Middaugh, C. R., Gerber-Jenson, B., Hurvitz, A., Paluszek, A., Scheffel, C., & Litman, G. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3440.
Moffat, K., & Gibson, Q. H. (1974) *Biochem. Biophys. Res. Commun. 61*, 237.
Oosawa, F., & Asakura, S. (1975) *Thermodynamics of the Polymerization of Protein*, Academic Press, New York.
Podell, D. N., & Abraham, G. N. (1978) *Biochem. Biophys. Res. Commun. 81*, 176.
Pruzanski, W., Jancelewicz, Z., & Underdown, B. (1973) *Clin. Exp. Immunol. 15*, 181.
Saha, A., Edwards, M. A., Sargent, A. U., & Rose, B. (1968) *Immunochemistry 5*, 341.
Saluk, P. H., & Clem, W. (1975) *Immunochemistry 12*, 29.
Scoville, C. D., & Turner, D. H. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, Abstr. no. 3193.
Steiner, L. A., & Blumberg, P. M. (1971) *Biochemistry 10*, 4725.
Williams, R. C., Jr. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1506.
Yamashiro, D., Rigbi, M., Bewley, T. A., & Li, C. H. (1975) *Int. J. Pept. Protein Res. 7*, 389.

Purification and Properties of a Translation Inhibitor from Wheat Germ†

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ABSTRACT: A translation inhibitor from wheat germ has been purified more than 400-fold to apparent homogeneity. The inhibitor is a basic protein with a molecular weight of 30000. This protein effectively blocks protein synthesis in animal cell-free extracts but does not affect protein synthesis in intact cells. Inhibition occurs at a ribosome to inhibitor molar ratio of 100:1, indicating an enzymic mechanism of action. The wheat germ protein inhibits the translation of endogenous mRNA, exogenous mRNA, and poly(uridylic acid) at a step in polypeptide chain elongation and without breakdown of the polysomes. Neither the aminoacylation reaction nor mRNA degradation is affected by the inhibitor. An interesting feature of the inhibition reaction is that it requires, in addition to the wheat germ inhibitor, both ATP and tRNA. The function of these two compounds in the inhibition is presently unknown since neither the hydrolysis of the β,γ-pyrophosphate bond of ATP nor a modification of the tRNA can be demonstrated during the reaction.

Cell-free extracts from animal cells and plant embryos have become useful systems for carrying out the translation of isolated mRNAs and for studying possible mechanisms involved in the regulation of protein synthesis (Lodish, 1976; Busch et al., 1976). These extracts prepared from different eucaryotic cell types are generally regarded as being qualitatively similar with respect to the components of their protein-synthesizing machinery. However, certain components, such as initiation factors and isoaccepting tRNA species, do vary quantitatively, and perhaps qualitatively, between species. These variations could explain the observation that a given mRNA may be translated with unequal efficiency and fidelity in different cell-free systems. For example, EMC[1] viral RNA is translated more efficiently in an ascites cell-free system than in a wheat germ system, whereas the reverse is true for many animal cell mRNAs.

An interest in this difference between the wheat germ and ascites cell-free systems prompted us to do mixing experiments between the two systems. These experiments failed because very small amounts of components from the active wheat germ system completely inhibited protein synthesis in the ascites cell extracts. We found that wheat germ extract stopped the translation of ascites cell mRNA in the ascites cell system at a concentration less than 0.1% of that present during the translation of the same mRNA in the wheat germ system (Stewart et al., 1977). This suggested the presence in the wheat germ extract of an inhibitor with an enzymic mode of action and precise species or kingdom specificity. We felt that such an inhibitor could be useful as a reagent for studying the molecular mechanisms of protein synthesis, as a probe for differences in the translational machinery of eucaryotes, and as a means of identifying a reaction that might prove to be a general mechanism for regulating protein synthesis.

In an earlier report, we presented evidence indicating that the wheat germ inhibitor is a protein and that it acts at a step in polypeptide chain elongation (Stewart et all., 1977). Here, we describe the purification of the inhibitor to apparent homogeneity. Also, we present additional evidence concerning the requirements for inhibitor activity as well as the inhibitor's mechanism of action.

Experimental Procedures

Preparation of Extracts. Wheat germ was obtained fresh from a local mill and stored at 4 °C. A cell-free protein-synthesizing system was prepared from this material according to the procedure of Marcu & Dudock (1975) except that KCl was replaced with potassium acetate in the buffers. This extract was found to efficiently translate poly(A)-containing RNA from ascites cells, but to inhibit, at low concentrations,

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[1] Abbreviations used: EMC virus, encephalomyocarditis virus; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; poly(U), poly(uridylic acid); $NaDodSO_4$, sodium dodecyl sulfate; AMP-PCP, adenylyl (β,γ-methylene)diphosphonate.

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translation of the same mRNA in an ascites cell-free system.

Protein synthesis was normally carried out in S10 extracts prepared from Ehrlich ascites cells (Stewart et al., 1977). Endogenous protein synthesis in these extracts was measured as the routine assay for the wheat germ inhibitor. For the translation of exogenous mRNAs, the extracts were preincubated to reduce the extent of endogenous protein synthesis. The conditions used for the preincubation and various translations have been described (Stewart et al., 1977).

Wheat Germ Inhibitor Assay. Endogenous protein synthesis was measured in 25-μL samples containing 5 μL of S10 extract. The samples were incubated at 30 °C for 90 min, 10-μL aliquots were removed and spotted on filter paper, and the incorporation of ^{14}C-labeled amino acids into hot acid-insoluble material was determined (Kerr et al., 1976). To assay the inhibitor, 2-μL portions of various dilutions of an inhibitor-containing sample were added to parallel tubes at 0 °C containing the above protein synthesis mixture. Incubation at 30 °C was then begun and the effect of the addition on endogenous protein synthesis was measured (Stewart et al., 1977). One unit of wheat germ inhibitor is defined as the amount of wheat germ extract which will inhibit translation in the standard 25-μL reaction mixture by 50%.

Protein Assay. Protein concentrations were determined according to the method of Bradford (1976) by using the Bio-Rad Protein Assay Kit with bovine γ-globulin as the protein standard.

Purification of the Wheat Germ Inhibitor. (i) S100 Fraction. An extract was prepared from 60 g of wheat germ as outlined above. This extract was centrifuged at 100000*g* for 2 h to remove ribosomes and the supernatant fluid dialyzed for 12 h at 4 °C against two 1000-mL changes of 20 mM KCl, 20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM dithiothreitol. The extract was then centrifuged at 10000*g* for 20 min to remove turbid material and the supernatant fluid (68 mL) used to begin the purification.

All steps in the purification were carried out at 4 °C.

(ii) DEAE-cellulose Column Chromatography. The S100 fraction was passed into a DEAE-cellulose column (25 × 4.5 cm) equilibrated with the dialysis buffer used in step i above. Most of the protein in the S100 fraction was bound to the column, whereas the inhibitor passed through the column and appeared in the break-through volume. The effluent was collected in 20-mL fractions, and those fractions which had a faint yellow color were found to contain the remaining protein and virtually all of the inhibitor activity; these fractions were combined (100 mL, fraction ii).

(iii) Ammonium Sulfate Precipitation. Solid $(NH_4)_2SO_4$ was added over 30 min with stirring to fraction ii. The precipitate which had formed at 55% saturation was removed by centrifugation and additional solid $(NH_4)_2SO_4$ was added to 85% saturation. The precipitate that formed contained the inhibitor, and it was dissolved in 20 mL of 0.1 M KCl, 0.02 M Hepes (pH 7.6), and dialyzed against two 500-mL portions of the same buffer (fraction iii).

(iv) Phosphocellulose Column Chromatography. Fraction iii was added to a 60-mL phosphocellulose column which had been washed and equilibrated with fraction iii buffer. Fraction iii was washed through the column with an additional 120 mL of the buffer, resulting in most of the protein appearing in the effluent but the inhibitor remaining bound to the column. The inhibitor could then be eluted from the column by a shallow gradient of buffered 0.2 M KCl passing into buffered 0.1 M KCl (Figure 2). In practice, inhibitor with virtually the same specific activity could be obtained by eluting the inhibitor with 200 mL of 0.15 M KCl, 0.02 M Hepes (pH 7.6). The peak fractions containing the inhibitor (e.g., fractions 7 and 8 in Figure 1) were combined and used for the final purification step (38 mL, fraction iv).

(v) Gel Filtration on Sephadex G-75. Fraction iv was concentrated to 3 mL by ultrafiltration through a Diaflo PM10 membrane. This concentrate was added to a Sephadex G-75 column (2 × 30 cm) which had been equilibrated with 0.1 M KCl, 0.02 M Hepes (pH 7.6). Elution was carried out by using the same buffer with 3-mL fractions collected. Fractions containing peak inhibitor activity were combined (15 mL), and aliquot portions were stored at −70 °C for use in all subsequent experiments.

Polyphenylalanine Synthesis Assay. Polyphenylalanine synthesis was carried out in reactions containing sucrose-washed ribosomes at a concentration of 12 absorbancy units (260 nm) per mL as the source of ribosomes, elongation factors, and phenylalanyl-tRNA synthetase (Roberts & Coleman, 1971). In addition to sucrose-washed ribosomes, the reaction mixtures contained 100 mM KCl, 20 mM Hepes (pH 7.6), 4.0 mM $Mg(OAc)_2$, 0.3 mM spermidine, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 160 μg/mL creatine phosphokinase, 7 mM mercaptoethanol, 100 μg/mL of unfractionated yeast tRNA or 50 μg/mL of purified yeast $tRNA_{Phe}$, 0.04 μCi/mL of L-[4-^{3}H]phenylalanine (19 Ci/mmol), and 40 μg/mL of poly(U). Samples (25 μL) were incubated at 30 °C for the times indicated, and the Cl_3CCOOH-insoluble radioactivity in 10-μL aliquots was determined as before (Kerr et al., 1976; Stewart et al., 1977).

The effect of the wheat germ inhibitor on polyphenylalanine synthesis was usually studied by preincubating the inhibitor with a ribosome-containing mixture at 30 °C for 20 min prior to the initiation of polyphenylalanine synthesis. For the preincubation step, the reaction mixture was as usual except that [^{3}H]phenylalanine and poly(U) were omitted and the reaction was carried out at 60 mM KCl, 20 mM Hepes (pH 7.6), 1.5 mM $Mg(OAc)_2$, and 0.15 mM spermidine. Following preincubation, the samples were chilled, a mixture containing [^{3}H]phenylalanine, poly(U), and supplemental salts added, and polyphenylalanine synthesis initiated. During the investigation of the effects of ATP and tRNA on the inhibition, the concentration of ATP or tRNA was varied in the preincubation mixture as indicated, and then added to the usual concentration in the chilled samples just before the initiation of translation.

Phenylalanyl-tRNA Synthesis Assay. This assay was performed by using the same procedure as the polyphenylalanine synthesis assay except that poly(U) was omitted from the reaction mixture, and the reaction mixtures were spotted on filter paper squares that had been previously wetted with a solution containing 5 mg/mL of nonradioactive phenylalanine and dried. Also, the filter paper squares containing the Cl_3CCOOH-insoluble material were not washed in 5% Cl_3CCOOH at 95 °C for 20 min, as was true for the translation assays, but rather washed an additional two times at 0 °C with 5% Cl_3CCOOH. Control samples lost over 90% of their radioactivity if they were washed in hot Cl_3CCOOH or if $tRNA_{Phe}$ were omitted from the reaction. This indicates that the assay is in fact measuring formation of the acid-labile phenylalanyl-tRNA bond.

Purification of AMP-PCP. The ATP analogue AMP-PCP was purchased from Boehringer Mannheim. Although there was no evidence that this compound contained any impurities, the fact that it was 5% as efficient as ATP in promoting the inhibition of translation (Figure 6) raised the possibility that

Table I: Purification of the Wheat Germ Translational Inhibitor[a]

fraction	sample vol (mL)	inhibitor concn (units/mL)	protein concn (mg/mL)	% inhibitor units recovered	sp. act. (units/mg of protein)
(i) S100	68	50 000	39	100	1 300
(ii) DEAE-cellulose	100	32 000	3.7	94	8 600
(iii) $(NH_4)_2SO_4$	21	125 000	5.4	77	23 000
(iv) phosphocellulose	38	40 000	0.15	45	270 000
(v) Sephadex	15	64 000	0.11	28	580 000

[a] Details of the purification are given in the Experimental Procedures section.

this activity was due to a small ATP contamination. Therefore, AMP-PCP was purified by using paper chromatographic systems (Paladini & Leloir, 1952) that separated the analogue from ATP. The systems and relative R_f's were: ethanol–1 M ammonium acetate (pH 7.5) (75:30), ATP 1.0, AMP-PCP 0.75; ethanol–1 M ammonium acetate (pH 4.7) (75:30), ATP 1.0, AMP-PCP 1.2. Chromatography was on Whatman 3MM paper (descending) at 22 °C for 4 days. Strips containing the AMP-PCP were cut out and extracted three times with ethanol to remove residual ammonium acetate, and the AMP-PCP was eluted and used in the inhibition reactions.

Results

Specificity of the Inhibition. Earlier work on the wheat germ inhibitor showed that the inhibitor blocked the translation of both endogenous and exogenous mRNA in a mouse ascites cell-free system (Stewart et al., 1977). The possibility existed that this inhibition was somehow unique to the ascites system. We have now found that low concentrations of wheat germ inhibitor also prevent the translation of endogenous mRNA in cell-free extracts from mouse brain and rabbit reticulocytes (results not shown). The wheat germ protein, therefore, appears to be a general inhibitor of protein synthesis in animal cell extracts.

Purification of the Inhibitor. Table I summarizes the results from a procedure used to purify the wheat germ inhibitor more than 400-fold. The details of this procedure are described in the Experimental Procedures section. The purification is achieved primarily because, unlike most proteins, the inhibitor passes through a DEAE-cellulose column at a relatively low ionic strength and binds to phosphocellulose at a relatively high ionic strength (Figure 1). These unusual properties indicate that the inhibitor is a basic protein.

Each of the five purification steps summarized in Table I was monitored by analyzing the inhibitor-containing fraction by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). The purification procedure yielded an apparently homogeneous inhibitor with a molecular weight of 30 000. This molecular weight was confirmed by sucrose gradient centrifugation and Sephadex G-75 chromatography of the inhibitor, together with marker proteins, followed by assays to locate the inhibitor activity (results not shown).

By knowing the specific activity of the purified inhibitor and the ribosome concentration in the standard assay, it can be calculated that one inhibitor molecule is sufficient to inhibit approximately 100 ribosomes. This strongly suggests an enzymic mechanism of inhibition.

Effect of the Wheat Germ Inhibitor on Polysome Size Distribution. Inhibitors of polypeptide chain elongation can be divided into two main classes depending upon their effect on polysomes. One class, typified by puromycin and nucleases, causes a breakdown of the polysomes. The other class, of which emetine is an example, "freezes" the ribosome on the mRNA and no change in polysome size distribution is seen following inhibition.

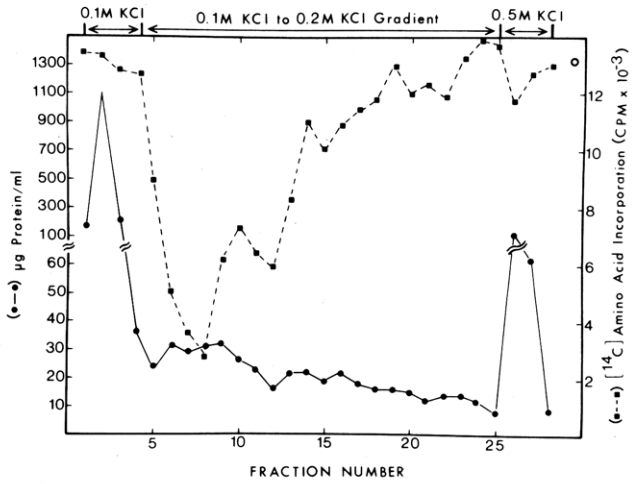

FIGURE 1: Phosphocellulose chromatography of the wheat germ inhibitor. A portion of fraction iii (14 mg of protein in 3 mL) was added to a 0.9 × 15 cm phosphocellulose column in 0.1 M KCl–0.02 M Hepes (pH 7.6). Elution was with buffered KCl as indicated with a 5-mL fraction collected. The gradient was linear with 50 mL of 0.2 M KCl passing into 50 mL of 0.1 M KCl. For inhibitor assays, aliquots from each fraction were diluted 1:5, 2-μL samples of each were added to 25-μL protein synthesis reactions, and amino acid incorporation was measured. O represents the control reaction without addition of a column sample.

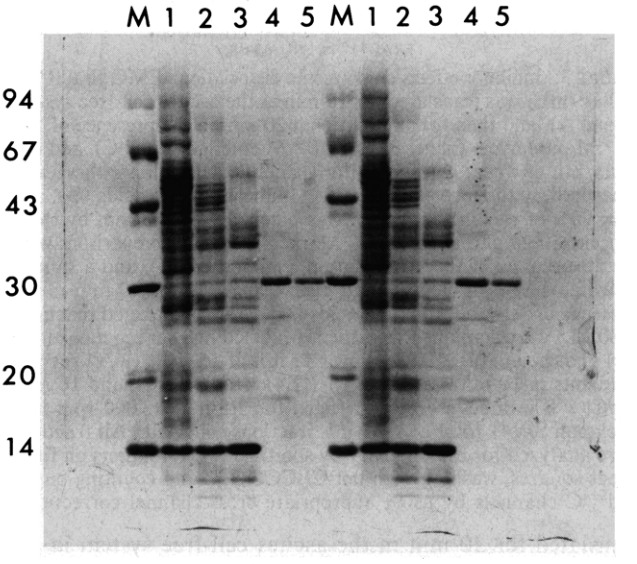

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified wheat germ inhibitor fractions. Fractions were analyzed in duplicate. Samples 1–5 correspond to fractions i–v shown in Table I. M refers to marker proteins from an electrophoresis calibration kit from Pharmacia; molecular weights are 94 000, 67 000, 43 000, 30 000, 20 100, and 14 400 as indicated. Samples (1–5 μL) from each fraction were analyzed by electrophoresis on a 12% polyacrylamide slab gel containing 0.1% $NaDodSO_4$ according to the procedure of Laemmli (1970).

Accordingly, the wheat germ inhibitor was investigated with respect to its effect on polysomes. EMC viral RNA was

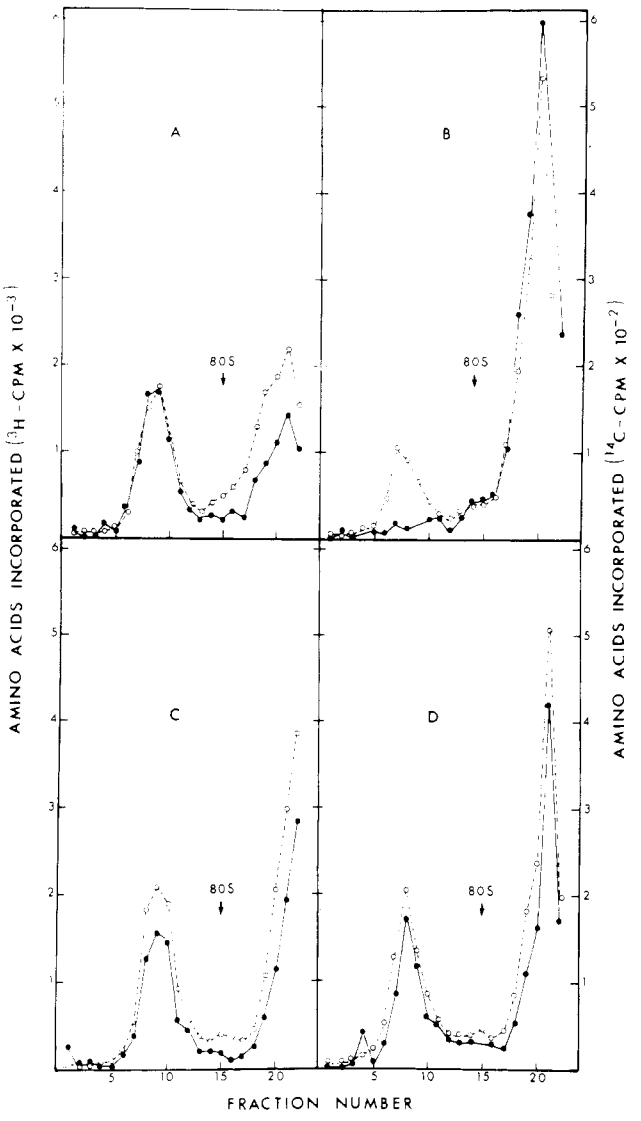

FIGURE 3: Inhibitor effects on polysome dissociation. EMC viral RNA (20 μg/mL) was translated for 20 min in the ascites cell-free system (panel A), and then for an additional 20 min in the presence of 5×10^{-4} M puromycin (panel B), 1×10^{-4} M emetine (panel C), and 400 units/mL of wheat germ inhibitor (panel D). Protein synthesis was measured by the incorporation of ^{14}C-labeled amino acids (●), and over 90% of this incorporation was blocked within 5 min by these concentrations of the inhibitors. Markers for panel comparisons were the absorbancy of the 80 S ribosome monomers (↓), and a sample in which EMC viral RNA had been translated for 40 min in the presence of ^{3}H-labeled amino acids (○). The ^{14}C-labeled reactions (100 μL) were combined with the ^{3}H-labeled marker reactions (100 μL) and the mixtures layered over 11 mL of 15–50% (w/v) sucrose gradients in 10 mM Tris-HCl (pH 7.4), 10 mM KCl, and 10 mM $MgCl_2$. The tubes were centrifuged for 2.5 h at 33000 rpm in a Beckman SW41 rotor and 0.5-mL fractions collected. All fractions were analyzed for radioactivity by spotting 25-μL aliquots on filter paper squares, washing with hot Cl_3CCOOH, and counting on ^{3}H and ^{14}C channels by using appropriate cross-channel corrections.

translated for 20 min in the ascites cell-free system in the presence of ^{14}C-labeled amino acids. In parallel samples, sufficient inhibitor (puromycin, emetine, or purified wheat germ inhibitor) was then added to prevent subsequent translation, and the incubations were continued for an additional 20 min. Marker ^{3}H-labeled polysomes were added to all samples and the samples analyzed by zonal centrifugation (Figure 3). Compared with the control polysomes (panel A), puromycin caused polysome breakdown (panel B), whereas emetine and the wheat germ inhibitor caused no change in the polysome profile (panels C and D). We conclude, therefore,

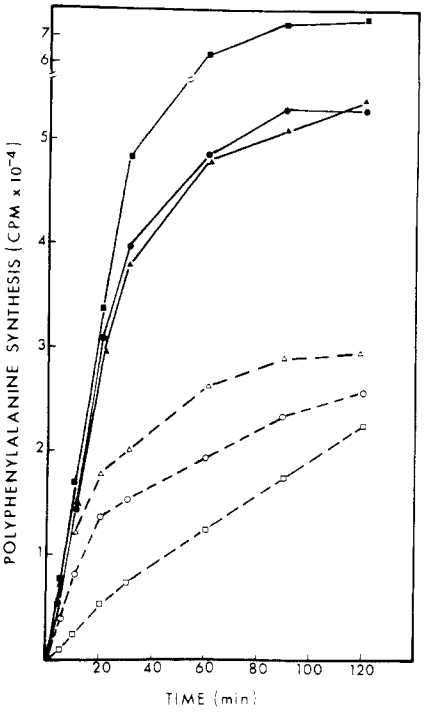

FIGURE 4: Inhibition of polyphenylalanine synthesis by the wheat germ inhibitor. Polyphenylalanine synthesis by sucrose-washed ribosomes was carried out as described in Experimental Procedures, either in the absence (closed symbols) or presence (open symbols) of 400 units/mL of wheat germ inhibitor. Inhibition was measured in samples that had been preincubated for 20 min at 30 °C (squares), preincubated for 20 min at 0 °C (circles), and nonpreincubated samples (triangles).

that the wheat germ inhibitor does not act by causing the dissociation of growing polypeptide chains or ribosomes from the mRNA, nor does it induce degradation of the mRNA.

Inhibition of Ribosome-Mediated Polyphenylalanine Synthesis. Earlier experiments concerning the effect of the wheat germ inhibitor on translation, including the inhibition of polyphenylalanine synthesis, were all carried out utilizing S10 extracts (Stewart et at., 1977). Implicit in these experiments is the possibility that the wheat germ inhibitor does not act directly to inhibit translation, but rather acts indirectly by modifying an animal cell supernatant factor which in turn mediates the inhibition. To test this possibility, and to obtain a more defined system for studying the mechanism of inhibition, the wheat germ inhibitor was tested for its effect on polyphenylalanine synthesis by sucrose-washed ribosomes. As previously reported (Roberts & Coleman, 1971), these ribosomes have bound to them sufficient phenylalanyl-tRNA synthetase, EF-1, and EF-2 to efficiently carry out polyphenylalanine synthesis without the addition of other cellular macromolecules except tRNA.

After a brief lag period, the wheat germ inhibitor reduced the rate of polyphenylalanine synthesis by sucrose-washed ribosomes (Figure 4, triangles). The inhibition of translation in this system was not as complete as the inhibition of endogenous protein synthesis, and required an approximately 20-fold higher concentration of inhibitor to achieve a 50% reduction in translation. As discussed earlier (Stewart et al., 1977), this appears to be due to the fact that the inhibitor does not act efficiently at the relatively high salt concentrations required for polyphenylalanine synthesis.

The above results suggested that the lag period might be eliminated and the inhibition of polyphenylalanine synthesis made more pronounced by preincubating the system with inhibitor at a lower ionic strength prior to initiating translation.

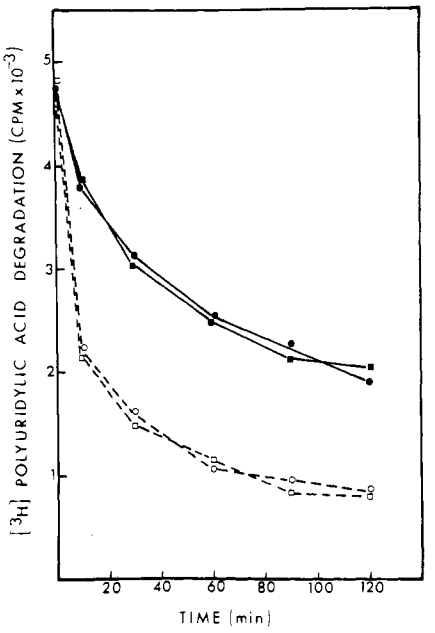

FIGURE 5: Poly(U) degradation in the presence and absence of wheat germ inhibitor. Polyphenylalanine synthesis was carried out as usual except that nonradioactive phenylalanine was used in the reaction and the inhibitor, when present, was at a concentration of 4000 units/mL. (Squares represent reactions with inhibitor present; circles represent reactions without inhibitor.) Also, polyphenylalanine synthesis was directed by [^{3}H]poly(U) (1 μCi/mL) to which nonradioactive poly(U) was added to make final concentrations of poly(U) of 30 μg/mL (closed symbols) or 70 μg/mL (open symbols). The samples (75 μL) were incubated at 30 °C, and at the times indicated 10-μL aliquots were removed, spotted on filter paper squares, washed by using the cold Cl_3CCOOH procedure, and counted to determine the remaining acid-insoluble poly(U).

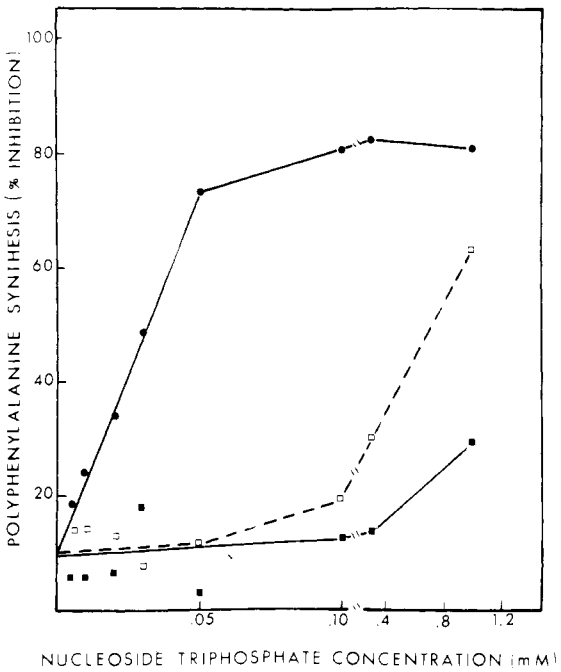

FIGURE 6: Effects of nucleoside triphosphates on inhibition. Preincubations were carried out with and without inhibitor as before (Experimental Procedures, Figure 4), except that the triphosphate mixture was replaced by the indicated concentrations of ATP (●), GTP (■), or AMP-PCP (□). Following preincubation at 30 °C for 20 min, poly(U), [^{3}H]phenylalanine, supplemental salts, and triphosphate mixture were added, and polyphenylalanine synthesis was measured after 10 min of additional incubation. Results are expressed as percent inhibition of polyphenylalanine synthesis in inhibitor-containing reactions compared with the respective controls which were preincubated with the indicated concentration of nucleoside triphosphate in the absence of inhibitor.

Accordingly, the system was preincubated at 30 °C for 20 min and then polyphenylalanine synthesis was begun by adding poly(U), [^{3}H]phenylalanine, and additional salts to the incubation mixture (Figure 4, squares). Preincubation at 30 °C in the absence of inhibitor caused a small but reproducible increase in polyphenylalanine synthesis. If inhibitor was included in the mixture during preincubation at 30 °C, a dramatic inhibition of polyphenylalanine synthesis was observed, particularly early in the reaction. This early inhibition of translation was much less pronounced if the preincubation was carried out at 0 °C instead of 30 °C (Figure 4, circles). These results are consistent with the notion that the inhibitor acts by an enzymic mechanism, rather than by a stoichiometric inhibitory binding to the target site. They also indicate that this enzymic reaction can occur during preincubation of the ribosomes with inhibitor at 30 °C in the presence of nucleoside triphosphates and tRNA, and in the absence of translation and cell supernatant factors.

Effect of the Inhibitor on the Degradation of Poly(U). The absence of polyribosome breakdown following the inhibition of protein synthesis (Figure 2) suggests that the wheat germ inhibitor does not act directly or indirectly as a nuclease in the reaction. This was confirmed by following the degradation of [^{3}H]poly(U) during polyphenylalanine synthesis by sucrose-washed ribosomes in the presence and absence of purified wheat germ inhibitor (Figure 5). No difference could be detected between the inhibited and uninhibited systems in the rate of disappearance of acid-insoluble radioactivity. The [^{3}H]poly(U) resistant to degradation probably represents that fraction of poly(U) bound to ribosomes and, thus, protected from endogenous nuclease activity (Kerr et al., 1976); this fraction would be less, in terms of radioactivity, in the samples containing the larger concentration of nonradioactive poly(U) (open symbols).

ATP Requirement for Inhibition. The effect of preincubation with inhibitor on polyphenylalanine synthesis at early reaction times (Figure 4) permits the requirements for inhibition to be studied separately from translation. Thus, when ribosomes were preincubated with wheat germ inhibitor and tRNA, but in the absence of nucleoside triphosphates, no increase in the inhibition of polyphenylalanine synthesis was observed in a 10-min reaction. A detailed analysis of this phenomenon revealed that inhibition required a low concentration of ATP in the preincubation mixture and that the ATP could not be replaced by similar concentrations of GTP or the ATP analogue AMP-PCP (Figure 6). However, higher concentrations of the analogue (1 mM) did promote inhibition by the wheat germ protein. Similar results were obtained by using the ATP analogue AMP-PNP, as well as AMP-PCP which had been purified by using paper chromatographic procedures that separate the analogue from a possible ATP contaminant (Experimental Procedures). We conclude, therefore, that ATP is required for the wheat germ inhibitor to block translation, but that hydrolysis of the β,γ-prophosphate bond of ATP probably does not occur during the inhibition reaction.

tRNA Requirement for Inhibition. The possible involvement of tRNA in the inhibition reaction was tested utilizing a preincubation procedure similar to that used above to identify the requirement for ATP. Sucrose-washed ribosomes were preincubated with nucleoside triphosphates, with and without tRNA, and with and without purified wheat germ inhibitor, and the effect of the preincubation conditions on reducing early polyphenylalanine synthesis noted. Preincubation in the

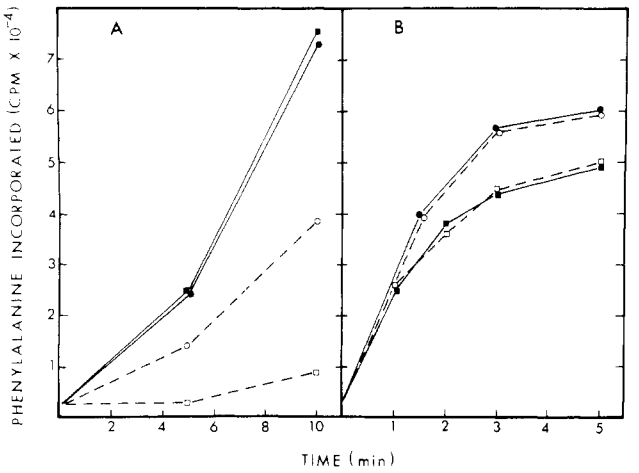

FIGURE 7: Effect of $tRNA_{Phe}$ on the inhibition of polyphenylalanine synthesis and on the lack of inhibition of aminoacylation. Samples were preincubated to augment the wheat germ protein-induced inhibition of polyphenylalanine synthesis (Figure 4), except that the preincubations were carried out in the presence (open symbols) and absence (closed symbols) of 400 units/mL of wheat germ inhibitor, and in the presence (squares) and absence (circles) of 50 μg/mL of purified yeast $tRNA_{Phe}$. Samples which were preincubated without the tRNA were supplemented with 50 μg/mL $tRNA_{Phe}$ after the preincubation step and just prior to the assay. Panel A shows phenylalanine incorporated into polyphenylalanine, whereas panel B displays incorporation into phenylalanyl-tRNA. Both assays (Experimental Procedures) were performed by using parallel samples which had been prepared and preincubated together.

absence of tRNA resulted in only a moderate inhibitor-induced reduction in translation (Figure 7, panel A, circles), similar to that observed in the absence of a preincubation step when the inhibition reaction occurs concomitantly with translation. In contrast, preincubation in the presence of inhibitor and tRNA caused a marked immediate reduction in polyphenylalanine synthesis (Figure 7, panel A, squares). Thus, tRNA appears to be required for efficient inhibition.

The simplest interpretation of these results is that tRNA itself is the target site for the wheat germ inhibitor and that it is inactivated during the preincubation. To test this possibility, preincubation reactions were run in parallel with those above and then assayed for tRNA charging instead of polyphenylalanine synthesis. The rate of formation of phenylalanyl-tRNA (Figure 7, panel B) was the same in samples in which inhibitor was present or absent, regardless of whether the $tRNA_{Phe}$ substrate was added before (squares) or after (circles) preincubation. This demonstrates that the reduction in polyphenylalanine synthesis seen in Figure 7, panel A, was not a consequence of the inhibitor affecting the aminoacylation of tRNA.

The experiment shown in Figure 7, panel A, was corroborated by using as a source of radioactive label [^{3}H]-phenylalanyl-tRNA instead of [^{3}H]phenylalanine. The synthesis of radioactive polyphenylalanine in reactions containing [^{3}H]phenylalanine-tRNA and excess nonradioactive phenylalanine is complete within several minutes after initiation; thus, inhibition of polyphenylalanine synthesis by the wheat germ protein is very dependent upon proper preincubation of the system. When the above preincubation experiment was repeated substituting [^{3}H]phenylalanyl-tRNA plus nonradioactive phenylalanine for [^{3}H]phenylalanine during the translation, synthesis of radioactive polyphenylalanine was inhibited only 23% in samples preincubated with inhibitor but without tRNA. Inhibition increased to 87% when tRNA was added to the preincubation mixtures. This shows again that tRNA is required for the inhibition and that the inhibition does not involve an inactivation of the tRNA, since the [^{3}H]phenylalanyl-tRNA substrate was not present during the inhibition-promoting preincubation.

Discussion

A number of other inhibitors of protein synthesis have been isolated from plant sources. Extracts from the seeds of a variety of plants have been found to stop translation in animal cell-free extracts (Gaspari-Campani et al., 1977). The best studied of the seed inhibitors are ricin, from the seeds of *Ricinus communis*, and abrin, from the seeds of *Abrus precatorius*. Both of these inhibitors are lectins which appear to act by an enzymic modification of the 60S ribosomal subunit to prevent polypeptide chain elongation (Montanaro et al., 1975; Olsnes et al., 1975; Nolan et al., 1976). These inhibitors differ from the wheat germ inhibitor in their chemical properties, the absence of a cofactor requirement (such as ATP or tRNA) for their inactivation of ribosomes, and their ability to inhibit protein synthesis in intact animal cells (Lin et al., 1971). We have been unable to demonstrate any effect of the wheat germ inhibitor on cultured animal cells (unpublished observations), in contrast to the toxic properties of ricin and abrin on animal cells in vivo and in vitro.

The wheat germ inhibitor appears most similar to a protein isolated from the leaves of pokeweed (*Phytolacca americana*) which has been shown to inhibit protein synthesis in a number of eucaryotic cell-free systems (Obrig et al., 1973; Owens et al., 1973). This protein is a basic protein with a molecular weight of 31 000 (Robertus et al., 1977), and it does not block protein synthesis in intact cells (Ussery & Irvin, 1974). In common with ricin and abrin, the pokeweed protein appears to act enzymatically on the 60S ribosomal subunit (Obrig et al., 1973; Irvin, 1975). In contrast to the wheat germ inhibitor, no cofactor requirements have been reported for the pokeweed protein.

The functions of ATP and tRNA in the inhibition reaction catalyzed by the wheat germ protein are currently unknown. The ability of relatively high concentrations of the analogue AMP-PCP to substitute for ATP in the reaction (Figure 6) suggests that the inhibition does not occur via a phosphorylation mechanism. Furthermore, when the inhibition reaction was carried out in the presence of [γ-^{32}P]ATP and in the presence and absence of purified inhibitor, no difference could be detected in the patterns of phosphorylated ribosome-associated proteins when these proteins were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis (results not shown). The apparent positive results from similar experiments, which were carried out earlier by using partially purified inhibitor (Stewart et al., 1977), now seem to have been caused by the phosphorylation of a protein impurity in the inhibitor preparation. Thus, the wheat germ inhibitor does not appear to be closely analogous to those protein kinases which have been found to inhibit protein synthesis, often at the level of initiation, in reticulocyte lysates (Levin, et al., 1976; Kramer et al., 1976; Farrell et al., 1977), extracts from interferon-treated animal cells (Lebleu et al., 1976; Roberts et al., 1976; Zilberstein et al., 1976), and extracts from a variety of other eucaryotic cell types (Clemens et al., 1976; Sierra et al., 1977; Pinphanichakarn et al., Delaunay et al., 1977). The remaining possible roles of ATP in the inhibition reaction include serving as a cofactor in an adenylation reaction, and acting as an inducer of an allosteric effect on the inhibitor or of a conformational change by the substrate.

The role of tRNA in the inhibition may also be one of inducing a conformational change in the inhibitor or in the ribosome target site. tRNA is required in the preincubation

reaction in order for the subsequent inhibition of polyphenylalanine synthesis to immediately be expressed (Figure 7, panel A). Yet tRNA isolated from mixtures preincubated with inhibitor functions as well as control tRNA in carrying out the polymerization of phenylalanine in the tRNA-dependent system (unpublished observations). Thus, preincubation of tRNA with ribosomes, ATP, and inhibitor neither functionally inactivates the tRNA (beyond the control) nor converts the tRNA to an inhibitor molecule.

The remaining candidate target sites for the wheat germ inhibitor include the elongation factors EF-1 and EF-2, and the core ribosome itself. Currently, we are attempting to identify the target site and determine the mechanism by which this site is enzymically modified by the inhibitor acting in conjunction with ATP and tRNA.

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References

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Busch, H., Choi, Y. C., Daskal, Y., Liarakos, C. D., Rao, M. R. S., Ro-choi, T. S., & Wu, B. (1976) *Cancer Res. 13*, 101.
Clemens, M. J., Pain, V. M., Henshaw, E. C., & London, I. M. (1976) *Biochem. Biophys. Res. Commun. 72*, 768.
Delaunay, J., Ranu, R. S., Levin, D. H., Ernst, V., & London, I. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2264.
Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J., & Trachsel, H. (1977) *Cell 11*, 187.
Gaspari-Campani, A., Barbieri, L., Lorenzoni, E., & Stirpe, F. (1977) *FEBS Lett. 76*, 173.
Irvin, J. D. (1975) *Arch. Biochem. Biophys. 169*, 522.
Kerr, I. M., Brown, R. E., Clemens, M. J., & Gilbert, C. S. (1976) *Eur. J. Biochem. 69*, 551.
Kramer, G., Cimadevilla, M., & Hardesty, B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3078.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lebleu, B., Sen, G. C., Shaila, S., Cabrer, B., & Lengyel, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3107.
Levin, D. H., Ranu, R. S., Ernst, V., & London, I. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3112.
Lin, J. Y., Lin, K., Chen, C. C., & Tung, T. C. (1971) *Cancer Res. 31*, 921.
Lodish, H. F. (1976) *Annu. Rev. Biochem. 45*, 39.
Marcu, K., & Dudock, B. (1975) *Nucleic Acids Res. 1*, 1385.
Montanaro, L., Sperti, S., Mattioli, A., Testoni, G., & Stirpe, F. (1975) *Biochem. J. 146*, 127.
Nolan, R. D., Grasmuck, H., & Drews, J. (1976) *Eur. J. Biochem. 64*, 69.
Obrig, T. G., Irvin, J. D., & Hardesty, B. (1973) *Arch. Biochem. Biophys. 155*, 278.
Olsnes, S., Fernandez-Puentes, C., Carrasco, L., & Vazquez, D. (1975) *Eur. J. Biochem. 60*, 281.
Owens, R. A., Bruening, G., & Shepherd, R. J. (1973) *Virology 56*, 390.
Paladini, A. C., & Leloir, L. F. (1952) *Biochem. J. 51*, 426.
Pinphanichikarn, P., Kramer, G., & Hardesty, B. (1977) *J. Biol. Chem. 252*, 2106.
Roberts, W. K., & Coleman, W. H. (1971) *Biochemistry 10*, 4304.
Roberts, W. K., Hovanessian, A. G., Brown, R. E., Clemens, M. J., & Kerr, I. M. (1976) *Nature (London) 264*, 477.
Robertus, J. D., Monzingo, A. F., & Irvin, J. D. (1977) *Biochem. Biophys. Res. Commun. 74*, 775.
Sierra, J. M., Haro, C. D., Datta, A., & Ochoa, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4356.
Stewart, T. S., Hruby, D. E., Sharma, O. K., & Roberts, W. K. (1977) *Biochim. Biophys. Acta 479*, 31.
Ussery, M. A., & Irvin, J. D. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 33*, 1544.
Zilberstein, A., Federman, P., Shulman, C., & Revel, M. (1976) *FEBS Lett. 68*, 119.