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Glutamine Synthetase: Isolation of Isoforms, Poly (A) +RNA and In Vitro Translation

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GLUTAMINE SYNTHETASE: ISOLATION OF ISOFORMS, POLY(A)+RNA AND *IN VITRO* TRANSLATION

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Master of Science

by

Stanley L. Silver

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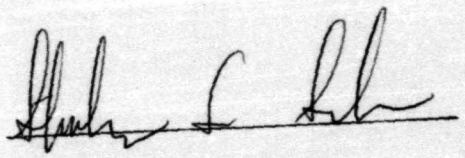
Signed 

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GLUTAMINE SYNTHETASE: ISOLATION OF ISOFORMS, POLY(A)+RNA AND IN VITRO TRANSLATION

Recommended August 21, 1987
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Elmer Gray
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I dedicate this thesis to my parents and my three brothers. Without their never ending moral and spiritual support, this research would have never been possible.

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Stanley L. Silver August, 1987 60 pages

Directed by: Valgene L. Dunham, Martin R. Houston and Frank R. Toman

Department of Biology Western Kentucky University

Two forms of glutamine synthetase (GS) have been isolated from three-day old etiolated soybean hypocotyls by ammonium sulfate precipitation and DEAE-cellulose chromatography. The GS isoforms were eluted from a 30 ml DEAE-cellulose column using 0.14 M KCl and 0.175 M KCl and from a 1.0 ml DEAE-cellulose column using 0.08 M KCl and 0.14 M KCl. The two isoforms of soybean GS have a molecular weight of about 392,000 daltons as indicated by in gel enzyme assays and staining of protein bands.

The poly(A)+RNA specific for GS synthesis was isolated using phenol and chloroform followed by Hybond mAP paper. Translation was performed using a wheatgerm cell-free lysate. Following the application of the *in vitro* translated products to a 1 ml DEAE-cellulose column, GS was successfully eluted at 0.14 M KCl.

INTRODUCTION

Glutamine synthetase (L-glutamate; ammonia ligase [ADP forming], E.C. 6. 3. 1. 2.) is the enzyme necessary for the assimilation of ammonia from nitrate reduction and photorespiration. The reaction catalyzed by glutamine synthetase (GS) is the following:

L-glutamate + ATP + NH_3 $\rightleftharpoons$ L-glutamate + ATP + ADP + Pi

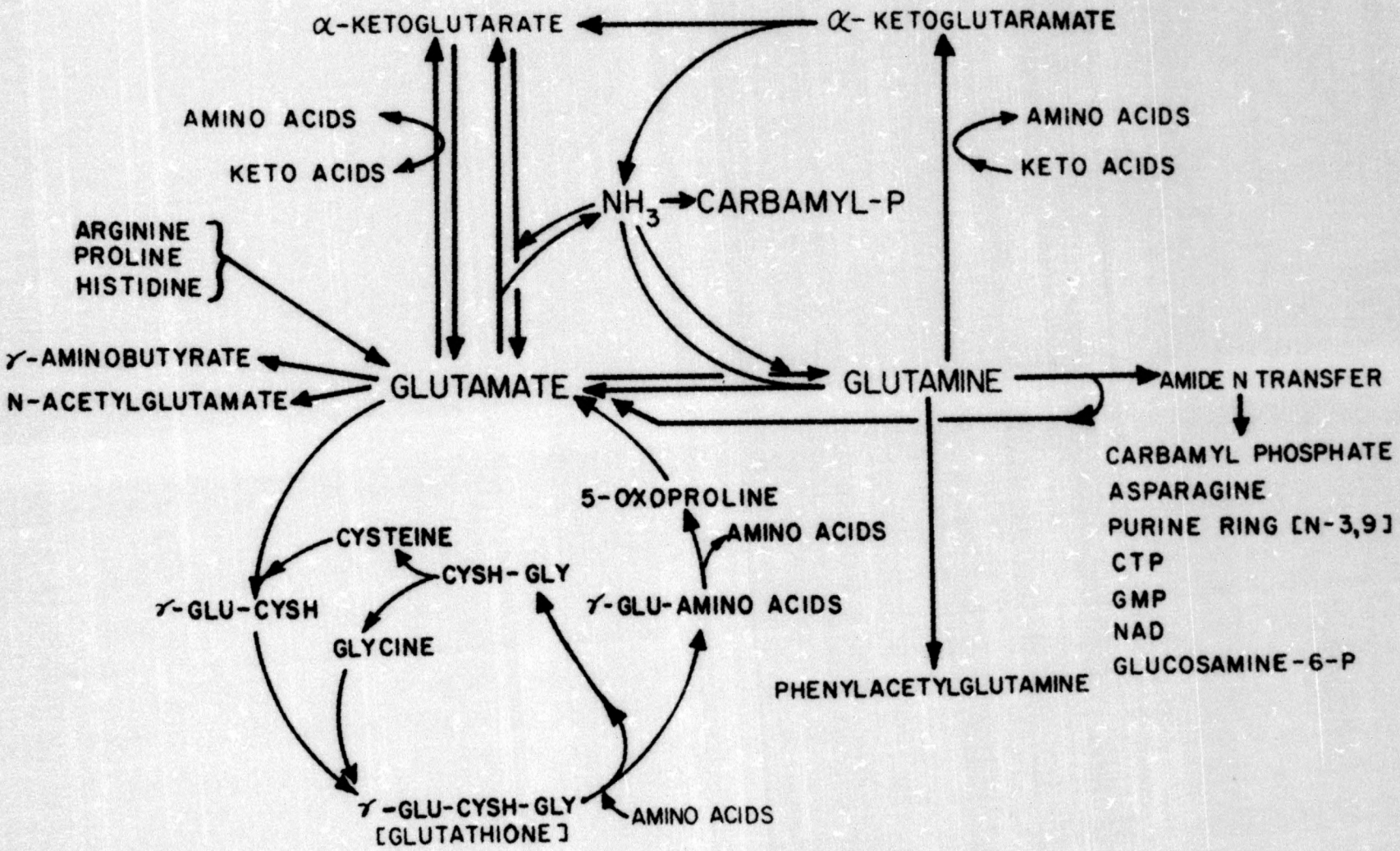
The reaction in Escherichia coli involves a two-step process in which glutamate and ATP bind to the enzyme resulting in the hydrolysis of ATP to ADP. The inorganic phosphate is not released but remains bound to the glutamate. This activated intermediate is known as gamma-glutamyl-phosphate (Pamiljans et al., 1961; Gass and Meister, 1969; Todhunter and Purich, 1974). It is now sterically possible for the ammonia to attack the carbonyl carbon of the glutamate molecule followed immediately by the release of ADP and glutamine from the enzyme complex. Evidence for this reaction comes from experiments using rapid-quench and isotope partitioning techniques to identify the intermediate as gamma-glutamyl-phosphate (Meek et al., 1982).

In living tissue, GS is important for two reasons. First, GS catalyzes the reaction forming glutamine from L-glutamate, an amino acid present in all organisms. In higher plants, glutamine acts as a storage and transport form of both ammonia and glutamate (Meister, 1980). In

addition, glutamine is a key source of nitrogen for use in higher organisms. The amide nitrogen from glutamine is transferred during the biosynthesis of asparagine, CTP, GMP, NAD and glucosamine-6-phosphate (Figure 1). The alpha nitrogen of glutamine also acts as an important supply of nitrogen. Glutamine is converted to alpha-ketoglutaramate and several amino acids including phenylalanine, tyrosine and methionine. The resulting alpha-ketoglutaramate is a secondary source of ammonia for the synthesis of carbamyl-phosphate. In addition to the above reactions, glutamine is readily converted to glutamate which can either undergo a deamination reaction to form the Krebs' cycle intermediate alpha-ketoglutarate or can be utilized as an initial molecule in the formation of amino acids. Therefore, it is obvious that significant interrelationships exist between glutamine and glutamate.

The second reason for the importance of GS is in nitrogen assimilation. Nitrogenase catalyzes the conversion of atmospheric molecular nitrogen to a biologically useful compound, ammonia. Since ammonia is then utilized in the biosynthesis of amino acids and nucleic acids, the supply of useful nitrogen to plants is critical. GS has been implicated as a possible regulator of nitrogenase in Rhodopseudomonas capsulata, Rhodospirillium rubrum and Salmonella typhimurium (Kustu et al., 1979; Yoch, 1980). By using a mutant of R. capsulata which had no detectable GS activity, nitrogenase appeared to be unregulated.

Figure 1. Glutamine metabolism


α-KETOGLUTARATE
α- KETOGLUTARAMATE
AMINO ACIDS
KETO ACIDS
AMINO ACIDS
KETO ACIDS
NH3 → CARBAMYL-P
ARGININE
PROLINE
HISTIDINE
γ-AMINOBUTYRATE
N-ACETYLGLUTAMATE
GLUTAMATE
GLUTAMINE
AMIDE N TRANSFER
CARBAMYL PHOSPHATE
ASPARAGINE
PURINE RING [N-3,9]
CTP
GMP
NAD
GLUCOSAMINE-6-P
5-OXOPROLINE
AMINO ACIDS
CYSTEINE
CYSH-GLY
γ-GLU-CYSH
γ-GLU-AMINO ACIDS
GLYCINE
PHENYLACETYLGLUTAMINE
γ-GLU-CYSH-GLY
[GLUTATHIONE]
AMINO ACIDS

Therefore, it is obvious that GS is not only involved in the synthesis of glutamine, but may also regulate ammonia levels and subsequently nitrogenase activity.

Glutamate is a key molecule in the assimilation of nitrogen *in vivo*. There are two conceivable mechanisms for the formation of glutamate. The first involves the amination of 2-oxo-glutarate to glutamate by the enzyme glutamate dehydrogenase (E.C. 1.4.1.3). The second mechanism involves the transfer of the amino group from ammonia to glutamate to form glutamine. This reaction is catalyzed by GS. In turn, the amide nitrogen from glutamine is utilized in the synthesis of other biomolecules or is transferred to 2-oxo-glutarate to form two molecules of glutamate (Stewart and Rhodes, 1976). This second reaction is catalyzed by glutamate synthase (EC. 1.4.1.14). The first mechanism, catalyzed by glutamate dehydrogenase (GDH), was initially thought to be the major route for glutamate synthesis in both prokaryotes and eukaryotes. The enzyme is now thought to be involved in the detoxification of cells with extremely high levels of ammonia (Givan, 1979). In addition, the GDH mechanism may be involved in the reassimilation of ammonia from photorespiration (Miflin and Lea, 1976). Therefore, the glutamine synthetase/glutamate synthase cycle appears to be the mechanism for ammonia assimilation. Evidence for this conclusion is based on the fact that GDH has a K_m for ammonia that is greater than physiological levels, whereas GS has a K_m for ammonia that

is typical of *in vivo* levels (Vargas and DeJimenez, 1986). When *Lemna minor* tissue was incubated with methionine sulphoximine, an inhibitor of GS, a decrease in both GS activity and nitrogen assimilation was observed (Stewart and Rhodes, 1976). Therefore, it can be concluded that GS is the major enzyme involved in nitrogen assimilation rather than GDH.

In prokaryotes, GS is regulated by an adenylation/deadenylation cycle of the GS subunits. When the subunits are adenylated GS is subject to feedback inhibition by several different end products of glutamine metabolism including ATP, CTP, glucosamine-6-P, histidine and carbamyl-phosphate (Woolfolk et al., 1966). The adenylation of the GS subunits in enteric bacteria is enhanced by the presence of a high concentration of ammonia. Also, when *E. coli* was grown on an ammonia rich media, the gene(s) for GS was repressed, whereas the gene for GDH is expressed (Howitt and Gresshoff, 1985). Therefore, the amount of available ammonia in prokaryotes controls the expression of the GS gene(s) and the level of adenylation of the GS subunits.

At the present time, there is no well-defined regulation scheme for GS in eukaryotes. Since glutamine concentrations in the cell are much greater in higher plants than in prokaryotes, it has been hypothesized that the reactions utilizing glutamine should be regulated rather than the reactions synthesizing glutamine (Meister, 1980).

Regulation of GS in eukaryotes, however, is well documented. For example, when Neurospora crassa is grown in a nitrogen limited environment, an increase in GS activity is observed. In this same study, the specific poly(A)+RNA for GS was shown to increase as the level of usable nitrogen was decreased (Palacios, 1980). The typical octameric form of eukaryotic GS has been shown to be replaced by a smaller tetrameric form under these nitrogen limited conditions (Mora, 1980). Subsequent studies using soybean have shown that feedback inhibition of GS occurs by such molecules as alanine, pyrophosphate and AMP (McMaster and Dunham, 1980).

The evidence to date implies that, in addition to feedback inhibition, GS can be regulated at two other levels: either by post-translational modification or by the transcriptional inhibition of the specific poly(A)+RNA for GS.

Glutamine synthetase has been found to exist in several forms in both prokaryotes and eukaryotes. In Bacillus brevis, two isoforms of GS have been isolated depending on the composition of the growth media. The optimal pH for GSa isolated from B. brevis cultured on an alanine medium is 7.0, whereas the optimal pH for GSp isolated from B. brevis cultured on a medium containing pyruvate was 8.0 (Gaur et al., 1981). Inhibition studies indicated that GSa is inhibited by Mg^{++} but insensitive to amino acid and nucleic

acid regulation. GSp was stimulated by Mg^{++} and much more sensitive to alanine and glycine inhibition (Gaur et al., 1981).

In the green algae Stichococcus bacillaris two isoforms of GS have been isolated. GS1 was found to be the major isoform in light-grown S. bacillaris and was inhibited by thiol reagents such as 2-mercaptoethanol. GS2, however, was the major isoform in etiolated algae and was stimulated by thiol reagents (Ahmad and Hellebust, 1987). Therefore, GS1 is important in light-mediated glutamine synthesis as earlier suggested by Hirel and Gadal (1980).

In higher plants, two major forms of GS have been isolated from root, stem and leaves (Table 1). The two forms have been characterized using enzyme kinetics and gel electrophoresis. GS1, the more stable isoform at higher temperatures, is found in the cytosol, and GS2 is found in the chloroplast (Hirel and Gadal, 1980; Mann et al., 1979). GS1 and GS2 were found to have molecular weights of 349,000 and 363,000 daltons respectively (Mann et al., 1979). Inhibition studies show GS1 to be more sensitive to AMP, CTP and GTP, whereas GS2 was inhibited by NADH and glucosamine-6 phosphate (Hirel and Gadal, 1980). In soybeans two isoforms of GS have been isolated in dark-grown tissue and four isoforms in light-grown tissue (Dyviniak and Dunham, 1983). Two of these isoforms from light-grown tissue were located in chloroplasts, whereas the other two forms were cytosolic

Table 1. Tissue specificity for plant glutamine synthetase isoforms.

		ROOT	LEAF		STEM	
TISSUE		GS_R	GS_1	GS_2	GS_1	GS_2
LOCATION		CYTOSOL	CYTOSOL	CHLORO-PLAST	CYTOSOL	CYTOSOL
MLC. WT. (Kd)		330	330	330	365	365
OPTIMUM pH		7.5	7.5	7.9	7.0	6.5
THERMAL STABILITY		LABILE	STABLE	LABILE	STABLE	LESS STABLE
K_m (mM)	GLUT-AMINE	2.0	2.0	20.0	36.0	17.0
	NH_2OH	1.0	1.0	1.0	4.5	5.3
* INHIBITORS	HMP	+	++	++	0	0
	NADH	0	0	+++	0	0
	CTP	0	++	++	0	0
	GTP	N.A.	++	+	N.A.	N.A.
	GLC-6-P	+	0	0	0	0
	ALANINE	N.A.	N.A.	N.A.	++	++
	PPi	N.A.	N.A.	N.A.	+++	+++
	MSO	+++	+++	+++	++	++

* INHIBITORS:

+++	Extremely Sensitive
++	Very Sensitive
+	Sensitive
0	Not Sensitive
N.A.	Not Available

and identical to the previously isolated GS isoforms from etiolated soybean tissue (Stasiewicz and Dunham, 1979).

To understand the regulation of GS in eukaryotic systems with respect to the presence of isoforms, the gene or genes coding for the different isoforms of GS must be determined. Once the gene(s) is isolated, then it will be possible to address the questions of when and what environmental stimulus regulates the gene for GS.

The initial approach to this problem was to isolate the poly(A)+RNA specific for GS and then translate the message *in vitro* using a cell-free wheat germ translation system. From the translated proteins, the different isoforms of GS might be isolated and partially characterized.

MATERIALS AND METHODS

Plant Material

Soybean seeds (*Glycine max* variety SB 4000) were purchased from Stewart Seed Company, Greensburg Indiana. The seeds were spread on the surface of extremely moist vermiculite and lightly covered with a thin layer of vermiculite.

Seeds were germinated in the dark at 31°C for three days in a temperature controlled incubator or under black plastic in a greenhouse.

Assay Procedure

GS activity was determined using the transferase assay as basically described by Woolfolk et al. (1966). The assay mixture consisted of 20 mM imidazole, pH 7.0; 20 mM $NaHAsO_4$; 3 mM $MnCl_2$; 60 mM hydroxylamine; 30 mM L-glutamine, pH 7.0; 0.4 mM ADP, pH 7.0 and water. After the reactants were combined in the above order, 0.1 ml of enzyme (1.5 mg) was added to initiate the reaction. The reaction was carried out at 37°C for 15 minutes in a shaking water bath. A solution (0.5 ml) consisting of 4% trichloroacetic acid, 1 N HCl and 1.67% $FeCl_3$ was added to terminate the reaction. The product, gamma-glutamyl-hydroxamate, in the presence of the termination solution, resulted in a colored complex that absorbed maximally at 540 nm. The absorbance of the

solution was determined using a Gilford Response spectrophotometer. The control tube consisted of the above assay mixture minus ADP.

A unit of GS activity was defined as that amount of enzyme producing 1 μmole of gamma-glutamyl-hydroxamate in 15 minutes at 37°C. In order to convert absorbance to activity, a standard curve was produced using gamma-glutamyl-hydroxamate (Sigma Chemical Company, St. Louis).

Isolation of Glutamine Synthetase

Hypocotyl sections (1-2 cm) from three-day old etiolated plants were harvested and placed in cold homogenization buffer (5.0 ml/g of tissue) consisting of 0.2 M imidazole-HCl, pH 7.5; 0.01 mM $MnCl_2$ and 2.0 mM 2-mercaptoethanol. The imidazole-HCl must be at pH 7.5 and cold before the addition of the $MnCl_2$. The tissue was macerated with a Sorval Omni-mixer at setting six for 45 seconds. The mixture was filtered through four layers of cheesecloth into chilled centrifuge tubes and centrifuged at 22,000 x g for 20 minutes at 4°C. The pellet was discarded and the volume of the supernatant determined. Ammonium sulfate was added slowly over 30 minutes to obtain 70% saturation. Following centrifugation at 22,000 x g for 20 minutes at 4°C, the protein pellet was resuspended in

homogenization buffer and desalted using a 30 ml G50 Sephadex column. The fractions with the highest GS activity as determined by the transferase assay were combined.

Ion-Exchange Chromatography

The desalted pooled fraction was applied to a 30 ml DEAE-cellulose column. The column was washed with two volumes of homogenization buffer. During the wash, 2 ml fractions were monitored for protein at 280 nm. Elution of the enzyme was accomplished either by a two step gradient of KCl (0.1 M and 0.8 M) or by a 10 ml linear gradient of KCl (0.05 M to 1.0 M). Fractions containing protein were subsequently assayed for GS activity using the transferase assay.

Gel Electrophoresis

The procedure for gel electrophoresis was as basically described by Farkas et al. (1986). A 54 ml solution containing 6% acrylamide-bis acrylamide, buffer (0.09 M Tris-HCl, pH 8.4 and 0.08 M boric acid), 0.05% ammonium persulfate, and 0.05% N,N,N',-tetramethylethylenediamine was allowed to polymerize in a 16 x 20 cm gel. Both the upper and lower electrode buffer chambers were filled with the same Tris-borate buffer (TB) as above with the addition of 20 mM 2-mercaptoethanol. A continuous circulation of water (4^{o}C) from an ice bath through the inner core was initiated 30 minutes prior to the beginning of the run. Ammonium persulfate, a strong oxidizing agent, was removed prior to

loading the gel by applying a current of 50 mA for at least 30 minutes. The protein was made 10% w/v sucrose in TB buffer and then loaded into the wells of the gel using a 100 µl Hamilton syringe. After allowing electrophoresis to continue for 2-4 hours, the gel was stained by placing it in a solution of a 0.1% Coomassie Blue in 40% methanol and 10% acetic acid for 30 minutes. The gel was destained to remove background using a 40% methanol, 10% acetic acid solution for 1-3 hours. Other gels were stained with silver (Bio-Rad, New York) as follows: the gel was fixed in 400 ml of 40% methanol/10% acetic acid for 60 minutes followed by two washes of a 10% ethanol/5% acetic acid solution for 30 minutes each wash. The gel was oxidized in 200 ml of a nitric acid and potassium dichromate solution (Bio-Rad) for 10 minutes. Following washing with distilled water, the gel was stained in silver reagent for 30 minutes. The gel was developed using a sodium carbonate and paraformaldehyde solution (Bio-Rad). A 5% acetic acid solution terminated the development reaction.

Siliconization

All glassware used in the isolation and translation of RNA was siliconized using a solution of 5% dichlorosilane in chloroform. The glassware was immersed in the siliconization fluid for 15 minutes and then rinsed thoroughly with distilled water. The glassware was baked at 180°C overnight.

Isolation of Total RNA

Hypocotyl sections (1-2 cm) from three-day old etiolated plants were harvested and placed in a solution (2 ml per gram of tissue) of 50 mM Tris-HCl (pH 8.8) and 2% sodium-dodecyl sulfate (SDS) preheated to 60°C. An equal volume of phenol/chloroform/iso-amyl alcohol (24:24:1) was added and then the mixture homogenized with a Sorvall Omni-mixer at setting 6 for 2 minutes. The mixture was filtered through four layers of cheesecloth and centrifuged at 9500 x g for 10 minutes. The aqueous layer was removed and brought to 0.15 M NaCl. Precipitation of the RNA was accomplished by addition of two volumes of cold ethanol (-20°C). After two hours, the precipitated RNA was pelleted by a 10 minute centrifugation at 12,000 x g and then resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA) in a total volume of 5 ml. An equal volume of phenol/chloroform/isoamyl alcohol was added and the mixture centrifuged at 3000 x g for 5 minutes. The aqueous layer was removed and stored at 4°C. TE buffer (5 ml) was added to extract additional RNA from the organic layer. Following centrifugation at 3000 x g for 5 minutes, the resultant aqueous layer was combined with the previously stored RNA fraction and brought up to 0.15 M NaCl. The RNA was precipitated by addition of 2 volumes of cold (-20°C) ethanol. The mixture was centrifuged at 12,000 x g for 10 minutes and the RNA pellet resuspended in TE buffer. The

high molecular weight RNA (greater than 5S) was precipitated by addition of 3 M NaCl overnight. After centrifugation at 14,000 x g for 10 minutes, the pellet was resuspended in sterile water and a spectral analysis from 220 nm to 310 nm was performed. A 280/260 ratio of 0.49 or less and a peak absorbance at 260 nm indicated a purified nucleic acid solution.

Isolation of Poly(A)+RNA

Poly(A)+RNA was selected from total RNA by means of Hybond mAP paper (Amersham, Arlington Heights). A small piece of the paper (2-4 cm^2) was equilibrated in 0.5 M NaCl. Before spotting the RNA onto the mAP paper, the RNA was heated in distilled water to 60°C for 5 minutes and brought up to 0.15 M NaCl. The paper was placed in a weigh dish and the RNA solution spotted slowly onto the paper. After 2 minutes, the paper was placed on several layers of paper towels and the excess RNA from the weigh dish was spotted onto the paper. After all the RNA was applied, the paper was air dried and washed three times with 0.5 M NaCl. The residual NaCl was removed using 70% (v/v) ethanol/water for 2 minutes. The paper was then placed in a test tube containing 1 ml of sterile water and heated to 70°C for 5 minutes. The mAP paper was removed and a spectral analysis from 220 to 310 nm was performed on the RNA solution.

In Vitro Translation

Translation of the poly(A)+RNA was performed using a nuclease-treated wheat germ cell-free extract (Bethesda Research Laboratories, Gaithersburg). The translation system contained all the enzymes and amino acids necessary to translate protein. After combining potassium acetate, magnesium acetate, the reaction mixture, wheat germ extract, and poly(A)+RNA (0.5 µg), the mixture was incubated at 25°C for 60 minutes. The translated proteins were then assayed for GS activity.

In Gel Enzyme Assay

GS activity in 6% polyacrylamide gels was determined using a modified transferase assay. The volume of the in gel assay was 10 times the standard protocol. The gel lane with GS was excised and placed in a 20 cm cuvette containing the assay mixture. The assay reaction was allowed to proceed for 30 minutes at 37°C, after which the gel was scanned to 600 nm.

Gel Scan

Gel scans of RNA and protein were performed using the Gilford Response spectrophotometer. For RNA, the gel was silver stained and the lane of interest was excised and placed in a 20 cm quartz cuvette. The gel was scanned at 400 nm.

Protein gels were stained with Coomassie Blue and the lane with protein was excised and placed in a quartz cuvette. The gel was scanned at 576 nm.

RESULTS

Partial Purification of GS Isoforms

Partial purification of the isoforms of GS included ammonium sulfate precipitation, G-50 Sephadex and DEAE-cellulose chromatography. In general, activity in post-DEAE fractions was 26-fold more purified when compared to the crude homogenate.

Initial experiments were performed to determine the chromatographic characteristics of GS. Using a 30 ml column of DEAE-cellulose, 2.8 mg of the desalted GS preparation was applied to the column. GS was eluted in one major peak of enzyme activity with 1.0 M KCl (Figure 2). Further characterization of GS was performed by using a linear salt gradient between 0.05 and 0.5 M KCl to elute the possible isoforms of GS (Figure 3). The first peak of GS activity was eluted from the column at 0.14 M KCl and the second peak at 0.175 M KCl. Since future work involved the possible *in vitro* translation of GS and, therefore, less protein, a 1 ml column of DEAE-cellulose was prepared using a pasteur pipette with a glass wool plug. Elution of two major peaks of GS activity from this small column was accomplished by a two step gradient of KCl at 0.08 M and 0.14 M (Figure 4). Although elution of the first fraction (0.08 M KCl) was continued until $A_{280}=0$, all of the GS activity eluting at this KCl concentration may not have been released from the

Figure 2: Step gradient elution of GS activity from a DEAE-cellulose column. One ml of soybean protein was loaded onto a 30 ml DEAE-cellulose column resulting in one peak of GS activity eluted using 1.0 M KCl. Fractions were monitored for protein at 280 nm (——●——) and GS activity at 540 nm (---O---)

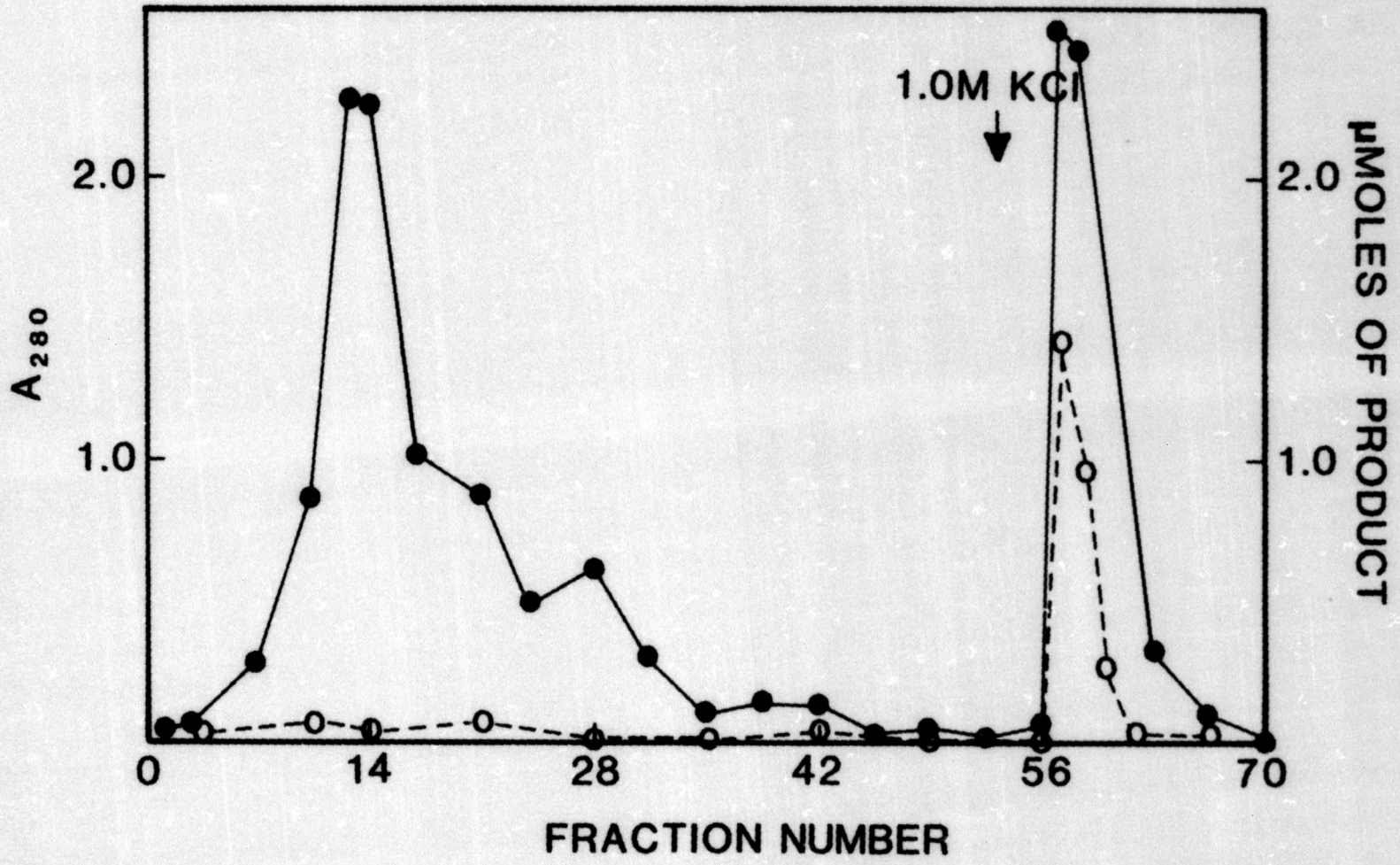

1.0M KCl
2.0
1.0
A_{280}
2.0
1.0
μMOLES OF PRODUCT
0
14
28
42
56
70
FRACTION NUMBER

Figure 3. Linear gradient elution of GS activity from a DEAE-cellulose column. One ml of soybean protein was loaded onto a 30 ml DEAE-cellulose column. Using a linear KCl gradient from 0.05 to 0.5 M KCl, two peaks of GS activity were eluted. Peak 1 was eluted at 0.14 M KCl and peak 2 at 0.175 M KCl. Fractions were monitored for protein at 280 nm (——●——) and GS activity at 540 nm (---O---).

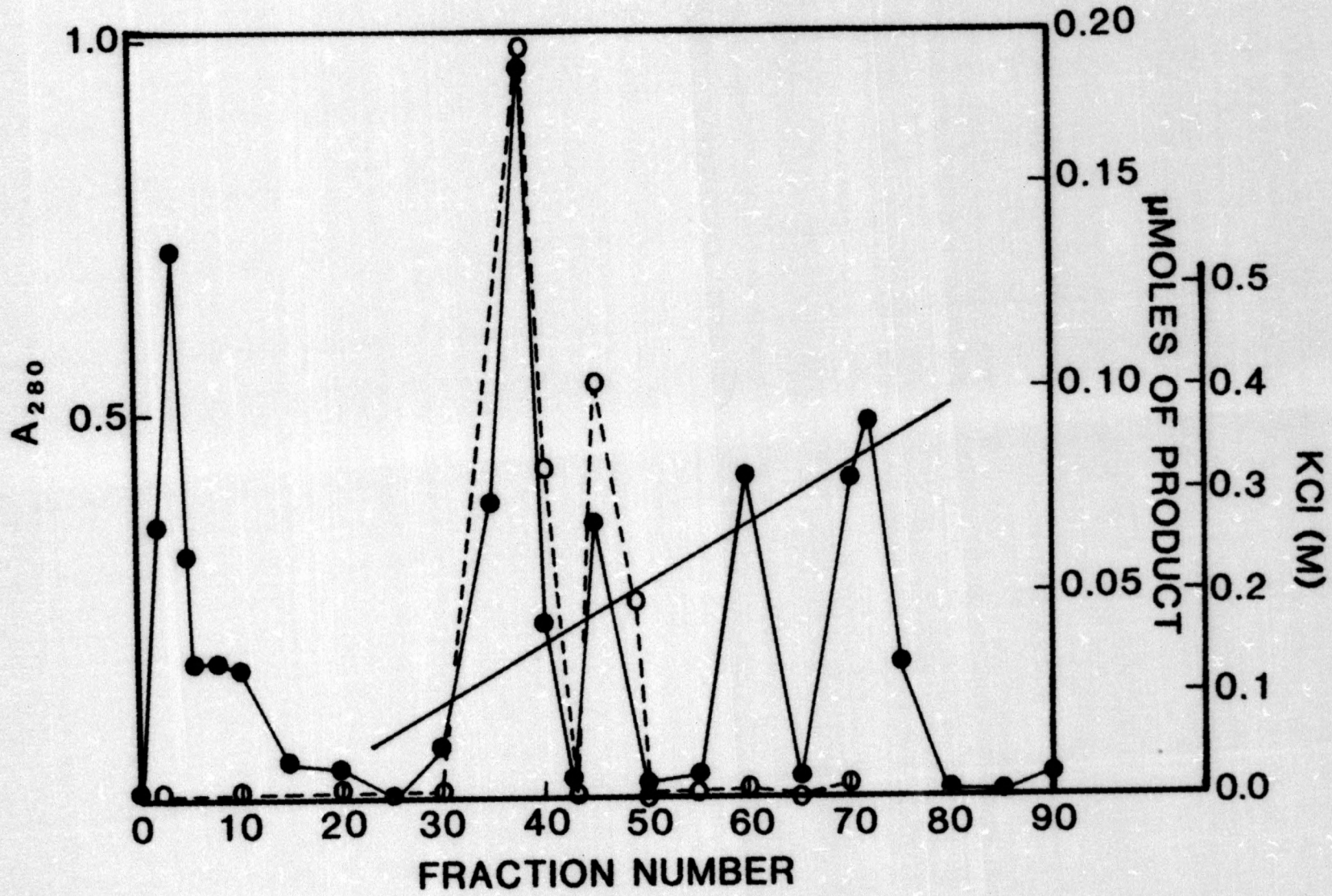
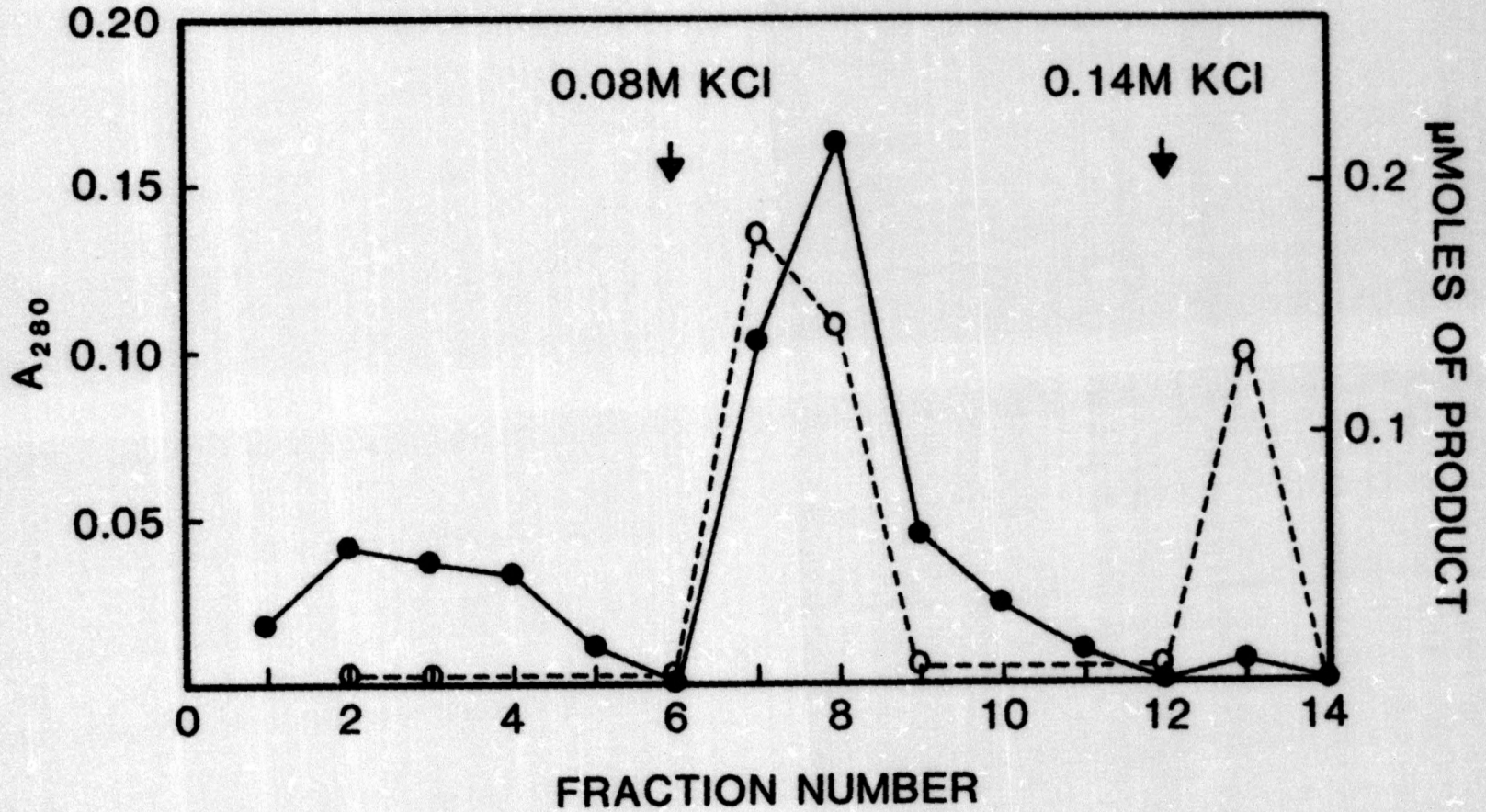

1.0
0.5
A_{280}
0.20
0.15
0.10
0.05
μMOLES OF PRODUCT
0.5
0.4
0.3
0.2
0.1
0.0
KCl (M)
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 4. Step gradient of GS activity from a small DEAE-cellulose column. Three hundred µl of soybean protein was loaded onto a 1 ml DEAE-cellulose column resulting in two peaks of GS activity. Peak 1 was eluted using 0.08 M KCl and peak 2 using 0.14 M KCl. Fractions were monitored for protein at 280 nm (——●——) and GS activity at 540 nm (---O---).


0.20
0.15
0.10
0.05
A_{280}
0.08M KCl
0.14M KCl
0.2
0.1
μMOLES OF PRODUCT
0
2
4
6
8
10
12
14
FRACTION NUMBER

column and could result in an additional peak of activity at a higher salt concentration. To insure that two isoforms were being isolated, the enzymes were eluted with a linear gradient of KCl (Figure 5). Two separate peaks of GS activity were again eluted from the colum at 0.21 M and 0.25 M KCl. These results indicated that GS is a major protein in three-day old etiolated soybean hypocotyls because sufficient enzyme was present to allow separation of two isoforms on the small column using only 1 ml of the post G-50 Sephadex preparation (45 µg of protein).

Each peak of GS activity eluted from the large ion-exchange column was placed in separate Gibco concentrator vials and concentrated to a volume of 200 µl. The samples were run on a 6% polyacrylamide gel and stained with 0.05% Coomassie Blue (Figure 6). Sheep brain GS (392,000), urease (272,000), albumin (66,000) and carbonic anhydrase (29,000) were loaded into lane 1 as molecular weight standards. Lanes 3 and 5 were GS peaks 2 and 1, respectively. A major protein in both peaks was observed to migrate the same distance as the sheep brain GS in lane 1. From the gel GS was determined to be a major protein in plants. Since both protein peaks of GS from three-day old etiolated soybean hypocotyls migrated the same distance as the sheep brain GS, it was concluded that soybean GS has a molecular weight of 392,000.

Figure 5. DEAE-cellulose chromatography of GS activity from etiolated soybean hypocotyls. Three hundred µl of soybean protein was loaded onto a 1 ml DEAE-cellulose column resulting in elution of 2 peaks of GS activity. Using a linear KCl gradient from 0.075 and 0.4 M KCl, peak 1 was eluted using 0.21 M KCl and peak 2 was eluted using 0.25 M KCl. Fractions were monitored for protrein at 280 nm (——●——) and GS activity at 540 nm (---O---).

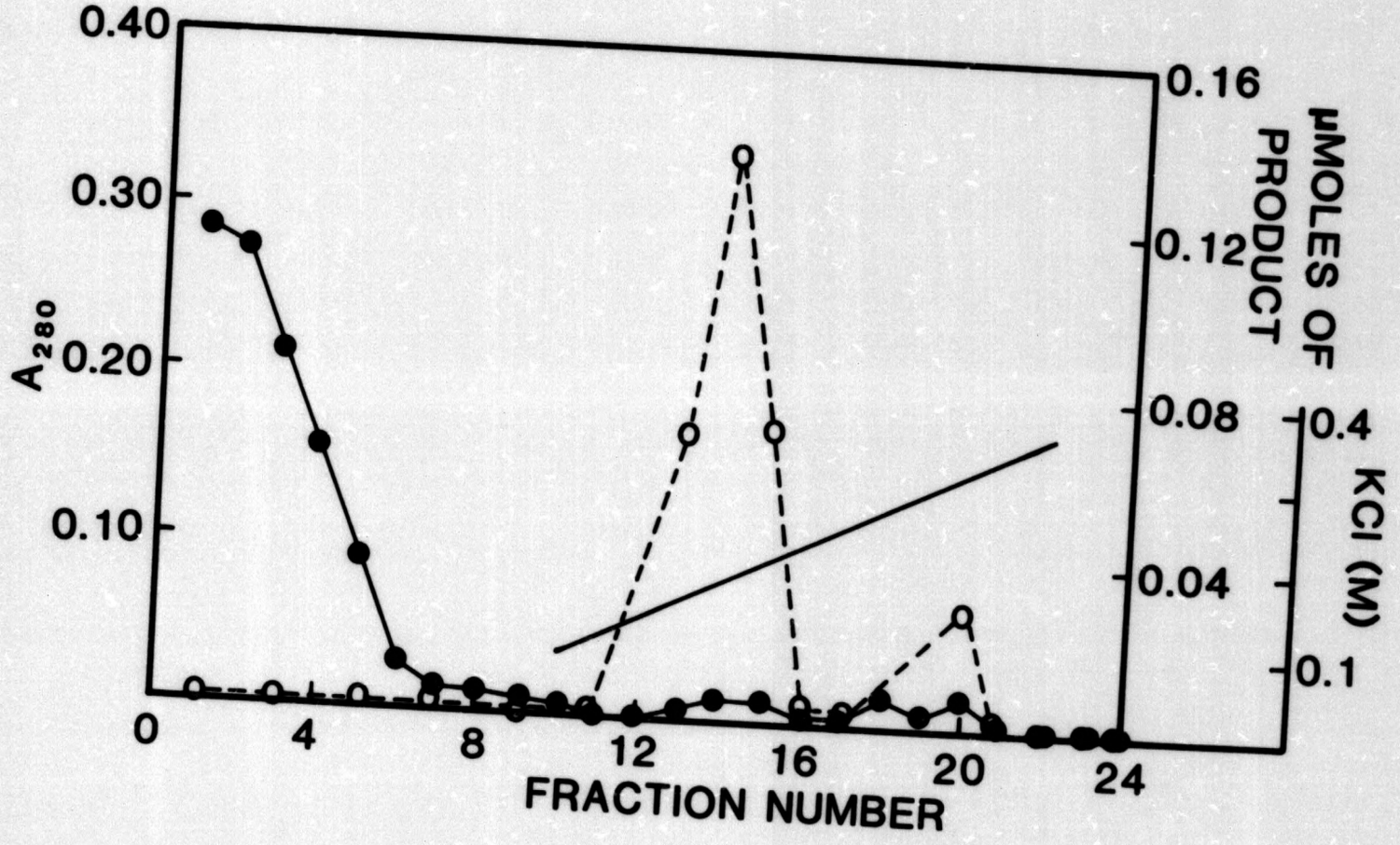
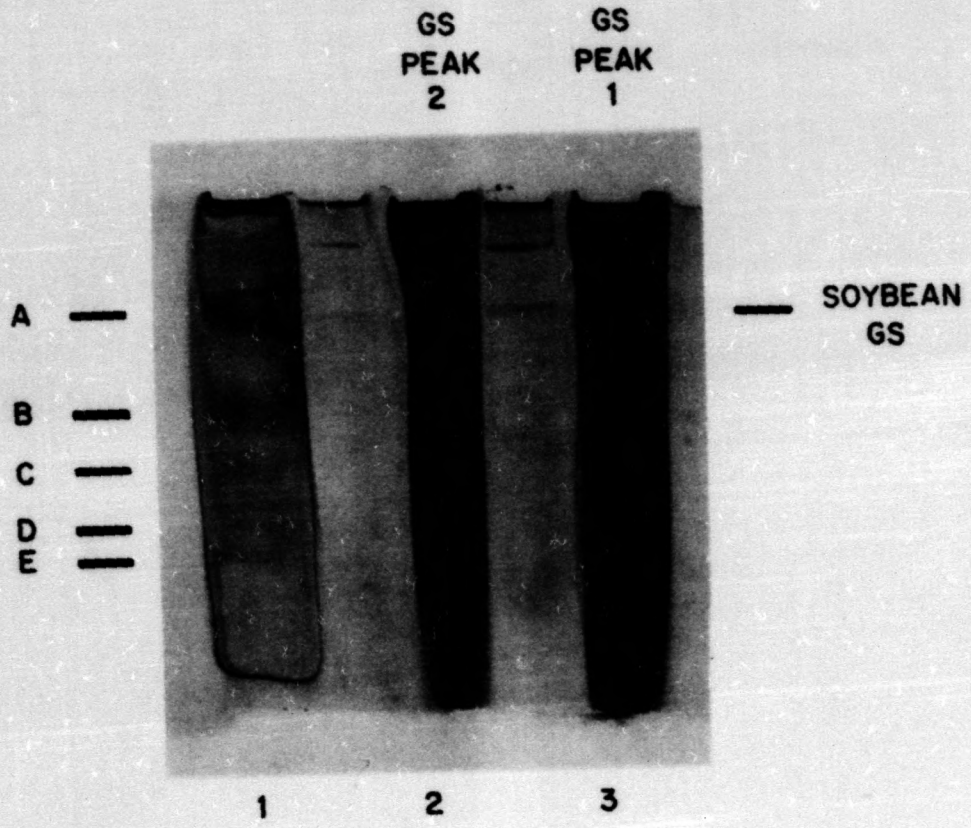

0.40
0.30
0.20
0.10
A_{280}
0.16
0.12
0.08
0.04
μMOLES OF PRODUCT
0.4
0.1
KCl (M)
0
4
8
12
16
20
24
FRACTION NUMBER

Figure 6. Characterization of soybean GS peaks 1 and 2 on a 6% polyacrylamide gel. GS isolated from soybeans was passed through a DEAE-cellulose column to select soybean GS. Lane 1 contained (A) sheep brain GS (392,000), (B) urease (272,000), (C) bovine albumin (66,000), (D) chicken albumin (45,000) and (E) carbonic anhydrase (29,000). Lane 2 and 3 are GS peaks 2 and 1, respectively.


GS
PEAK
2
GS
PEAK
1
A
B
C
D
E
SOYBEAN
GS
1
2
3

Isolation of Total RNA

Isolation of total RNA from three-day old etiolated soybean hypocotyls was accomplished utilizing the phenol-chloroform method. A typical yield of RNA isolated was 55 µg per gram of tissue. If the sample was not pure nucleic acid as determined by the 280/260 ratio, the preparation was discarded and the RNA isolation repeated. A spectral analysis between 200 and 300 nm was performed on the pure RNA. A total of 50 µg of RNA or less was analyzed at one time. A characteristic total RNA curve is represented in Figure 7. The region where RNA absorbs maximally was approximately 260 nm. With decreasing amounts of protein in the sample, the peak absorbance moved closer to 255 nm. The RNA was then size fractionated on a 6% polyacrylamide gel (Figure 8). Lane one was loaded with 25 µg of lamda DNA excised with HindIII and lane 2 was loaded with 25 µg of the RNA. The DNA fragments ranged in size from 6 to 9.5 kilobases (kb). The RNA migrated as a smear with the exception of at least two major bands of RNA. The smearing effect was due to RNA molecules with varying numbers of bases. The two bands of RNA migrated to the same region of the gel as the lamda DNA fragments. Therefore, the two major bands of RNA were approximately 6 to 9.5 kb in size.

Figure 7. Spectral analysis of total RNA isolated from etiolated soybean hypocotyls. Fifty µg of total RNA was analyzed using the Gilford Response spectrophotometer between 200 and 310 nm.

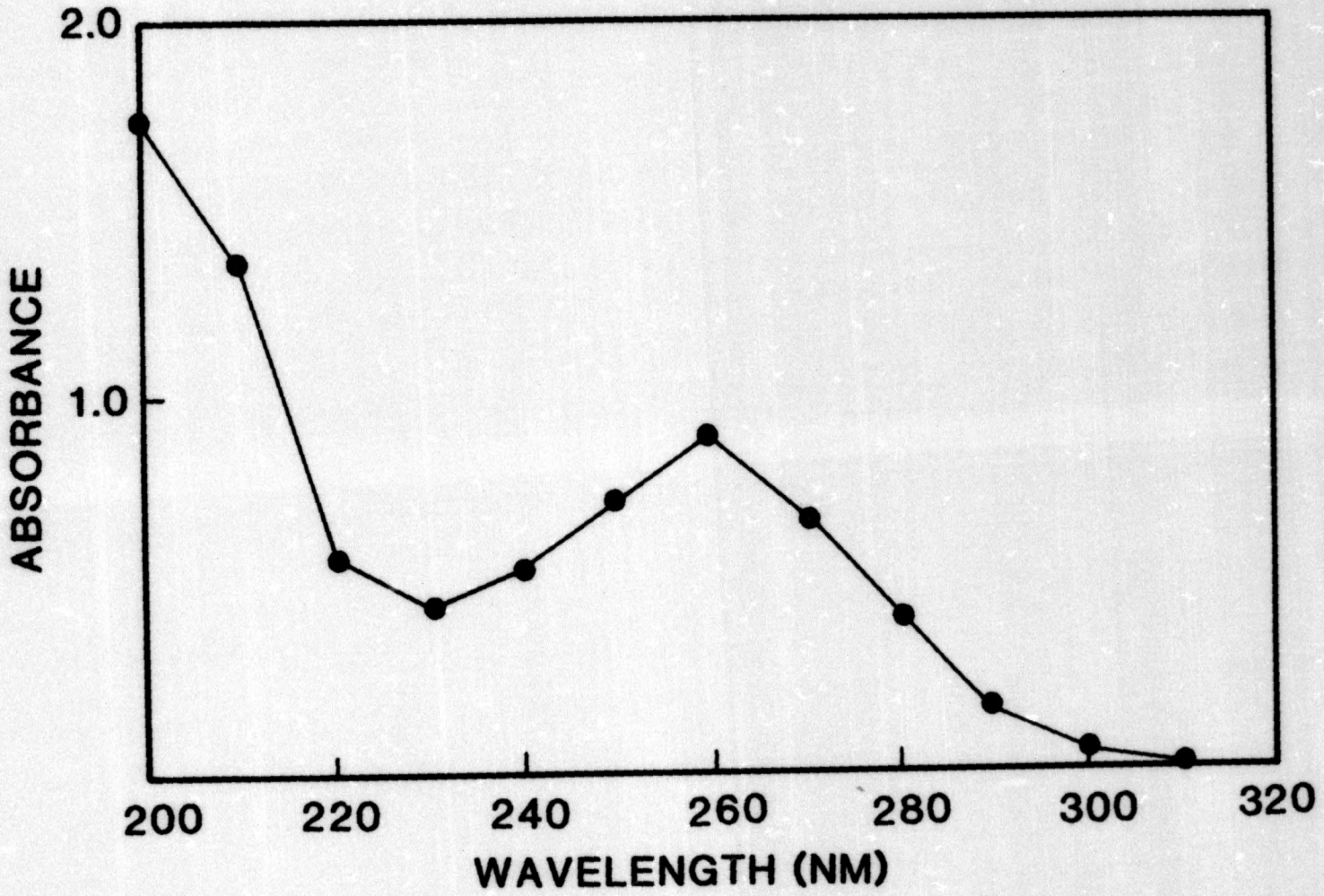
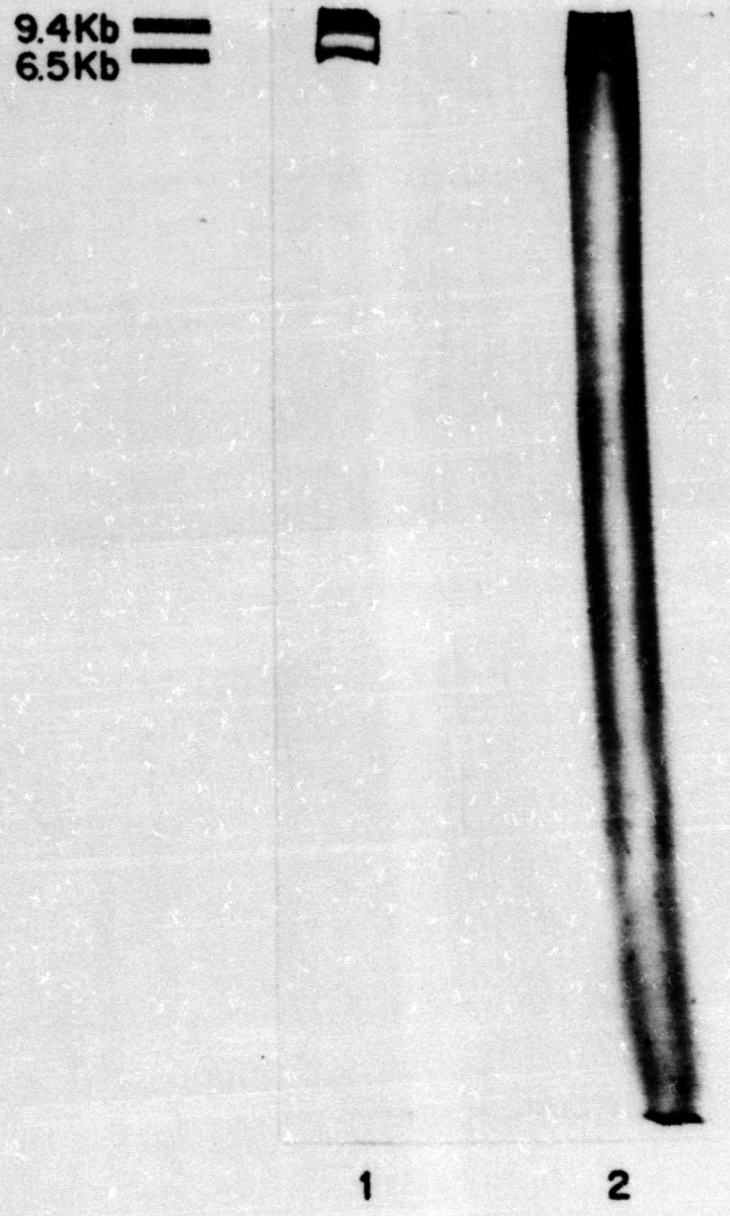

2.0
1.0
ABSORBANCE
200
220
240
260
280
300
320
WAVELENGTH (NM)

Figure 8. Separation of total RNA on 6% polyacrylamide. Five µg of lambda DNA digested with HindIII (lane 1) was electrophoresed and used as a molecular weight marker. Twenty-five µg of total RNA (lane 2) was electrophoresed and silver stained.


9.4Kb
6.5Kb
1
2

Isolation of poly(A)+RNA

Poly(A)+RNA was isolated using Hybond mAP paper (Amersham) impregnated with uracil residues. Since plant mRNA's have a segment composed entirely of adenosine residues, there is binding of the poly A tail to the uracil residues in the paper. A typical yield of mRNA from 20 grams of fresh tissue was 30 µg. The purity of the mRNA sample was determined using the 280/260 ratio and if the mRNA was not pure nucleic acid, the sample was discarded. A spectral analysis between 200 and 300 nm was performed on a 5.0 µg sample of the pure mRNA and the peak absorbance was determined to be 260 nm (Figure 9). A comparison of the total and poly(A)+RNA was performed by gel electrophoresis using a 6% polyacrylamide gel (Figure 10). Lane one was loaded with 25 µg of poly(A)+RNA and lane two was loaded with 100 µg of the total RNA. The total RNA migrated as a smear indicating RNA molecules of varying molecular weights. The poly(A)+RNA, unlike the total RNA, was composed primarily of large RNA molecules as was evident by a smear occuring only on the top half of the gel. Since GS has a large molecular weight, a large poly(A)+RNA molecule would be expected. In addition, the procedure apparently had eliminated any small molecular weight transfer RNA and ribosomal RNA.

Figure 9. Spectral analysis of poly(A)+RNA isolated from etiolated soybean hypocotyls. Five g of the poly(A)+RNA was analyzed using the Gilford Response spectrophotometer between 200 and 300 nm.

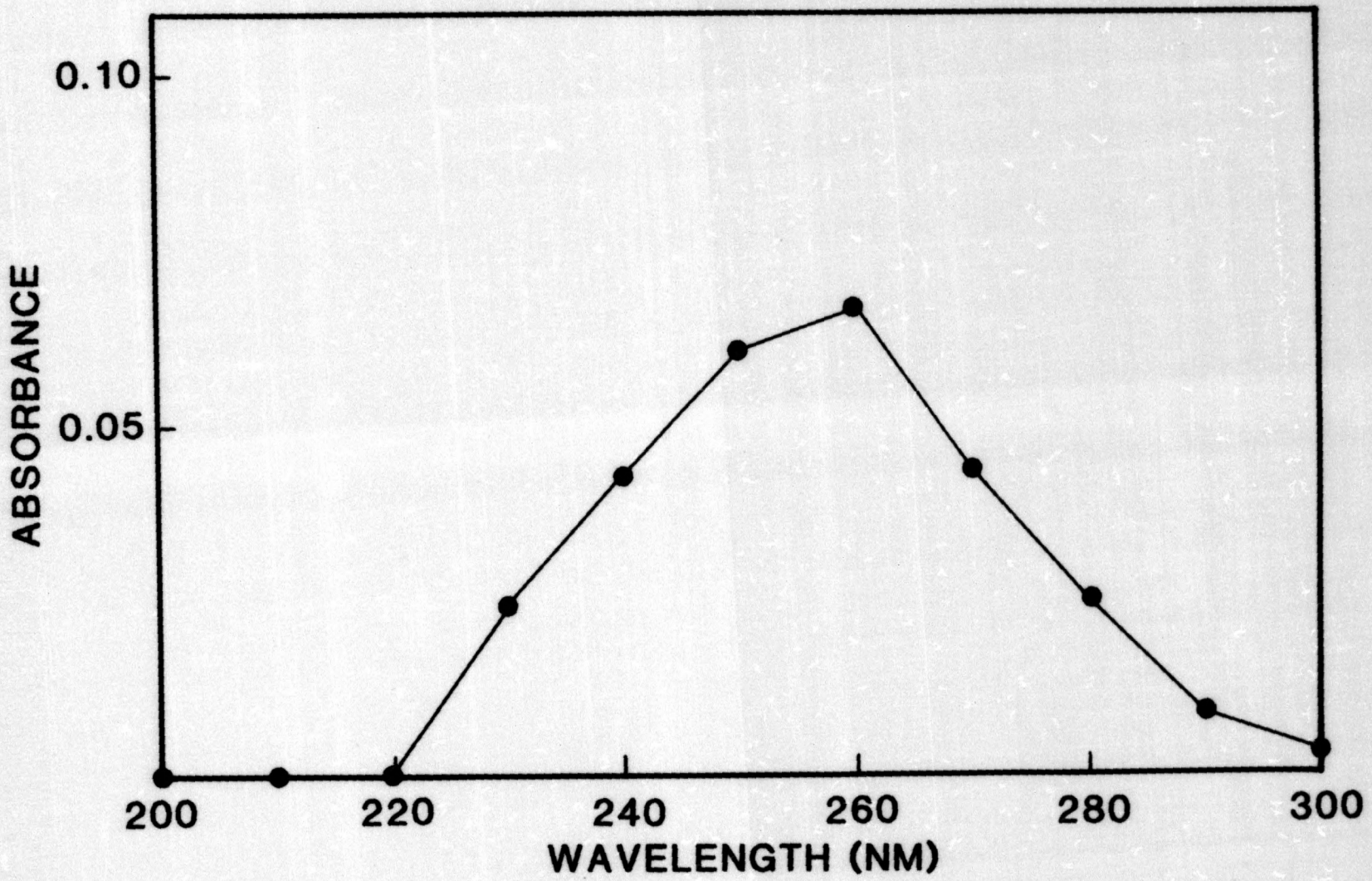

0.10
0.05
ABSORBANCE
200
220
240
260
280
300
WAVELENGTH (NM)

Figure 10. Separation of poly(A)+RNA and total RNA on 6% polyacrylamide. Twenty-five µg of poly(A)+RNA (lane 1) and 100 µg of total RNA (lane 2) was electrophoresed on 6% polyacrylamide. The gel was silver stained.


mRNA
TOTAL RNA
1
2

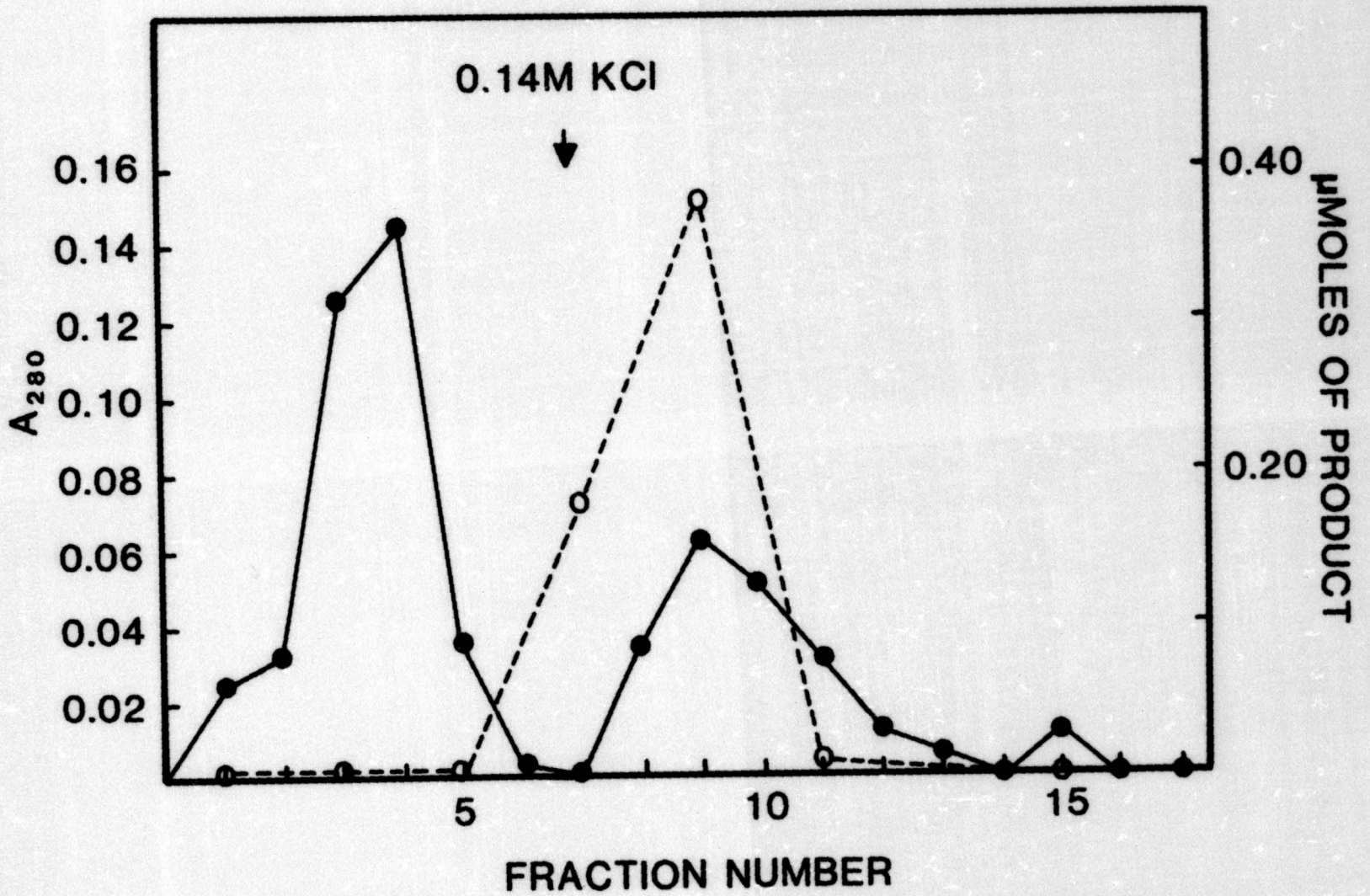
Translation

Translation of the poly(A)+RNA was performed using the nuclease-treated wheat germ system (BRL). A typical reaction volume was 600 µl or 20 times the volume recommended by the protocol. The amount of poly(A)+RNA used was 10 µg. After reacting the poly(A)+RNA with all the necessary enzymes and amino acids, the entire translation mixture was applied to a 1 ml DEAE-cellulose column. The RNA sample contained the poly(A)+RNA of other proteins beside GS. Therefore, it was expected that translation not only produced GS but other proteins as well. One major peak of protein was eluted by a one-step gradient using 0.14 M KCl. This peak also was shown by the transferase assay to have GS activity (Figure 11). Therefore, the mRNA specific for the synthesis of GS was isolated and subsequently translated. The elution of the possible isoforms of GS from the 1 ml column was attempted using a linear salt gradient from 0.05 to 0.5 M KCl. Due to the extremely low levels of GS however, only one peak of GS was eluted.

In Gel Activity Assay

An in gel assay was developed to aid in the identification of specific protein bands attributed to GS. The presence of GS activity in the gel was indicated by a pink-brown band. The typical wavelength used in the *in vitro* assay to detect GS activity was 540 nm. In the case of the in gel assay, wavelengths between 500 and 750 nm were

Figure 11. DEAE-cellulose chromatography of GS activity recovered from *in vitro* translation of poly(A)+RNA. The isolated poly(A)+RNA was translated using a wheat germ cell-free lysate. The translation mixture was loaded onto a 1 ml DEAE-cellulose column and GS was eluted using 0.14 M KCl. Fractions were monitored for protein at 280 nm (——●——) and at 540 nm for GS activity (---O---).


0.14M KCl
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
A_{280}
0.40
0.20
μMOLES OF PRODUCT
5
10
15
FRACTION NUMBER

tested and a wavelength of 600 nm was determined to be the absorbance maximum. To compare the location of the band of enzyme activity to the actual protein, the gel was stained with Coomassie Blue and densitometric analysis performed. The Coomassie Blue was found to absorb maximally at 592 nm (Figure 12). To test the in gel assay conditions, sheep brain GS was used initially. After running the gel for 3 hours, the sheep brain GS was still active according to visual inspection of the gel and densitometric analysis (Figure 13). After spectral analysis of the gel stained with Coomassie Blue was completed, the band of GS activity and the protein band were found to exist at the same location in the gel (Figure 13). The transferase assay specific for GS was not inhibited by the gel. Once the in gel assay conditions were determined, the soybean GS (7.0 units) was loaded into a well. The lane with GS was exposed to the assay mixture and then analyzed spectrophotometrically. After analysis, the lane was stained with Coomassie Blue and a second densitometric analysis of the lane was performed. As can be seen in Figure 14, the GS activity band and the protein band are identical. Therefore, the protein band on a 6% polyacrylamide gel responsible for GS activity was unequivocally determined. The location of the soybean GS in relation to the sheep brain GS after gel electrophoresis can be visualized in Figure 15. Lane 1 was loaded with a partially purified GS preparation (7.0 units) and lane 2 was loaded with 2.0

Figure 12. Spectral analysis of Coomassie Blue stain. One ml of Coomassie Blue was analyzed using the Gilford Response spectrophotometer between 530 and 625 nm.

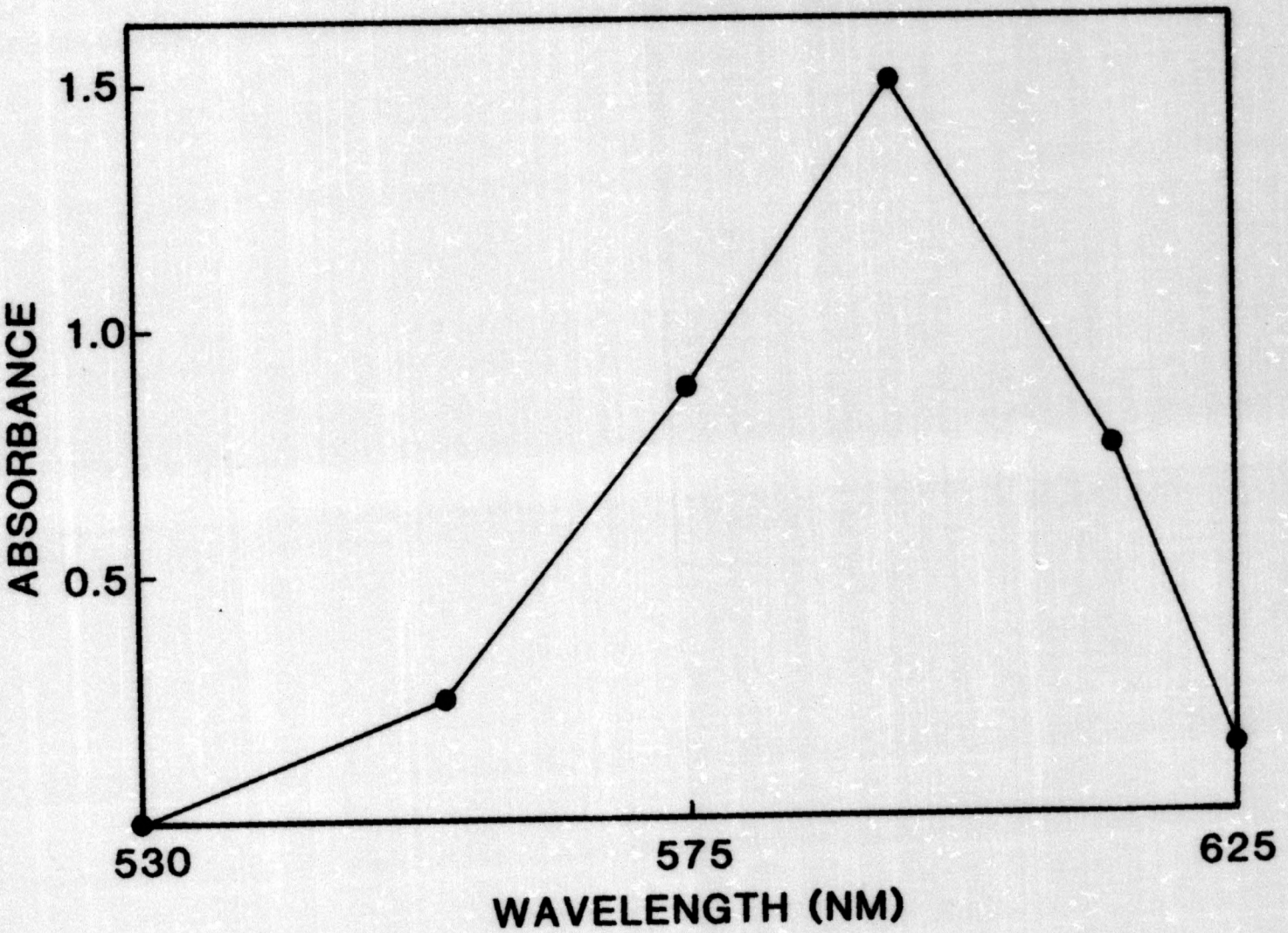
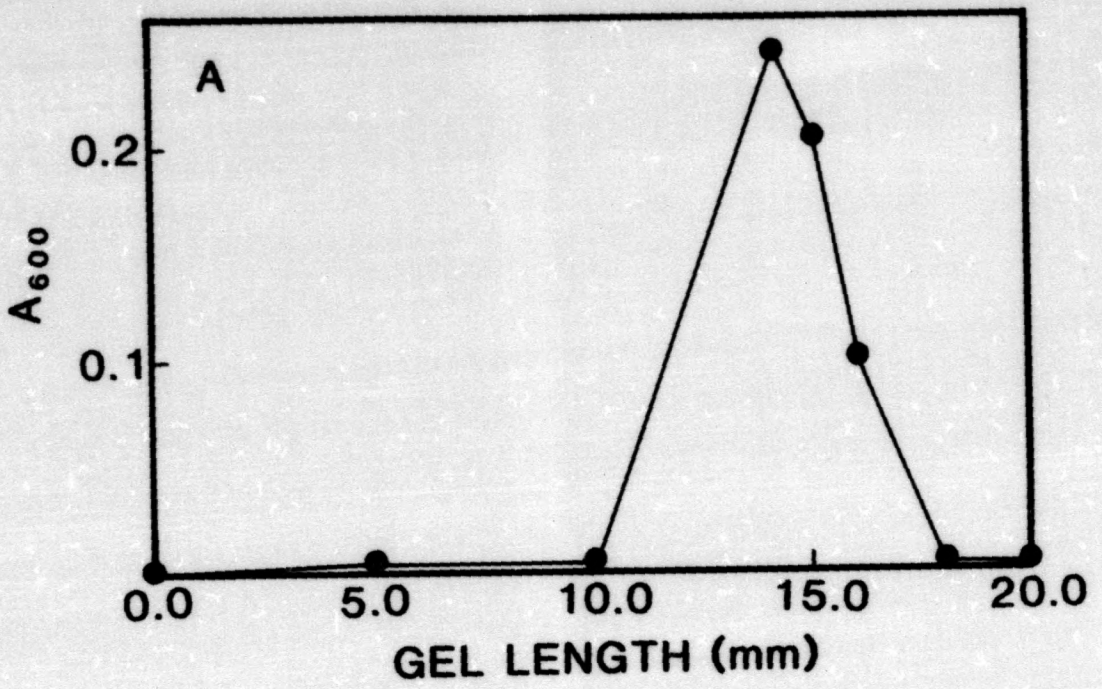

1.5
1.0
0.5
ABSORBANCE
530
575
625
WAVELENGTH (NM)

Figure 13: In gel analysis of soybean GS on 6% polyacrylamide. The soybean GS was passed through a DEAE-cellulose column. Fractions containing GS activity were concentrated and loaded onto a 6% polyacrylamide gel.
Graph A: The lane with GS (7.0 units) was assayed after which the gel was scanned at 600 nm using the Gilford Response spectrophotometer.
Graph B: After analyzing the gel for GS activity, the gel was stained with Coomassie Blue and scanned at 592 nm.


A
0.2
0.1
A_{600}
0.0
5.0
10.0
15.0
20.0
GEL LENGTH (mm)

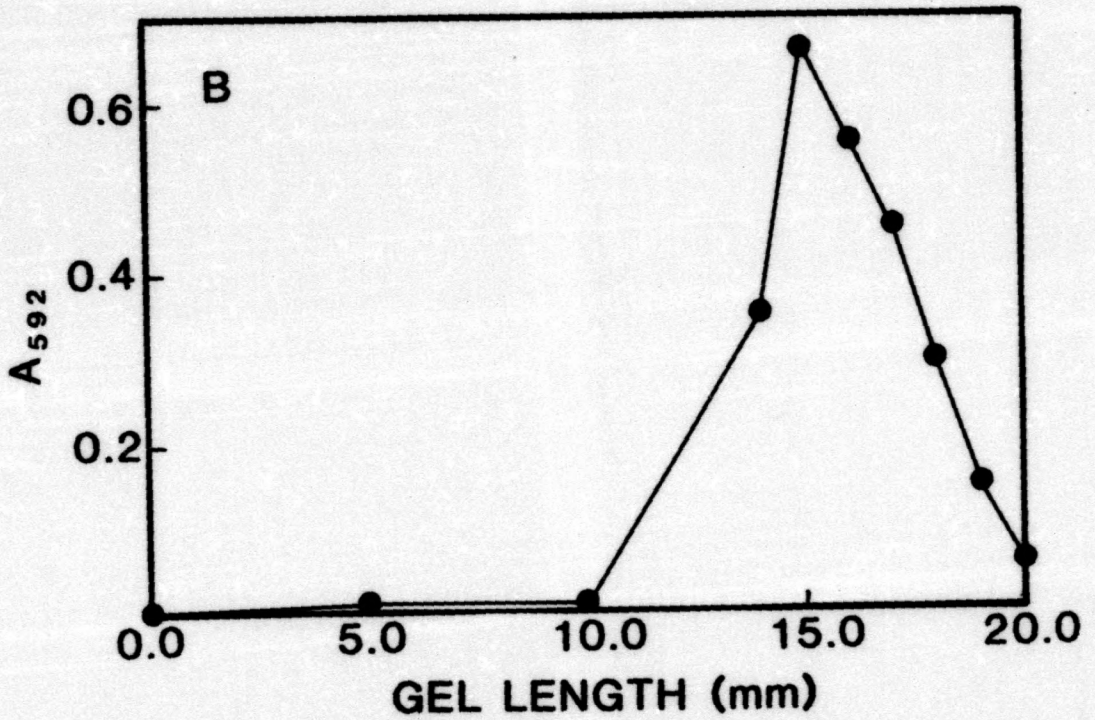
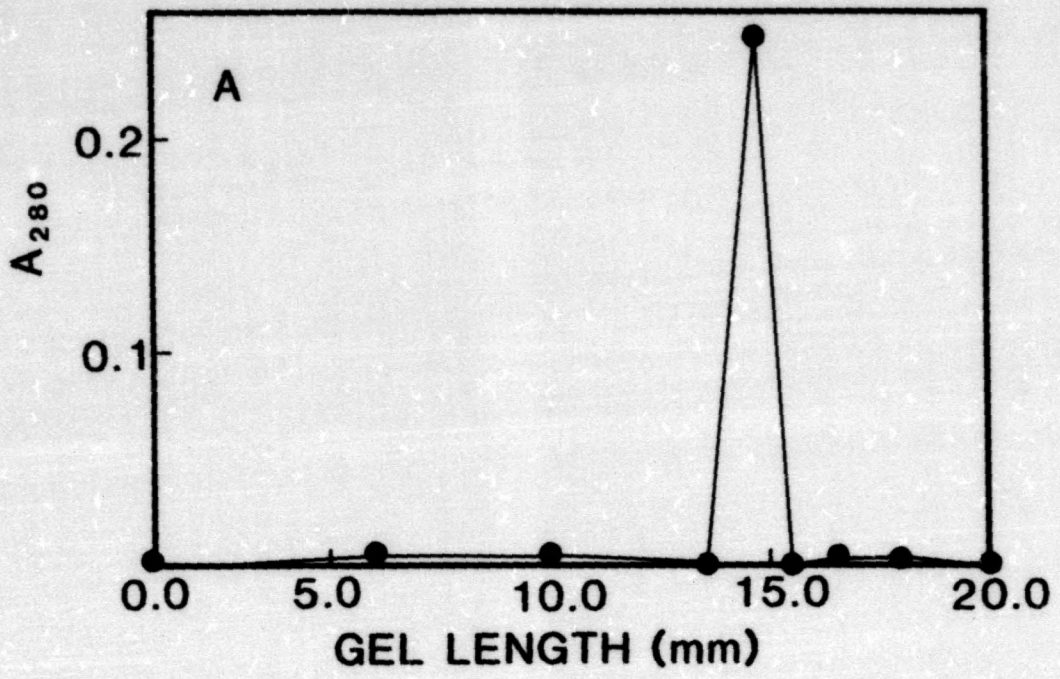

B
0.6
0.4
0.2
A_{592}
0.0
5.0
10.0
15.0
20.0
GEL LENGTH (mm)

Figure 14. In gel analysis of sheep brain GS on 6% polyacrylamide. The sheep brain GS (Sigma) was loaded onto a 6% polyacrylamide gel.
Graph A: The lane with GS (2.0 units) was assayed and scanned at 600 nm using the Gilford Response spectrophotometer.
Graph B: After analyzing the gel for GS activity, the gel was stained with Coomassie Blue and scanned at 592 nm.


A
0.2
0.1
A_{280}
0.0
5.0
10.0
15.0
20.0
GEL LENGTH (mm)

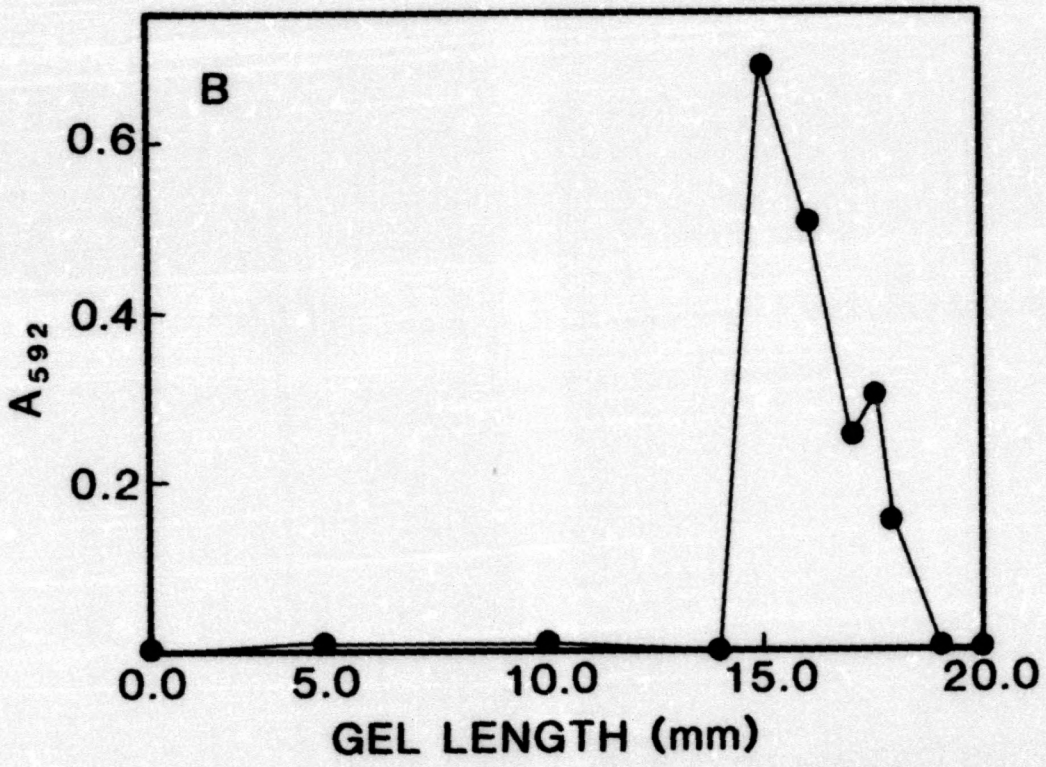
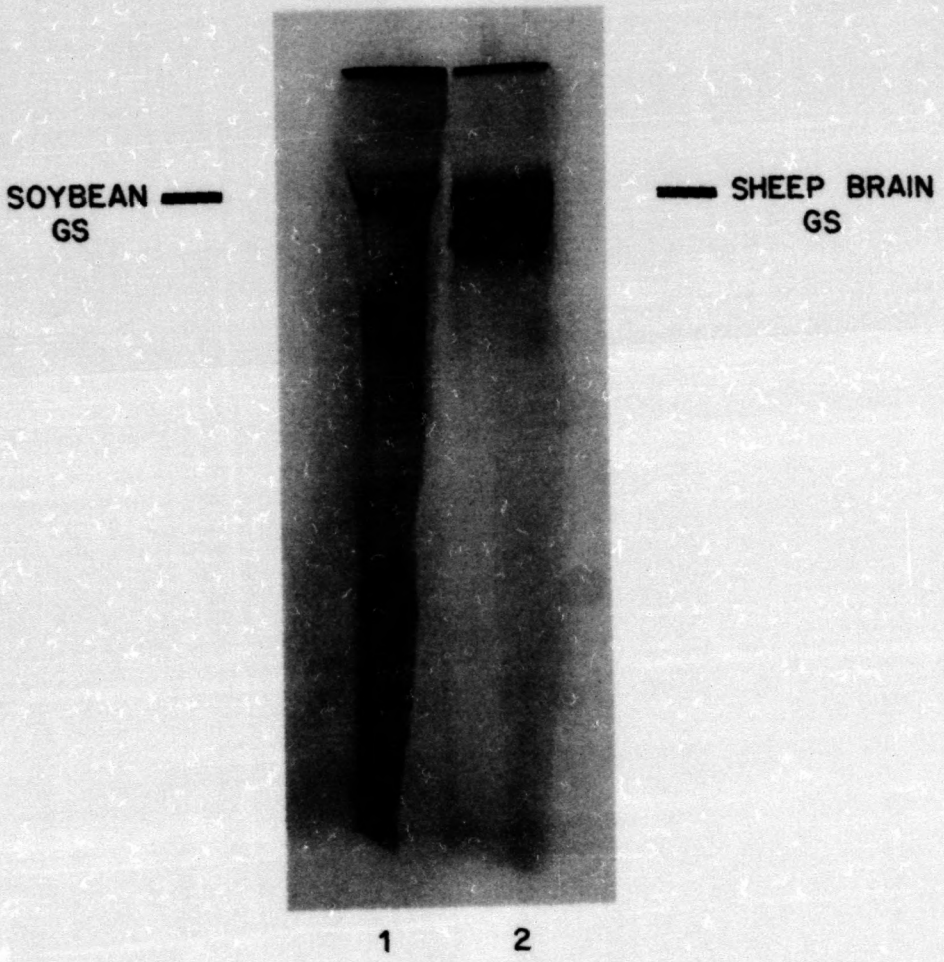

B
0.6
0.4
0.2
A_{592}
0.0
5.0
10.0
15.0
20.0
GEL LENGTH (mm)

Figure 15. Characterization of soybean and sheep brain GS on a 6% polyacrylamide gel. Protein isolated from soybean was passed through a DEAE-cellulose column to select for GS. Seven units of soybean GS (lane 1) and two units of sheep brain GS (lane 2) were electrophoresed on a 6% gel. Proteins were visualized with Coomassie Blue.


SOYBEAN
GS
SHEEP BRAIN
GS
1
2

units of sheep brain GS. A major protein band migrated the same distance in both lanes. Therefore, the soybean GS has a molecular weight similar to the sheep brain GS (392,000) as also determined (Figure 15) by gel electrophoresis.

DISCUSSION

To measure the amount of GS isolated from soybean tissue or the amount of GS translated from isolated poly(A)+RNA, the gamma-glutamyl-hydroxamate assay was employed. Such enzymes as glutaminases, amidases and GS are capable of producing the compound gamma-glutamyl-hydroxamate (Meister et al., 1954; Meister, 1963). Glutaminases and amidases have been subsequently discovered to be independent of the presence of a divalent cation or ADP (Meister, 1963); but GS activity is highly dependent on both a divalent cation and ADP (Stasiewicz and Dunham, 1979). In addition, a pH optima of the glutaminases is 6.0 (Meister et al., 1954), whereas the pH optimum for soybean GS is 7.5 (Stasiewicz and Dunham, 1979). Therefore, the control reaction employed in this research was the assay mixture minus ADP at a pH of 7.5. Since the requirements for the amidases and glutaminases are different from those for GS, it was possible to accurately measure GS activity.

The elution of two isoforms of GS from both large (30 ml) and small (1.0 ml) columns is consistent with recent research (Stasiewicz and Dunham, 1979; Cummings, 1985). Further characterization of the two isoforms of GS was performed by gel electrophoresis. Since the two peaks of GS co-migrated to the same location on a 6% polyacrylamide gel, the two isoforms of GS are not significantly different in

the number of subunits or molecular weight. Although the molecular weight of the two isoforms are similar, peaks 1 and 2 are different in some aspects. Studies have shown the K_m values and the isoelectric points of the two GS peaks as well as the regulatory properties of the two peaks as differing significantly (Stasiewicz and Dunham, 1979; McMaster and Dunham, 1980). The two bands corresponding to GS migrated in the gel the same distance as sheep brain GS. Therefore, since sheep brain GS has a molecular weight of 392,000 (Meister, 1974), soybean GS has a similar molecular weight. Additional studies on other higher plant tissue have shown the molecular weight of GS to lie between 330,000 and 376,000 daltons (O'Neal and Joy, 1973; McParland et al., 1976).

From the intensity of the protein bands, GS is a major protein in three-day old etiolated soybean hypocotyls. Therefore, the isolation of the poly(A)+RNA specific for GS was straightforward. The gene responsible for the synthesis of GS has been isolated and sequenced from Chinese hamster cells and alfalfa. The poly(A)+RNA responsible for GS in Chinese hamster cells is composed of 2800 base pairs (Hayward et al., 1985), whereas the alfalfa poly(A)+RNA is 2000 base pairs in size (Tischer et al., 1986). Gel electrophoresis of the isolated total RNA indicated two major bands of RNA between 6 and 8 kb in size. In addition, RNA molecules varying in size from a few base pairs up to 5

kb were also isolated. Therefore, the RNA isolated from soybean was sufficiently large to contain the information necessary for the translation of GS.

Poly(A)+RNA was isolated using the Hybond mAP paper. As indicated by gel electrophoresis, the poly(A)+RNA fraction was composed mainly of medium to high molecular weight RNA molecules. However, a band of poly(A)+RNA in the size range of the one isolated from alfalfa was also visualized on the gel. Therefore, the poly(A)+RNA isolated using the Hybond mAP paper was sufficient in size to encode GS. The amount of poly(A)+RNA isolated from soybean using the Hybond mAP paper was consistent with recent findings by Werner et al. (1984) who isolated poly(A)+RNA from Ehrlich ascites tumor cells. When compared to the oligo (dt) cellulose method of poly(A)+RNA isolation, the paper method resulted in a 50% decrease in poly(A)+RNA isolation per gram tissue (Cummings, 1985). For translation experiments, however, the poly(A)+mAP paper was adequate. Translation of the isolated poly(A)+RNA resulted in only one form of GS. As indicated earlier, the fact that so little protein was actually translated was reason to believe that only the major isoform could be detected.

In order to determine the specific location of GS in a polyacrylamide gel, an in gel assay procedure was utilized. When the GS was active, a pink-brown coloration of the gel occurred in the immediate location of the suspected GS band. After allowing the pink-brown color to develop for 10

minutes, an immediate spectral analysis of the gel was performed since gel retention of the color was approximately 30 minutes. From the results, the bands of protein specific for sheep brain and soybean GS have been identified using the in gel assay. Since GS activity was able to be detected in the gel, GS and its isoforms are in sufficiently high quantity at this stage of soybean growth.

The results of this study have shown that two isoforms of GS occur in significant quantities in three-day old etiolated soybean hypocotyls. Since the poly(A)+RNA was abundant, as judged by the amount of GS translated in vitro, GS is a major protein in hypocotyl cells at this stage of development. Since the messenger RNA that encodes GS is abundant, the synthesis of a complete cDNA of the GS gene(s) should prove straightforward. The GS cDNA can then be cloned into an appropriate vector, making it possible to determine the number of gene or genes responsible for the synthesis of the isoforms of GS. Also, cloning and transcription of the cDNA for GS will provide enough poly(A)+RNA to translate the two isoforms and to study the differences between the two enzymes.

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B6, F9