Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1124-1132

# ISOLATION OF A TRANSLATIONAL INHIBITOR FROM WHEAT GERM WITH PROTEIN KINASE ACTIVITY THAT PHOSPHORYLATES INITIATION FACTOR eIF-2

Rajinder Singh Ranu

Department of Microbiology and
the Graduate Program in Cellular and Molecular Biology
Colorado State University
Fort Collins, Colorado 80523

Received October 31,1980

Summary: A translational inhibitor (WGI) has been partially purified from wheat germ extracts. WGI inhibits protein synthesis in rabbit reticulocyte lysates with inhibition kinetics that are similar to those observed in heme-deficiency or by the addition of purified heme-regulated translational inhibitor (HRI). Initiation factor eIF-2 from rabbit reticulocytes overcomes this inhibition. This finding suggests that WGI inhibits protein chain initiation. WGI induced inhibition is enhanced by ATP (2 mM), and overcome by GTP (2 mM) and cyclic-AMP (10 mM). WGI preparations contain a cyclic-AMP independent protein kinase activity that phosphorylates the 38,000-dalton subunit of rabbit reticulocyte eIF-2. The phosphopeptide analyses of eIF-2 phosphorylated by WGI or HRI show that they phosphorylate the same site(s) of eIF-2. HRI phosphorylates the corresponding 38,000-dalton subunit of wheat germ eIF-2. These results obtained with WGI are similar to that of HRI. HRI has been identified as a cyclic-AMP independent protein kinase that phosphorylates the 38,000-dalton subunit of eIF-2 [for review see Ochoa, S. and de Haro, C. (1979) Ann. Rev. Biochem. 48, 549]. Hence, these findings with wheat germ-a phylogenetically distant eukaryote, raise further the possibility that phosphorylation-dephosphorylation of eIF-2 may be an important general mechanism in the regulation of eukaryotic protein biosynthesis.

Wheat germ extracts are extensively used to study mechanism of protein synthesis in vitro and as a tool in the translation of mRNA from diverse eukaryotes (1-7). There is little information available, however, on the mechanism(s) that may regulate protein synthesis in this sytem. This report describes the isolation and partial purification of a translational inhibitor (WGI) from wheat germ extracts. The WGI activity is assayed in rabbit reticulocyte lysates. WGI inhibits protein chain initiation. This inhibition is overcome by initiation factor eIF-2 that forms a ternary complex (eIF-2·GTP·Met-tRNA$_f$) with Met-tRNA$_f$ and GTP (8). WGI is associated with an adenosine 3':5' cyclic monophosphate (cyclic-AMP) independent protein kinase activity that phosphorylates the 38,000-dalton subunit of eIF-2 from wheat germ and rabbit reticulocytes. A preliminary account of this work was reported earlier (9).

0006-291X/80/231124-09$01.00/0

## Materials and Methods

The following have been described: preparation of rabbit reticulocyte lysates, protein synthesis mixtures, assay of protein synthesis, preparation of highly purified heme-regulated protein kinase (specific activity 5000 units/mg); preparation of eIF-2 from rabbit reticulocytes, protein kinase assay, SDS-polyacrylamide gel electrophoresis and the autoradiography of polyacrylamide gel (10).

Partial purification of wheat germ translational inhibitor. Wheat germ extracts were prepared by gently stirring wheat germ (30 g) in 250 ml of Buffer A [Tris-HCl, 20 mM (pH 7.8); KCl, 20 mM; NaCl, 10 mM; Mg $(Ac)_2$, 1 mM and dithiothreitol (DTT), 1 mM] for 2 hr. The extract was centrifuged at 8,000 xg for 30 min. The supernate was then centrifuged at 13,000 RPM in a Sorvall SS 34 rotor for 20 min. A ribosome-free supernate was prepared (10). The supernate (420 ml) was brought to 40% saturation by the addition of solid $(NH_4)_2SO_4$. After 10 min the precipitate was collected by centrifugation. The precipitate from 0-40% $(NH_4)_2SO_4$ saturation cut was extracted with 25% saturated solution of $(NH_4)_2SO_4$ in Buffer B [Tris-HCl, 20 mM (pH 7.8); KCl, 100 mM, EDTA 0.1 mM, β-mercaptoethanol (β-ME) 10 mM]. The protein fraction which did not go in solution by extraction with 25% saturated solution of $(NH_4)_2SO_4$ was discarded. The fraction which went in solution was precipitated by the addition of solid $(NH_4)_2SO_4$ to 60% saturation. The precipitate was collected; it was dissolved in 20 ml of Buffer B and dialyzed against the same buffer.

The protein preparation (1170 mg) in 130 ml of Buffer B was applied to a DEAE-cellulose column (2.5 x 30 cm) preequilibrated with Buffer B. The column was extensively washed with Buffer B. WGI binds to DEAE-cellulose and was eluted with Buffer B containing 0.3 M KCl. The protein fraction from DEAE-cellulose was dialyzed against Buffer C [potassium phosphate 20 mM (pH 6.8) and β-Me, 5 mM]; 217 mg of protein in 31 ml of Buffer C was applied to a phosphocellulose column (1.6 x 30 cm) preequilibrated with Buffer C. The column was extensively washed with Buffer C and WGI was eluted with Buffer C containing 250 mM KCl; 18 mg of protein was recovered. Further purification of WGI was carried out by gel filtration (5 mg of protein preparation in 0.6 ml of Buffer B) on a Sephacryl S-300 column (0.9 x 55 cm) equilibrated with Buffer B; 0.65 μg of WGI preparation obtained by gel filtration on Sephacryl S-300 inhibited protein synthesis in lysates (25 μl) by 50 percent.

Preparation of wheat germ eIF-2: Wheat germ eIF-2 was partially purified with minor modifications by the procedure described by Treadwell et al. and Walthall et al. (4,5). Wheat germ ribosomes prepared by the extraction of wheat germ in low salt buffer (Buffer A) was the source of eIF-2. We, and others have previously shown that under these conditions eIF-2 is ribosome bound (5,11). A 40-60% $(NH_4)_2SO_4$ saturation cut of high salt ribosomal wash (from 39,000 $A_{260}$ units of ribosomes) was used and purification was carried out by chromatography on DEAE-cellulose, CM-Sephadex A50 and finally on phosphocellulose as described (4,5). This material was impure as judged by electrophoresis in SDS-polyacrylamide gel. However, it was free of any protein kinase activity that phosphorylates the low molecular weight subunit of wheat germ eIF-2 (comparable to the 38,000-dalton subunit of rabbit reticulocyte eIF-2). This polypeptide of eIF-2 (of primary interest in the present study) was clearly resolved from others by electrophoresis in SDS-polyacrylamide gel (Fig 2, lanes 2,4).

Phosphopeptide analysis: The detials of the phosphopeptide analyses of eIF-2 phosphorylated by WGI or HRI has been described (12).

## Results

The translational inhibitor isolated from wheat germ ribosome free supernate inhibits protein synthesis in rabbit reticulocyte lysates (Fig 1, A). In the presence of WGI protein synthesis proceeds at the control rate during an

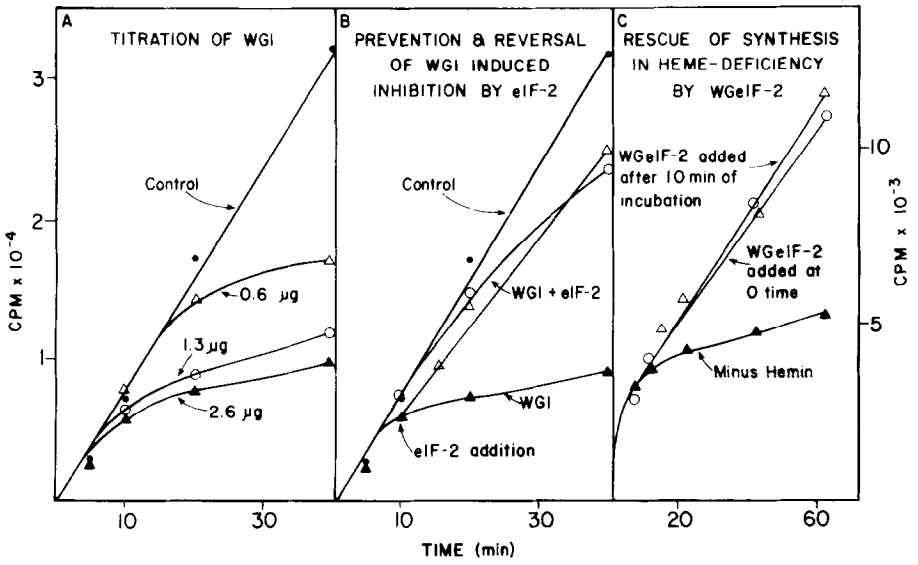


Fig. 1 Kinetics of inhibition of protein synthesis by WGI and its rescue by eIF-2. Protein synthesis reaction mixtures (25 µl) containing 10 µM hemin were incubated at 30° with : A, indicated concentrations of WGI, B, WGI (2.6 µg) and eIF-2 (1 µg) added either at the start of incubation (O——O) or after 10 min of incubation (Δ——Δ); and C, wheat germ eIF-2 (2.8 µg) added to heme-deficient protein synthesis reaction mixture (25 µl) either at the start (O——O) or after 10 min of incubation (Δ——Δ). At intervals aliquots (5 µl) were taken out and protein synthesis was assayed (10).

initial period of 5-10 min followed by a sharp decline in the rate of synthesis (Fig 1, A). The degree of inhibition increases with increasing concentrations of WGI (Fig 1, A). The initiation factor eIF-2 from rabbit reticulocytes prevents inhibition by WGI when added at the start of incubation and reverses inhibition when addition is made after the onset of inhibition (Fig 1, B). The wheat germ eIF-2 (WGeIF-2) is similarly effective in rescuing protein synthesis in heme-deficiency in rabbit reticulocyte lysates (Fig 1, C).

Inhibition of protein synthesis by WGI is enhanced by ATP (2 mM), and high levels of GTP (2 mM) and cyclic-AMP (10 mM) block the inhibitory effect of WGI (Table 1).

WGI preparations contain a protein kinase activity that phosphorylates the 38,000-dalton subunit of eIF-2 from rabbit reticulocytes and wheat germ (Fig 2, lanes 6 and 9). The heme-regulated inhibitor from rabbit reticulocytes phosphorylates the 38,000-dalton subunit of wheat germ eIF-2 (Fig 2, lanes 4 and 8).

A comparison of the phosphopeptides of reticulocyte eIF-2 phosphorylated by WGI and eIF-2 phosphorylated by the homologus inhibitor (HRI) show that they phosphorylate the same site(s) (Fig 3, A,B and C,D). The phosphopeptides ob-

Table 1. Effect of ATP, GTP and Cyclic-AMP on WGI induced inhibition of protein synthesis.

| WGI | Additions | CPM x $10^{-3}$ | % Inhibition |
|---|---|---|---|
| - | none | 20 | - |
| + | none | 11 | 45 |
| + | ATP (2 mM) | 6 | 70 |
| + | GTP (2 mM) | 20 | - |
| + | Cyclic-AMP (10 mM) | 19 | 5 |

Protein synthesis reaction mixture (30 µl) was prepared without the addition ATP and $Mg^{++}$ (10). The mixtures were incubated with or without WGI (13 µg, fraction from phosphocellulose) and the indicated nucleotide concentrations at 30° for 40 min. The values represent CPM of [$^{14}$C]leucine incorporated in 5 µl aliquot.

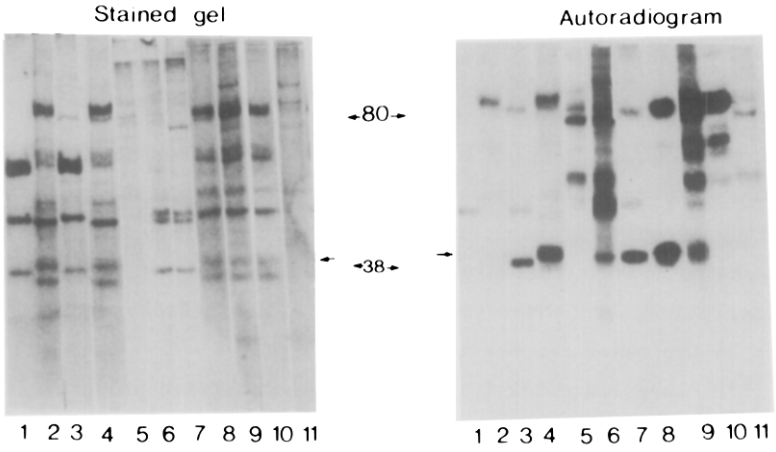


Fig. 2 Phosphorylation of eIF-2 from wheat germ or rabbit reticulocytes by WGI or HRI: Reaction mixture (10 µl) in 20 mM Tris-HCl, pH 7.8; 60 mM KCl; 2 mM $Mg(Ac)_2$; 1 mM DTT containing 0.1 mM [$\gamma^{32}$P]ATP (specific activity 4000 cpm/pmol) were incubated at 30° for 25 min with: lane 1, eIF-2 (1 µg); lane 2, WGeIF-2 (4.4 µg); lane 3, eIF-2 (1 µg) and HRI (0.1 µg); lane 4, WGeIF-2 (4.4 µg) + HRI (0.1 µg); lane 5, WGI (1.5 µg); lane 6, WGI (1.5 µg) + eIF-2 (0.7 µg); lane 7, eIF-2 (0.7 µg) + HRI (0.1 µg); lane 8, WGeIF-2 (4.4 µg) + HRI (0.1 µg); lane 9, WGeIF-2 (4.4 µg) + WGI (1.5 µg); lane 10, WGeIF-2 (4.4 µg); and lane 11, WGI (2.5 µg). Reaction was terminated (10). Samples were electrophoresed in SDS-polyacrylamide gel (10%); the proteins were stained (left) and an autoradiogram (right) was prepared (10). eIF-2 in lane 1 and 3 contain bovine serum albumin. The position of the 80,000-dalton polypeptide of HRI (selfphosphorylated), 38,000-dalton subunit of eIF-2 are indicated. The arrows (alone) indicate the position of the "38,000-dalton" subunit of wheat germ eIF-2.

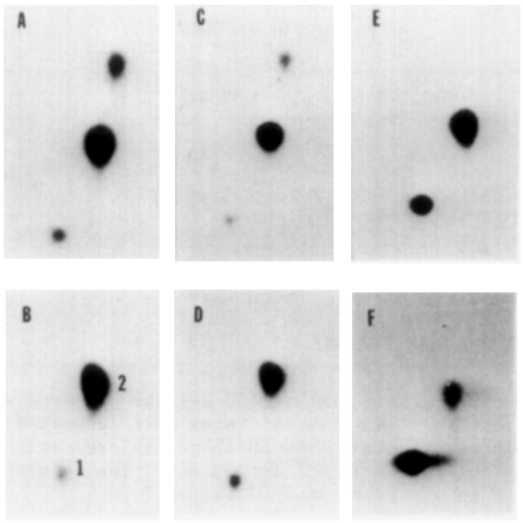


Fig. 3 Phosphopeptide analyses of the 38,000-dalton subunit of rabbit reticulocyte eIF-2 (A, B and C,D) and WGeIF-2 (E, F) phosphorylated by HRI (A, B and E, F) or WGI (C, D). Rabbit reticulocyte eIF-2 or wheat germ eIF-2 were phosphorylated as described in Fig 2. The 38,000-dalton subunit was resolved by electrophoresis in SDS-polyacrylamide gel (10%). The 38,000-dalton subunit of eIF-2 was cut out and subjected to proteolytic digestion with proteinase K (top panel) or thermolysin (bottom panel). The phosphopeptides were analyzed as described (12). The section of the autoradiograms containing the phosphopeptides are presented.

tained by proteolytic digestion of wheat germ eIF-2 phosphorylated by HRI with proteinase K, however, show only two phosphopeptides. The third main phosphopeptide is either completely missing (Fig 3,E) or is a very minor component (results not shown). When thermolysin is used for proteolytic digestion the two main phosphopeptides are observed (Fig 3, F). The phosphopeptide #1, however, constitutes as the major component.

When [$^{32}$P] labelled eIF-2 (prepared by HRI catalyzed phosphorylation of the 38,000-datlon subunit) is added to wheat germ extracts, there is a slow and progressive dephosphorylation of added eIF-2 (Fig 4). This finding suggests that wheat germ extracts contain a phosphoprotein phosphatase that dephosphorylates eIF-2. The presence of a similar phosphoprotein phosphatase activity has been described in rabbit reticulocyte lysates (13).

## Discussion

There are many features of WGI that bear close resemblance to HRI (reviewed in 8). They include: I, WGI like HRI is present in the ribosome free supernate; II, chromatographic behavior of WGI on DEAE-cellulose and phosphocellulose are very similar to HRI, and WGI like HRI is precipitated with 25-40% saturated

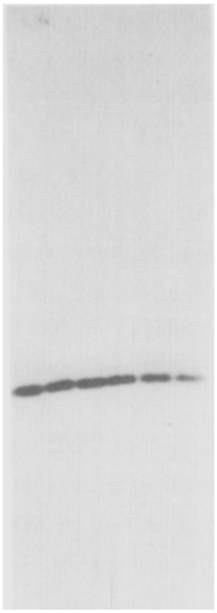


Fig. 4 Dephosphorylation of eIF-2 by wheat germ extracts. Rabbit reticulocyte eIF-2 was phosphorylated in the presence of [$\gamma^{32}$P]ATP using HRI as described (10). [$^{32}$P]eIF-2 was isolated by chromatography on phosphocellulose and was added (16,000 CPM) to wheat germ extract (33 µl). The incubation was at 30°. At intervals aliquots (4 µl) were removed and subjected to electrophoresis in SDS-polyacrylamide gel. The autoradiogram shows samples (1 to 6) incubated for 0, 4, 8, 12, 17 and 25 min.

$(NH_4)_2SO_4$ solution (14); III, the biphasic kinetics of inhibition of protein synthesis, potentiation of inhibition by ATP, and the prevention of inhibition by high levels of GTP and cyclic-AMP are similar to those observed with HRI (15,16). The restorative and preventative effects of exogeneously added eIF-2 on protein synthesis in lysates inhibited by WGI point further to the close relationship of these inhibitors in the mechanism by which they inhibit protein synthesis (8). This observation is supported further by the findings that WGI phosphorylates the site(s) on the 38,000-dalton subunit of eIF-2 from reticulocytes that are indistinguishable from those phosphorylated by HRI (Fig 3, A,B and C,D).

The findings discussed in the preceding suggest basic similarities in the two systems. Some differences, however, start to emerge from the phosphopeptide analyses of wheat germ eIF-2 phosphorylated by HRI (Fig 3, E and F). These differences in phosphopeptides may be a reflection of the resistance of phosphopeptides of wheat germ eIF-2 to complete proteolytic digestion. In an earlier study, however, we (17) and subsequently others (6) had observed that HRI concen-

trations that inhibit protein synthesis in rabbit reticulocyte lysates do not inhibit protein synthesis in wheat germ extracts. The present finding of differences in the phosphopeptides of wheat germ eIF-2 phosphorylated by HRI may be related to this lack of inhibition of protein synthesis by HRI. Other explanations for this lack of inhibition are also possible, e.g., wheat germ extracts may contain a very large pool of eIF-2 or one or more factors in wheat germ extract may block the complete phosphorylation of endogeneous eIF-2 or that wheat germ extracts contain excess amount of reversing factor which can also block inhibition of protein synthesis by HRI (18-21). Further study is warranted before a definitive basis for this apparent lack of inhibition of protein synthesis by added HRI can be provided. Another difference in WGI and HRI involve the lack of sensitivity of WGI to sulfhydryl agents, e.g. N-ethylmaleimide (NEM). Treatment of HRI with NEM markedly enhances the activity of HRI (22). However, treatment of WGI with NEM has no detectable effect on WGI activity (results not shown). This lack of sensitivity to NEM has been noted with other protein kinases that phosphorylate 38,000-dalton subunit of eIF-2 and inhibit initiation of protein synthesis (R. S. Ranu, unpublished results). They have been isolated from rat liver (23), Krebs ascites cells, erythroid precursor cells and chicken reticulocytes (24).

The finding that the wheat germ eIF-2 can rescue protein synthesis in heme-deficiency in rabbit reticulocyte lysates and the similar results of Benne et al (6) suggest that basic similarities exist in the wheat germ and reticulocyte systems. It needs to be noted that the wheat germ eIF-2 "38,000-dalton subunit" has a higher molecular weight (about 39,000-40,000-daltons) than the rabbit reticulocyte eIF-2. Furthermore it is resolved into two polypeptides by electrophoresis in SDS-polyacrylamide gel (Fig 2, lane 2 and 4)consisting of , a major component of about 39,000 and a minor polypeptide of about 40,000-daltons. This resolution of the "38,000-dalton subunit" of wheat germ eIF-2 into two polypeptides results in a broad band in the autoradiogram when eIF-2 is phosphorylated by HRI (Fig 2, lanes 4 and 8). The reason for these two polypeptides which correspond to the 38,000-dalton subunit of reticulcoyte eIF-2 is not clear. They do not appear to be generated by proteolysis. Since the same polypeptide pattern is observed when eIF-2 is prepared in the presence of protease inhibitor phenylmethylsufonyl fluoride.

The finding of a translational inhibitor of initiation with protein kinase activity that phosphorylates the 38,000-dalton subunit of eIF-2 in wheat germ-a phylogenetically distant eukaryote, raises further the possibility that phosphorylation-dephosphorylation of eIF-2 may be an important general mechanism in eukaryotic protein biosynthesis (24). Modulators of at least two of these protein kinases from rabbit reticulocytes are known: they are heme for the HRI

and double stranded RNA (dsRNA) for the dsRNA-activated protein kinase (for review see 8). How and what regulates the activity of some these other protein kinases (8,23,24) is an open and intriguing question.

Finally, it should be pointed out that the translational inhibitor described here bears no relationship to the reported inhibition of protein synthesis in wheat germ extracts by the addition of the catalytic subunit of the cyclic-AMP dependent protein kinase (25). The site and the mechanism of this inhibition is not known. WGI is also not related to an inhibitor of polypeptide chain elongation from wheat germ reported by Roberts and Steward (26).

Acknowledgements: This work was supported by an NIH Biomedical Research Support Grant 2 SO 7 RR-05458-18. The author wishes to thank Dr. P. Azari for the TLC apparatus, Drs. B. J. Treadwell and W. Robinson, for a wheat geram eIF-2 sample and Dr. J. Bagby, Deans R. D. Phemister and F. Murphy for encouragement.

## References

1. Marcus, A., Seal, S. N. and Week, D. P. (1974) Methods Enzymol. 30, 94-101.
2. Marcu, K. and Dudock, B. (1975) Nucleic Acid Res. 1, 1385-1391.
3. Roberts, B. E. and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330-2334.
4. Treadwell, B. V., Mauser, L. and Robinson, W. G. (1979) Methods Enzymol 60, 181-193.
5. Walthall, B. J., Spremulli, L., Lax, S. R. and Ravel, J. (1979) Methods Enzymol. 60, 193-204.
6. Benne, R., Kasperaitis, M., Voorma, H. O., Ceglarz, E. and Legocki, A. B. (1980) Eur. J. Biochem. 104, 109-117.
7. Taylor, J. M. (1979) Ann. Rev. Biochemistry 48, 681-718.
8. Ochoa, S. and deHaro, C. (1979) Ann. Rev. Biochem. 48, 549-580.
9. Vander-linden, J. and Ranu, R. S. (1980) Fed. Proc. Fed. Am. Soc. Exp. Biol. 39, 2028.
10. Ranu, R. S. and London, I. M. (1979) Methods in Enzymol. 60, 459-484.
11. Ranu, R. S. and Wool, I. G. (1976) J. Biol. Chem. 251, 1926-1935.
12. Ranu, R. S. (1979) Biochem. Biophys. Res. Commun. 91, 1437-1444.
13. Safer, B. and Jagus, R. (1979) Proc. Natl. Acad. Sci. USA 76, 1094-1098.
14. Ranu, R. S. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA. 73, 4349-4353.
15. Balkow, K., Hunt, T. and Jackson, R. J. (1975) Biochem. Biophys. Res. Commun. 67, 366-375.
16. Ernst, V., Levin, D. H., Ranu, R. S. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA 73, 1112-1116.
17. Ranu, R. S., Glass, J., Delaunay, J., Ernst, V. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 1566.
18. Ranu, R. S. and London, I. M. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 868.
19. Gross, M. (1976) Biochem. Biophys. Acta. 447, 445-459.
20. Ralston, R. O., Das, A., Grace, M., Das, H. and Gupta, N. K. (1979) Proc. Natl. Acad. Sci. USA 76, 5490-5494.

21. Amesz, H., Goumans, H., Haubrick-morree, T., Voorma, H. O. and Benne, R. (1979) Eur. J. Biochem. 98, 513-520.
22. Gross, M. and Rabinovitz, M. (1972) Biochem. Biophys. Acta. 287, 340-352
23. Delaunay, J., Ranu, R. S., Levin, D. H., Ernst, V. and London, I. M. (1977) Proc. Natl. Acad. Sci. USA 74,2264-2268.
24. Ranu, R. S. (1980) FEBS Lett. 112, 211-215.
25. Sierra, J. M., deHaro, C. D., Datta, A. and Ochoa, S. (1977) Proc. Natl. Acad. Sci. USA 74, 4356-4360.
26. Roberts, W. K. and Stewart, S. T. (1979) Biochemistry 18, 2615-2621.