0.3 protein by radioimmune precipitation

1. Preliminary experiments. Rabbit antibody was raised to purified 0.3 protein. This was allowed to react with $^{35}$S-methionine labeled lysates of T7-infected *E. coli* cells. In our initial RIP experiments protein A-containing *S. aureus* was used to precipitate immune complexes. On visualization of the immune complexes by fluorography following PAGE, 0.3 protein was the major band present; however, other polypeptides were also present. Exhaustive attempts were made to remove these extraneous polypeptides by varying conditions of the assay. Improvements were seen, especially when protein A Sepharose was used instead of *S. aureus* for precipitation of immune complexes (fig. 3). We were, however, unable to completely eliminate the other polypeptides. This may be due to nonspecific adherence of these polypeptides to protein A Sepharose or to the presence in our 0.3 antiserum of rabbit antibody to *E. coli* or to other T7 proteins.

2. Specificity of the RIP-PAGE assay for the 0.3 protein. Specificity was demonstrated by reacting normal or immune serum with lysates of *E. coli* strains infected with a T7 amber mutant (CR10b) in the 0.3 gene. These strains were either capable or incapable of suppressing the nonsense mutation in the 0.3 gene (fig. 4). With normal serum, protein was not seen in the 0.3 protein position in either case, but with immune serum the 0.3 protein was seen, and only when the nonsense mutation-suppressing strain was used.

3. Increase in misincorporation of cysteine into 0.3 protein with drugs. The RIP-PAGE assay was used to detect an increase in $^{35}$S-cysteine incorporation into the 0.3 protein after treatment with streptomycin or gentamicin. An increase in incorporation was detected by both scintillation counting and autoradiography using either drug at all concentrations tested (5, 10, 20 and 40 ug/ml). In the absence of drugs $^{35}$S-cysteine incorporation into 0.3 protein was detectable by autoradiography in only some experiments and in those cases exposure times in excess of 2 weeks were required. When

RIP-PAGE: $^{35}$S-Methionine Labelled E. coli B Extract

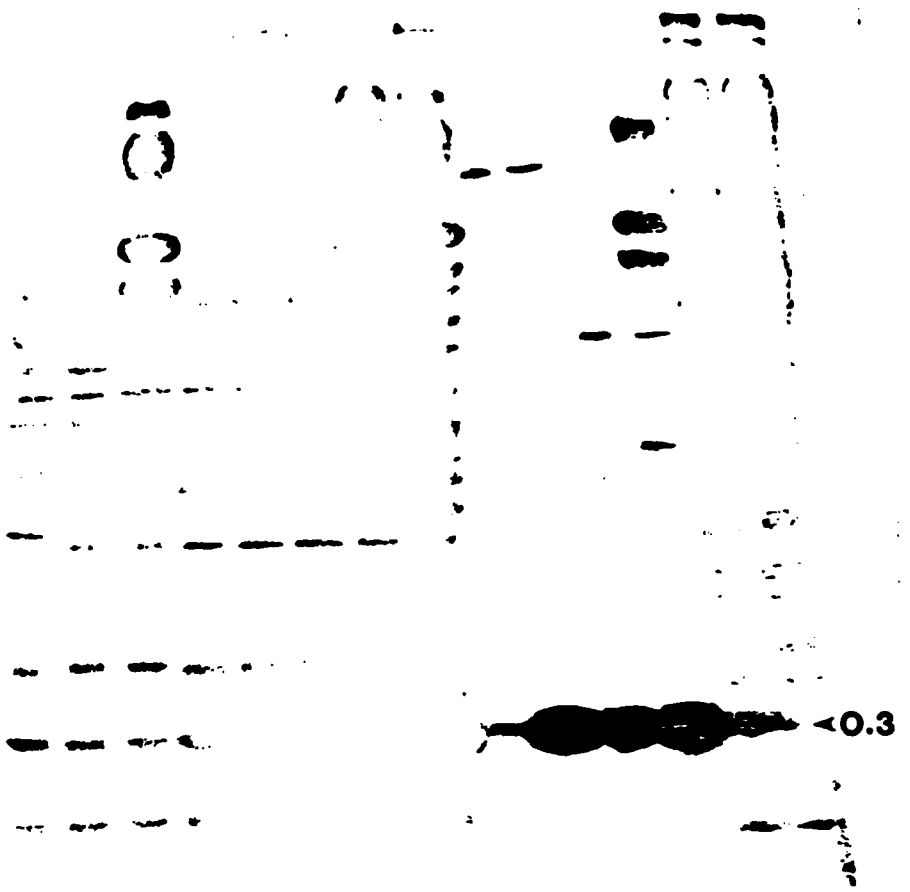


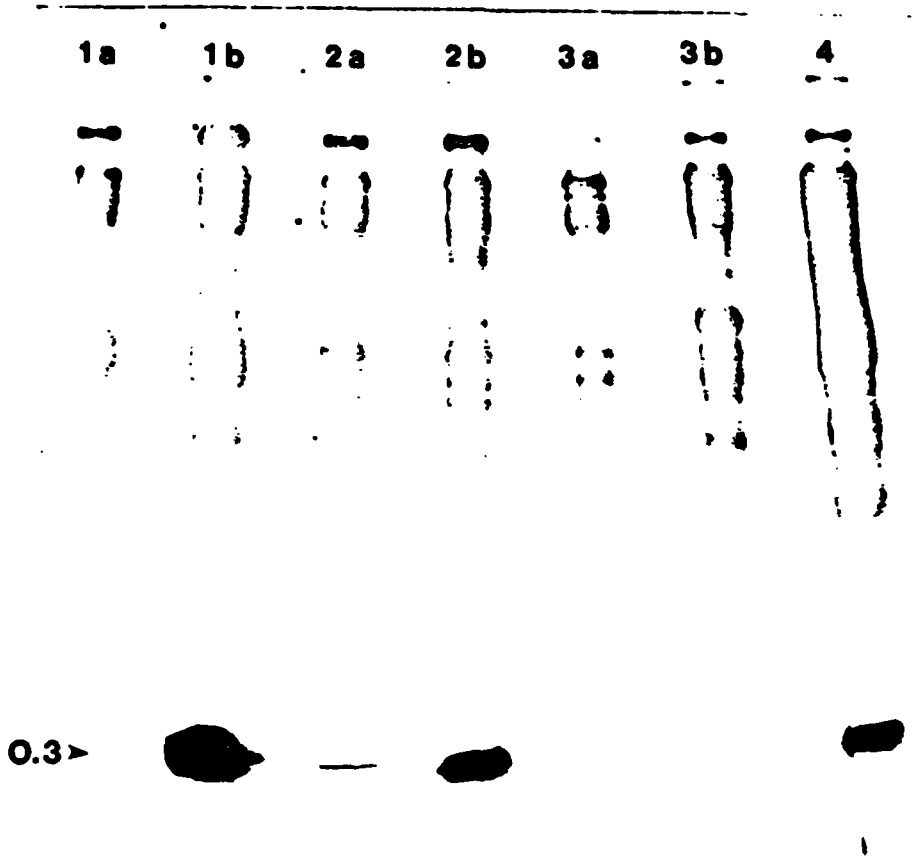

FIGURE 3

1a = Uninfected E. coli + normal serum + S. aureus
1b = Uninfected E. coli + immune serum 1 + S. aureus
1c = Uninfected E. coli + immune serum 2 + S. aureus
2a = T7 infected E. coli + normal serum + S. aureus
2b = T7 infected E. coli + absorbed immune serum 1 + S. aureus
2c = T7 infected E. coli + immune serum 1 + S. aureus
2d = T7 infected E. coli + absorbed immune serum 2 + S. aureus
2e = T7 infected E. coli + immune serum 2 + S. aureus

3a = T7 infected *E. coli* + normal serum + protein A Sepharose
3b = T7 infected *E. coli* + absorbed immune serum 1 + protein A Sepharose
3c = T7 infected *E. coli* + immune serum 1 + protein A Sepharose
3d = T7 infected *E. coli* + immune serum 2 + protein A Sepharose
4a = Extract of T7 infected *E. coli* B cells used in this experiment
4b = Extract of T7 infected *E. coli* B cells used in this experiment

## Specificity of the RIP-PAGE Assay for 0.3 Protein

# FIGURE 4




T7 infected E. coli labelled with $^{35}$S-methionine was allowed to react with normal or immune serum and then protein A Sepharose.

1a = T7 infected E. coli B + normal serum
1b = T7 infected E. coli B + immune serum
2a = T7 (0.3 amber mutant) infected $Su^+$ strain of E. coli + normal serum
2b = T7 (0.3 amber mutant) infected $Su^+$ strain of E. coli + immune serum
3a = T7 (0.3 amber mutant) infected $Su^o$ strain of E. coli + normal serum
3b = T7 (0.3 amber mutant) infected $Su^o$ strain of E. coli + immune serum
4 = Extract of T7 infected E. coli

streptomycin or gentamycin were added, however, $^{35}$S-cysteine incorporation was always detectable and the darkness of the band (amount of $^{35}$S-cysteine incorporation) was proportional to the concentration of the drug used (fig. 5). To quantitate the increase in misincorporation of $^{35}$S-cysteine into 0.3 protein we will subject this autoradiogram to scanning densitometry. An isolated cysteine-containing protein will be used to correct for the overall decrease in protein synthesis resulting from the use of gentamicin at these high concentrations.

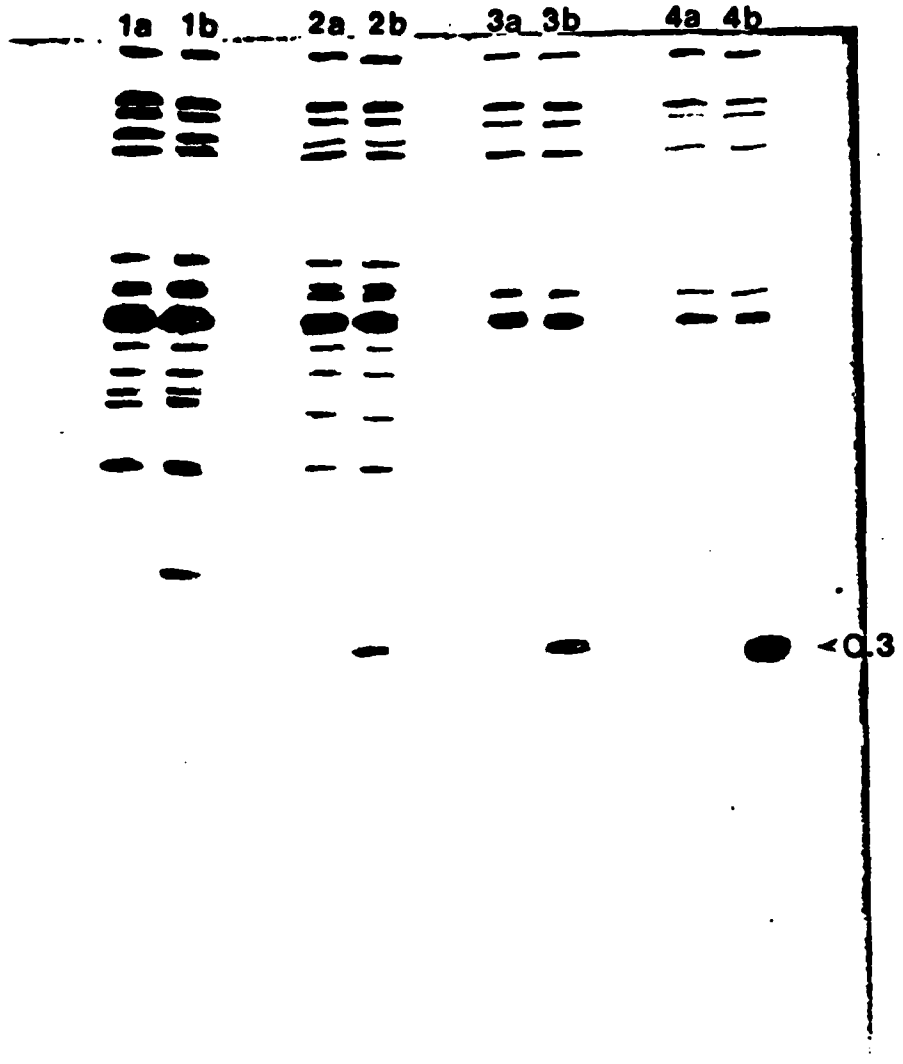
C. Determination of the normal mistranslation level in vivo

To estimate the increased error frequency in translation in vivo when drugs or toxic chemicals are present, it is necessary to know the natural error level. The T7 0.3 protein system provides an advantage over other systems used to estimate mistranslation levels in vivo. The T7 0.3 protein is made only after T7 infection and therefore all the 0.3 protein made is under the conditions of the experiment, i.e., all 0.3 protein synthesized is labeled. It is therefore unnecessary to determine the amount synthesized during the labeling period relative to the amount existing before label was added. In addition, we have simplified the purification procedure for 0.3 protein and made it suitable for the small amounts of protein obtained in labeling experiments.

1. Purification of small amounts of labeled 0.3 protein. Two methods were compared: affinity chromatography using rabbit anti- 0.3 antibody conjugated to CNBr-activated Sepharose 6B and DEAE cellulose anion exchange chromatography followed by ethanol extraction (scaled down from our procedure to purify milligram amounts of 0.3 protein). We found that the 0.3 protein, eluted from the immunoaffinity column, was impure and required additional ethanol extraction steps for a complete purification. In addition, the capacity of the immunoaffinity columns was much less than that of the DEAE columns and not sufficient to retain all the 0.3 protein made in a labelling experiment. We, therefore, decided against the use of immunoaffinity chromatography and have used anion exchange chromatography for the initial purification steps.

2. Experimental conditions necessary for calculations of mistranslation levels. To calculate the normal mistranslation frequency, i.e., the number of cysteine residues per molecule of 0.3 protein, it is necessary to know the specific activity of cysteine available to the cell for protein synthesis. This means that the amount of radioactively labelled cysteine provided should not be diluted by endogenous cysteine synthesis nor should the precursor $^{35}$S-cysteine be converted to methionine. An excess of methionine must be provided to block cysteine conversion into methionine by feedback inhibition and the endogenous pathway of cysteine biosynthesis must be repressed by providing a saturating level of cysteine in the absence of sulfate. Experiments were performed to determine minimum saturating levels of cysteine and methionine. In Figures 6 and 7 cysteine and methionine uptake can be seen to be at the saturating level at $5 \times 10^{-4}$ M. Additional experiments showed that the minimum saturating level for both amino acids was $2 \times 10^{-4}$ M.

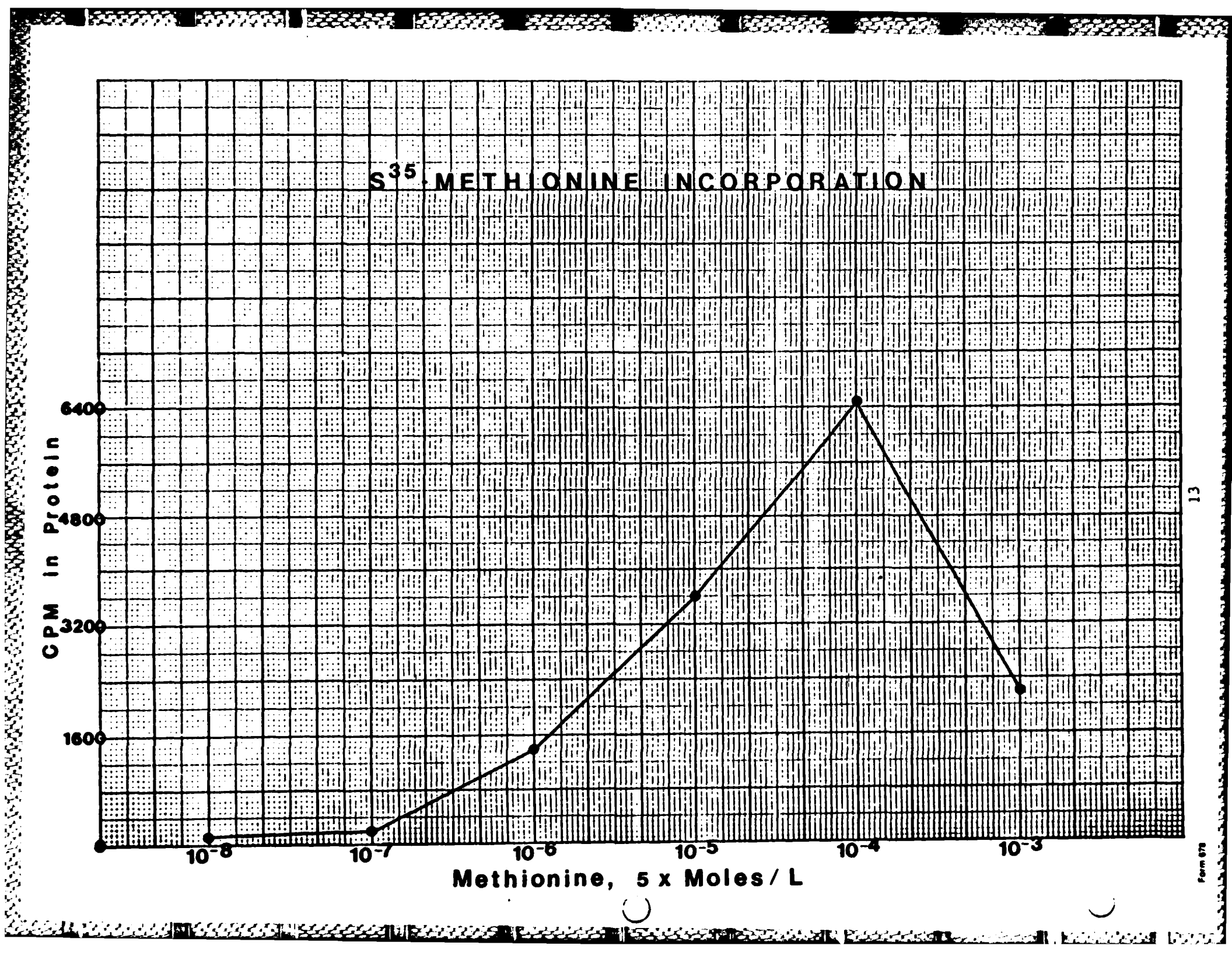
3. Demonstration that $^{35}$S is not transferred from cysteine to methione. If $^{35}$S-cysteine were converted to $^{35}$S-methionine the calculated error level in the synthesis of 0.3 protein would be higher

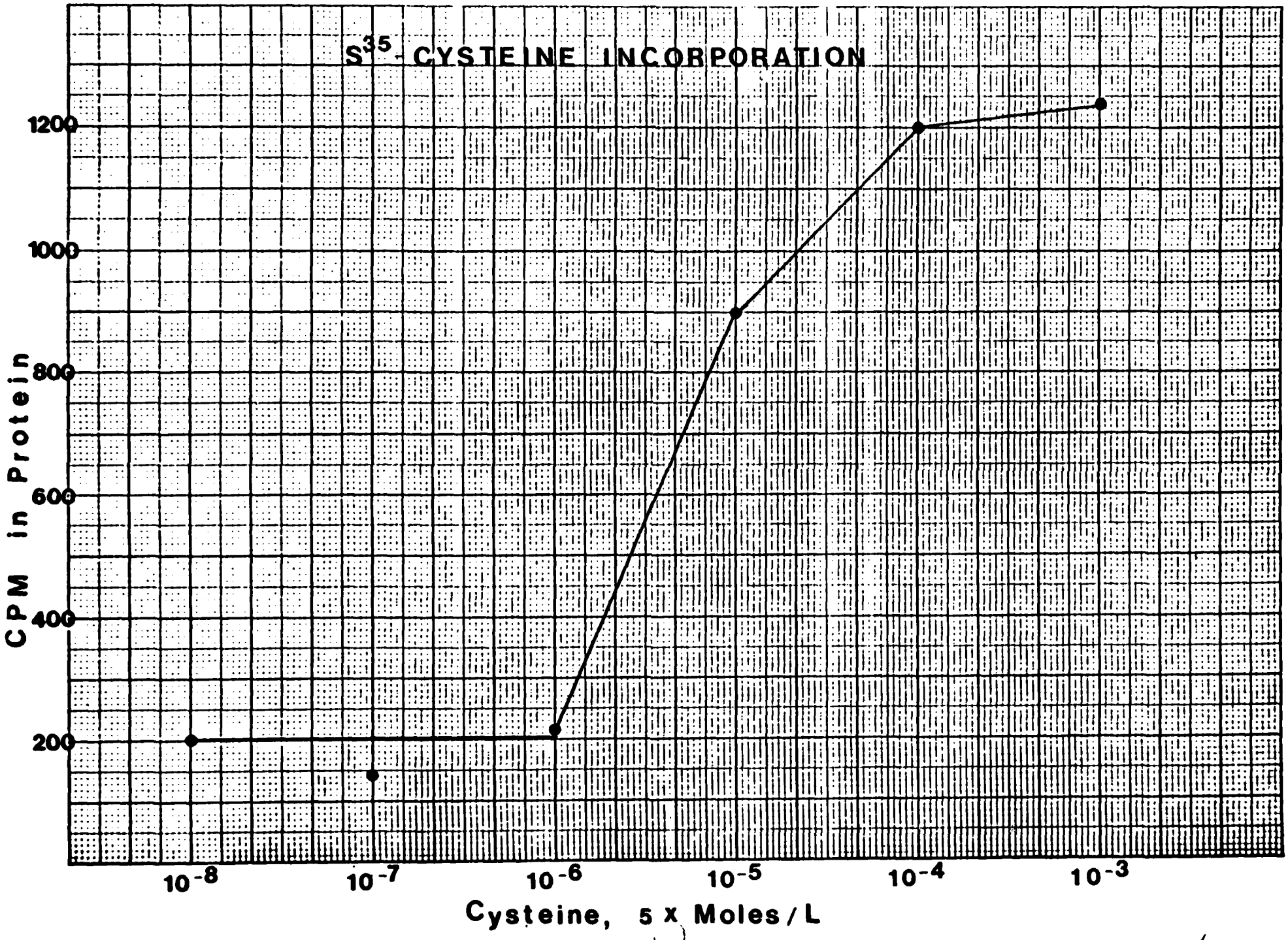



# FIGURE 5

| Lanes | Gentamicin (ug/ml) |
|---|---|
| 1a,1b | 0 |
| 2a,2b | 10 |
| 3a,3b | 20 |
| 4a,4b | 40 |

Lanes 1a, 2a, 3a + 4a are extracts of T7 infected *E. coli* B, labelled with $^{35}$S-cysteine, and allowed to react with normal serum and then protein A Sepharose. Lanes 1b, 2b, 3b + 4b are extracts of T7 infected *E. coli* B, labelled with $^{35}$S-cysteine and allowed to react with antiserum and then protein A Sepharose.


S35-METHIONINE INCORPORATION
CPM in Protein
6400
4800
3200
1600
10-8
10-7
10-6
10-5
10-4
10-3
Methionine, 5 x Moles/L


S35- CYSTEINE INCORPORATION
CPM in Protein
1200
1000
800
600
400
200
10-8
10-7
10-6
10-5
10-4
10-3
Cysteine, 5 x Moles / L

than the true error level since methionine is present in 0.3 protein. It is, therefore, necessary to show that methionine in the 0.3 protein contains no $^{35}S$ when $^{35}S$-cysteine is supplied and that all the radioactivity is present in cysteine. Thin layer chromatography (TLC) was chosen as the best way to determine this. Several solvent systems and stationary phase solid supports were examined in preliminary experiments to determine the best way for separation of methioine and cysteine. We found that two-dimensional TLC provided no advantage over one-dimensional TLC and that N-propanol: ammonium hydroxide was the solvent system best suited to our needs. Silica gel, cellulose, or paper can be used as the solid support. The specific activity of $^{35}S$-cysteine-labeled 0.3 protein is naturally very low and therefore long exposure times of the TLC chromatograms to x-ray flm are needed for satisfactory autoradiography.

We expect to obtain a precise determination of the normal error level for translation in vivo during the synthesis of 0.3 protein within the next few months.

## III. References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# PROGRESS REPORT

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Title: Development of an *in vivo* assay for mistranslation-including activity of pollutants and characterization of amino acid substitutions

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REPORT FOR RESEARCH UNDERTAKEN
BETWEEN AUGUST 1, 1982 - JULY 31, 1983

I. Research Undertaken Between August 1, 1982 - February 1, 1983

A. Abstract

B. Details of experiments completed between 8/1/82 and 2/1/83

1. Determination of the error level in 0.3 protein translation by $^{35}$S-cysteine misincorporation

2. Demonstration that $^{35}$S-cysteine is not converted to $^{35}$S-methionine

II. Research Undertaken Between February 1, 1983 - July 31, 1983

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III. Manuscripts Submitted for Publication

A. Cloning using bacteriophage SPP1v as the vector: Vector development, stability, and expression, A.I. Desmyter, J.B. Rice, J.N. Reeve. (In Press.)

B. Mistranslation of a T7 bacteriophage protein, J.B. Rice, R. T. Libby, J.N. Reeve (Submitted to J. Biol. Chem.)

I. Report for research undertaken between August 1, 1982 - February 1, 1983

A. Abstract

As part of our efforts to develop a simple in vivo assay for the ability of pollutants to increase mistranslation, we have determined the normal frequency of mistranslation resulting in misincorporation of amino acids into the T7 0.3 protein. An average value of 0.019 cysteine residues per molecule was found in this non-cysteine-containing protein. Since there are 116 amino acids in the protein, this represents 1 cysteine misincorporated per 5,000 amino acids: a mistranslation frequency of $3 \times 10^{-3}$ per codon. Control experiments were carried out in which the number of methionine residues in 0.3 protein was measured. Our experimental results indicated that 0.3 protein has 5.7 methionine residues per molecule. The DNA sequence indicates that 0.3 protein actually contains 6 methionines per molecule.

Additional evidence to support our value for normal mistranslation in 0.3 protein was the demonstration that $^{35}S$ is not transferred from cysteine to methionine. Experiments using a methionine-cysteine double auxotrophic strain, E. coli KL266, gave values for methionine and cysteine incorporation into 0.3 protein similar to those obtained with the prototrophic E. coli strain B. Autoradiograms of thin layer chromatograms of material produced by hydrolysis of $^{35}S$-cysteine-labeled 0.3 protein showed that the radioactivity was still in cysteine and had not been metabolized to methionine.

B. Details of experiments completed between 8/1/82 and 2/1/83

1. Determination of the error level in 0.3 protein translation by $^{35}$-cysteine misincorporation

Our last progress report described preliminary experiments which determined conditions for calculations of misincorporation of cysteine into the non-cysteine-containing T7-encoded 0.3 protein. Based on these experiments we have determined the mistranslation frequency during normal T7 infection of E. coli (Table 1). In similar experiments substituting $^{35}S$-methionine for $^{35}S$-cysteine, and adding $2 \times 10^{-4}$ M methionine to saturate the methionine transport system, we have experimentally determined the number of methionine residues per 0.3 molecule (Table 1). The latter experiments

represent an ideal control for all parameters of the experiments since it is known (1) that the 0.3 protein contains 6 methionine residues.

Calculations used to determine the number of cysteine (or methionine) residues incorporated into the 0.3 protein are as follows:

(1) Specific activity of $^{35}$S-cysteine (total number of cys residues/CPM) =

$$\frac{\text{No. molecules } ^{35}\text{S-cys} + \text{No. molecules unlabeled cys in culture}}{\text{CPM of } ^{35}\text{S-cysteine added to culture}}$$

(2) Specific activity of 0.3 protein (Number cys residues per molecule 0.3 protein) =

$$\frac{\text{CPM of 1 ml of 0.3 protein} \times \text{specific activity of } ^{35}\text{S-cys}}{\text{molecules of 0.3 protein in 1 ml}}$$

The calculation requires labeling of 0.3 protein with $^{35}$S cysteine followed by biochemical purification of the protein and radiochemical measurements on the purified protein. The following scheme has been developed to purify the 0.3 protein and obtain the required measurements:

E. coli B is grown to $8 \times 10^8$ cells/ml ($A_{580} = 0.8$) (exponentially growing cells which are most susceptible to phage infection.)

↓

Cells are washed twice in M9 minimal medium.

↓

Cells are resuspended at $2 \times 10^9$/ml in 50 ml M9 minimal medium containing $2 \times 10^{-4}$ M cysteine + 0.1 mg methionine/ml + 10% (v/v) Cysteine Assay Medium (Difco) (for $^{35}$S-cysteine labeling) or $2 \times 10^{-4}$ M methionine + 10% (v/v) Methionine Assay Medium (Difco) (for $^{35}$S-methionine labeling).

↓

The T7 mutant H3 1am193 LG3 is added at MOI = 3.

↓

$^{35}$S-cysteine (or $^{35}$S-methionine) is added at 10 μCi/ml.

↓

Infected cells are incubated for 1 hr at 37°C with shaking.

↓

Cells are centrifuged for 10 min at 10,000 x g.

↓

Cells are resuspended in 2 ml lysis buffer + 20 μl 2 mg lysozyme/ml + 10 μl 0.5 mM PMSF (protease inhibitor).

↓

Cells are incubated 30 min at 32°, then frozen and thawed 5 times.

↓

DNA is digested with 10 μl 2 mg DNase/ml + 20 μl 1 M $MgSO_4$ + incubation for 30 min at 32°C.

The volume is measured and $NH_4$ Cl is added to 0.3 M.

↓

The liquid is centrifuged for 15 min at 17,000 x g.

↓

The clarified supernatant is passed through a DEAE column equilibrated with 0.3 M $NH_4Cl$ - 20 mM Tris buffer, pH 8. The column (volume = 10 ml) is washed with 100 ml of 0.3 M $NH_4Cl$-Tris buffer. Acidic proteins are eluted with a 40 ml 0.3 M - 1.0 M $NH_4Cl$-Tris buffer gradient. Fractions (3 ml) are collected.

↓

Fractions are tested for 0.3 protein by the Ouchterlony immunodiffusion technique using rabbit anti-0.3 antiserum, raised as part of this project.

↓

Fractions containing the 0.3 protein are pooled, and protein is precipitated with 10% (w/v) trichloroacetic acid (TCA).

↓

The precipitate is dissolved in Tris buffer, pH 8.8. The volume of buffer added is determined by the $A_{280}$ sum of the pooled fractions. (Example: if the $A_{280}$ sum is 4.0, 0.40 ml of buffer is added.)

↓

Four volumes of 95% ethanol are added, and the solution is placed on ice for 30 min.

↓

The solution is centrifuged for 15 min at 17,000 g.

↓

The pellet is discarded because 0.3 protein is soluble in ethanol. An equal volume of 10% TCA is added to the supernatant.

↓

The solution is placed on ice for 1 hr.

↓

The solution is centrifuged for 15 min at 17,000 x g.

↓

The pellet is dissolved in 0.5 ml Tris buffer, pH 8.8.

↓

The pellet is tested for purity of 0.3 protein by SDS-PAGE (10-20% gradient) and stained for protein with the silver nitrate staining technique.

↓

The quantity of pure 0.3 protein is determined using the Bio-Rad Protein Assay kit.

↓

10 µl of pure 0.3 protein is applied to filters (in triplicate) and counted in a scintillation counter. 5 µl of a 1:1000 dilution of the $^{35}$S-cysteine (as supplied by the manufacturers) is also counted in triplicate.

↓

Calculations are then performed.

2. Demonstration that $^{35}$S-cysteine is not converted to $^{35}$S-methionine

If $^{35}$S were transferred from cysteine to methionine, the calculated error level in the synthesis of 0.3 protein would be higher than the actual error level since methionine is present in 0.3 protein. We demonstrated $^{35}$S was not transferred from cysteine to methionine in two ways.

(1) The hydrolysate of 0.3 protein, synthesized in the presence of $^{35}$S-cysteine, was resolved into individual amino-acid spots by thin layer chromatography (cellulose matrix using N-propanol: ammonium hydroxide (70:30) as solvent). Autoradiography was used to locate $^{35}$S on the chromatograms. The radioactivity was located in the cysteine spot and not in the methionine spot.

(2) An E. coli strain auxotrophic for methionine and cysteine was used as host for T7 infection. (E. coli KL266). This strain is defective in met E (tetrahydropteroyltriglutamate methyltransferase) and cys C (adenylsulfate kinase) and cannot convert cysteine to methionine. The levels of $^{35}$S-cysteine incorporation into 0.3 in T7-infected E. coli KL266 were not significantly different from the levels of incorporation found with prototrophic E. coli strains (Table 1).

Table 1. Incorporation of Cysteine and Methionine into the 0.3 Protein

| Experiment | *E. coli* strain[1] | No. cys residues[2] per molecule | No. met residues[2,3] per molecule |
|---|---|---|---|
| 6 | B | .021 | —— |
| 7 | B | .022 | —— |
| 9 | B | .028 | —— |
| 11 | B | .023 | —— |
| 10 | KL266 | .012 | —— |
| 13 | KL266 | .016 | —— |
| 3 | B | —— | 5.2 |
| 5 | B | —— | 6.4 |
| 4 | KL266 | —— | 3.7 |
| 8 | KL266 | —— | 4.3 |

[1] *E. coli* B is prototrophic.
*E. coli* KL266 is F⁻, *leu* B6, *proC32*, *hisF80*, *cysC43*, *thyA54*, *metE70*, *thi-1*, *ara-14*, *lac236*, *xyl-5*, *mtl-1*, *malA38*, *rpsE2115*, (= *spc* A15), *rpsL109* (= *strA109*).

[2] Values calculated on the basis of $^{35}$S-cysteine or $^{35}$S-methionine incorporation.

[3] Direct amino acid sequencing and the known DNA sequence predict 6 methionines per molecule of 0.3 protein (1).

II. Research undertaken between February 1, 1983 and July 31, 1983

A. Overall Objectives

1. To develop a simple, quantitative *in vivo* assay for the mistranslation-inducing activity of pollutants.

2. To determine the molecular basis for mistranslation resulting in the incorporation of cysteine into a protein which normally contains no cysteine.

B. Precise objectives for the period 2/1/83 - 7/31/84

1. Production of a monoclonal antibody to the 0.3 gene product and use of this antibody in development of a radioimmune precipitation assay.

2. Direct determination of whether cysteine substitutes only for arginine in mistranslation during synthesis of 0.3 protein.

C. Abstract

We have, in experiments directed toward the first overall objective, established the natural level of cysteine misincorporation into the bacteriophage T7 encoded 0.3 protein. We have also shown that this level can be increased by altering the environment of the translation machinery. This can be accomplished either by growing cells in the presence of mistranslation-inducing antibiotics or by introducing mutations which cause defective ribosomal proteins into the cells being studied (see attached preprint). The above results were obtained using purified 0.3 protein. Additional experiments directed toward the first objective have led to a second procedure for quantitating cysteine misincorporation into 0.3 protein. A radioimmune precipitation (RIP) assay was developed which used polyclonal antibodies to 0.3 protein, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and scanning densitometry. We are currently preparing monoclonal antibody to 0.3 protein to obviate the need for SDS-PAGE and scanning densitometry. Experiments directed toward the second overall objective have provided interesting preliminary results. Trypsinization of cysteine-labeled 0.3 protein and analysis of fragments by SDS-PAGE have shown that new peptide fragments are produced. This indicates that cysteine is substituting for arginine. Cleavage of cysteine-labeled 0.3 protein with CNBr and analysis of the peptide fragments by gel filtration, however, results in 3-4 labeled fractions which should not occur if cysteine were substituting only for arginine. This indicates that cysteine substitutes for at least one other amino acid besides arginine. These results will be confirmed and refined by HPLC (high performance liquid chromatography).

D. Details of experiments completed between 2/1/83 and 7/31/83

1. Progress in preparation of monoclonal antibody to the 0.3 protein

We have twice constructed hybridomas which produce the desired antibody but on both occasions the positive clones did not survive

continued serial passage. Problems of fungal contamination were also encountered. We are now into our second month of the third preparation, have no signs of fungal contamination, and we have several positive hybridomas. If these clones prove to be stable and we are successful in preparing pure monoclonal 0.3 protein antibody-producing clones, we will use this antibody in the RIP assay which we previously developed using polyclonal antibody. We anticipate that the pure antibody will precipitate only 0.3 protein ($^{35}$S-cysteine labeled) allowing quantitation of cysteine incorporation by simple scintillation counting of the immune precipitate.

2. Experiments to determine whether cysteine substitutes only for arginine during 0.3 protein synthesis under normal (non-drug, non-pollutant) conditions

Two types of experiments have been performed - trypsinization of $^{35}$S-cysteine-labeled 0.3 protein and chemical cleavage of $^{35}$S-cysteine-labeled 0.3 protein with CNBr. The 0.3 protein fragments were analyzed by SDS-PAGE (20% acrylamide) and by gel filtration (Sephadex G-50, Sephadex G-25, Bio-gel P6 and Bio-gel P4).

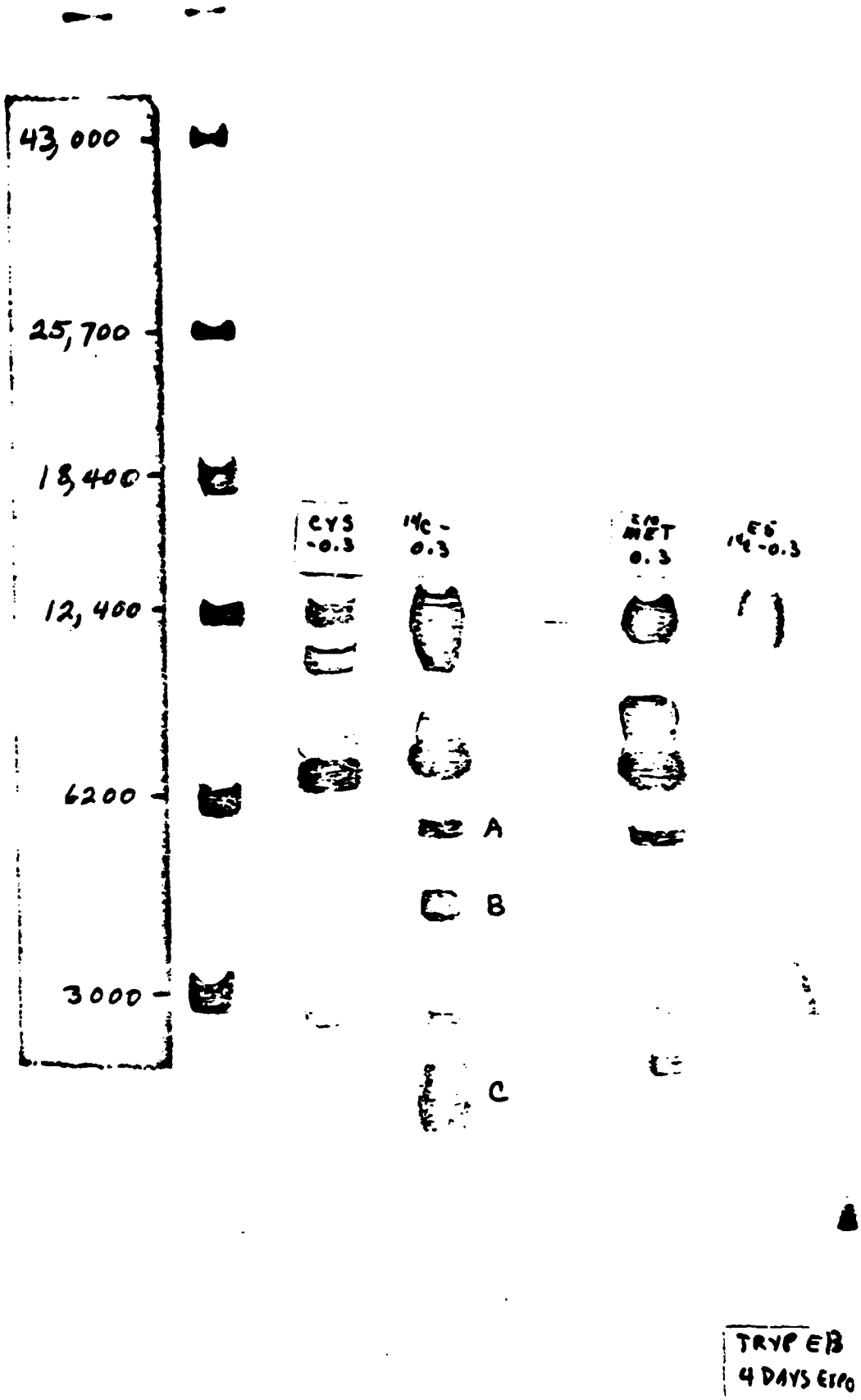
Analysis of 0.3 protein fragments by SDS-PAGE has been unsatisfactory because of lack of resolution of the lower MW peptide fragments. A smear is seen instead of discrete bands. We have been using the Laemli "Tris discontinuous buffer system" with 20% acrylamide. A continuous sodium phosphate buffer system has been found to give somewhat better resolution of bands but the 0.3 peptide fragments migrate to different positions relative to the MW standards using this buffer system. Results of an experiment in which 0.3 protein was trypsinized and peptide fragments separated by SDS-PAGE using the sodium phosphate buffer system are shown in figure 1. The major peptide band of $^{35}$cys-, $^{14}$C-, and $^{35}$S met-labelled 0.3 proteins has an apparent MW slightly larger than 6000. Since the largest complete cleavage peptide should contain 47 amino acids, equivalent to a MW of 4700, this band is probably a partial cleavage peptide. It can be seen however, that whereas the major bands of $^{35}$S met- and $^{14}$C-labelled 0.3 protein have identical mobilities, that of $^{35}$S cys-labelled 0.3 protein is slightly faster. The bands marked "A", "B", and "C" are 47 amino acid, 32 amino acid, and 19 amino acid complete tryptic digestion products of 0.3 protein. There is no methionine in the 32 amino-acid fragment, and therefore this band does not show up in the lane containing $^{35}$S met-labeled 0.3 protein. The 19 amino-acid peptide (which contains methionine) can be seen on the original autoradiogram but is not easily seen on the photographic reproduction shown in Figure 1. Interestingly, there is no cysteine in the 32 amino acid peptide, whereas cysteine is found in the 19 amino acid peptide and in a smaller MW peptide.

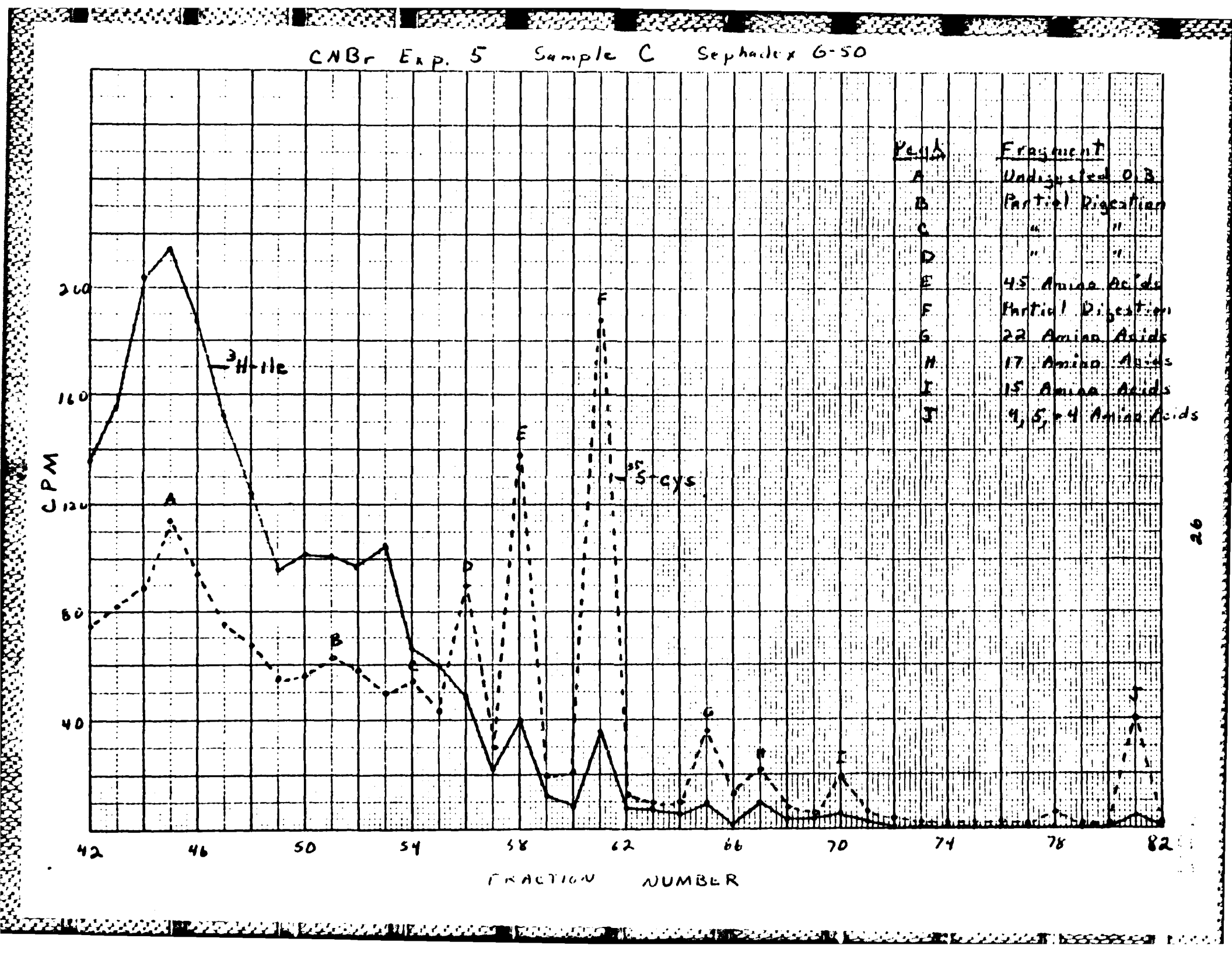
There are six amino acids for which cysteine could possibly substitute by misreading of a single base in the codon: arginine, tyrosine, tryptophan, serine, phenylalanine, and glycine. From the trypsinization experiments we can deduce the following: We can tentatively say that cysteine substitutes for arginine because a

band with a different MW is seen only when 0.3 protein is labelled with cysteine. Relative to the major band of $^{14}C$- and $^{35}S$-met-labelled 0.3 protein, this band has an increased mobility (decreased size) whereas if an arginine site were no longer available for cleavage by trypsin because of cysteine substitution for arginine the cysteine-containing tryptic peptide should have an increased size. However, the possibility remains that this major $^{35}S$-cys-labeled band is the higher MW band predicted to be formed by lack of an arginine site N-terminal to the 47 AA peptide (band A). 2) The 32 amino acid peptide contains no cysteine (a result confirmed by gel filtration) and therefore cysteine is probably not substituting for tyrosine or tryptophan. (These are the only two amino acids of the possible six for which cysteine could substitute.) 3) Cysteine is found in the 19 amino acid peptide (determined by gel filtration) and therefore it appears that cysteine does substitute for serine and/or phenylalanine.

When cysteine-labelled 0.3 protein was cleaved with CNBr and fragments examined by gel filtration, similar results were observed. Figure 2 shows a typical experiment. 0.3 protein labeled with $^{3}H$-isoleucine or with $^{35}S$-cysteine were mixed, cleaved with CNBr, and analyzed on Sephadex G-50. If cysteine were substituting only for arginine, as suggested by Edelmann and Gallant (Cell, 10:131-137, 1977) there should be peaks corresponding only to the 45 and 22 amino acid peptides (the only CNBr fragments which contain arginine), and any peptides resulting from partial digestion which contain one or both of these fragments. Peaks B, C, D and F contain peptides produced by partial digestion. Also seen are the 45 amino acid peptide (peak E) and the 22 amino acid peptide (peak G). Three additional peaks are however present (H, I, and J) and these correspond to peptides which do not contain arginine. Of the 6 amino acids for which cysteine could most likely substitute, these fragments instead contain tyrosine, phenylalanine, and serine; serine, glycine, and phenylalanine; and serine, respectively. Very similar peak profiles were obtained when peptide fragments were analyzed by Sephadex G-25, and Bio-gel P6 or P4. Our experiments to date therefore suggest that in mistranslation of the T7 0.3 protein, cysteine can at least substitute for arginine, and probably also for serine. Substitutions for other amino acids are, of course, still possible. Improved separation and identification ofpeptide fragments of 0.3 protein is needed before the precise substitution pattern can be obtained. We therefore plan to use high pressure liquid chromatography (HPLC) to better separate peptide fragments. In addition, we plan to include the use of reagents to cleave the 0.3 protein at other residues to confirm our present results.

FIGURE 1


43,000
25,700
18,400
12,400
6200
3000
CYS -0.3
14C - 0.3
S35 MET 0.3
ES 14C -0.3
A
B
C
TRYP EB
4 DAYS EIPO


CNBr Exp. 5 Sample C Sephadex G-50
Peak
Fragment
A
Undigested O.B.
B
Partial Digestion
C
"
"
D
"
"
E
45 Amino Acids
F
Partial Digestion
G
22 Amino Acids
H
17 Amino Acids
I
15 Amino Acids
J
4, 5, + 4 Amino Acids
$^3$H-Ile
$^{35}$S-cys
CPM
200
160
120
80
40
42
46
50
54
58
62
66
70
74
78
82
FRACTION NUMBER
A
B
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H
I
J

REPORT FOR RESEARCH UNDERTAKEN

BETWEEN AUGUST 1, 1983 - JULY 31, 1984

I. Research undertaken between August 1, 1983 - February 1, 1984

A. Abstract

B. Details of experiments completed between 8/1/83 and 2/1/84

1. Experiments to determine whether cysteine substitutes only for arginine during 0.3 protein synthesis under normal (non-drug, non-pollutant) conditions.

2. Progress in preparation of monoclonal antibody to 0.3 protein.

3. Experiments to determine whether the T7 0.3 gene cloned in the *B. subtilis* phage $Spp1_{\gamma}$ and into the *E. coli* plasmid pAR324 is expressed *in vivo*.

II. Research undertaken between February 1, 1984 and August 1, 1984

A. Abstract

B. Details of experiments completed between 2/1/84 and 8/31/84

1. Purification of monoclonal antibody to 0.3 protein from culture media and from ascites.

2. Use of monoclonal antibody to 0.3 protein in radioimmune precipitation experiments.

3. Progress in experiments to quantitate cysteine misincorporation in 0.3 protein encoded in the plasmid BpAR324.

4. Experiments directed toward determination of the molecular basis of mistranslation.

III. References

I. Research Undertaken between August 1, 1983 - February 1, 1984

A. Abstract

Experiments directed toward characterization of amino acid substitutions during mistranslation using our model of cysteine misincorporation into 0.3 protein have suggested that cysteine substitutes for arginine. This was demonstrated by the generation of novel peptides upon trypsinization of [$^{35}$S] cysteine labeled 0.3 protein. Arginine is not however, the only amino acid for which cysteine substitutes as shown by the location of [$^{35}$S] cysteine-labeled peptides in the same electrophoretic positions as peptides from trypsinized [$^{14}$C] labeled 0.3 protein. Similar conclusions were drawn from experiments utilizing CNBr treatment of [$^{35}$S] cysteine-labeled 0.3 protein followed by analyses of peptides by gel filtration. Experiments in which the 0.3 protein was cleaved by two other reagents also provided results which suggested that cysteine was being misincorporated in more positions than those of only arginine.

As part of our efforts to develop a simple *in vivo* assay for mistranslation we have succeeded in producing a hybridoma which synthesizes 0.3 antibody. The monoclonal antibody will be used in developing a simple, quantitative *in vivo* assay for mistranslation-inducing activity of pollutants.

In experiments directed toward determining the influence of cellular environment on mistranslation, we previously cloned the 0.3 gene (normally a constituent of the coliphage T7) into the *B. subtilis* phage SPP1 (1). We have now found that this phage does synthesize 0.3 protein but at a very low level. We have also found that the 0.3 gene cloned into the *E. coli* plasmid pAR 324 (2) is expressed in *E. coli* cells. Currently, we are attempting to purify the 0.3 protein from both of these microorganisms as a first step in quantitating mistranslation of 0.3 protein synthesized in cellular environments different from that in which our original observations were made, namely in T7-infected *E. coli* cells.

B. Details of Experiments Completed Between 08/01/83 and 02/01/84.

1. Experiments to determine whether cysteine substitutes only for arginine during 0.3 protein synthesis under normal (non-drug, non-pollutant) conditions.

These experiments to date have been based on cleavage of purified [$^{35}$S] cysteine labeled 0.3 protein and analysis of resulting peptides for radioactivity. Cleavage has been accomplished by treatment of 0.3 protein with CNBr (7 peptides produced), or with trypsin (7 peptides produced or 5 when arginine residues are blocked). We have also cleaved 0.3 protein at cysteine residues by cyanylation and at tryptophan residues with dimethylsulfoxide/HCl/HBr. Peptides have been analyzed by SDS-PAGE or gel filtration or both.

In our last progress report we discussed difficulties observed in resolution of small MW peptides by SDS-PAGE using the Laemli "Tris discontinuous buffer system" and 20% acrylamide. We had found that SDS-PAGE using the sodium phosphate buffer system and 20% acrylamide resolved these low MW peptide bands more effectively. Recently, we have been using both the sodium phosphate buffer with 20% acrylamide and the Tris discontinuous buffer system with a 13% to 22% acrylamide gradient. The latter technique provides clearer resolution of peptide bands while the former allows us to calculate MW based on [$^{14}$C] labeled standards.

To identify the various peptide fragments observed both by SDS-PAGE and by gel filtration we have used 0.3 protein labeled with [$^{14}$C] amino acids, [$^{3}$H] isoleucine, [$^{3}$H] arginine, and [$^{35}$S] methionine. The rational is that because the amino acid sequence of 0.3 protein is known, particular peptides should be observed when one amino acid is labeled and not observed when another amino acid is labeled. This helps to identify the peptides generated when 0.3 protein is cleaved.

We have also treated radiolabeled 0.3 protein with a reagent which cleaves at tryptophan. Since there is only one tryptophan residue in 0.3 protein, these experiments were designed to determine whether cysteine was misincorporated into both halves of the protein. Unfortunately, we discovered that the reagent cuts not only at tryptophan but also at at least one other position in the molecule probably at the triplet, his-tyr-tyr. Our results show that cysteine is probably misincorporated in the C-terminal 1/5 of the molecule substitating for tyrosine and concentrated in the middle section or N-terminal half. (These two peptides were too similar in size to distinguish between them.)

We have used two different labels to identify cysteine residues in 0.3 protein: *in vivo* labeling with [$^{35}$S] cysteine, and *in vitro* labeling of cysteine by carboxymethylation with [$^{14}$C] iodoacetic acid. With either method the specific activity is low, which was expected since our previous work (3) has shown that for every molecule which contains one cysteine residue there are fifty which contain none.

We have accumulated much data which suggest that cysteine substitutes mainly for arginine in 0.3 protein but not exclusively. However, clear cut answers have been hampered by technical problems caused by the resistance of 0.3 protein to denaturation, cross-linking of peptides during treatment with CNBr, the low specific activity of [$^{35}$S] cysteine-labeled protein, and problems in resolution of low MW peptides.

The 0.3 protein is very hard to denature; this results in a mixture of peptides due to partial as well as complete digestion with trypsin. Preliminary evidence suggest that blockage of lysine residues of 0.3 protein helps denature it. Following trypsinization the number of partial digestion products is decreased (or even abolished). In addition, the total number of peptides expected is reduced, i.e., cleavage is at the four arginine residues instead of at the four arginine plus two lysine residues. Therefore, in future experiments, we plan to block lysine residues in the 0.3 protein before trypsinization.

Another problem we have encountered is poor resolution of tryptic peptides of similar MWs by gel filtration. We have tried four different gel types (Bio-gel P4 and P6, and Sephadex G-25 and G-50) on columns of different sizes. We hope and expect that reverse phase high performance liquid chromatography (RP-HPLC) will provide better resolution of 0.3 peptides. All materials have been ordered for these experiments and purified 0.3 protein labeled with different radionuclides prepared.

Separation by gel filtration of 0.3 peptides resulting from CNBr treatment (vs. trypsinization) has provided clearer results. Figure 1 shows typical results obtained. 0.3 protein labeled with [$^{3}$H] isoleucine was mixed with [$^{35}$S] cysteine-labeled 0.3 protein and the mixture cleaved with CNBr. Peptides were analyzed by Sephadex G-25 gel filtration. The [$^{35}$S] label was found in peptides with MWs of 5750, 4070, 2700, 1860, 1580, and $\leq$ 1000. These MWs correspond to the following CNBr fragments (numbered from the $NH_2$-terminus): fragment number 7, a probable partial cleavage product composed of fragments three and four, fragment four, fragment five, fragment three, and fragments one and/or two. Arginine is found in fragments four and seven only. Therefore, these results indicate that cysteine is substituting for amino acids other than the predicted arginine substitution, as indicated by the presence of [$^{35}$S] cysteine in fragments 5, 3, and 2. (Fragment 1 contains only alanine and methionine - neither amino acid a likely candidate for cysteine substitution.)

We have recently prepared [$^{35}$S] cysteine-labeled 0.3 protein of relatively high specific activity and sent a portion to Dr. Alfin Vaz of the Protein Sequencing Facility at the University of Michigan. He will sequence the first twenty to forty residues of 0.3 protein from the N-terminus and examine each run (amino acid) for radioactivity. This should not only confirm our finding of cysteine misincorporation in the amino terminal portion of the protein (CNBr fragments two and three) but will allow us to pinpoint the precise amino acid substitutions.

The complete separation of peptides we expect to be afforded by RP-HPLC should extend and confirm our previous findings as to which peptides contain [$^{35}$S] cysteine. After these peptides are purified, they will then also be sent to Dr. Vaz for amino acid sequencing. Sequencing will identify the peptides unambiguously as well as determine the exact amino

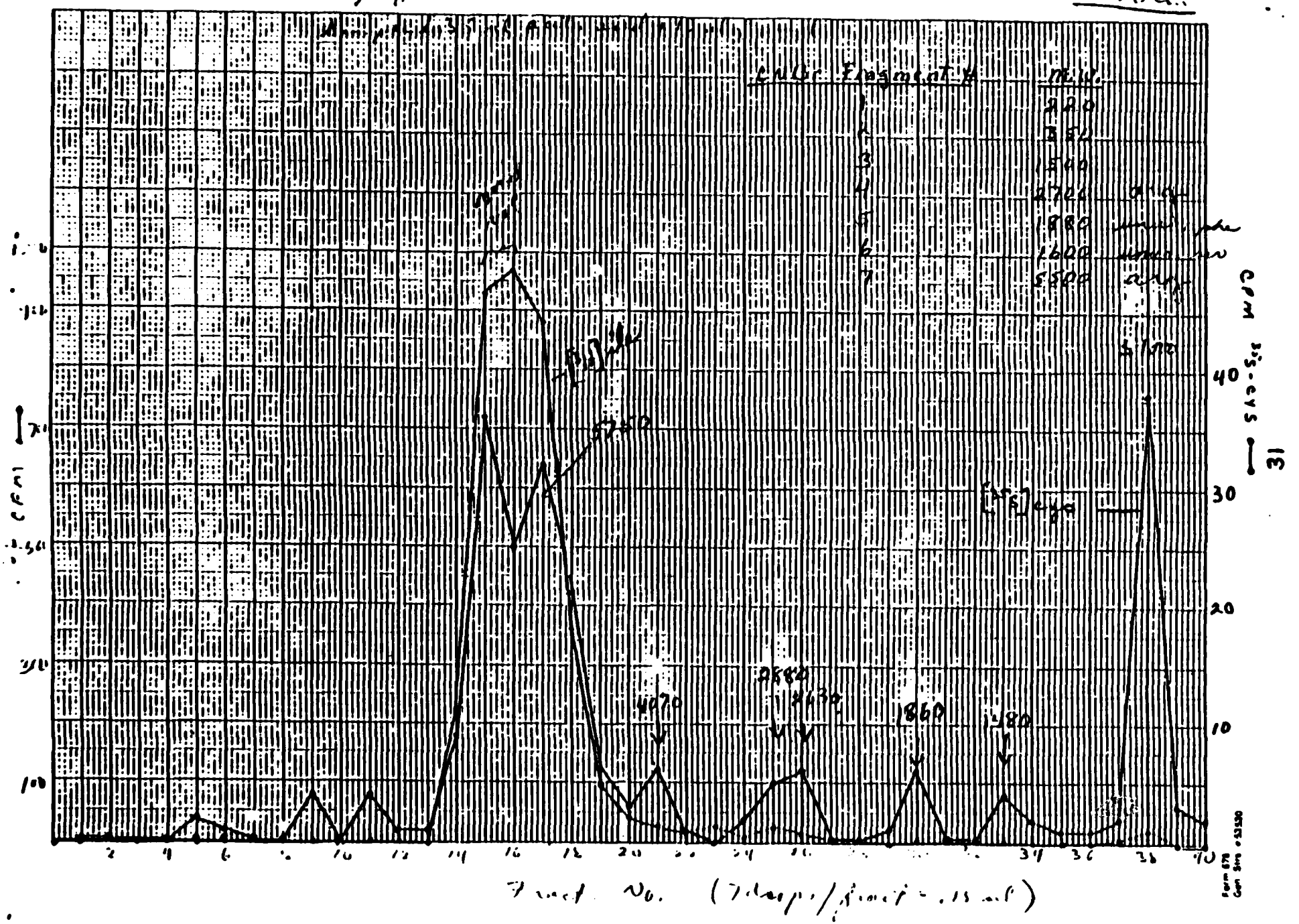



Figure 1

acids in the remainder of the 0.3 protein (middle and C-terminal end) for which cysteine substitutes. An added bonus afforded by sequencing will be determination of the relative frequency of cysteine substitution for the various amino acids and their corresponding codons. This knowledge should contribute significantly to the understanding of *in vivo* mistranslation.

2. Progress in preparation of monoclonal antibody to 0.3 protein

At this point we do have a hybridoma which is producing 0.3 antibody. We are currently attempting to grow enough cells to freeze. (This hybridoma is a slow grower which is often the case when the hybridoma is producing a lot of antibody). Once we have enough cells to insure a frozen stock, we will produce our monoclonal antibody in mice by growing the hybridoma as an ascites tumor, as well as in tissue culture. At that point we will be in a position to work out the parameters of the radioimmune precipitation assay with which we hope to detect 0.3 protein and 0.3 protein only.

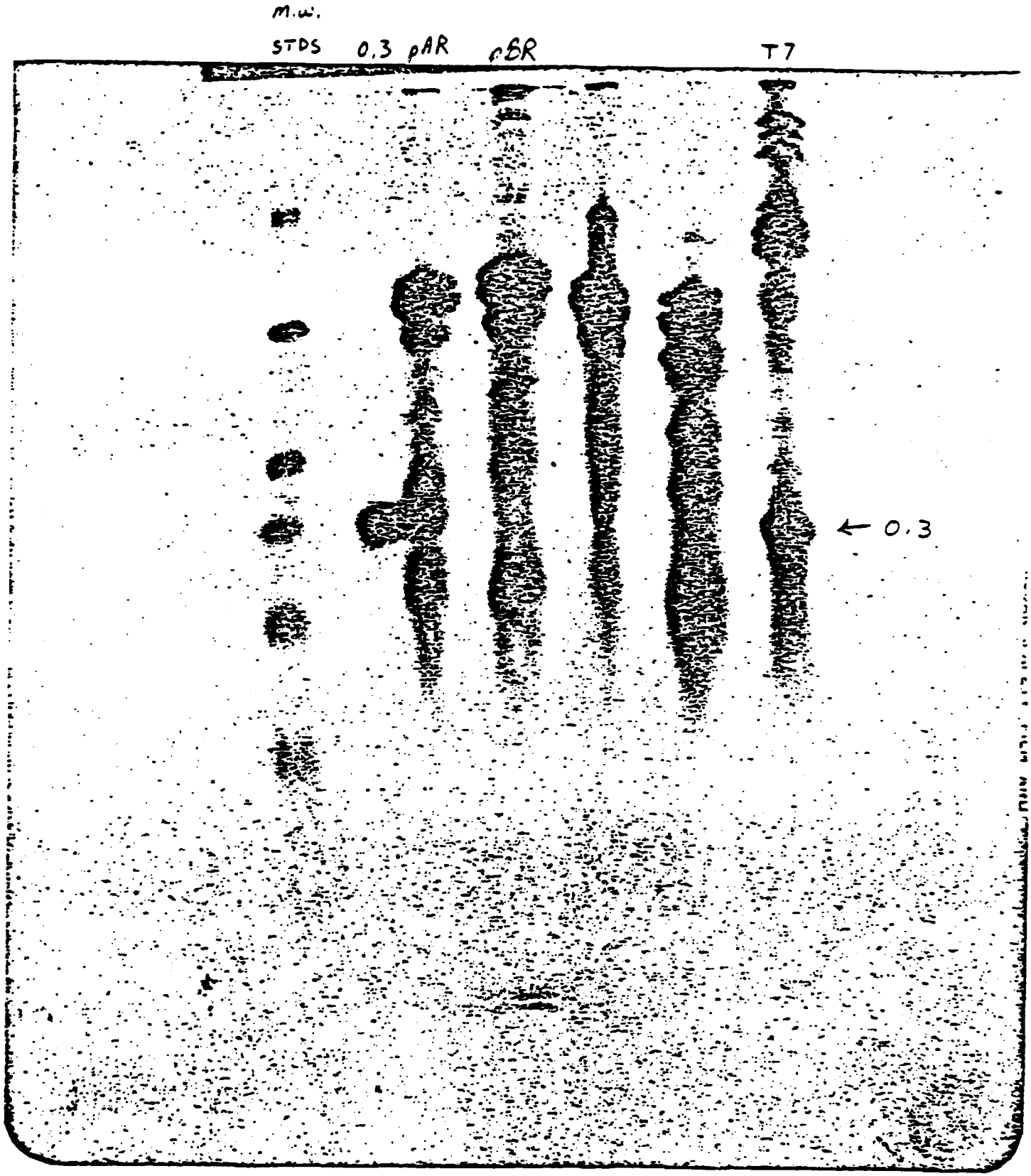
3. Experiments to determine whether the T7 0.3 gene cloned into the B. subtilis SPP1$_v$ phage and into the E. coli plasmid pAR324 is being expressed.

The usefulness of our model to measure levels of *in vivo* mistranslation by quantitation of cysteine misincorporation into the 0.3 protein in T7 infected *E. coli* can be extended. We have cloned the 0.3 gene (in both orientations into the *B. subtilis* phage SPP1$_v$ (1) and it has also been cloned in the *E. coli* plasmid pBR322, producing plasmid PAR324(2). We can, therefore, attempt to determine whether the mistranslation level which occurs during 0.3 gene expression is a constant or whether its position in the DNA and/or the cellular protein sythesizing machinery affect error levels. Before attempting to answer these questions we first had to determine whether the 0.3 gene was being expressed in its new locations.

We used the radioimmune precipitation-polyacrylamide gel electrophoresis assay (RIP-PAGE) for 0.3 protein to determine whether the 0.3 gene in SPP1$_v$ phage was being expressed in SPP1$_v$-0.3-infected cells of *B. subtilis*. These experiments showed that if it were being expressed at all, the amounts synthesized were too small to be detected. Next, we used an enzyme-linked immunospecific assay (ELISA) for 0.3 protein which is more sensitive that RIP-PAGE. As with the RIP-PAGE assay we tested both phages containing the 0.3 gene cloned in opposite orientations. Our results suggested that the infection of *B. subtilis* with SPP1$_v$ phage containing the 0.3 gene oriented correctly so that it could be transcribed from a known SPP1 promotor results in synthesis of 0.3 protein whereas the phage containing the 0.3 gene in the opposite orientation does not direct 0.3 protein synthesis. We must still resolve the problem of low levels of 0.3 gene expression - a problem which makes purification of 0.3 protein and therefore quantitation of mistranslation difficult.

To determine whether the 0.3 gene cloned in an *E. coli* plasmid (pAR324) was being expressed in *E. coli* we used both the ELISA assay and expression in minicells. The use of minicells eliminates the problem of synthesis of

Figure 2



cellular proteins; only those proteins encoded by the plasmid are synthesized. Therefore, RIP was unnecessary in this case. Minicells were obtained from E. coli strains, containing either pAR324 (which contains the 0.3 gene) or the parent plasmid pBR322 (which contains all the genes of pAR except 0.3) and were allowed to incubate in media containing [$^{35}$S] methionine. The minicells were lysed and the plasmid-encoded proteins analyzed by SDS-PAGE. Figure 2 shows that plasmid pAR324 does, indeed, direct the synthsis of the 0.3 gene product while the parent plasmid pBR322 does not.

Once we had determined that plasmid pAR324 does express the 0.3 gene, we tranformed it into the E. coli strains we had previously used to determine 0.3 protein mistranslation levels via T7 infection, i.e., E. coli B, KL266 and DS410. (3). This provides identical cellular environments so that the position of the 0.3 gene in a DNA molecule can be evaluated in regard to its effect on mistranslation. Results can be directly compared with our established observations. An ELISA assay was used to determine that the three transformed strains do indeed produce the 0.3 protein. Next, we attempted to determine the error level in 0.3 protein synthesized in E. coli B cells containing pAR324. We used an experimental protocol nearly identical to that used to measure cysteine misincorporation into 0.3 protein synthesized in T7 infected E. coli B cells. We have encountered an unexpected problem. In contrast to the situation in T7-infected E. coli where, following anion exchange chromatography, 0.3 protein is the only ethanol-soluble protein, in extracts from E. coli B pAR324 cells there is a second ethanol soluble protein which is, unfortunately, produced in greater quantity than 0.3 protein. We are currently working on methods to circumvent this problem allowing the complete purification of 0.3 protein from cells containing pAR324.

B. Details of experiments completed between 2/1/84 and 8/31/84.

Our stated objectives for the period 2/1/83 to 7/31/84 (Progress Report, Fall, 1983) were:

1) Production of monoclonal antibody to the 0.3 gene product and use of this antibody in development of a radioimmune precipitation assay.

2) Direct determination of whether cysteine substitutes only for arginine in mistranslation during synthesis of 0.3 protein.

1. Purification of monoclonal antibody to 0.3 protein from culture media and from ascites.

As reported in our last progress report, we have obtained a monoclonal antibody to the 0.3 protein. This antibody was obtained primarily to use in a simple, quantitative radioimmune precipitation assay (RIP) for 0.3 protein. The monoclonal antibody was to eliminate the need for SDS-PAGE and densitometry. The latter 2 steps were necessary in addition to RIP using polyclonal antibody because 0.3 protein was not the only protein precipitated. Monoclonal antibody produced in cell culture is too dilute to use in a RIP assay. We, therefore, attempted to purify and concentrate it with Protein A Sepharose affinity chromatography using standard procedures (4). Antibody titers before and after spent media were run through the column were obtained by ELISA. In addition, the amount of immunoglobulin retained by the column was calculated from the absorbance at 280 nm and estimated from SDS-PAGE (Fig. 3A). We found that approximately 90% of the monoclonal antibody was not retained by the column. Therefore, Protein A Sepharose chromatography was not regarded as a suitable technique for purification of 0.3 monoclonal antibody from culture media.

Next, we tried a technique involving growing the hybridoma in serum-free medium (5). This eliminates the necessity of removing albumin from the media and involves only concentration of proteins (mainly immunoglobulin) to obtain the monoclonal antibody in usable form. Unfortunately, our hybridoma is one which apparently cannot survive without fetal calf serum in the medium.

Two additional techniques to purify 0.3 monoclonal antibody from cell culture media were also tried: DEAE cellulose chromatography and ammonium sulfate precipitation. DEAE chromatography didn't work because the column became overloaded by albumin in the media. Results of ammonium sulfate precipitation are shown in figure 3B. It is clear that the major portion of immunoglobulin was not precipitated by ammonium sulfate. As with Protein A Sepharose chromatography we obtained some purification of the monoclonal antibody but at the cost of at least 90% antibody loss.

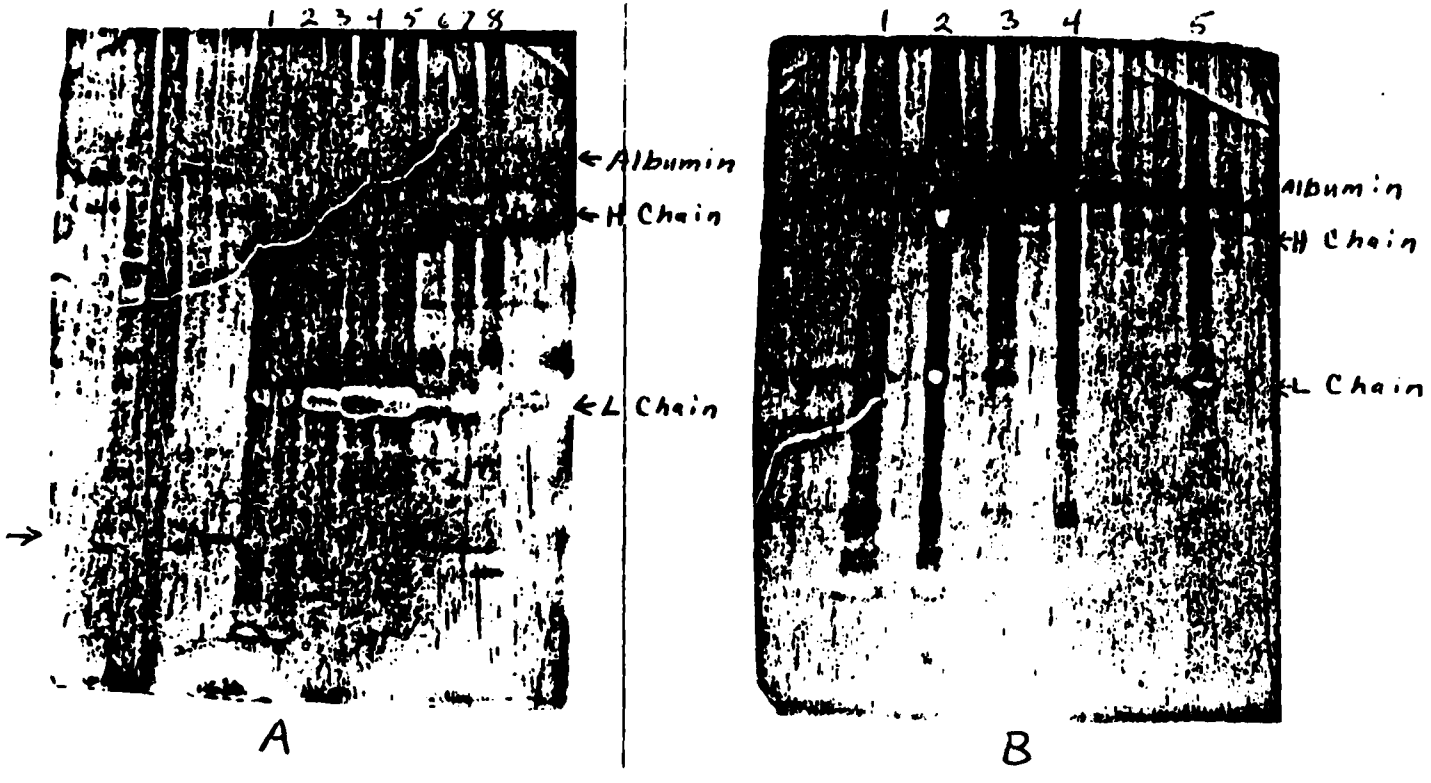

1 2 3 4 5 6 7 8
←Albumin
←H Chain
←L Chain
A
1 2 3 4 5
Albumin
H Chain
L Chain
B

Figure 3

Legend to Figure 3

SDS-polyacryamide gels stained for protein with the silver nitrate technique. A: Comparison of proteins in hybridoma culture medium and ascites before and after Protein A Sepharose chromatography and comparison with proteins eluted from the column. 1 = Medium before chromatography; 2 = Medium after chromatography; 3 = 1 µl untreated ascites; 4 = 5 µl octanoic acid - purified ascites before chromatography; 5 = 5 µl octanoic acid-purified ascites after chromatography; 6 = Pool A of ascites proteins retained by column; 7 = Pool B of ascites proteins retained by column; 8 = Pool B of medium proteins retained by column.

B: Purification of monoclonal antibody from hybridoma culture medium by ammonium sulfate precipitation. 1 = Medium before precipitation; 2 = Supernatant of 1st precipitation with ammonium sulfate; 3 = supernatant of second precipitation with ammonium sulfate; 4 = Proteins in second ammonium sulfate precipitate; 5 = Monoclonal antibody from ascites purified by Protein A Sepharose chromatography (used for H and L chain markers).

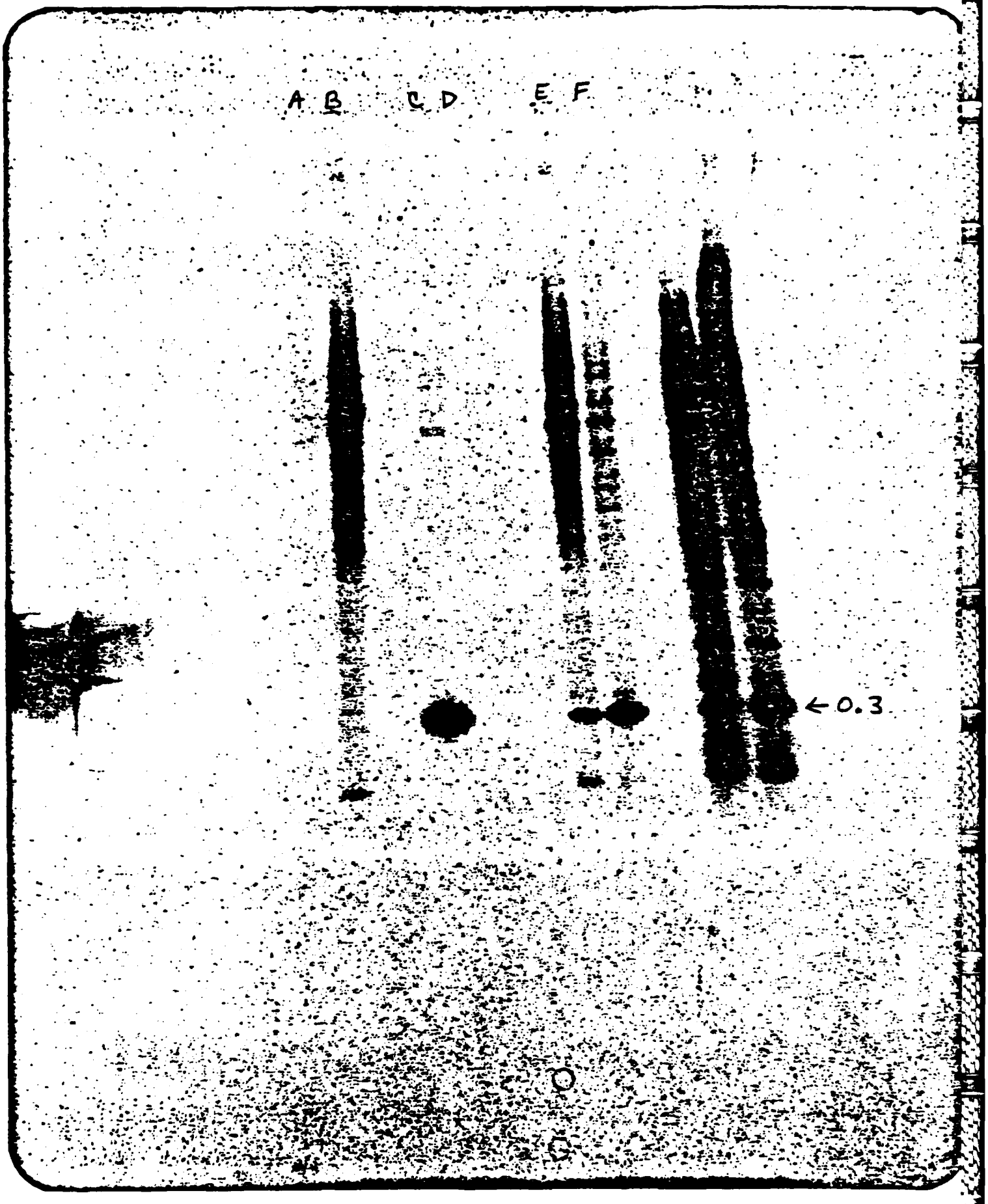
We also grew the hybridoma as an ascites tumor in mice and attempted to purify the antibody by Protein A Sepharose Chromatography. Partial purification was first achieved by the octanoic acid technique. Results are shown in Fig. 3A. It is clear that, as with antibody in culture media, the major portion of immunoglobulin was not retained by the column. Next, we tried purification of antibody from ascites by DEAE cellulose chromatography. We found that virtually all the proteins in the octanoic acid-treated ascites were retained by the column and thus this method was no improvement over octanoic acid treatment alone.

2. Use of monoclonal antibody to 0.3 protein in radioimmune precipitation experiments.

Unpurified monoclonal antibody in spent media and untreated ascites were compared with rabbit antiserum in our standard RIP-PAGE assay for 0.3 protein. Results are shown in Figure 4. Lanes A and C contain proteins precipitated by rabbit antibody from uninfected (lane A) and T7-infected E. coli (lane B). Only trace amounts of extraneous proteins (proteins other than 0.3) are precipitated. Lane D shows the proteins precipitated by monoclonal antibody in spent media. No proteins are visible indicating insufficient antibody is contained in 100 µl to precipitate 0.3 protein by this technique. Lanes B, E and F contain proteins precipitated by antibody in untreated ascites. It is clear that 0.3 protein is precipitated by monoclonal antibody in untreated ascites, and more by 50 µl (lane F) than by 10 µl (lane E), but the number of extraneous proteins precipitated is larger than found with rabbit antiserum. Whether these proteins are precipitated by antibody in ascites or are nonspecifically adsorbed to Protein A is unknown. However, we can say that rabbit antiserum contains more antibody to 0.3 protein than does untreated ascites and, in addition, fewer extraneous proteins are precipitated by rabbit antiserum.

Next we compared monoclonal antibody purified from spent media and from ascites by Protein A-Sepharose chromatography with rabbit antiserum in RIP-PAGE. Results are shown in Figure 5. Lane A shows proteins which are adsorbed nonspecifically by Protein A. BSA prevents this adsorption only slightly (lane B). Lanes C, D and E show proteins precipitated by decreasing amounts of rabbit antiserum. Clearly, the more antiserum added the more 0.3 protein precipitated and the less extraneous protein precipitated. Lanes F, G and H show proteins precipitated by monoclonal antibody purified from spent media. 50 µl of Pool B from the column eluate precipitates a barely visible amount of 0.3 protein but there are several extraneous proteins present (lane G). In lane H, there is not enough antibody present to prevent non-specific adsorption of proteins to Protein A. Lanes I, J and K show proteins precipitated by Pools A and B of monoclonal antibody purified from ascites. Only Pool B (lanes J and K) has sufficient antibody to precipitate 0.3 protein. However, 2 or 3 minor contaminating proteins are also present. From this experiment we concluded that 10 µl of rabbit antiserum was more

Figure 4



Legend to Figure 4

Autoradiogram of [$^{35}$S] methionine-labeled proteins in radioimmune precipitation - polyacrylamide gel electrophoresis (RIP-PAGE) experiment. Comparison of rabbit antiserum and unpurified monoclonal antibody in precipitation of 0.3 protein. *E. coli* cells were left uninfected or were infected with T7 phage and [$^{35}$S] methionine was added. Cells were lysed after 1 hour, and either rabbit antiserum, hybridoma culture medium or hybridoma - induced ascites was added. Following incubation, Protein A Sepharose was added to precipitate immune complexes. The beads were washed 5 times and eluted proteins examined by SDS-PAGE.

A = uninfected cells and 10 µl rabbit antiserum; B = uninfected cells + 10 µl ascites; C = infected cells + 10 µl rabbit antiserum; D = infected cells + 100 µl hybridoma culture medium; E = infected cells + 10 µl ascites; F = infected cells + 50 µl ascites.

efficient at precipitating 0.3 protein than 50 µl of purified monoclonal antibody and the amount of background (extraneous proteins precipitated by antibody or Protein A) was comparable.

We attempted to reduce the background of contaminating proteins to negligible levels by substituting rabbit immunobeads (immunobeads containing α-rabbit IgG antibody) or mouse immunobeads for Protein A Sepharose. Results of the experiment utilizing rabbit immunobeads are shown in Figure 6. In lanes A-E increasing amounts of immunobeads were added and increasing amounts of 0.3 protein plus extraneous proteins were precipitated. The background was significantly higher with immunobeads than with Protein A Sepharose (lane G). This is also seen by comparing proteins non-specifically adsorbed by immunobeads (lane F) with those adsorbed by Protein A (lane H).

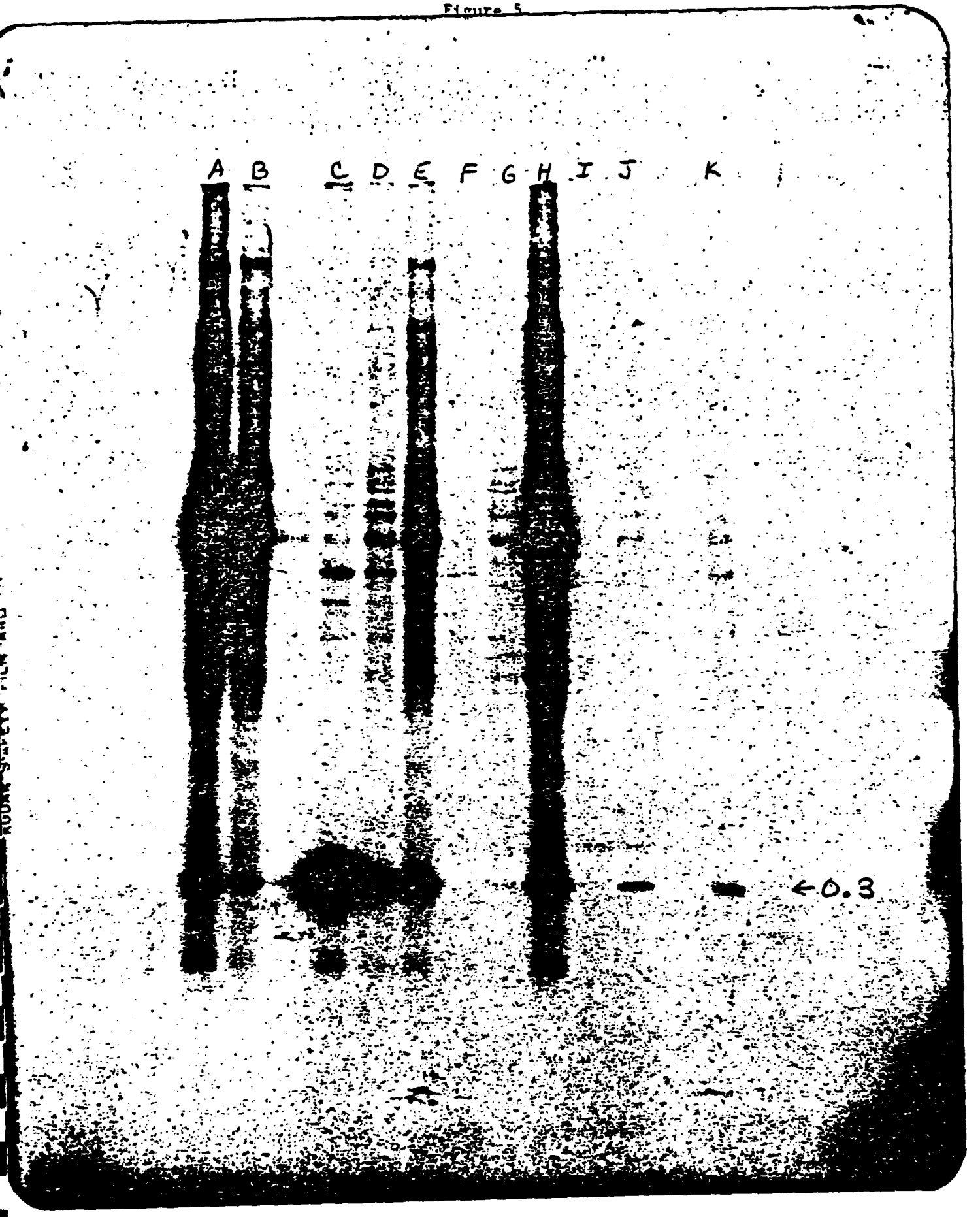
Our overall conclusion from these (and other) experiments is that rabbit antiserum is superior to monoclonal antibody in the RIP assay. Only 1 or 2 trace contaminating proteins are seen when rabbit antiserum plus Protein A Sepharose are used, but these appear to be due to precipitation by antibody rather than nonspecific adsorption by Protein A (Figure 5, lane C). It is unfortunate that we have been unable to adequately concentrate monoclonal antibody from cell culture. The background proteins seen in RIP-PAGE are undoubtedly caused only by nonspecific adsorption by Protein A. However, background proteins seen with monoclonal antibody from ascites are probably the result of precipitation by antibodies to E. coli in ascites. Even if we were successful in eliminating all background proteins when using monoclonal antibody in RIP the quantity of 0.3 protein precipitated is only a fraction of that precipitated by rabbit polyclonal antibody. This is not an uncommon finding. Monoclonal antibodies generally would not be expected to precipitate as much antigen as avid polyclonal antibody.

3. Progress in experiments to quantitate cysteine misincorporation in 0.3 protein encoded in the plasmid pAR324.

We have proposed to determine whether the mistranslation level as determined by cysteine misincorporation into 0.3 protein synthesized in T7-infected E. coli cells ($4 \times 10^{-3}$) is a constant value or whether it can be altered by a change in the cellular environment, i.e., when the protein is encoded in a plasmid gene (plasmid pAR324) or in a B. subtilis phage gene ($SPP1_{\gamma}$-0.3). These experiments are important for genetic engineering as it is possible, and even expected, that expression of a gene in a foreign cellular environment, which has a different set of preferred codons and tRNA abundances, could lead to increased misreading.

We previously showed that the 0.3 gene is expressed in cells containing the plasmid pAR324 and in B. subtilis cells infected with phage $SPP1_{\gamma}$-0.3. However, expression was at a much lower level than in T7-infected E. coli cells and this precluded 0.3 purification by our standard procedure. Consequently, we have been working on a means to quantitate the 0.3 protein other than purification. We

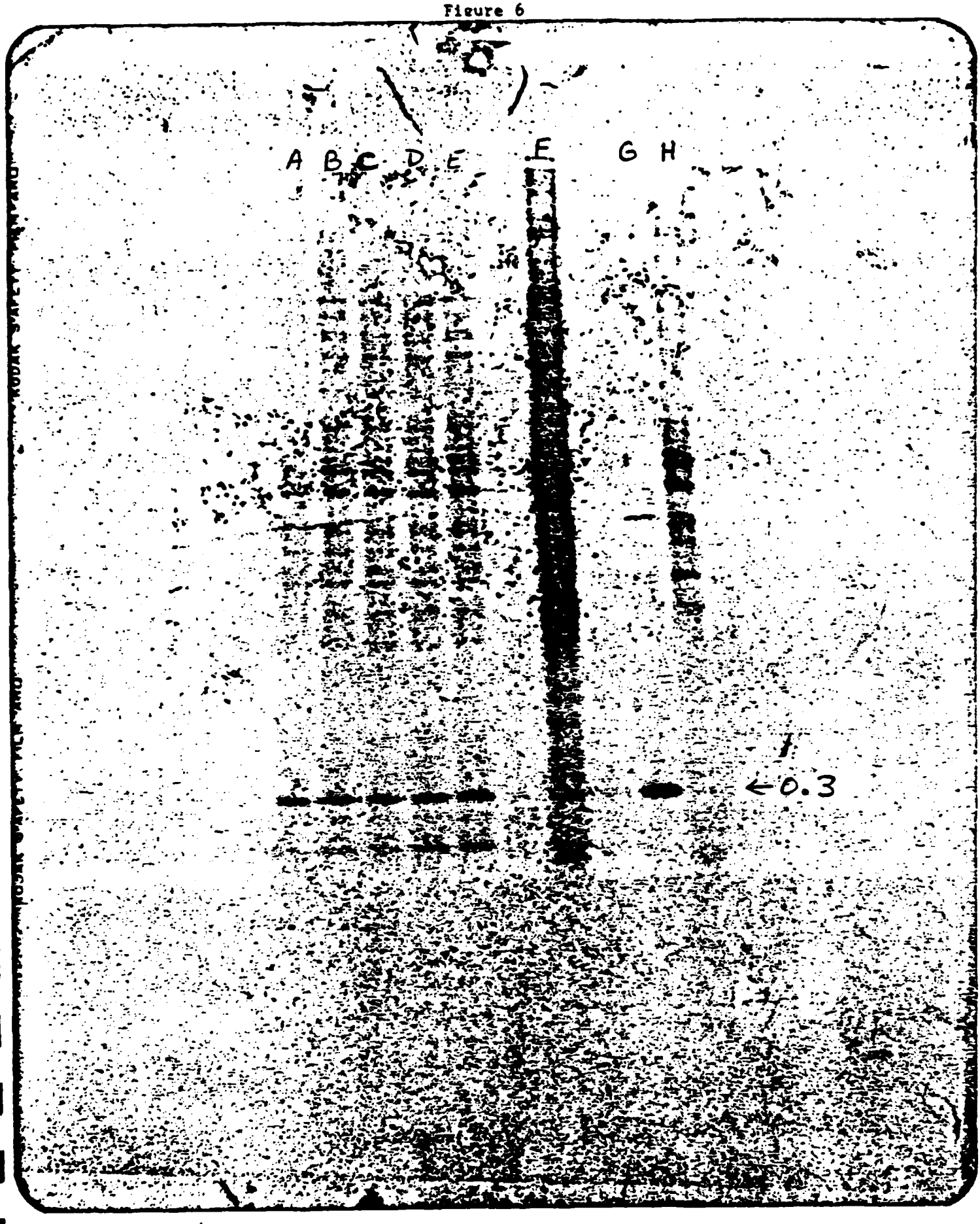
Figure 5



Legend to Figure 5

Comparison of rabbit antiserum and purified monoclonal antibody in RIP-PAGE. A = 30 µl Protein A Sepharose only; B = Protein A Sepharose + 10 µl of 20 mg BSA/ml; C = 10 µl rabbit antiserum + Protein A Sepharose; D = 1 µl rabbit antiserum + Protein A Sepharose; E = 0.3 µl rabbit antiserum + Protein A Sepharose; F = 100 µl Pool A proteins (from medium) eluted from Protein A Sepharose column + Protein A Sepharose; G = 50 µl Pool B proteins (from medium) eluted from Protein A Sepharose column + Protein A Sepharose; H = 10 µl Pool B proteins (from medium) eluted from Protein A Sepharose column + Protein A Sepharose; I = 100 µl Pool A proteins (from ascites) eluted from Protein A Sepharose column + Protein A Sepharose; J = 50 µl Pool B proteins (from ascites) eluted from Protein A Sepharose column + Protein A Sepharose; K = same as J plus BSA.

Figure 6



Legend to Figure 6

Comparison of anti-rabbit Ig-immunobeads and Protein A Sepharose in RIP-PAGE.

A = 5 µl rabbit antiserum + 12 µl immunobeads;

B = 5 µl rabbit antiserum + 25 µl immunobeads;

C = 5 µl rabbit antiserum + 50 µl immunobeads;

D = 5 µl rabbit antiserum + 100 µl immunobeads;

E = 5 µl rabbit antiserum + 200 µl immunobeads;

F = 50 µl immunobeads only; G = 5 µl rabbit antiserum + 25 µl Protein A Sepharose;

H = 25 µl Protein A Sepharose only.

plan to precipitate the double-labeled ($^{3}$H-leu and $^{35}$S-cys) 0.3 protein using rabbit antiserum, cut it out of a gel stained for protein, solubilize the gel and count radioactivity. Preliminary experiments will establish $^{3}$H radioactivity to be equal to a certain amount of 0.3 protein and the $^{35}$S CPM will allow us to quantitate cysteine in that amount of protein. We are directing our initial efforts at experiments in E. coli BpAR324 because there is more 0.3 protein produced in this strain than in SPP1$_{y}$-0.3-infected B. subtilis cells (unpublished data).

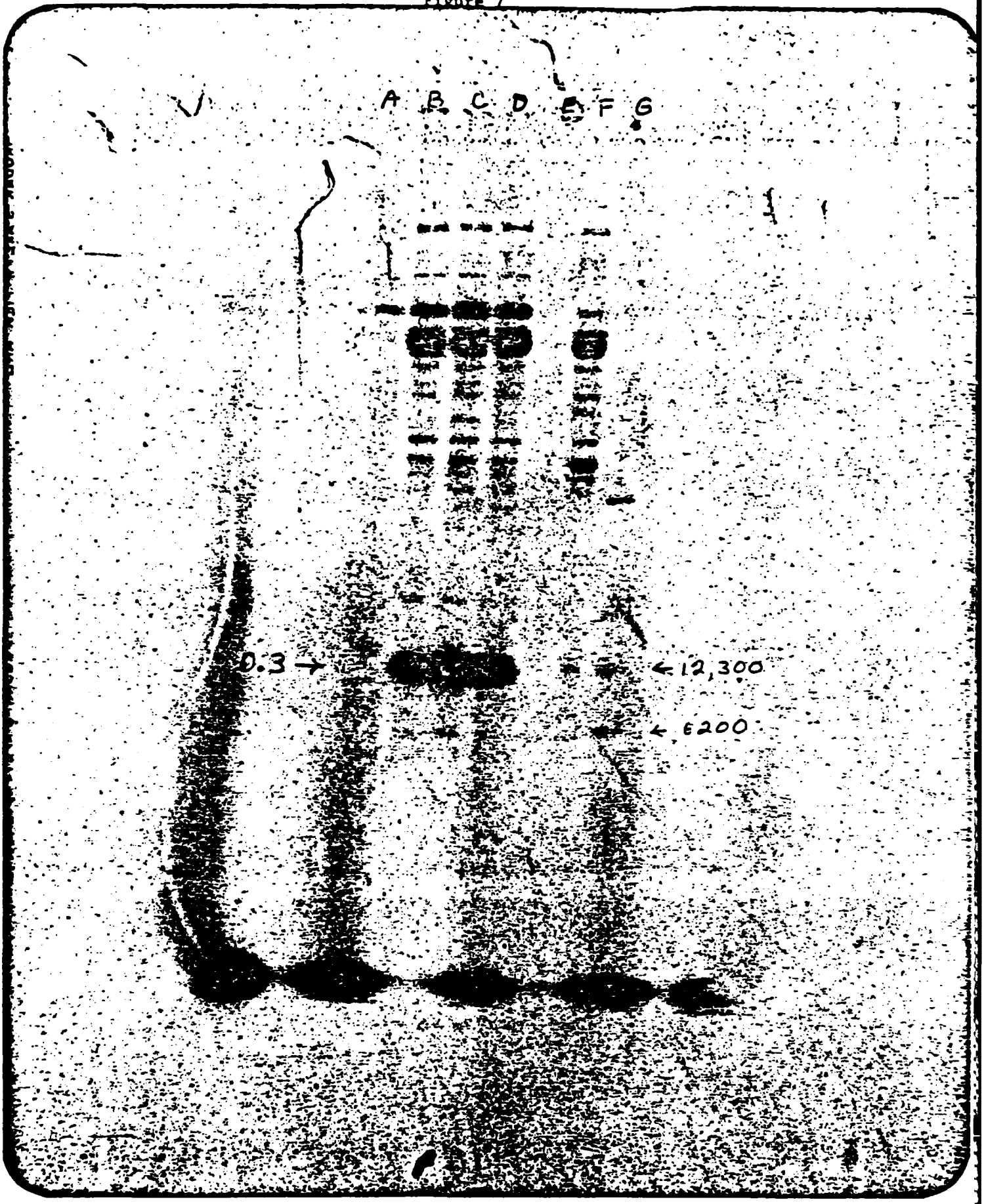
Figure 7 (lanes E and F) shows the proteins precipitated from [$^{35}$S]-met-labeled E. coli BpAR324 by normal rabbit serum and by rabbit antiserum 489, respectively. 0.3 protein can clearly be seen in lane F, although as a faint band compared to the 0.3 protein precipitated by antiserum 489 from T7-infected E. coli cells (lanes B, C, D). The new rabbit antiserum, as compared to that used in experiments shown in Figures 4-6, has much more antibody to E. coli proteins. However, this is of no importance in the present experiments because no other precipitated protein has a M.W. similar to 0.3 protein and we plan to cut the protein out of the gel before quantitation. Should this technique prove successful with E. coli BpAR324, we will also quantitate mistranslation in the other E. coli strains we have transfected with this plasmid (DS410 and KL266). Results can then be compared to the 0.3 protein mistranslation level already determined in the T7-infected parent strains. Should the amount of 0.3 protein synthesized in SPP1-0.3-infected B. subtilis cells be insufficient for even this technique, we plan to increase 0.3 expression by cloning the gene into a B. subtilis plasmid which has a strong promoter.

4. Experiments directed toward determination of the molecular basis of mistranslation.

One of the major advantages in utilization of the T7 0.3 protein to study mistranslation is that the complete nucleotide sequence of the gene has been determined (6). Thus, it should only be necessary to determine the specific sites of misincorporation of a particular amino acid (as cysteine) to deduce important patterns of misreading. It would be even more advantageous if relative (or absolute) frequencies of amino acid misincorporation at particular positions could be determined. This would provide information as to the hierarchy of misreading patterns which do occur. In particular, it would provide valuable information pertinent to the current debate of whether codon or context exerts more influence on misreading frequency (7, 8, 9, 10). Thus, with our mistranslation model utilizing 0.3 protein we have an opportunity to generate the kind of data which are sorely needed as a prerequisite to understanding the mechanisms of mistranslation (7).

Our initial approach to determining the sites of cysteine misincorporation into the 116 amino acid 0.3 protein has been to treat [$^{35}$S] cysteine-labeled 0.3 protein with reagents which cleave

Figure 7



Legend to Figure 7

Detection of 0.3 protein synthesized in E. coli BpAR324 by RIP-PAGE; 30 µl Protein A Sepharose was added to precipitate immune complexes.

A = Proteins precipitated by normal rabbit serum (492) from lysate of T7-infected E. coli B;

B = Proteins precipitated by rabbit antiserum 489 pi 1-84 from lysate of T7-infected E. coli B;

C = Proteins precipitated by rabbit antiserum 489 pi 2-84 from lysate of T7-infected E. coli B;

D = Proteins precipitated by rabbit antiserum 489 pi 3-84 from lysate of T7-infected E. coli B;

E = Proteins precipitated by normal rabbit serum (492) from lysate of E. coli BpAR324;

F = Proteins precipitated by rabbit antiserum 489 pi 1-84 from lysate of E. coli BpAR324.

proteins at only a few sites. Resulting peptides are then examined for radioactivity. The use of trypsin to digest 0.3 protein has an advantage over use of other reagents in that simple examination of the molecular weights of the peptides can distinguish between cysteine substitutions for arginine and for other amino acids. Figure 8 shows that only 3 major peptides are expected by tryptic digestion of 0.3 protein. However, 2 of these occur by cleavage at lysyl residues and it is known that trypsin cleaves somewhat slower at lysyl than at arginyl residues (11). In addition, the cleavage rate is slowed even more if lysine is adjacent to an acidic amino acid (11) -which is the case with both lysyl residues in 0.3 protein. Thus, we can normally expect an additional 2 peptides -one of 2800 daltons and one of 5700 daltons -for a total of 5 peptides visible by SDS-PAGE.

Samples of 0.3 protein synthesized with different radiolabels were used to help identify the peptides on SDS gels after autoradiography. As shown in Figure 8, when peptides generated by tryptic digestion of [$^{35}$S] met-labeled 0.3 protein are examined, all of the 5 peptides should be visible except the C-terminal 4000 dalton one (actual results shown in Figure 9). Likewise, tryptic digestion of [$^{3}$H] ile-labeled 0.3 protein should produce visible peptides of all but the N-terminal 2300 dalton one. (The 2800 dalton partial cleavage peptide should be visible, however (Figure 8).

To aid in identification of peptides which contained a cysteine for arginine substitution, we used [$^{3}$H] arginine-labeled 0.3 protein. Digestion with trypsin should result in a selected group of peptides visible by autoradiography. These are the 2800 dalton partial cleavage N-terminal peptide, a 5900 dalton partial cleavage product containing peptides 3 and 4, the 5700 dalton peptide, and a 4600 dalton partial cleavage product containing peptides 6 and 7. The peptides which should not be visible are the 2300, 5200 and 4000 dalton major peptides.

Results of tryptic digestion of 0.3 protein samples containing different radiolabels followed by SDS-PAGE and autoradiography are shown in Figure 10. The [$^{3}$H] ile-labeled sample contains the same visible peptides as the [$^{14}$C] aminoacid-labeled sample except that the 2300 dalton peptide is missing in the former, as expected. In contrast, the [$^{3}$H] arg-labeled sample has a quite different set of visible peptides: 2 major high M.W. peptides of approximately 5900 and 6400 daltons, another peptide at approximately 4000 daltons, (this is the 4600 dalton partial cleavage peptide -peptides between 3000 and 5000 daltons are difficult to resolve in this gel system) and another faint peptide at 2800 daltons. These peptides include all the arginine-containing peptides expected plus a larger peptide, (~ 6400 daltons) which is probably a partial cleavage product containing at least 2 arginine residues.

Results of a typical experiment in which trypsinized, [$^{35}$S] cys-labeled 0.3 protein is compared with trypsinized samples of 0.3 protein containing other radiolabels is shown in Figure 11. The 3

Figure 8

## 0.3 Peptides Obtained by Trypsin Digestion

| No. | MW | No. AA | No. MET | No. ILE |
|---|---|---|---|---|
| 1 | 2300 | 19(lys) | 3 | 0 |
| 2 | 500 | 4 | 0 | 1 |
| 3 | 700 | 5 | 0 | 1 |
| 4 | 5200 | 47(lys) | 3 | 3 |
| 5 | 500 | 4 | 0 | 1 |
| 6 | 600 | 5 | 0 | 1 |
| Z | 4000 | 32 | 0 | 2 |

Figure 9

TRYPTIC PEPTIDES OF [$^{35}$S]METHIONINE-LABELLED 0.3 PROTEIN

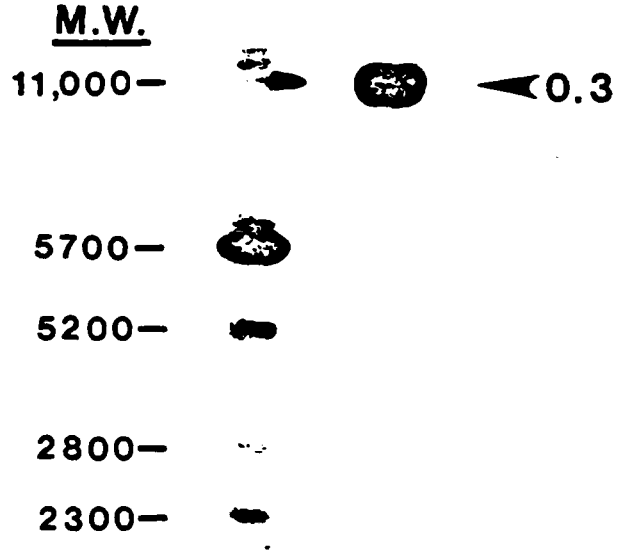


Figure 10

TRYPTIC PEPTIDES OF RADIOLABELLED 0.3 PROTEIN

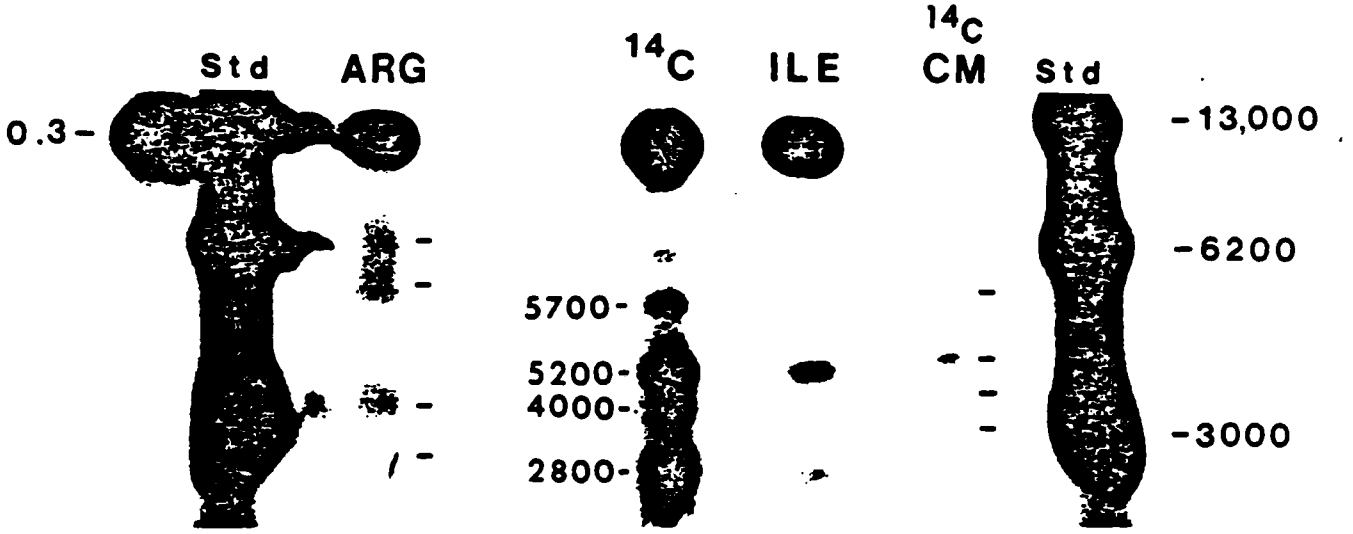
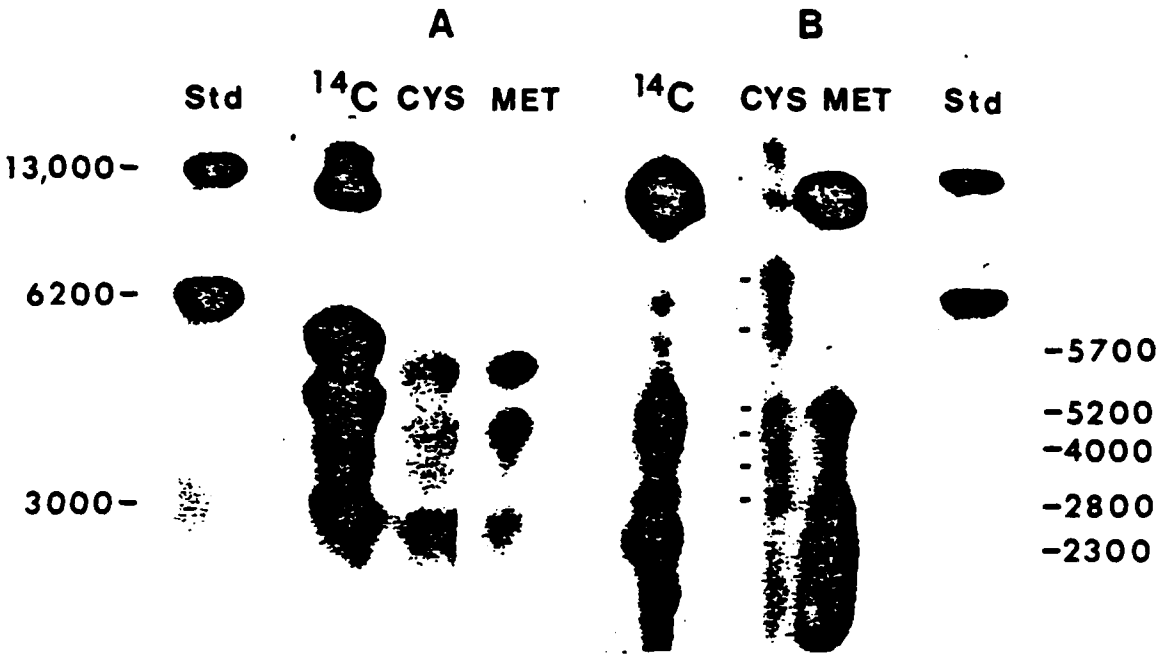


Figure 11

TRYPTIC PEPTIDES OF RADIOLABELLED 0.3 PROTEIN



lanes on the left half of the gel (side A) show peptides from 0.3 protein which had been succinylated to block lysyl residues. Thus, the 2300 dalton peptide generated by cleavage at lysine has disappeared, increasing the prominence of the 2800 dalton peptide. The 5700 dalton peptide expected when lysyl residues are blocked is also more prominent compared to the 5700 dalton peptide seen in unsuccinylated samples on the right half of the gel (side B).

On side A in Figure 11 it can be seen that the peptide pattern of all 3 radiolabeled samples of 0.3 protein, are similar. The differences are that the [$^{35}$S] cysteine-labeled sample has an additional (but faint) high M.W. peptide (about 6400 daltons), and the [$^{35}$S] methionine-labeled sample has no 4000 dalton peptide. All 3 samples contain visible 5700, 5200 and 2800 dalton peptides. Thus, it is clear that cysteine has been misincorporated into all the major tryptic peptides.

On side B in Figure 11 there are more peptides in each sample than on side A. This is because lysyl residues are not blocked in samples on side B and because succinylation aids digestion by trypsin. There are at least 7 [$^{35}$S] cys-labeled peptides and these appear to be the same as those in the [$^{14}$C]-labeled peptides with 2 or 3 exceptions. The 2 largest [$^{35}$S] cys-labeled peptides are in the same positions as the 2 largest [$^{3}$H] arg-labeled peptides seen in Figure 10 (approximately 5900 and 6400 daltons). (These peptides are larger than the 2 highest M.W. [$^{14}$C]-labeled partial-cleavage peptides.) The remaining [$^{35}$S] cys-labeled peptides have the following approximate M.W.s: 5200, 4600 or 4000 (or both), 3500 (perhaps), 2800 and 2300 daltons. We conclude from the trypsinization experiments that cysteine substitutes for arginine in at least one position (probably position 79) and perhaps more. (A cysteine for arginine substitution at position 84 would generate a 4600 dalton peptide.) However, the major sites of cysteine substitution as shown by the [$^{35}$S] cysteine label in peptides of 5200, and 2300 daltons (Figure 11A and B) are elsewhere. Assuming a mistake at one nucleotide base pair, cysteine could substitute for serine, glycine, tyrosine or phenylalanine in the 5200 dalton peptide, for serine, tyrosine, or phenylalanine in the 2300 dalton peptide, and for tryptophan or tyrosine in the 4000 dalton peptide.

One of the other reagents we used to cleave 0.3 protein was 2-nitro-5-thiocyanobenzoic acid. This reagent is specific for cysteine residues (11). Two experiments were performed. In one we used [$^{14}$C]-amino acid labeled 0.3 protein and separation of peptides by gel filtration, and in the other we used [$^{35}$S] methionine-labeled 0.3 protein and separation of peptides by SDS-PAGE. Similar results were obtained from both experiments. Results of SDS-PAGE and auto-radiography are shown in Figure 12. We expected to see a whole series of peptides because it was clear from the trypsinization and CNBr experiments that cysteine is misincorporated at several positions. We were, therefore, very surprised to find only 2 major peptides. Furthermore, the sum of the M.W.'s (5700 and 4600) is

approximately that of the native 0.3 protein, suggesting a cleavage at one position only. We deduced that cleavage occurred at one of 2 approximate positions: position 49 from the N-terminus or position 67 from the C-terminus. Position 49 is a tyrosine (see Figure 13) whereas position 67 is aspartate. These results are important because they suggest that there is a particular position in 0.3 protein where cysteine is misincorporated much more frequently than at any other position.

Very recently, we received some very interesting data from a collaborator, Dr. Edwin Beachey of the Veterans Administration Medical Center in Memphis. We had sent him several samples of [$^{35}$S] cysteine-labeled 0.3 protein for sequencing to determine the precise locations of cysteine misincorporation. They were able to sequence and count radioactivity in amino acids through position 42 (Figure 13). They found that the amino acid positions in 0.3 protein with the highest radioactivity were positions 9, 15 and 41, especially position 15 which had at least 3 times more counts than the other 2 positions. These results are very surprising and most interesting for several reasons: 1) Mis-incorporation of cysteine at both position 9 (asn) and 41 (ala) requires mistakes at 2 base pairs, which has been shown to be an unlikely occurrence <u>in vitro</u>. However, both these amino acids are the second of 2 identical amino acids in a row. This may be very significant. 2) Cysteine apparently did not substitute for arginine at positions 23 and 28. 3) Cysteine did not substitute for tyrosine at positions 7 and 24 but did so at a high level in position 15. Because the codon at position 7 is the same as at position 15, this suggests that context effects are very important in misreading. 4) The experiments with the reagent which cleaves at cysteine suggested that a hot spot for cysteine misincorporation might be position 49. This is tyr (UAC) preceded by another tyr with the identical codon. If the presence of 2 identical, or nearly identical, codons in a row predisposes to misreading of the second one, position 49 may well be the most likely site for the greatest frequency of cysteine misincorporation in 0.3 protein. It is the only site where there are 2 codons which differ from cysteine codons by only one base. We are presently attempting to purify by HPLC 0.3 peptides generated by CNBr or trypsin cleavage. Once we have these techniques perfected we will send [$^{35}$S] cys-labeled peptides to Dr. Beachey for sequencing and determination of cysteine misincorporation sites in the remainder of the protein. This data will be of utmost value in determination of the mechanisms of mistranslation and the relative importance of codon versus context effects.

Figure 12


Cleavage of [35S] Met-0.3 Protein at Cysteine Residue
m.w. Stds
[35S] met 0.3
12300
6200
3000
11,000
5700
4600

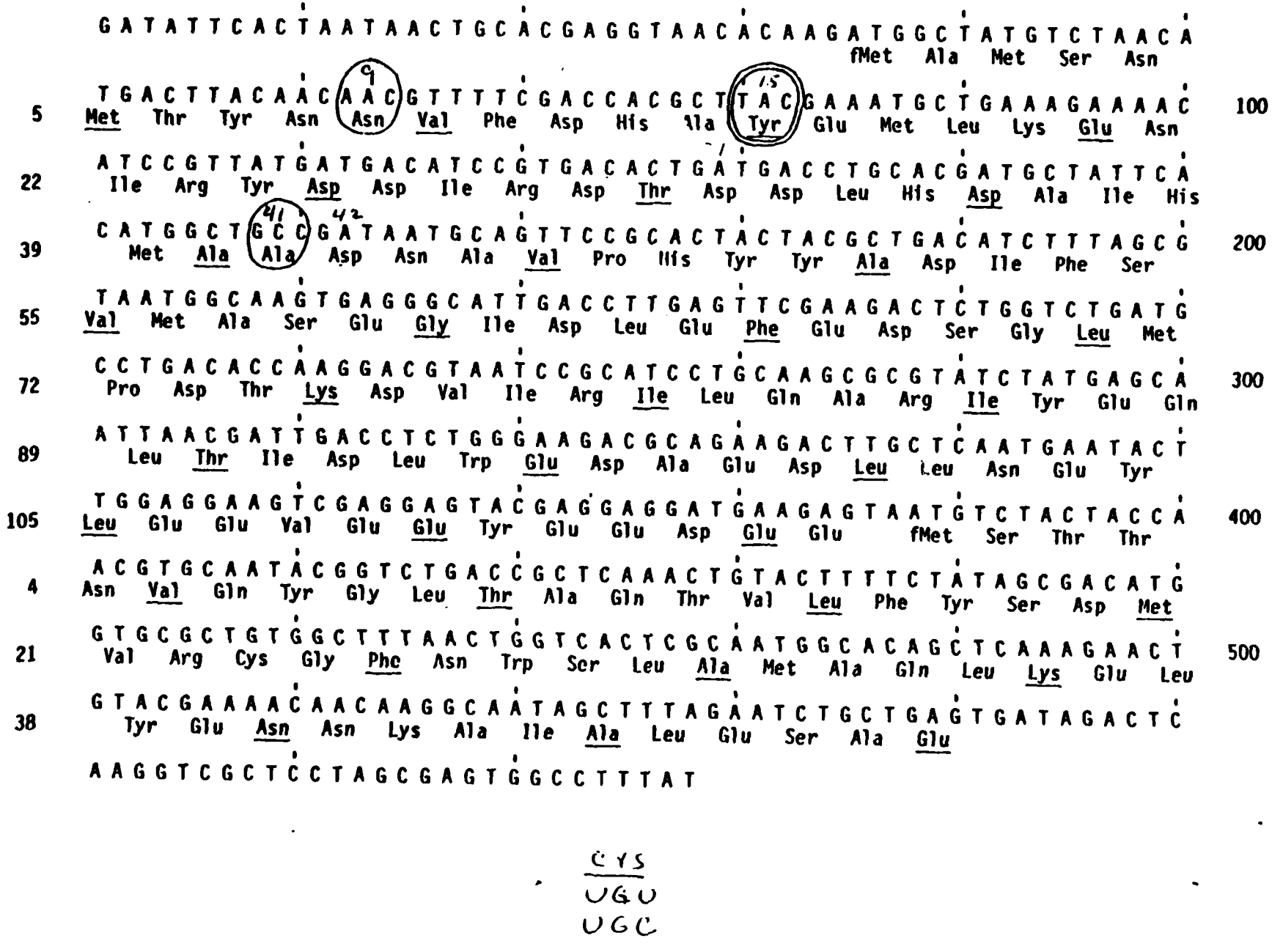

GATATTCACTAATAACTGCACGAGGTAACACAAGATGGCTATGTCTAACA
fMet Ala Met Ser Asn
TGACTTACAACAACGTTTTCGACCACGCTTACGAAATGCTGAAAGAAAAC
5 Met Thr Tyr Asn Asn Val Phe Asp His Ala Tyr Glu Met Leu Lys Glu Asn 100
ATCCGTTATGATGACATCCGTGACACTGATGACCTGCACGATGCTATTCA
22 Ile Arg Tyr Asp Asp Ile Arg Asp Thr Asp Asp Leu His Asp Ala Ile His
CATGGCTGCCGATAATGCAGTTCCGCACTACTACGCTGACATCTTTAGCG
39 Met Ala Ala Asp Asn Ala Val Pro His Tyr Tyr Ala Asp Ile Phe Ser 200
TAATGGCAAGTGAGGGCATTGACCTTGAGTTCGAAGACTCTGGTCTGATG
55 Val Met Ala Ser Glu Gly Ile Asp Leu Glu Phe Glu Asp Ser Gly Leu Met
CCTGACACCAAGGACGTAATCCGCATCCTGCAAGCGCGTATCTATGAGCA
72 Pro Asp Thr Lys Asp Val Ile Arg Ile Leu Gln Ala Arg Ile Tyr Glu Gln 300
ATTAACGATTGACCTCTGGGAAGACGCAGAAGACTTGCTCAATGAATACT
89 Leu Thr Ile Asp Leu Trp Glu Asp Ala Glu Asp Leu Leu Asn Glu Tyr
TGGAGGAAGTCGAGGAGTACGAGGAGGATGAAGAGTAATGTCTACTACCA
105 Leu Glu Glu Val Glu Glu Tyr Glu Glu Asp Glu Glu fMet Ser Thr Thr 400
ACGTGCAATACGGTCTGACCGCTCAAACTGTACTTTTCTATAGCGACATG
4 Asn Val Gln Tyr Gly Leu Thr Ala Gln Thr Val Leu Phe Tyr Ser Asp Met
GTGCGCTGTGGCTTTAACTGGTCACTCGCAATGGCACAGCTCAAAGAACT
21 Val Arg Cys Gly Phe Asn Trp Ser Leu Ala Met Ala Gln Leu Lys Glu Leu 500
GTACGAAAACAACAAGGCAATAGCTTTAGAATCTGCTGAGTGATAGACTC
38 Tyr Glu Asn Asn Lys Ala Ile Ala Leu Glu Ser Ala Glu
AAGGTCGCTCCTAGCGAGTGGCCTTTAT
Cys
UGU
UGC

Figure 13

57

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I. Report for Research Undertaken Between August 1, 1984 - July 31, 1985

A. Abstract

B. Details of experiments directed toward determination of the molecular basis of mistranslation

1. Determination of the sites of cysteine misincorporation in the N-terminal 1/3 of the 0.3 protein

2. Progress in experiments to purify peptides of proteolytically cleaved 0.3 protein

a. Separation of 0.3 peptides by preparative gel electrophoresis

b. Separation of 0.3 peptides by reverse phase high performance liquid chromatography

1) Separation of peptides from trypsinized 0.3 protein

2) Separation of peptides from S. aureus V8 protease-cleaved 0.3 protein

II. References

## A. Abstract

We have made significant progress toward determination of the molecular basis of mistranslation. Novel ideas concerning the "rules" of mistranslation are being generated from our present experiments. By sequencing the amino-terminal 1/3 of 0.3 protein we found the specific sites of cysteine misincorporation to be at positions 9 (asparagine), 15 (tyrosine) and 41 (alanine). Lower amounts of cysteine (less frequent substitutions) were also seen at positions 12 and 31 (aspartyl residues). From this data we can say that 1) context (position) effects are of primary importance in determining misreading, 2) contrary to what is believed, the cysteine for arginine substitution is not a common substitution, at least in the 0.3 protein. In fact, we found no substitution of cysteine at the 2 arginine positions in the amino-terminal 1/3 of 0.3 protein. 3) Misreading by mispairing of the codon-anticodon at 2 bases occurs fairly frequently (examples are cysteine substitutions for asparagine, alanine and aspartate). 4) The presence of tandem codons requiring the same tRNA may predispose the second (or later) codon to being misread (examples are the misreading events at positions 9 and 41).

We have purified an internal 0.3 peptide by digestion of 0.3 protein with S. aureus V8 protease and purification by RP-HPLC or by preparative gel electrophoresis. We are now in a position to obtain sufficient quantities of [$^{35}$S]cysteine-labeled peptide for sequencing. These studies will provide data concerning specific cysteine substitution sites and their frequencies in the middle section of 0.3 protein (39 residues, #21-#59).

Preliminary studies to determine the precise in vivo misreading effects of streptomycin have proved interesting. In addition to the mistakes made during translation of 0.3 protein under normal (non-drug) conditions, streptomycin induced misincorporation of cysteine at positions 10 (valine), 11 (phenylalanine), 16 (glutamate), 42 (aspartate), 43 (asparagine), and 45 (valine). Misreading of codons for valine and glutamate requires codon-anticodon mispairing at all 3 bases, while misreading of the phenylalanine codon requires C to be misread as G in the internal position - mistakes not usually seen during in vitro mistranslation experiments.

B. Details of experiments directed toward determination of the molecular basis of mistranslation

1. Determination of the sites of cysteine misincorporation in the N-terminal 1/3 of 0.3 protein

[$^{35}$S]cysteine-labeled samples of purified 0.3 protein were sent to the laboratories of Drs. Edwin Beachey and Jerome Seyer of the Veterans Administration Medical Center in Memphis, TN. They sequenced 0.3 protein up to position 43 and counted radioactivity in collected fractions to determine sites of cysteine misincorporation. Results are shown in Figure 1.

Despite the presence of excess unlabeled methionine (0.6 mg/ml) in growth media, a very small portion of the [$^{35}$S]cysteine was converted to [$^{35}$S]methionine. The presence of 4 methionine residues within the N-terminal 1/3 of 0.3 protein allowed us to calculate percent sample loss with each cycle of the automated Edman degradation procedure. Relative CPM at each amino acid position could then be obtained. The data clearly indicate that significant cysteine misincorporation occurred at positions 9 (asparagine), 15 (tyrosine), and 41 (alanine). Lower amounts of cysteine were misincorporated at positions 12 and 31 (both aspartyl residues).

The data lend themselves to the following conclusions and hypotheses:

1) Context (position) effects are of primary importance in determining whether cysteine substitutes for at least some amino acids. The evidence is as follows. The tyrosine codon at both positions 15 and 7 is UAC but cysteine was misincorporated only at position 15; the 5' and 3' codons at both these positions are different. The asparagine codon at positions 4, 8, 9, and 21 is AAC but cysteine was misincorporated only at position 9; the 5' and 3' codons at these positions are different. There are 4 alanine residues -at positions 14, 36, 40, and 41; GCU codes for alanine at positions 14, 36 and 40, while GCC codes for alanine at position 41 -the only position where cysteine was misincorporated. However, the same tRNA is used for GCC and GCU; therefore, context effects probably determine misreading in the case of alanine also.

2) Cysteine did not substitute for arginine at positions 23 and 28.

3) Of the 7 sites where cysteine could be misincorporated by mispairing of the codon-anticodon at only one base (2 serine, 3 tyrosine, 1 phenylalanine and 2 arginine sites), misreading actually occurred at only one site (tyrosine at position 15). Therefore, the presence of a codon with one base different from the codon of the misincorporated amino acid is not sufficient to predict misreading at that codon.

4) Cysteine substituted for asparagine, aspartate and alanine - mistakes which require mispairing of the anticodon at both the 5' and middle bases of the codon. This result was unexpected.

Figure 1. Automated Edman degradation of the N-terminal 42 positions of [$^{35}$S] cysteine-labeled 0.3 protein.

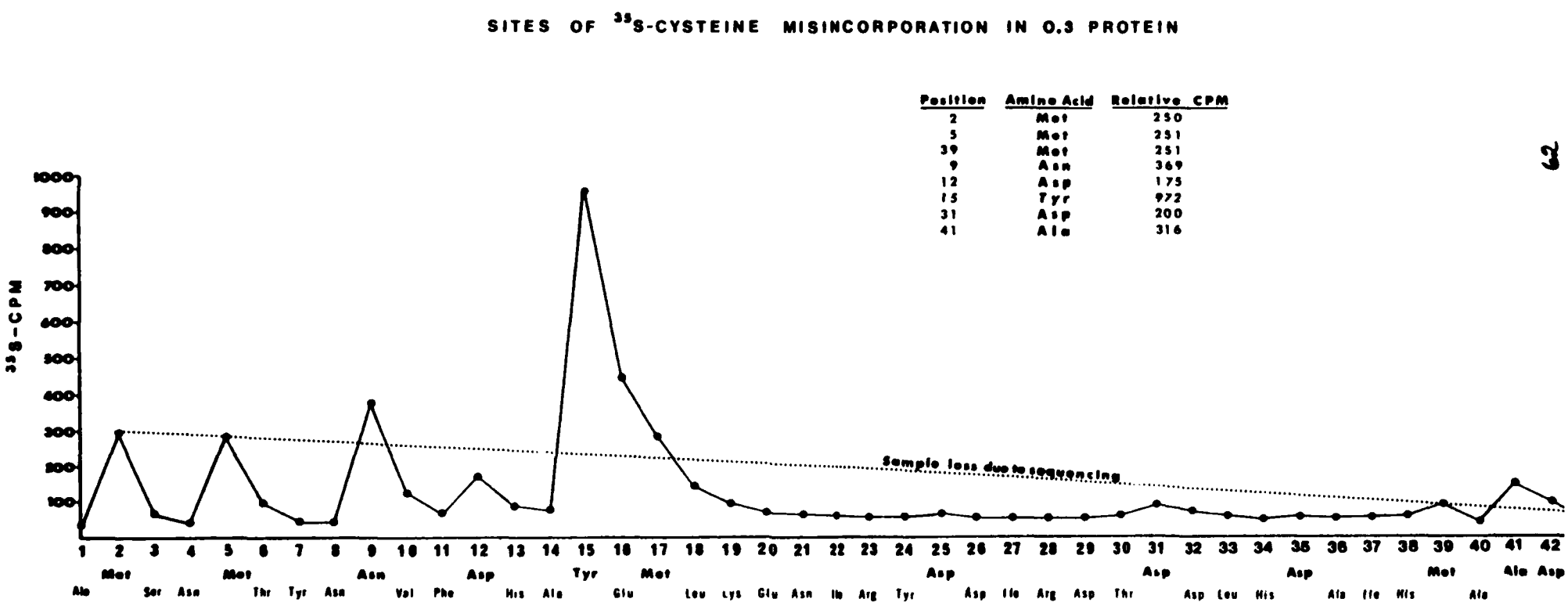


Figure 1. Automated Edman degradation of the N-terminal 42 positions of [$^{35}$S] cysteine-labeled 0.3 protein. Fractions corresponding to each amino acid position were collected and examined for radioactivity in a liquid scintillation counter. Radioactivity at each position was normalized by determining percent sample loss during each cycle of the sequencer. The presence of 3 methionine residues which were not adjacent to a "hot" position allowed calculation of percent sample lost.

5) In the cases of the relatively frequent (for 2-base-pair misreading events) cysteine substitutions at position 9 for asparagine and at position 41 for alanine, the preceding codons in the mRNA required the same tRNA as the misread codon. This suggests that the occurrence of tandem codons requiring the same tRNA may induce misincorporation errors in the amino acid position(s) corresponding to the second (or later) codon(s).

Some of these results and hypotheses have not heretofore been presented in the literature and, therefore, we believe this work is making significant contributions to the understanding of the mechanisms of mistranslation.

2. Progress in experiments to purify peptides of proteolytically cleaved 0.3 protein.

By sequencing analysis of 0.3 protein we have identified specific mistranslation events, and proposed several hypotheses to explain general types of translational errors. One drawback with this technique however, is that automated Edman degradation can only be performed accurately on the N-terminal 40 or so amino acid positions before the sample is lost. Therefore, in order to obtain more of this important kind of data on the remainder of the 0.3 protein molecule, it is necessary to produce significant quantities of purified [$^{35}$S] cysteine-labeled internal or C-terminal 0.3 peptides.

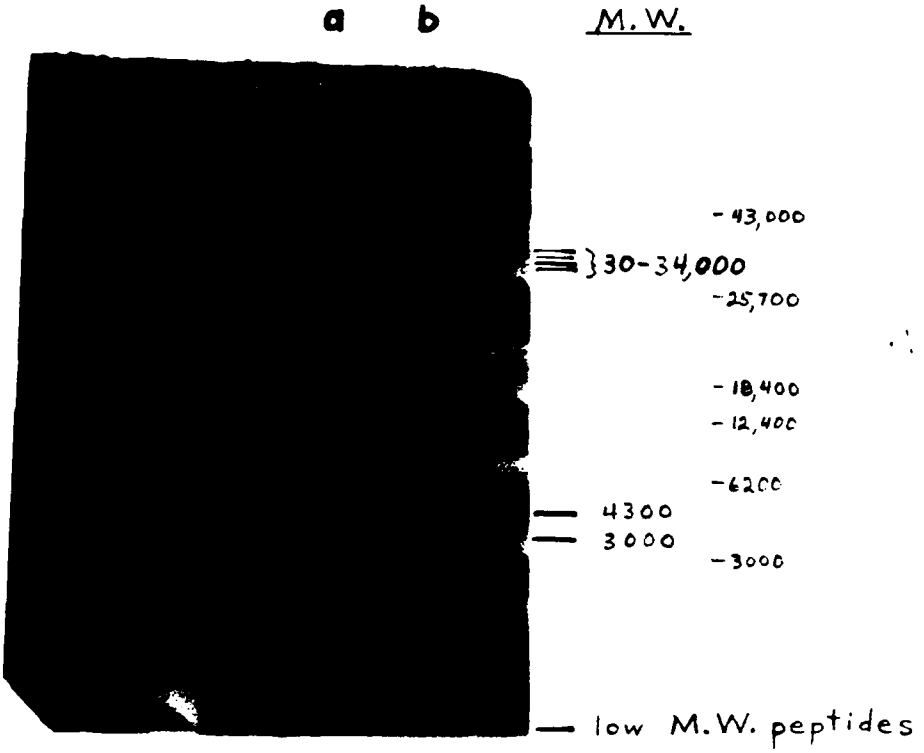
We have used many reagents which cleave the 0.3 protein at only a few sites, including CNBr, trypsin, arginine-specific protease, 2 reagents which are purported to cleave at only tryptophan (O-iodosobenzoic acid and DMSO/HCl/HBr) and Staphyloccous aureus V8 protease. To separate resulting peptides we have used gel filtration, reverse phase high performance liquid chromatography (RP-HPLC) and preparative gel electrophoresis.

Digestion of 0.3 protein with the arginine-specific protease of mouse salivary gland (1) resulted in less complete digestion than digestion with trypsin (analysis by SDS-PAGE). Iodosobenzoic acid is supposed to cleave at tryptophan specifically (2). Instead of obtaining 2 peptides (there is only 1 tryptophan in 0.3 protein), we found many peptides following treatment of 0.3 protein with iodosobenzoic acid. Likewise, cleavage of 0.3 protein with the "tryptophan-specific" reagent DMSO/HCl/HBr resulted in several peptides.

S. aureus V8 protease (3) has given us our best results to date. When used at its alkaline pH optimum (7.8) and with succinylated 0.3 protein (which facilitates complete digestion because succinylation causes molecule unfolding and hence accessibility to cleavage), 1 predominant peptide of 4300 D is seen on SDS gels. (Figure 2). In addition to the 4300 D peptide some high M.W. (30-34,000 D), apparently cross-linked, products are seen, as well as a peptide of 3000 D and other smaller peptides. The 4300 D peptide should contain residues 21-59 (see Fig. 6) and would be a good peptide in which to determine cysteine substitution positions since it contains the tyr-tyr doublet at positions 48 and 49 which we have predicted to be a hot spot for cysteine misincorporation. (See Progress Report for research undertaken between August 1, 1983 and February 1, 1984).

a. Separation of 0.3 peptides by preparative gel electrophoresis. 0.3 protein treated with trypsin or with S. aureus V8 protease was electrophoresced on a native, 13-22% acrylamide gel. A strip was cut off one end of the gel and stained with Coomassie Blue to locate positions of the peptides in the unstained gel. These locations were then excised, the gel mashed, and the peptides electroeluted into dialysis bags. Reservoir buffer was removed from the peptide samples either by dialysis or by desalting over Seph. G-15. Peptides in water (dialysis) or $NH_4HCO_3$ (desalting) were concentrated on a Savant Speed Vac Concentrator and analyzed by SDS-PAGE. The above

Figure 2. SDS-PAGE (13-22% acylamide) of S. aureus V8 protease-digested 0.3 protein.




250 µg of succinylated 0.3 protein were digested overnight at 37°C with 30 µg protease in 100 µl of 50mM $NH_4HCO_3$ buffer, pH 7.8. 5 µl of the digestion mixture (12.5 µg 0.3 protein) was loaded onto a SDS-polyacylamide gel and the samples were electrophoresced for 18 hrs at 100 V. The gel was then stained for protein with the sensitive silver stain (Sammons, D.W., et al., Electrophoresis 2:134-141, 1981). a = Low M.W. standards. b = S. aureus V8 protease-digested succinylated 0.3 protein. Four high M.W. bands, a predominant peptide at 4300 daltons, another peptide at around 3000 daltons, and low M.W. peptides are seen.

experiment has been done a number of times, changing the conditions somewhat each time, but we have been unable to eliminate the novel high M.W. bands which show up on the final analysis by SDS-PAGE. However, we have shown these bands to be artifacts as they show up even when pure 0.3 protein or no protein at all is cut out of a gel, electroeluted, dialyzed and analyzed on a SDS gel. They are also visible when a different silver stain method (4) or Coomassie Blue is used to stain the gel. In other words, if you ignore the high M.W. artifacts,we have been able to separate peptides of S. aureus V8 protease-cleaved 0.3 protein by preparative gel electrophoresis.

b. Separation of 0.3 peptides by reverse phase high performance liquid chromatography.

1) Separation of peptides from trypsinized 0.3 protein. Figure 3 shows the peptides eluted from the RP-HPLC column when 40 g of a trypsinized sample of 0.3 protein was chromatographed. The number of peptides expected if there had been complete digestion is 9 (7 0.3 peptides plus trypsin and trypsin inhibitor). There are obviously more than 9 peaks in the chromatograph, showing that partial digestion products are also present. Fractions corresponding to the numbered peaks were collected, concentrated, and analyzed for purity by SDS-PAGE (13-22% acrylamide). Only peaks 9, 10, 11 and 12 contained sufficient amounts of peptide to be visible with the sensitive silver stain. Peaks 9 and 10 contained a peptide of 3000 daltons. Peak 11 contained trypsin and trypsin inhibitor. Peak 12 contained trypsin inhibitor. When larger samples were chromatographed with monitoring at 220 nm for the peptide band, many of the peaks merged and separation became impossible. In addition, the 3000 D peptide which can be isolated is not useful since it is the N-terminal peptide and we already have the N-terminal sequencing data from use of the whole molecule.

2) Separation of peptides from S. aureus V8 protease-cleaved 0.3 protein. As mentioned above, the predominant peptide from S. aureus protease digestion of 0.3 protein is the largest one, 4300 D, and a very useful peptide for our purposes. Therefore, we hoped that with RP-HPLC we could separate it from the other lower MW peptides and from the high M.W. cross-linked products found after digestion of 0.3 protein with S. aureus V8 protease. Figure 4 shows the separation of these peptides achieved by RP-HPLC. Fractions corresponding to the numbered peaks were collected, evaporated to dryness in the Speed Vac Concentrator, and analyzed for purity by SDS-PAGE (13-22% acrylamide gradient). Results are shown in Figure 5. Ten lanes (peaks 1, 3, 10, 11, 12, 13, 14, 15, 16 and 17 on the chromatogram) contained visible bands. Lane 1 contained large amounts of very low MW peptides which migrated with the tracking dye; lane 3 contained a 23,000 D yellow (and therefore nearly invisible) protein; lane 10 contained a high MW ( 43,000 D) protein; lanes 11, 12 and 13

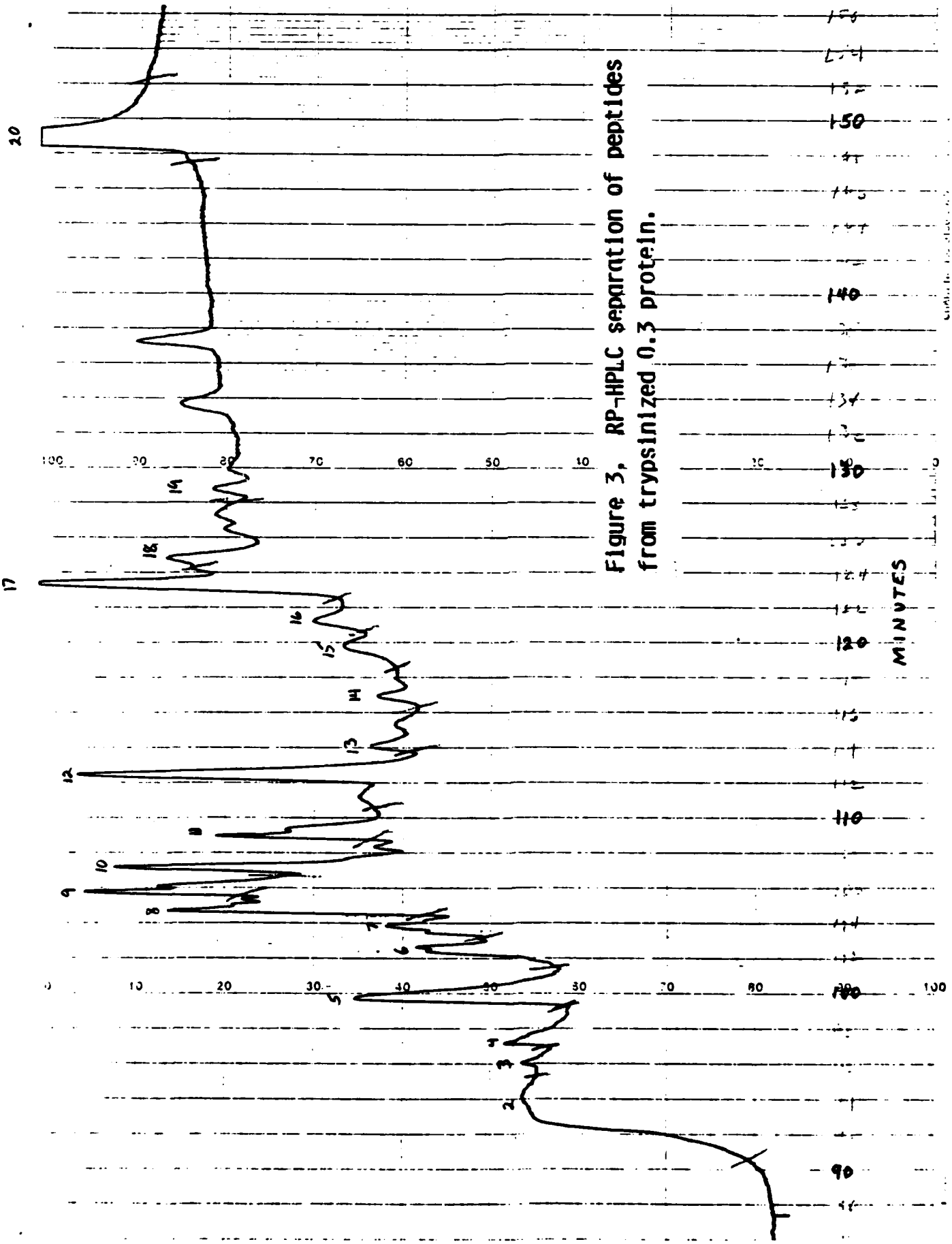



Figure 3, RP-HPLC separation of peptides from trypsinized 0.3 protein.

Figure 3. RP-HPLC separation of peptides from trypsinized 0.3 protein. Succinylated, trypsinized 0.3 protein (40$\mu$g) was chromatographed on a Bio-Rad RP-318 column. Buffer A was 0.1% trifluoroacetic acid (TFA) in water; buffer B was acetonitrile containing 0.1% TFA. The gradient was 0-100% B in 100 min, flow rate was 0.25 ml/min, range was 0.2, and absorbance was at 220 nm.

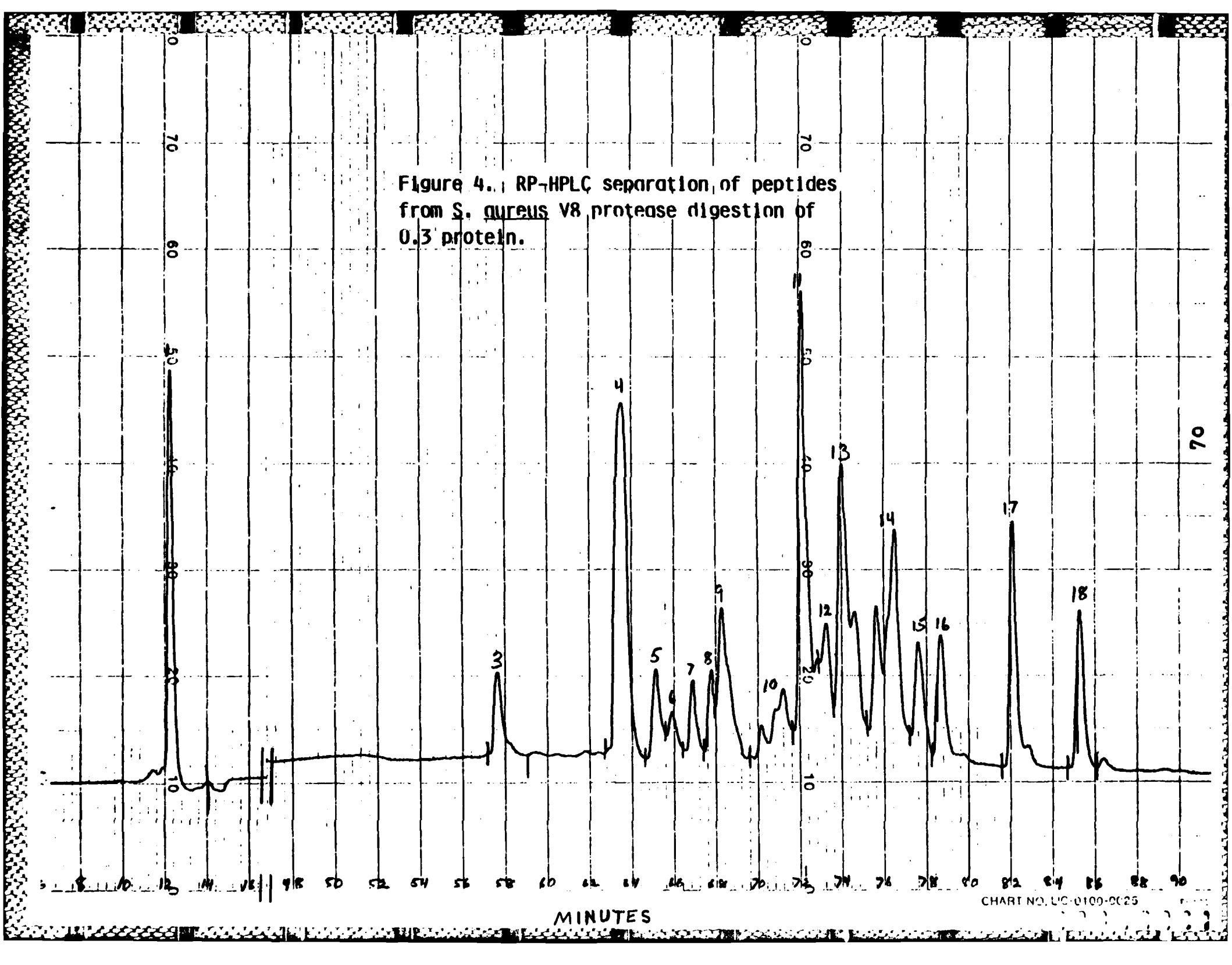
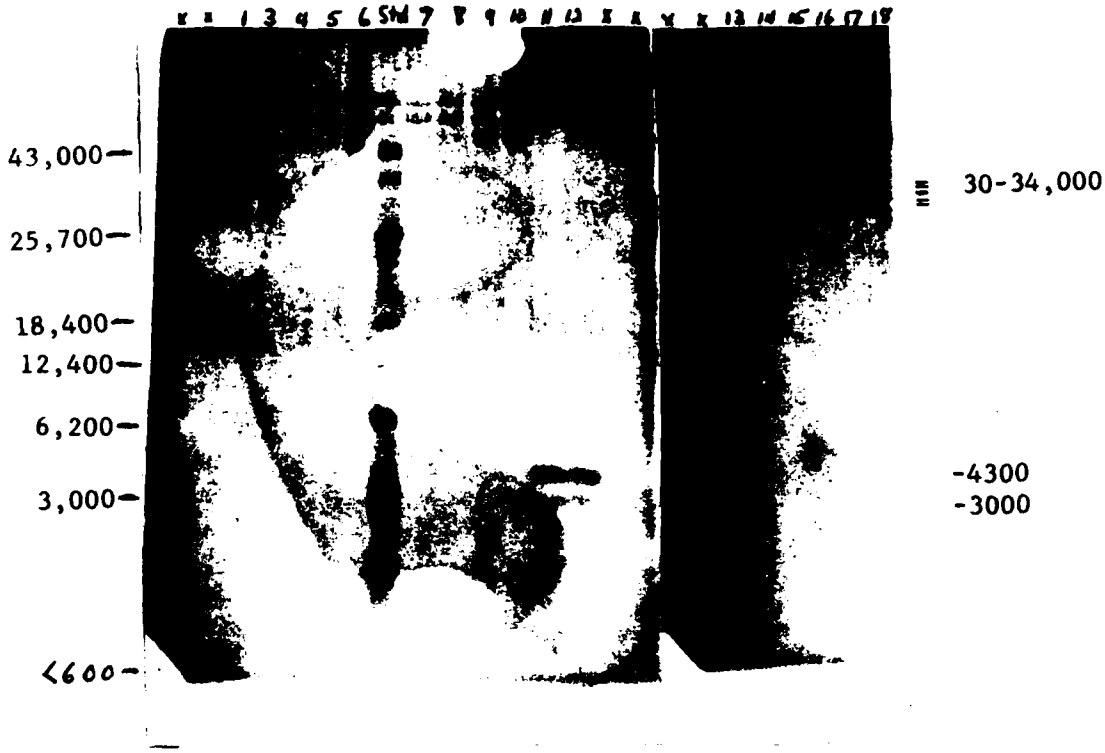


Figure 4.. RP-HPLC separation of peptides from S. aureus V8 protease digestion of 0.3 protein.
MINUTES
CHART NO. LC-0100-0025

Figure 4. RP-HPLC separation of peptides from S. aureus V8 protease digestion of 0.3 protein. Succinylated, protease-treated 0.3 protein (250 $\mu$g) was chromatographed on a Bio-Rad RP318 column. Conditions were the same as for figure 3, except that monitoring was done at 277 nm.

Figure 5. SDS-PAGE (13-22% acrylamide) of RP-HPLC fractions of S. aureus V8 protease-digested 0.3 protein.




The lanes numbered 1 through 18 correspond to the RP-HPLC fractions shown on Figure 4. The collected fractions were dried before sample buffer was added. Lane 1 contains large amounts of very low MW peptides which migrated with the tracking dye; lane 3 contains a ∿23,000 D yellow protein; lane 10 contains a high MW (> 43,000 D) protein; lanes 11, 12, and 13 contain decreasing amounts of the predominant 4300 D peptide plus another peptide ( ∿3000 D); lanes 14, 15, 16, and 17 contain decreasing amounts of the high MW (30-34,000) proteins.

contained decreasing quantities of the predominant 4300 D peptide plus another peptide at 3000 D; and lanes 14, 15, 16, 17 contained decreasing quantities of the high MW (30-34,000) proteins. Figure 6 shows the M.W.s of the peptides expected from cleavage by S. aureus protease.

Although we cannot separate the 4300 and 3000 D peptides by RP-HPLC, recovery is much better than by preparative gel electrophoresis. In addition, the 4300 D peptide represents at least 80% of the total protein in the fractions 11-13, and may therefore still be useful for sequencing. Separation of S. aureus V8 protease-cleaved 0.3 protein peptides by RP-HPLC is, therefore, the preferred method for purification of an internal peptide for sequencing studies. If sequencing is impossible because of the contaminating peptide, we will use preparative gel electrophoresis to purify the 4300 D peptide.

Figure 6. Peptides Expected from Cleavage of 0.3 Protein with S. aureus V8 Protease*

| Number | Residues from N-terminus | Molecular Weight |
|---|---|---|
| 1 | 1-16 | 1900 |
| 2 | 17-20 | 570 |
| 3 | 21-59 | 4300 |
| 4 | 60-64 | 530 |
| 5 | 65-66 | 300 |
| 6 | 67-87 | 2300 |
| 7 | 88-95 | 1000 |
| 8 | 96-98 | 320 |
| 9 | 99-103 | 580 |
| 10 | 104-106 | 400 |
| 11 | 107 | 130 |
| 12 | 108-109 | 230 |
| 13 | 110 | 130 |
| 14 | 111-112 | 290 |
| 15 | 113 | 130 |
| 16 | 114-115 | 240 |
| 17 | 116 | 130 |

* S. aureus V8 protease cleaves peptide bonds on the COOH-terminal side of only glutamic acid residues when the buffer used is ammonium bicarbonate, pH 7.8 (Houmard, J. and Drapeau, G.R., PNAS, 69:3506-3509, 1972).

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B.Sc. (1st Class Honors) University of Birmingham, England (1968). Major Field: Bacteriology
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Postdoctoral Training, Department of Microbiology, University of Arizona, Tucson, AZ. 1971-73.
Postdoctoral Training, Department of Microbiology, National Institute for Medical Research, Mill Hill, London, England, 1973-74.
Instructor in EMBO Advanced Techniques Course (Plasmids) Erlangen, West Germany, 1979.
Research Group Director, Max Planck Institut fur Moleculare Genetik, 1 Berlin 33, Germany. 1974-1979.
Adjunct Professor, University of Maryland, Extension Service, U.S. Armed Forces Teaching Program in Europe. 1976-79.
Associate Professor of Microbiology, Ohio State University, 1979-81.
Professor of Molecular, Cellular and Developmental Biology, 1981-present.
Professor of Microbiology, Ohio State University, 1981-present.
Chairman of the Department of Microbiology, Ohio State University, 1985-present.

HONORS

Commonwealth Universities Predoctoral Scholarship, 1968-1971.
NATO Postdoctoral Fellowship, 1971-1973.
EMBO Postdoctoral Fellowship, 1974.
NIH Research Career Development Award, 1980-85.
Distinguished Visiting Research Professorship, University of Adelaide, Adelaide, Australia, 1982.
Invited Judge, 1984 International Science Fair.
Invited Professor, Gray Freshwater Research Institute Summer Course, University of Minnesota 1985.

RESEARCH AREAS

Molecular genetics of Archaebacteria (methanogens).
Structure and regulation of genes responsible for methane biogenesis.
Fidelity of gene expression following gene cloning and introduction of recombinant DNA into foreign host cells.

RESEARCH GRANTS SINCE JOINING O.S.U. (1979).

P.I. on the following competitive grants:
National Institute for Health on Aging; 7/79-6/82. $153,000. Protein stability

and aging in anucleate cells.

National Science Foundation. 11/79 - 10/82 $160,000. Development and expression of bacterial viruses in anucleate cells.

National Institute for Health on Aging 12/80-11/85: $200,000. Enzyme aging and gene expression in anucleate cells.

Air Force Office of Scientific Research, 7/81-8/85; $355,000. Development of an in vivo assay for mistranslation-inducing activity of pollutants and characterization of amino-acid substitutions.

Department of Energy, 7/81-6/87; $395,000. Characterization of the organization of the genome of methanogens and development of genetic exchange systems for Methanococcus vannielii.

Environmental Protection Agency, 9/82-8/85; $142,000. Application of recombinant DNA technology to methane biogenesis.

Gas Research Institute, 4/84-3/86; $96,000. Construction of plasmids capable of replication in methanogens and in E. coli.

NATO Scientific Affairs Division 6/85-5/86; $3,200 Promoter structure and in vitro transcription of methanogen genes. Total Award $3,200.

PROFESSIONAL SERVICE

1) Current Panel Member for:
National Science Foundation
Genetic/Biology Program (1984-85 Panel Meetings)
(1985-86 Panel Meetings)

National Science Foundation
Selection of NATO Postdoctoral Fellows

Department of Defense
Chemical Research and Defense Command
Biotechnology Program Evaluation

Department of Energy
Biological Energy Research
Anaerobic Metabolism and Genetics Program Evaluation

Gas Research Institute
Biochemistry Program
C1 Metabolism, Methylotrophy Program Evaluation

2) Reviewer of Research Proposals Submitted to:
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Deutscheforschungs gemeinschaft (DFG)

3) Reviewer of Research Manuscripts Submitted to:
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Journal of Bacteriology
Journal of Virology
Molecular and General Genetics
European J. Biochemistry
Journal of Biological Chemistry
Current Microbiology

CONSULTANT ACTIVITIES

Battelle Memorial Research Institute.
Consultant to Research Management for evaluation of research and research planning in molecular biology and biotechnology.

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Addison-Wesley Publishing Co.
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UNIVERSITY ADMINISTRATIVE SERVICE

UNIVERSITY-WIDE COMMITTEES

Chairman Committee to evaluate and recommend plans for Biotechnology at O.S.U. Author of the Committee Report (submitted 1983). Activity required meeting with faculty from all Colleges of the University interested in biotechnology, co-ordinating this input and drafting an evaluation and recommendations for action by the Provost. Acceptance of the report has lead to the current search for a Director of Biotechnology.

Member Search Committee for Director of the University Biotechnology Center.

Member Committee to evaluate and make recommendations on University Research and its Relationship to External Sponsorship. An 18 month, in depth, evaluation of how O.S.U. obtains and responds to sponsorship of research was recently completed. An extensive written report (part authorship) was submitted to the President including recommendations for changes of policies related to patent issues, conflicts of interest and mechanisms of negotiation with potential sponsors. Action on these issues is currently in progress.

Member Committee to evaluate research proposals and facilities which may constitute potential Biohazards. Monitoring of experiments involving recombinant DNA.

Member University Promotion and Tenure Committee. Responsible for evaluation of all promotion and tenure cases from all Colleges. One of only six faculty members selected. Representative for all of Liberal Arts and Sciences.

COLLEGE OF BIOLOGICAL SCIENCES COMMITTEES

Chairman Research equipment/facilities committee. Responsible for determining major equipment needs of faculty in the College, co-ordinating multi-user purchases and negotiating with manufactures for multiple order discounts and service agreements. Committee collects and evaluates faculty equipment requests from faculty in all Departments in the College and makes an annual report (with priority recommendation) to the Dean. Chairs open College-wide-meetings to discuss purchases of very major equipment ($250K).

Member Committee to select Distinguished Visiting Research Professors and recommend recipients for Honorary Degrees. The College has funds for two or three Visiting Research Professorships each year. Candidates for these positions are solicited from the College Faculty, applications evaluated and recommendations made to the Dean.

Member College Promotion and Tenure Committee

Member Approximately ten Search Committees for College Faculty in the Departments of Biochemistry, Genetics and Microbiology.

Member Ad hoc committees on Graduate Student Programs, Seminar Programs, Departmental facilities, etc.

DEPARTMENT OF MICROBIOLOGY

Chairperson of Department.

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1. Reeve, J.N., D.H. Groves and D.J. Clark. 1970. Regulation of cell division of Escherichia coli. Characterization of temperature-sensitive division of mutants. J. Bacteriol. 104:1052-1064.

2. Reeve, J.N., R.A. Weigand and J.W. Greenwalt. 1971. Presence of bacteriophage-like inhibitory particles in Escherichia coli. J. Bacteriol. 107:926-928.

3. Reeve, J.N. and D.J. Clark. 1972. Cell division of Escherichia coli BUG-6: Effect of varying the growth period at the non-permissive temperature. J. Bacteriol. 110:117-121.

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5. Reeve, J.N., N.H. Mendelson and R.M. Cole. 197 Cell morphology of Bacillus subtilis: The effect of genetic background on the expression of a rod gene. Molec. Gen. Genet. 119:11-26.

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16. Mertens, G. and J.N. Reeve. 1977. Synthesis of cell envelope components by anucleate cells (minicells) of Bacillus subtilis. J. Bacteriol. 129:1198-1207.

17. Reeve, J.N. 1977. Mucopeptide biosynthesis by minicells of Escherichia coli. J. Bacteriol. 131:363-365.

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19. Reeve, J.N. 1977. A general method for the "in vivo" analysis of bacteriophage encoded products. Molec. Gen. Genetics 158:73-79.

20. Reeve, J.N., G. Mertens and E. Amann. 1978. Early development of bacteriophages SP01 and SP82G in minicells of Bacillus subtilis. J. Mol. Biol. 120:183-207.

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22. Magazin, M., J.N. Reeve, S. Maynard-Smith and N. Symonds. 1978. Bacteriophage Mu encoded polypeptides synthesized in infected minicells. FEMS Lett. 4:5-9.

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53. Hamilton, P.T. and Reeve, J.N. 1985. Sequence divergence of an archaebacterial gene cloned from a mesophilic and a thermophilic methanogen. J. Mol. Evol. Submitted for Publication 6/85.

54. Boeckh, C., Bade, E. G., Delius, H. and Reeve J.N. Inhibition of bacterial segregation by early functions of phage Mu is associated with the insertion of replication protein B into the inner cell membrane. Mol. Gen. Genet. Submitted for Publication 6/85.

# CURRICULUM VITAE

NAME: Jacqueline Bowers Rice

PII Redacted [redacted]

EDUCATION:

B.A. University of Wisconsin-Milwaukee, 1960.

Registered Medical Technologist, ASCP, 1961
Milwaukee Lutheran Hospital Internship, 1960-1961.

Graduate Program, Department of Medical Microbiology, 1967-1968, Marquette University, Milwaukee, Wisconsin.

Graduate Program in Molecular Biology, Department of Biology, Marquette University, Milwaukee, Wisconsin, 1968-1970.

Graduate Program, Department of Medical Microbiology, Ohio State University, Columbus, Ohio, 1972-1977.

Doctor of Philosophy: Research was directed by Dr. Raymond Lang whose work has involved several transplantation models.

Dissertation Title: Extraction of xenotransplantation antigens and their use in xenograft prolongation and studies of xenograft rejection.

TEACHING EXPERIENCE:

Medical Student Independent Study Program, Ohio State University, 1975. (Conducted laboratory exercises in medical microbiology and infectious diseases.)

Laboratory Instructor for the medical student Medical Microbiology course (MM 625), Ohio State University, 1973.

Teaching Assistant (Genetics), Department of Biology, Marquette University, 1969.

APPOINTMENTS:

Research Associate II, Ohio State University, Department of Microbiology, College of Biological Sciences, 1981-present.

Research Associate I, Ohio State University, Department of Veterinary Pathobiology, College of Veterinary Medicine, 1977-1981.

Graduate Research Associate, Ohio State University, Department of Medical Microbiology, 1973-1975.

Medical Technologist, Mount Carmel Hospital, Columbus, Ohio, 1971.

Medical Technologist, Deaconess Hospital, Milwaukee, Wisconsin, 1970.

NIH Traineeship in Developmental Biology, 1968-1970.

NDEA Title IV Fellowship (NIH), 1967-1968.

Medical Technologist, Milwaukee County Hospital, Milwaukee, Wisconsin, 1964-1967.

Medical Technologist, San Diego County Hospital, San Diego, California, 1961-1963.

HONORS AND AWARDS:

Science Honorary Fraternity (Delta Chi Sigma), University of Wisconsin-Milwaukee, 1958-1960.

Graduated With Honors, University of Wisconsin-Milwaukee, 1960.

NDEA Title IV Fellowship (NIH), Marquette University, 1967-1968.

NIH Traineeship in Developmental Biology, 1968-1970.

GRANTS AWARDED:

USPHS, NIH, I-RO1-CA30338-01, FeLV leukemogenesis and preneoplastic lesions, co-investigator, 3 yrs., $180,895 total, 11/81-10/84. (I wrote the entire grant proposal. Richard G. Olsen is listed as P.I. because of University regulations.)

The Ohio State Canine Research Funds, "Immunoprevention of Parvovirus-Induced Diarrhea of the Dog", 2 yrs., $20,000/yr., 1981-1982. (I wrote the entire grant proposal. Richard G. Olsen and G. Stephen Krakowka are listed as P.I.'s because of University regulations.)

PUBLICATIONS:


Rice, J.B.: Extraction of xenotransplantation antigens and their use in xenograft prolongation and studies of xenograft rejection. Ph.D. dissertation, 1977.

Rice, J.B. and Lang, R.W.: Solubilization and partial characterization of rat lymphocyte antigen. American Society for Microbiology, abstract, 1976.

Rice, J.B., Schaller, J.P., and Olsen, R.G.: FOCMA expression in preneoplastic FeLV-induced lesions. American Society for Cancer Research, abstract, 1980.

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Rice, J.B. Libby, R. T., & Reeve, J.N.: Mistranslation of the mRNA encoding bacteriophage T7 0.3 protein, J. Biol. Chem., 259: 6505-6510, 1984.

Rice, J.B. and Reeve, J.N.: A study of mistranslation using T7 0.3 protein. American Society for Microbiology abstract, 1985.

Rice, J.B. and Reeve, J.N.: Specific sites of cysteine misincorporation in mistranslated T7 0.3 protein. (In preparation)

Rice, J.B., Beachey, E., Seyer, J., & Reeve, J.N.: Streptomycin induces novel misreading errors in vivo. (In preparation)