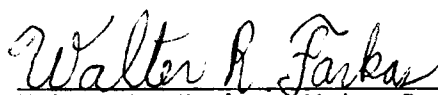


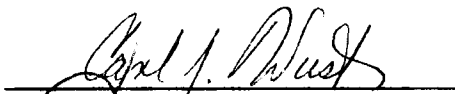
To the Graduate Council:

I am submitting herewith a thesis written by Thomas L. Walden, Jr. entitled "Purification of Wheat Germ Guanine Insertase and Observations Using an X-Irradiated Transfer RNA Substrate." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

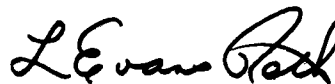


Walter R. Farkas, Major Professor

We have read this thesis and
recommend its acceptance:




Accepted for the Council:



Vice Chancellor

Graduate Studies and Research

PURIFICATION OF WHEAT GERM GUANINE INSERTASE AND
OBSERVATIONS USING AN X-IRRADIATED TRANSFER
RNA SUBSTRATE

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Thomas L. Walden, Jr.

March 1979

1781375

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The first items that must be acknowledged are my incredible good luck in coming to the University of Tennessee, the sound guidance that I have received, and the associations that I have made.

I would like to express my appreciation to my major professor, Dr. Walter Farkas, for whom I hold the highest esteem and admiration. His advice, trust, instruction, and support have made my graduate education an enjoyable and rewarding experience. I take pride in the skills and knowledge which I have acquired under his instruction.

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In skimming the surface with my thank you's, it is regrettable that these people will never be fully aware of the positive impression that

their examples, guidance, and friendships have had, and will have, on my life. Thank you.

ABSTRACT

Guanine insertase has been purified 3382-fold to homogeneity with a specific activity of $2,029 \text{ pmoles hr}^{-1} \text{ mg protein}^{-1}$. The enzyme, which has a molecular weight of 140,000, migrates as a single band on polyacrylamide gel electrophoresis. The enzyme substitutes guanine for guanine and is inhibited by Q base. A monovalent cation best met by 0.08 M K^+ and a divalent metal cation best met by $5 \times 10^{-3} \text{ M Mg}^{++}$ are required for guanylation. The divalent metal obviates the monovalent metal requirement. The K_m for guanine is $1.05 \times 10^{-6} \text{ M}$ and the optimum pH is 7.6. Seven tRNAs found in wheat germ are substrates for guanine insertase.

Observations on the guanine accepting ability of irradiated wheat germ tRNA shows 50% inactivation at 60,000 Roentgens with an unusual saw tooth pattern at low doses. The saw tooth has peaks at 2,000 and 4,000 Roentgens and can be modified by Tris or bovine serum albumin. RPC5 profiles of guanylated irradiated and unirradiated tRNA are identical.

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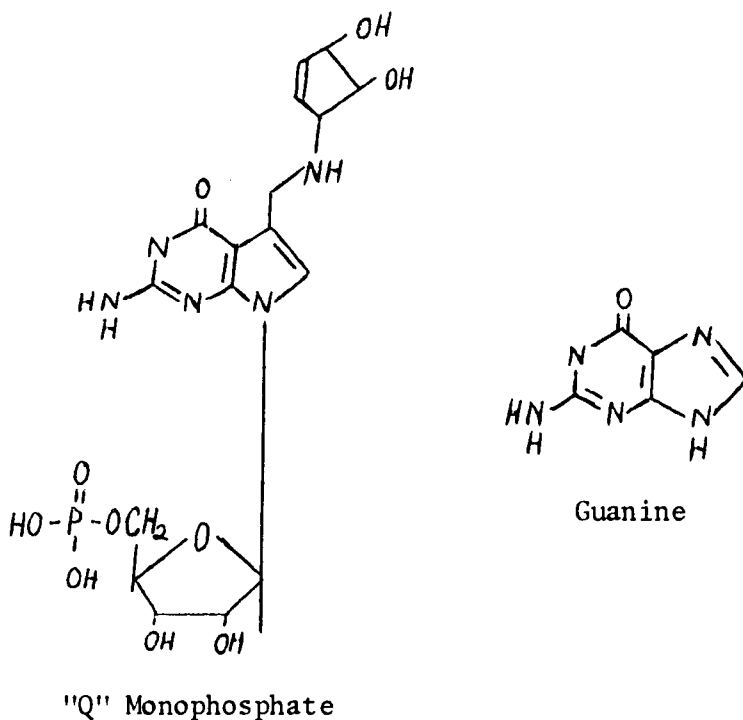
I. INTRODUCTION

The guanylation enzyme was recently purified to homogeneity from rabbit erythrocytes by Farkas and Howes (1). This enzyme replaces guanine in the first position (5' end) of the anticodon in certain isoaccepting transfer RNAs with either guanine or Q base (2,3,4,5). Studies by Nishimura showed that Q, a hypermodified derivative of guanine, was present in bacteria, many animal tissues and also in wheat germ (6). Unpublished work by Farkas demonstrated that wheat germ possessed the guanylation enzyme (7). It was decided to purify the plant enzyme from wheat germ and compare it to the mammalian enzyme.

II. LITERATURE REVIEW

"Q"

The hypermodified base "Q" was first reported in 1968 by groups sequencing *E. coli* tyrosine accepting transfer RNA (8). It was pinpointed to be in the first position of the anticodon (8,9) which is the "wobble" position proposed by Crick. Early reports indicated that Q was a modified derivative of guanine which possessed an additional positive charge.



The structure of Q was elucidated in 1975 on the basis of mass spectra analysis, nuclear magnetic resonance spectroscopy, and certain

chemical properties to be 7-(4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine (10). Q has a molecular weight of 409 based on the formula $C_{17}H_{23}N_5O_7$. The cyclopentene cis diol allows Q-containing tRNA to be bound to DBAE (dihydroxyboryl aminoethyl cellulose) (11). The cis diol on the 3' end of the transfer RNAs must be aminoacylated to prevent binding to the column by the 3' terminal adenosine. Transfer RNA containing Q nucleotides can be identified by the reaction of sodium periodate with the cis diol (12). Diols on the ribose sugars of the nucleotides are protected by their phosphate linkages. Reaction with cyanogen bromide causes the Q-containing peak to be eluted after untreated tRNA during reverse phase column-5 chromatography (RPC-5). Reaction with cyanogen bromide alone is not conclusive of Q since 4-thiouridine and (3-(3-amino-3-carboxyl-n-propyl)uridine), also known as X (13), react with cyanogen bromide (12,14). Tyrosyl and aspartyl tRNA contain X (15). Reaction with sodium periodate causes the Q-containing peak to bind very tightly to RPC-5. Salt concentrations over 1 M will elute periodate-treated Q(+) tRNA. Aspartyl tRNA does not react with sodium periodate probably because a derivative known as Q* is present instead of Q (12). (Q* is a modified derivative of Q discussed later.) Unlike most purine nucleosides, which are hydrolyzed to the free base with 1 N HCl, Q nucleoside is resistant to hydrolysis of the N-glycoside bond (10). The extra positive charge possessed by Q is due to the presence of the secondary amino group at C-7. When passed through a Dowex 1 or DEAE Sephadex A-25 column at slightly alkaline pH, the extra positive charge of QMP causes it to

elute prior to most of the other nucleosides (9,10). The absorbance spectrum of Q has peaks at 220 and 262 nanometers.

"Q" was found to be present only in those transfer RNAs which recognize an XA_C^U codon where X may be A,G,U, or C (1). Examination of a genetic code chart shows that only $tRNA^{(Tyr)}$, $tRNA^{(Asp)}$, $tRNA^{(Asn)}$ and $tRNA^{(His)}$ contain "Q." Each of these amino acids has a synonym codon with U or C in the third position. "Q" is found in the animal, plant, and monera kingdoms, but its distribution is not universal (6). It is absent in yeast and the bacterium Thermus thermophilus.

Modified derivatives of Q have also been discovered; they are represented in the literature as Q* (6). Q* contains either mannose or galactose bound in glycosidic linkage to the OH group on carbon number four of A's cyclopentene ring (16). Rat liver is an abundant source of Q* (6,17). Transfer RNA containing the two types of Q* may be isolated by utilizing the affinity of plant lectin for the sugar moieties (17). $tRNA^{(Asp)}$ which contains the mannose derivative was isolated with concanavalin A-sepharose and $tRNA^{(Tyr)}$ which contains Q with the galactose modification was isolated by Ricinus communis lectin-sepharose. Q* has not been found in plants or bacteria (17).

Those tRNAs which contain Q bind to XAU template better than XAC template (9,18). When guanine replaces Q in the wobble position, the anticodon has a higher binding affinity for the XAC template. "It is unlikely that replacement of G by U has any drastic effects on in vivo protein synthesis, since (few) code words end with U" (9). The first or wobble position is not as flexible as first thought. In the same column

of the genetic code as the Q-associated codons are those with adenosine and guanosine in the third position, XA_G^A . 2-thiouridine restricts the wobble in these codons by recognizing adenosine above guanosine (19). The wobble is restricted in other parts of the chart by 5 carbamoylmethyl uridine (20), and other modified bases in the first position.

Modified bases in the first position of the anticodon, in particular Q, may have some function other than preferential recognition of code words. The exact function of Q is not known at this time. One function postulated for Q is to prevent mistakes in the recognition of the second or third letter of the triplet (9). High magnesium concentrations ($10^{-2}M$) have been shown to induce miscoding. Poly U-G, which directs the synthesis of a valine cysteine copolypeptide, is misread at $0.02 M Mg^{++}$, and a valine tyrosine copolypeptide is produced (21). The G is misinterpreted as an A resulting in "UGU" coding for tyrosine instead of cysteine. Earlier work showed that polyuridylic acid which normally codes for phenylalanine is misread as isoleucine or tyrosine at $2.6 \times 10^{-2}M Mg^{++}$ (22). Polyadenylic acid, on the other hand, required $1.6 \times 10^{-2}M Mg^{++}$ for maximum efficiency in interpretation as lysine (22). Modified bases restrict the flexibility of the anticodon region, altering the stacking of the bases and the conformation of the tRNA. This difference in conformation alters the available sites for hydrogen bonding between corresponding bases in the anticodon. Since thiouridine also is found in the anticodon of tRNA recognizing XA in the first two codon positions, it too could function to prevent misreading of the second codon position at high Mg^{++} concentrations.

The steps comprising the biosynthesis of Q have not been determined; however, facts have emerged from the study of bacterial mutants such as methionine deficient E. coli which also lack Q (23). By varying the amounts of methionine given to these mutants, several different precursors of Q have been isolated (24). Each of the precursors were found to have absorbance spectra identical to Q indicating that the 7-deazaguanosine ring was already present and the side chain at C-7 was attached. The exact structures of the Q precursors have not been determined. Because Q is a modified derivative of guanine, guanine is considered to be the starting point in Q biosynthesis. Radioactive labeling showed that positions C-8 and N-7 of guanine were lost during Q synthesis and position C-2 was retained (25). Use of E. coli mutants with temperature sensitive ribonuclease P resulted in the accumulation of precursor RNA molecules (26). These precursor transfer RNAs were substrates for Q modification. Evidence indicates a likely role for the guanine insertase enzyme in the synthesis of (Q+tRNA). A literature review section on guanylation follows this section.

An enzyme has been discovered in rat liver lysates which catalyzes the formation of Q* from E. coli RNA (^{Asp}) and GDP-mannose (27). This enzyme requires Mg^{++} .

For each amino acid, there is more than one tRNA. The set of tRNAs which are specifically esterified by the same amino acid are known as isoacceptor tRNAs. A useful method for isolating these isoaccepting tRNAs is by reverse phase chromatography which uses a quarternary amine to bind the negatively charged tRNAs (28). Transfer RNAs are

eluted by raising the salt concentration. The chromatography system that we have used is known as RPC-5. Rabbit reticulocyte tRNA^(His) consists of three isoacceptors, only one of which contains Q. Transfer RNAs for histidine, aspartic acid, asparagine, and tyrosine consist of a (Q+) form which contains Q in the wobble position and an undermodified form in which G has not been replaced by Q in the wobble position. Research has shown that the relative amounts of (Q+) and (Q-)tRNA differ in Drosophila (29), silkworms (6), calf serum (15,30), rabbit reticulocytes (7), and humans (7) between developmental stages. In addition, concentrations of Q have been found to be tissue dependent in animals, with the liver and kidney showing the highest concentration (6). Tests showed five times as much Q in the liver of the starfish compared to the testes (6). Abnormally high levels of Q or Q* have been demonstrated in rat ascites hepatoma (6).

Contrary to reports of abnormally high levels of Q* in rat ascites hepatoma, RPC-5 elution profiles of many tumor tRNAs show increased levels of (Q-)tRNA (15,31). The (Q-) peak of tRNA^(Asp) can increase by as much as 50%. In most cases (Q-)tRNA is less than 50% of the total and the (Q+) is more abundant than the (Q-) form. Curiously, increases in the (Q-) peak (tRNA^(Asp)_{IV}) are found "in most human tumors classified as carcinomas or adenocarcinomas . . . [but not] melanomas or sarcomas" (31). The reasons for this phenomena are not known at present. The tRNA^(Asp)_{IV} peak is not unique to tumors, indicating that it does not occur exclusively in cancer cells. A possible explanation is that the enzymatic modification of Q biosynthesis cannot keep up with the

increased rate of tRNA synthesis resulting in a buildup of the unmodified (Q-) form. However, several slowly growing cancers also have more (Q-) than (Q+) tRNA in disagreement with this observation (32).

Relative amounts of (Q-) and (Q+) tyrosine, aspartic, asparagine, and histidine tRNA vary with developmental state. The most thoroughly studied of the changes in the level of Q with development has been done using Drosophila (29). The (Q-)tRNA^(Tyr) form in Drosophila was once thought to cause inhibition of tryptophan pyrrolase resulting in a vermilion mutant coded for by the gene (Su(S))² (33,34). Recent evidence has failed to support these findings. At the time the first studies on inhibition were presented, another study showed that the elution profiles on RPC-5 of tRNA^(Tyr), tRNA^(Asn), tRNA^(Asp), and tRNA^(His) differed between these mutants and normal wild type flies (29). The (Q-) form was absent in the wild type. Variations in (Q-) and (Q+) were thought to control the tryptophan pyrrolase enzyme. This same study reported that the percentage of (Q-) and (Q+) within each tRNA species varied with development of the Drosophila from egg to parent. From egg until late third instar larve, the (Q+) form decreased while the (Q-) form proportionately increased. After this period, the (Q+) increased until (Q-) and (Q+) were equal in the adult. This change was observed for all four tRNAs and has also been reported in other insects such as L. sericata (12). It was proposed that the (Q+) form may be essential for development of prepupa to adult (29). This change in the levels of Q from fetus or egg to adult has been observed in other animals including rabbits (7), humans (7), and hamsters (31).

Recent studies have indicated that changes in (Q+) and (Q-) levels are controlled by growth conditions and not by genotype at the $su(s)^2$ locus in Drosophila (15,35). Attempts to repeat the tryptophan pyrrolase inhibition have been unsuccessful (35) and an artifact due to EDTA rather than a specific $tRNA^{(Tyr)}_{(Q-)}$ is responsible for the inhibition (36). Several other types of Drosophila mutants, Minute mutations and suppressor of purple exhibit the same variation in (Q+) and (Q-) levels (37). Temperature and nutrients affect the (Q+) and (Q-) levels (35). Flies raised at 25°C had less $tRNA^{(Tyr)}_{(Q-)}$ than those grown at 22°C. Flies raised on a carbohydrate medium showed less $tRNA^{(Tyr)}_{(Q-)}$ than those raised on a yeast medium, regardless of genotype. Tissue culture studies also show a variation of Q containing tRNAs depending on the growth medium (15). Cells grown on fetal calf serum produce higher percentage (Q+)tRNA than cells grown on calf serum. Work to be published by Farkas and Katze indicates that this is because Q is present in fetal calf serum (30). The observations of variations in (Q+)tRNA with development are the basis for speculation that Q might have some function in development. Enzymes that interconvert the (Q+) and (Q-)tRNAs have been postulated (29), and it is appropriate to examine guanylation in such a process.

Guanylation

The incorporation of guanine into transfer RNA was first observed by Marks who interpreted it to be an addition of adenosine to the 3' end after conversion of guanine to adenine (38). Farkas showed that the mechanism was implausible (2) and that guanylation involved replacement of the base in the first anticodon position of certain isoaccepting RNAs

by guanine (2,3,4,5). Guanylation is a post-transcriptional modification involving removal of a base by cleavage of the N-glycoside linkage rather than by cleavage of the phosphodiester bonds in the tRNA chain. This reaction occurs only in species which recognize the XA_C^U codon (3). Guanylation appears in more organisms, occurring in humans (39), rabbits (2), E. coli (3), Ehrlich ascites tumors (5,40), chickens (3), sheep (39), and wheat germ, but it is absent in yeast (41). The enzyme or enzymes which carry out guanylation are called guanine insertase.

Guanylation was first postulated to participate in the biosynthesis of Q by Farkas (4). Guanine insertase isolated from rabbit reticulocytes was shown to replace guanine within the $tRNA_{(Q-)}^{(His)}$ but not the $tRNA_{(Q+)}^{(His)}$ of rabbit tRNA (4). When the same enzyme was used on E. coli tRNA which contains only (Q+)tRNA, it replaced Q with guanine (3). The enzyme isolated from Ehrlich ascites tumor cells also replaces Q with guanine when E. coli tRNA is used (5). In homologous tRNA, the Ehrlich ascites tumor enzyme exchanges guanine for guanine as does the reticulocyte enzyme. The guanine insertase isolated from E. coli only exchanged guanine for guanine (32). The literature doesn't contain any studies showing the ability of the enzyme to incorporate Q in place of guanine due to the unavailability of Q. Yeast tRNA which does not contain Q is an excellent acceptor of guanine in the reaction catalyzed by guanine insertase (4).

Guanine insertase was recently purified to homogeneity from rabbit erythrocytes (1). The enzyme has a molecular weight of 104,000 and is composed of two subunits whose molecular weights are 60,000 and 43,000.

The optimum pH for the enzyme is 7.8 (42). The only cofactor requirement is for a monovalent cation such as Na^+ or K^+ (42). The E. coli enzyme can utilize a divalent metal such as Mg^{++} in place of the monovalent cation (32). The guanylation enzyme from Ehrlich ascites tumor cells also can utilize $1.6 \times 10^{-2}\text{M}$ MgCl_2 or Mn^{2+} in place of K^+ or Na^+ (40). It may be significant that the MgCl_2 at $2 \times 10^{-2}\text{M}$ induced miscoding in an in vitro protein synthesizing system (21,22). Prevention of magnesium induced miscoding has been postulated as a function of Q (9). Research presented in this thesis also demonstrates a magnesium requirement for wheat germ guanine insertase.

Guanylation has not been observed in adult rat and mouse reticulocyte lysates due to the presence of guanine aminohydrolase which destroys the free guanine available for guanine insertase (43). Rat fetuses do exhibit guanylation (3), presumably the guanine aminohydrolase is either inhibited or not present.

Farkas and Katze have isolated Q from fetal calf serum and have shown that it is a competitive inhibitor of the rabbit guanine insertase (30). The K_i for Q is one-third of the K_m for guanine indicating that the true substrate for guanine insertase may be Q. Further proof that Q was a substrate for guanine insertase was shown by an experiment in which his tRNA(Q-) was isolated and incubated with the compound isolated from fetal calf serum plus pure guanine insertase. The his tRNA was now analyzed on an RPC-5 column, and it was eluted in the position where his tRNA (Q+) eluted (7,30). The function of guanine insertase is to replace G in the wobble position with Q or a Q precursor (7,30).

Guanine insertase was reported to be incapable of removing [^3H] labeled guanine once it was incorporated into tRNA by replacing it with cold guanine (4). However, studies by Howes and Farkas with pure guanine insertase showed that guanine exchange does indeed occur (7). The reason for the discrepancy was the low activity of the enzyme used in the first study. In the first study, the percentage of cold guanylatable tRNA was greater than the [^3H]guanine labeled tRNA, and the activity of the enzyme was not sufficient to release measurable levels of labeled guanine. An unanswered question is whether the enzyme has the ability to remove damaged Q or guanine molecules thereby acting as a repair enzyme. Attempts to isolate the enzyme from yeast have failed (41).

Many types of tumors show altered tRNA elution profiles on RPC-5 (32). An alteration frequently observed in the (Q+):(Q-) tRNA is elevated levels of the (Q-) form. Both of the bacterial and mammalian insertases readily exchange guanine for guanine in (Q-)tRNAs. Since many human tumors exhibit higher levels of (Q-)tRNA, there is promise for the development of a test for malignant cells based on detecting elevated levels of (Q-)tRNA with guanine insertase (32).

III. MATERIALS AND METHODS

Materials

[¹⁴C]Histidine (specific activity 312 mCi/mmmole), [¹⁴C]-aspartic acid (specific activity 208 mCi/mmmole), [¹⁴C]asparagine (specific activity 79 mCi/mmmole), [¹⁴C]tyrosine (specific activity 460 mCi/mmmole), and Omnifluor were purchased from New England Nuclear. [³H]guanine (specific activity 1 Ci/mmmole) was purchased from ICN. Sephadex G-25 medium grade, Blue sepharose CL-6B, and sephacryl S-200 were from Pharmacia; the DEAE cellulose was from Eastman and the cellulose phosphate was from Sigma. The RPC-5 column was prepared according to Pearson (28). The wheat germ was type II untreated from Sigma. Wheat germ tRNA was prepared according to Holley (44) and wheat germ aminoacyl synthetase was prepared by the method of Roe et al (45). Yeast tRNA (unfractionated) was purchased from Miles. Memphis factor (Q base isolated from fetal calf serum) was a gift from Dr. Jon Katze. The protein assay kit was purchased from Bio-Rad.

Methods

Guanylation assay. Analysis for guanylation was a modification of the procedure of Howes and Farkas (1). Each assay tube contained 10-200 µl of enzyme, 1.25 A₂₆₀ units of wheat germ tRNA, 55 µmoles of KCl, 10 µmoles TES, pH 7.4, 2 nm of [³H]guanine, and 1 µmole MgCl₂ in a volume of 0.6 ml. The solution was incubated at 37°C in a shaking water bath for times ranging from 45 to 150 min. The tRNA was precipitated

with cold 3 M HCl and collected on glass filters. Free guanine remains soluble in HCl. Samples were washed with ethanol, dried and counted in a Beckman liquid scintillation counter using 5 ml of omnifluor-toluene per vial.

Gel electrophoresis. Gel electrophoresis was carried out using 7.5% polyacrylamide gels according to the procedure of Jovin et al. (47) and those previously used in this lab (1).

Protein determinations. Protein determinations were made by the method of Bradford (48) as previously described (1).

RPC-5 separation of transfer RNAs. A reverse phase 5 column prepared by the method of Pearson (28) was washed with RPC-5 buffer consisting of 10 mM $MgCl_2$, 1 mM EDTA, 10 mM NaAc, pH 4.5, 5 mM β mercaptoethanol (EtSH) and 0.5 M NaCl. Five-tenths to 10 A_{260} units of tRNA were loaded onto the column and washed in 20 ml of 0.5 M NaCl in RPC-5 buffer. A 150 ml linear gradient from 0.5 M NaCl to 0.8 M NaCl in RPC-5 buffer was used to elute the tRNA. One ml fractions were collected. Each fraction was placed in a vial containing 10 ml of scintiverse and counted in a liquid scintillation counter.

X-irradiation of wheat germ transfer RNA. X-irradiation of transfer RNA was performed with a G.E. Maximar 250III X-ray machine with 250 KVP and a current of 15 mA. The target to object distance was approximately 6 cm and no filter was used. The exposure rate was corrected for temperature and pressure differences and calculated to be 919 Roentgens per min.

Wheat germ tRNA was isolated by the method of Holley and resuspended in distilled H_2O to a concentration of 60 A_{260} units per ml. The tRNA was irradiated by placing samples ranging from 100 to 500 μl on a sterile plastic petri dish set on ice. Exposures ranged from 500 to 75,000 Roentgens. Each sample was removed from the petri dish and placed in a vial kept at $0^\circ C$. Samples were tested as substrates for guanylation within 30 min of irradiation or stored at $-80^\circ C$ for later use.

In order to rule out the possibility of manipulative artifacts during irradiation, a second method of irradiating the wheat germ tRNA was also used. A sample was placed on the plastic petri dish and put on ice. This sample was then subjected to split doses of X-irradiation with aliquots being removed after each exposure. In this manner, exposures from 0 to 5,500 Roentgens were given to the sample.

IV. RESULTS

Purification of Wheat Germ Guanine Insertase

Preparation of crude extract. Three hundred seventy-five grams of wheat germ, 375 grams of silica sand, and 1.28 liters of grinding solution were homogenized in a Waring blender. The grinding solution consisted of 50 mM Tris, pH 7.6, 10 mM EDTA, pH 7.0, 10 mM EtSH, 10 mM MgCl_2 , and 10% glycerol in distilled H_2O . The preparation was cooled to 4° in a dry ice ethanol bath, then blended at high speed for 2 min. The prep was cooled again in the ice bath until it partially froze, and the ice was broken into small chunks with a spatula. This blending cooling procedure was repeated 6 times. All other steps of the purification procedure were carried out at 4°C .

The sand and other particulate matter were removed by centrifugation at $13,200 \times g$ for 20 min. The prep was filtered through glass wool and then centrifuged at $30,900 g$ for 20 min. Higher speeds and longer centrifuging times did not produce clearer preps or increase enzyme activity. The supernatant fluid was filtered through glass wool.

Sephadex G-25 chromatography of crude wheat germ lysate. A 1.5 liter column of Sephadex G-25 (medium grain) $5 \text{ cm} \times 80 \text{ cm}$ was equilibrated with 50 mM Tris, pH 7.6, 10 mM EDTA, pH 7.0, 10 mM EtSH, and 1 mM MgCl_2 . The wheat germ preparation was loaded onto the column. Four hundred seventy-five ml of eluate was collected in fractions of 20 ml. The eluate was monitored for protein content by determining

absorbance at 280 nm and assayed for guanylate activity. The enzyme was located in the first fractions which were clear and straw colored. Fractions with high enzymatic activity were pooled for further purification. The results are shown in Fig. 1.

DEAE chromatography of pooled G-25 fractions. The G-25 fractions were loaded onto a 2.5 cm × 40 column of DEAE previously washed with 0.1 M NaOH, 0.1 M HCl, distilled water, and then equilibrated with 50 mM Tris, pH 7.06, 10 mM EDTA, 10 mM EtSH, and 10% glycerol. The DEAE column was attached to a peristaltic pump. After the G-25 fraction was loaded onto the column, the DEAE was washed with the equilibration buffer until the eluate was devoid of material absorbing at 280 nm. This usually requires about 500 ml. The guanylation enzyme was then eluted with 0.1 M KCl dissolved in the equilibration buffer. Twenty ml fractions were collected and assayed for activity and absorbance at 280 nm. The activity peak usually coincided with the protein peak as shown in Fig. 2. Peak tubes were pooled for the next purification step.

Ammonium sulfate precipitation. Ammonium sulfate was added to 45% of saturation by addition of a mixture containing 0.1% (KHCO_3) plus solid ammonium sulfate. The solution was stirred for 45 min and then centrifuged at $10,000 \times g$ for 20 min. The pellet, a yellow paste, was dissolved in a minimal amount of S-200 buffer (0.5 M NaCl, 10% glycerol, 50 mM Tris, pH 7.4, 10 mM EDTA, pH 7.0, 10 mM EtSH, and 1 mM MgCl_2); usually 4 to 8 ml was sufficient.

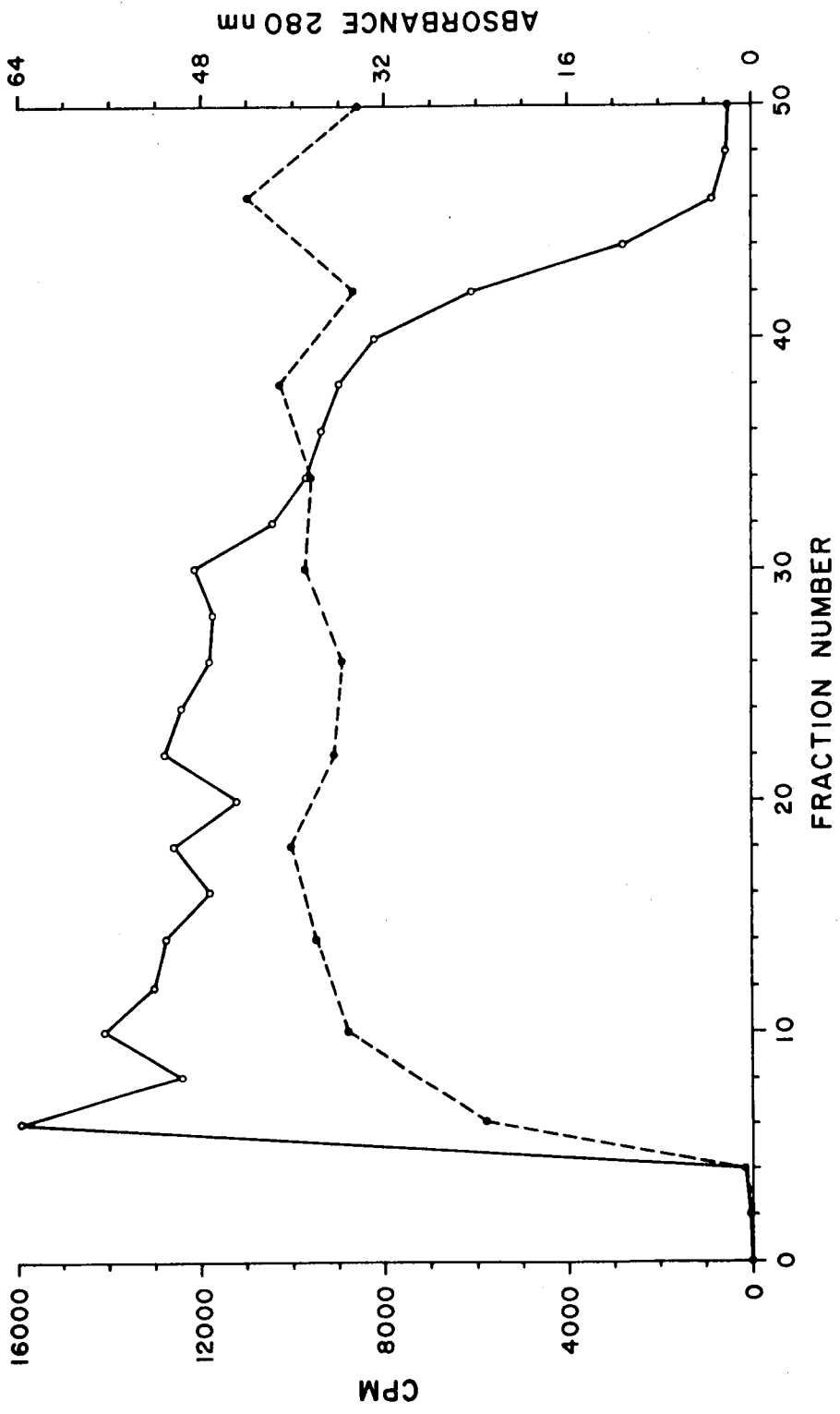


Figure 1. G-25 Chromatography.

(o—o) CMP [³H]guanine
 (●---●) Absorbance 280 nm

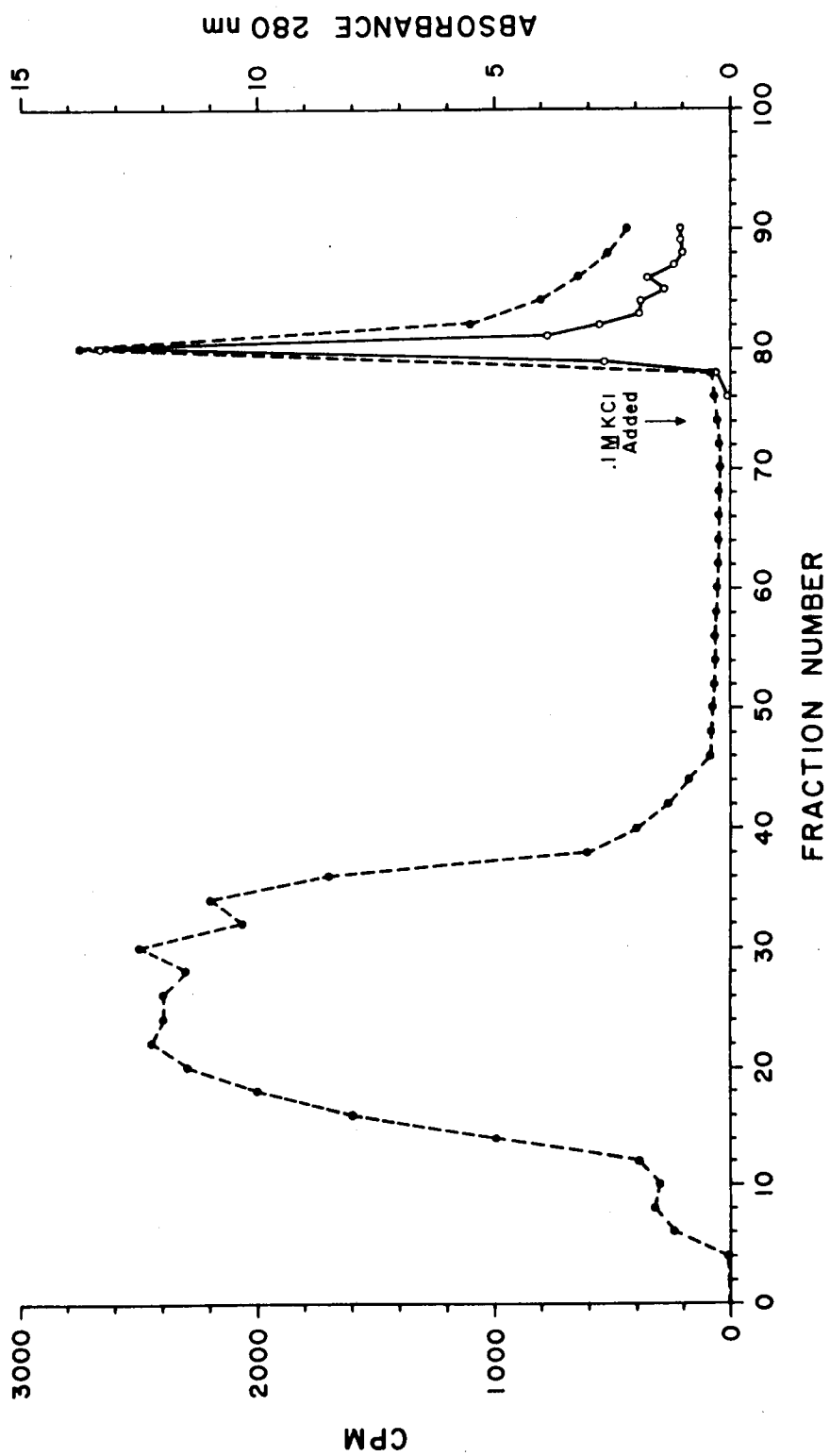


Figure 2. DEAE Chromatography.
(o—o) CPM of [³H]guanine incorporated
(●---●) Absorbance at 280 nm

Chromatography on sephacryl S-200. The enzyme solution was added to a column of sephacryl S-200 2.5 cm × 85 cm previously equilibrated with S-200 buffer. The enzyme was eluted with this buffer. After 100 ml had passed through the column, fractions of 4 ml were collected and assayed for absorbance at 280 nm and activity. The enzyme was eluted at 243 ml. Fifty μ l of enzyme is sufficient for assay at this step. The results are shown in Fig. 3.

Chromatography on blue sepharose CL-6B. The enzyme was purified further by a modification of the blue sepharose procedure described by Howes and Farkas (1). It was necessary to modify their procedure by adding MgCl_2 to maintain the enzyme activity. Pooled S-200 fraction was dialyzed against 50 mM Tris, pH 7.6, 10 mM EtSH, and 1 mM MgCl_2 3 hr at 4°C to remove salt and glycerol. A 1 cm × 10 cm column of blue sepharose CL-6B was previously equilibrated with 50 mM Tris, pH 7.6, 10 mM EtSH, and 1 mM MgCl_2 . After the enzyme was added, the column was washed with 20 ml of the buffer and then successively with 20 ml each of 0.1 M KCl, 0.2 M KCl, 0.3 M KCl, and 0.5 M KCl dissolved in the equilibration buffer. Assay for enzyme activity showed that the enzyme was eluted at 0.3 M KCl. The fractions containing activity were pooled and stored at -80°C in 10% glycerol. The results are shown in Fig. 4.

Cellulose phosphate chromatography. The pooled fractions were dialyzed against 50 mM Tris, 10 mM EtSH, and 1 mM MgCl_2 for 3 hr. A column of cellulose phosphate 1 cm × 10 cm was washed with 100 ml 0.1 M NaOH, followed by 100 ml water, 100 ml 0.1 M HCl, water then with 2 M

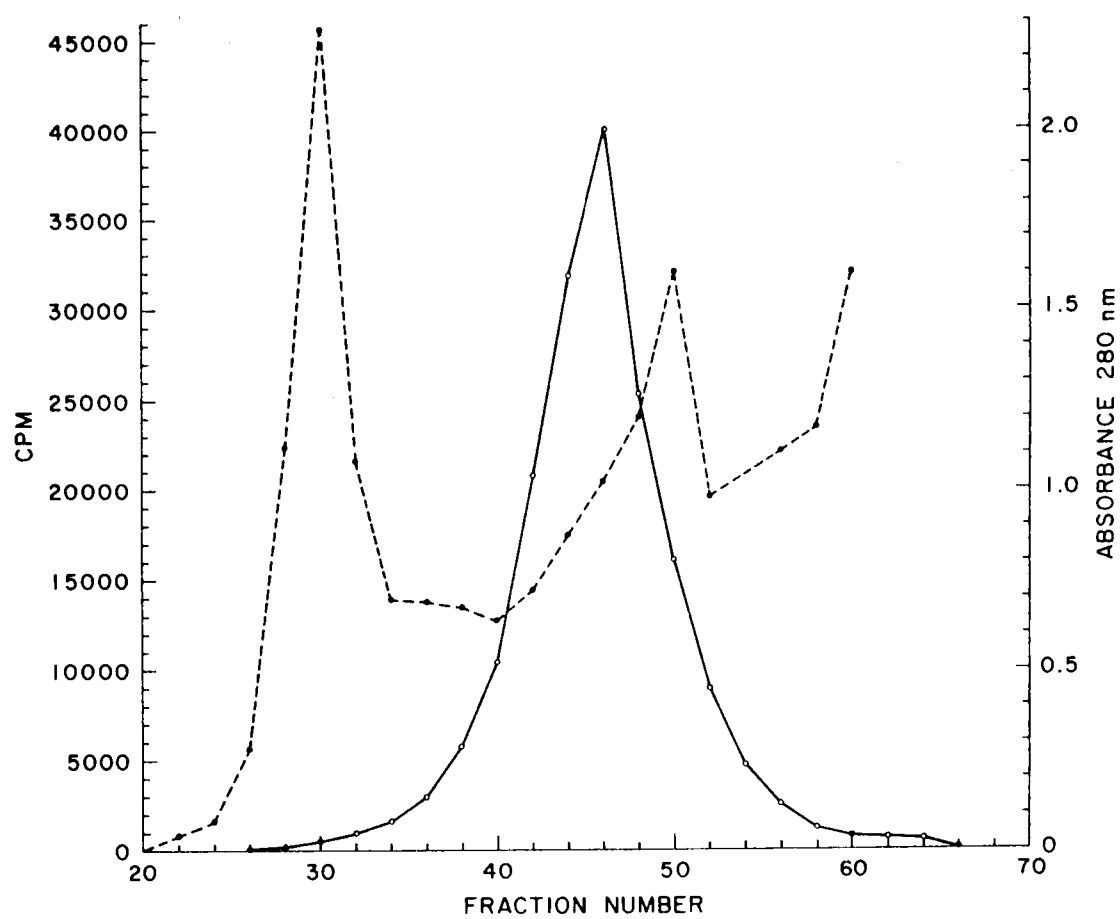


Figure 3. S-200 Chromatography.

(o—o) CPM of [³H]guanine incorporated
(●---●) Absorbance at 280 nm

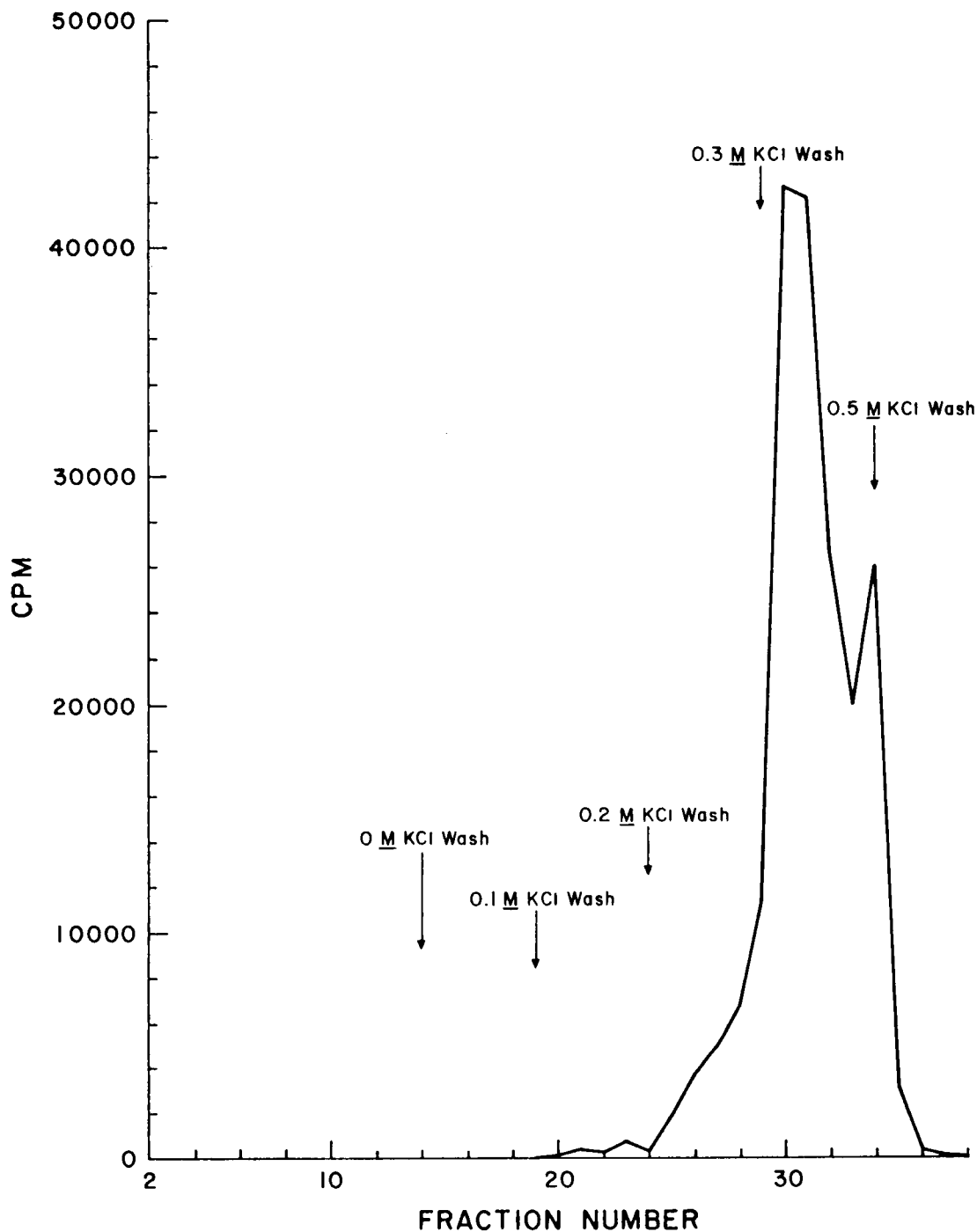


Figure 4. Blue Sepharose CL-B6 Chromatography.
[³H]Guanine Incorporation.

KCl dissolved in 50 mM Tris, pH 7.6, 100 mM EtSH, and 1 mM MgCl_2 and finally equilibrated with this buffer but without KCl. The dialyzed fraction was added to the column and washed with 40 ml of the buffer. Five-tenths M KCl dissolved in the equilibration buffer was used to elute the enzyme. Two ml fractions were collected and assayed for enzyme activity using 50 μl of each fraction. Ten percent glycerol was added to each fraction prior to storage at -80°C .

Gel electrophoresis of the pooled S-200 fractions indicated that 7 proteins were present. Only 2 proteins remained after the blue sepharose CL-6B column and only one protein, the guanylate enzyme, was observed after the cellulose phosphate column. Fig. 5 shows gels from the cellulose phosphate column at two different protein concentrations 10 and 12.9 μg , respectively. Fig. 6 shows that the protein band coincides with the guanylate activity in the gel.

The purified enzyme has a specific activity of 2029 pmoles guanine $\text{hr}^{-1} \text{mg protein}^{-1}$ and is purified 3382 fold. Using the procedure which is summarized in Table I, milligram quantities of the homogeneous enzyme may be obtained. Stability tests on the purified enzyme have not been performed although the S-200 stage remains active for several months when stored with 10^{-3}M Mg^{++} and 10% glycerol at -80°C .

Molecular weight determination of guanine insertase. The molecular weight of wheat germ guanine insertase was measured by comparing the void volume of an S-200 column 2.5 cm $\times$ 85 cm with the volume at which the enzyme peak was eluted. The results shown in Fig. 7 show that the

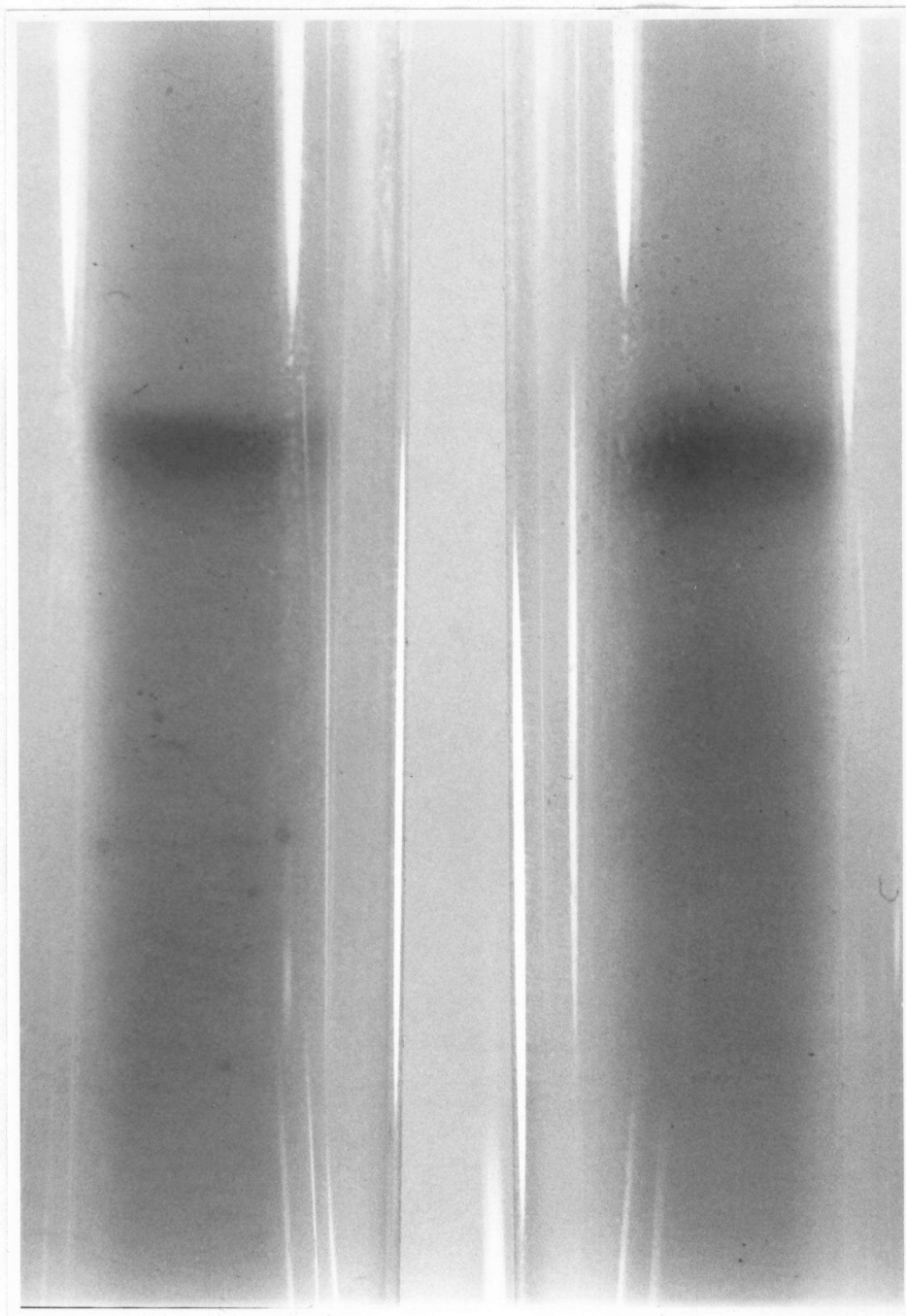


Figure 5. Polyacrylamide Gel Electrophoresis of Guanine Insertase. The Appearance of Two Gels after Loading with Different Amounts of Protein: 10 μ g (Left) and 12.9 μ g (Right) after Phosphocellulose Chromatography.

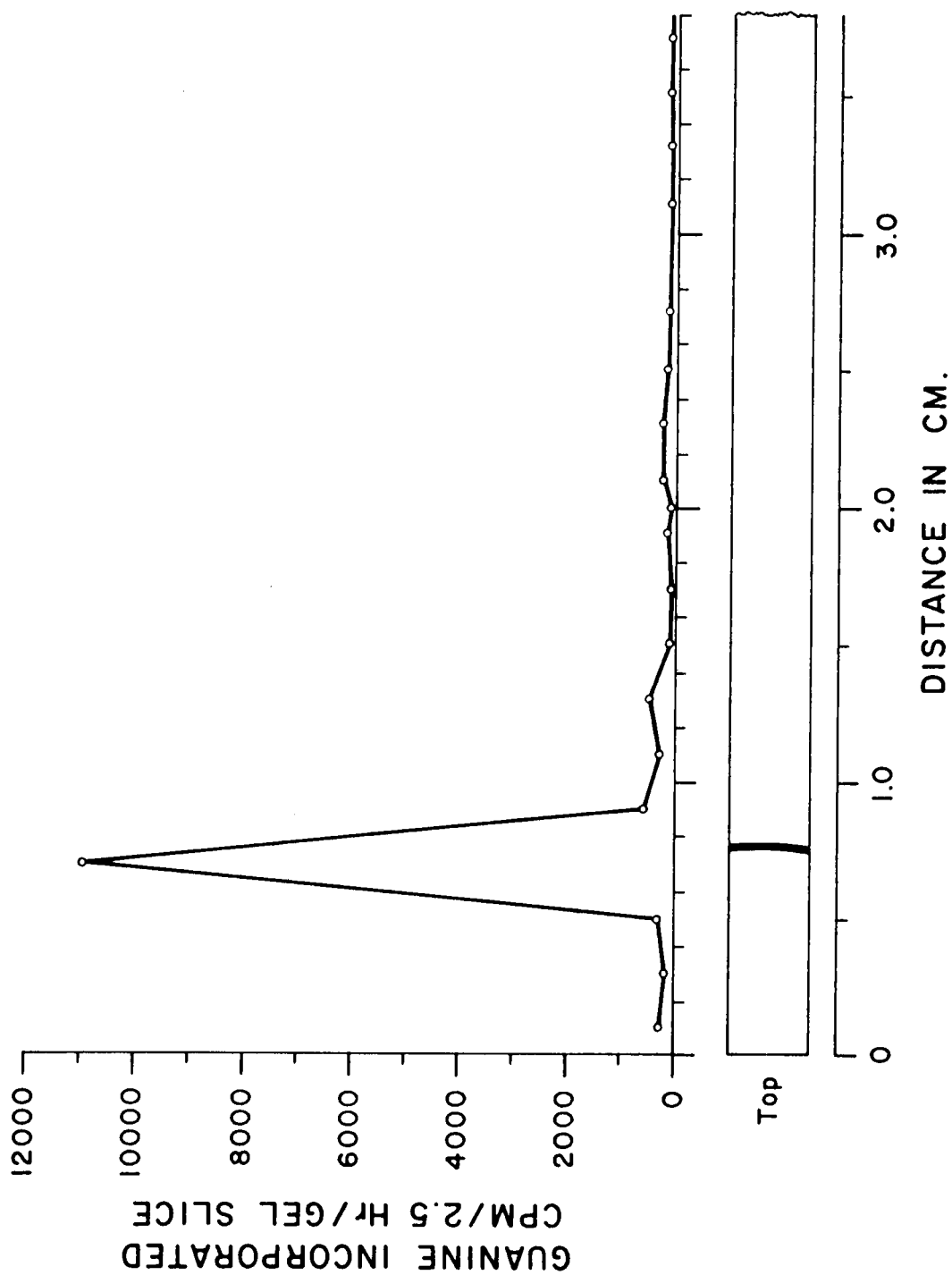


Figure 6. Single P.A.G.E. Band Is Guanine Insertase.

TABLE I
PURIFICATION OF GUANINE INSERTASE FROM WHEAT GERM

Stage	Total Protein in mg	Specific Activity pmoles hr ⁻¹ mg Protein ⁻¹	Purifi- cation	% Yield*
crude lysate	33401160	0.6006	1	-
G-25	29972167	2.318	3.86	100
DEAE	3915614	2.318	3.82	12.9
0-45%(NH ₃) ₂ SO ₄ precipitation	773545	8.632	14.38	9.6
S-200	232141	52.94	88.23	17.6
blue sepharose	11738	739.48	1232.4	12.4
phosphocellulose	353	2029.63	3382.7	1.03

*Percentage of yield was determined from the G-25 stage because of inhibition present in the crude lysate. The percentage of increase in the S-200 stage is probably due to renaturation or removal of inhibitors.

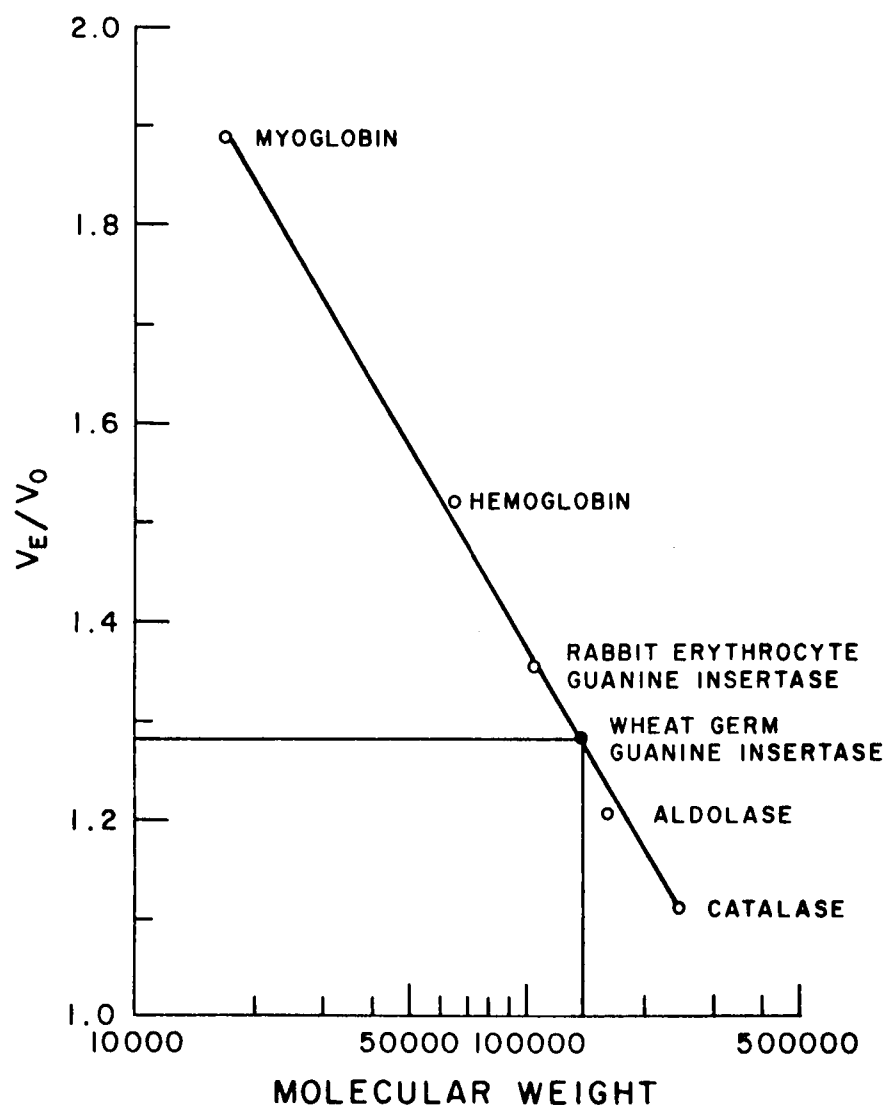


Figure 7. Molecular Weight Determination.

enzyme was eluted with a V_e/V_o corresponding to a molecular weight of 140,000. Catalase, aldolase, hemoglobin, and myoglobin were used as markers.

Determination of enzyme K_m for guanine. The K_m was determined by determining the rate of guanine incorporation at different concentrations of guanine using the S-200 stage enzyme. Incubation was for 45 min at which time guanylation was linear with time (unpublished results). The K_m was determined by plotting [^3H]guanine incorporation vs. guanine concentration and determining the guanine concentration at $1/2 V_{\max}$. A Lineweaver Burk plot was made of the results as indicated in Fig. 8. The K_m as determined from the Lineweaver Burk plot is $1.05 \times 10^{-6}\text{M}$ for guanine.

Determination of optimum pH. The effect of pH was determined by varying the pH using a mixed buffer of TES and Tris. The pH was tested over a range of 6.50 to 8.56. The optimal pH was at 7.6. At pH 6.9 activity was 8% of the optimal activity while 50% of the optimal activity was observed at pH 7.2 and 8.4.

Effect of different tRNA substrates. The ability of the S-200 pooled fractions to guanylate transfer RNAs from different sources was tested using yeast, rabbit liver, and wheat germ tRNA. The results, indicated in Table II, show that the wheat germ enzyme utilized its own tRNA better than the other tRNAs tested in the following order: wheat germ > yeast > rabbit liver tRNA.

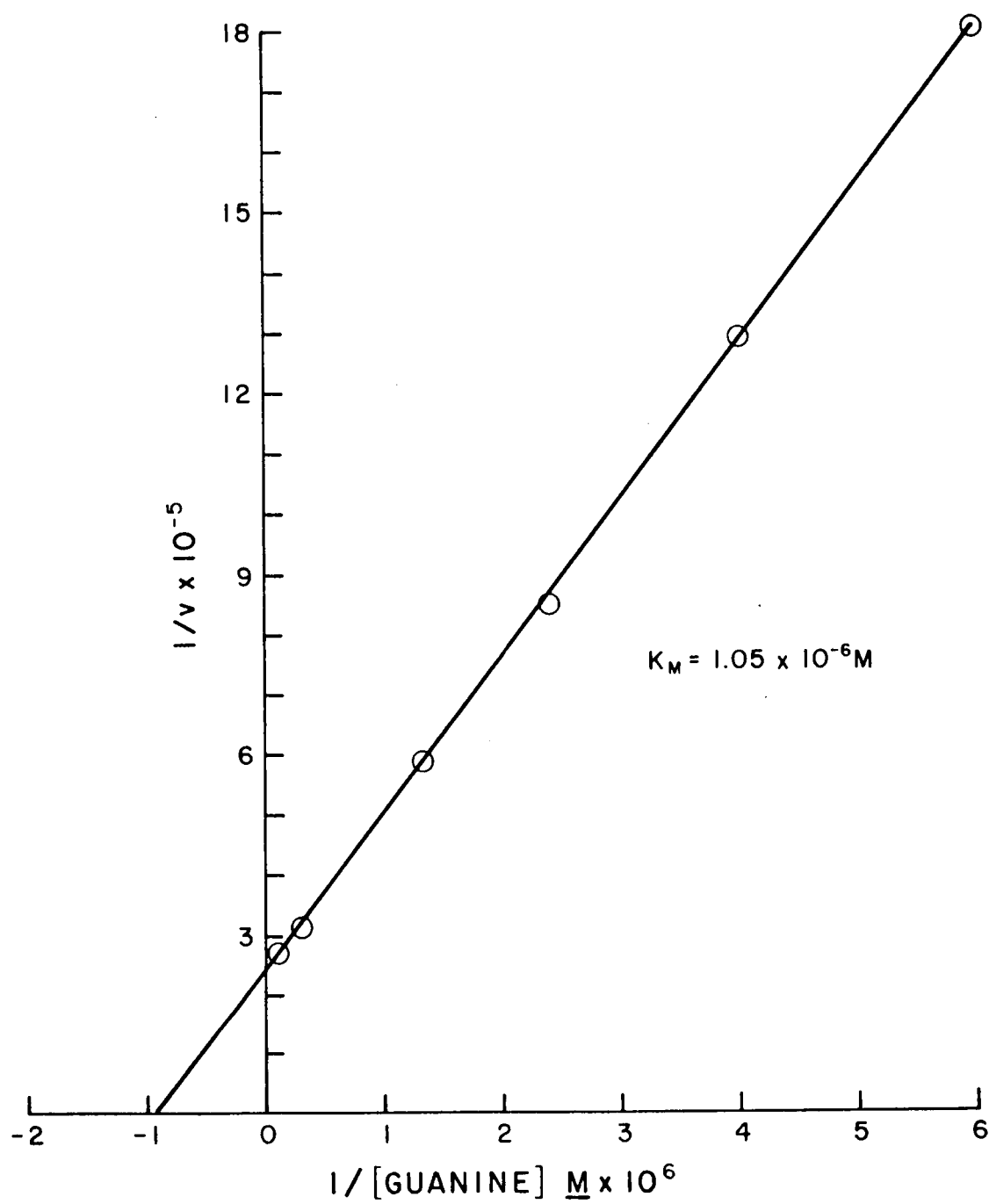


Figure 8. K_m of Guanine.

TABLE II
COMPARISON OF DIFFERENT tRNAs FOR GUANYLATING ABILITY

Source of tRNA	cpm/A ₂₆₀	% Difference from Wheat Germ
Yeast	2,984	72
Rabbit liver	1,240	88
Wheat germ	10,670	-

Inhibition of guanine insertase by Q base. The S-200 stage enzyme was incubated with 0, 0.016, and 0.04 A₂₆₀ units of Q base using wheat germ or yeast tRNA. The results in Table III show that Q base inhibits the wheat guanine insertase. Four-tenths A₂₆₀ units of Q base inhibits guanylation of wheat germ tRNA by 18% and yeast tRNA by 27.8%. Inhibition by Q base from 0.016 to 0.04 A₂₆₀ units is linear.

TABLE III
INHIBITION OF GUANYLATION BY Q BASE

tRNA Source	Amount in A ₂₆₀ Units	Concentration of Q Base in A ₂₆₀ Units	cpm	% Inhibition
Wheat germ	1.25	0	17,083	0
	1.25	0.016	15,844	7.25
	1.25	0.04	13,877	18
Yeast	1.25	0	8,097	0
	1.25	0.016	7,140	11.8
	1.25	0.04	5,846	27.8

Identification of guanylated tRNA. Cochromatography of [³H]guanylated wheat germ tRNA with [¹⁴C]aminoacyl tRNA shows that all four of the Q-containing family of tRNAs are guanylated to some degree

by the wheat germ guanine insertase. Fig. 9 shows the results with [^{14}C]aspartic acid. The [^3H]guanine cochromatographed with the second aspartyl-tRNA peak. Two isoacceptors for tRNA^(Asp) were observed; they are probably homogeneous. Fig. 10 shows the results with [^{14}C]asn-tRNA. The second tRNA^(Asn) cochromatographs with the first major [^3H]guanylated peak. Wheat germ asparaginyl tRNA has three isoaccepting peaks on RPC-5 chromatography. Tyrosyl tRNA chromatography is shown in Fig. 11. In the case of tyrosyl-tRNA, four isoacceptor peaks exist. The first peak, presumably Q+, is very low in wheat germ. More guanylation occurred in peak 2 than in isoacceptors 3 and 4, even though the latter two contained more tyr-tRNA. Note the total absence of guanylation in the fourth tyrosyl-tRNA peak. In this case the amino acid on the tRNA caused the elution to be earlier; otherwise the [^3H]guanine and [^{14}C]tyrosyl peaks would have coincided. This effect has been observed in chromatography of [^3H]guanylated and [^{14}C]histidyl tRNA. Notice in Fig. 12 the ridge which follows the histidyl peaks indicating their slightly early elution. It is of interest that only two histidyl peaks occur in wheat germ and that both are guanylated. This unexpected result has been repeated.

Based on RPC-5, there are two major guanylated peaks, tRNA^(Asp)_{II} and tRNA^(Asn)_{II}; and five minor guanylated peaks comprising of tRNA^(His)_{II}, tRNA^(Tyr)_{II}, tRNA^(Tyr)_{III}, and tRNA^(Tyr)_{IV}. Fig. 13, a combination of the data in Figs. 9 through 12, shows the identity of each of these peaks.

Cofactor requirements for the wheat germ enzyme. A monovalent cation is required for the rabbit enzyme. The wheat germ enzyme was assayed with different monovalent cations using the G-25 pool.

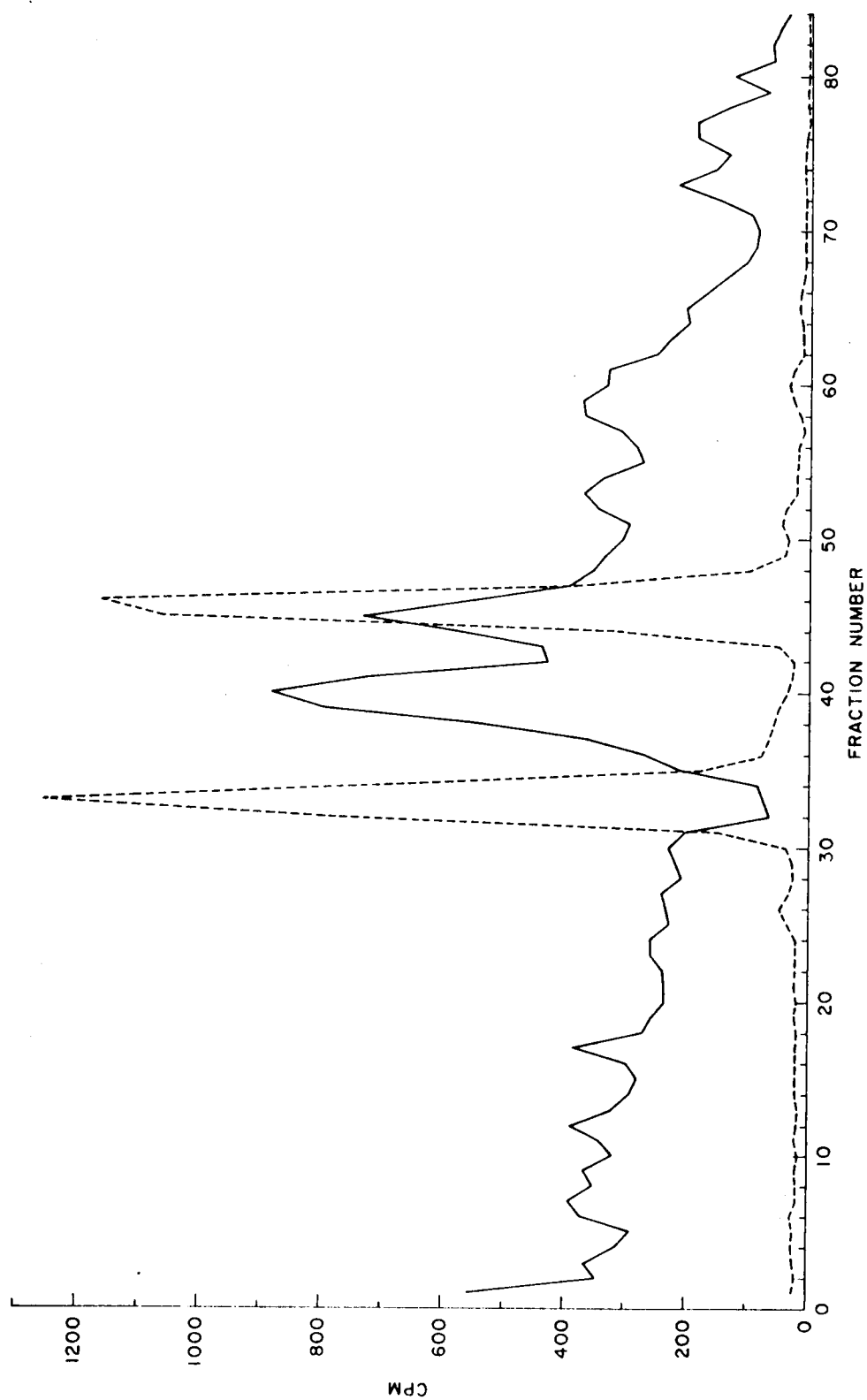


Figure 9. RPC-5 of Wheat Germ tRNA [^3H]Guanylated, [^{14}C]Aspartyl.

Note: Symbols used in Figures 9-13: Solid line represents [^3H]guanine; broken line represents [^{14}C]amino acids.

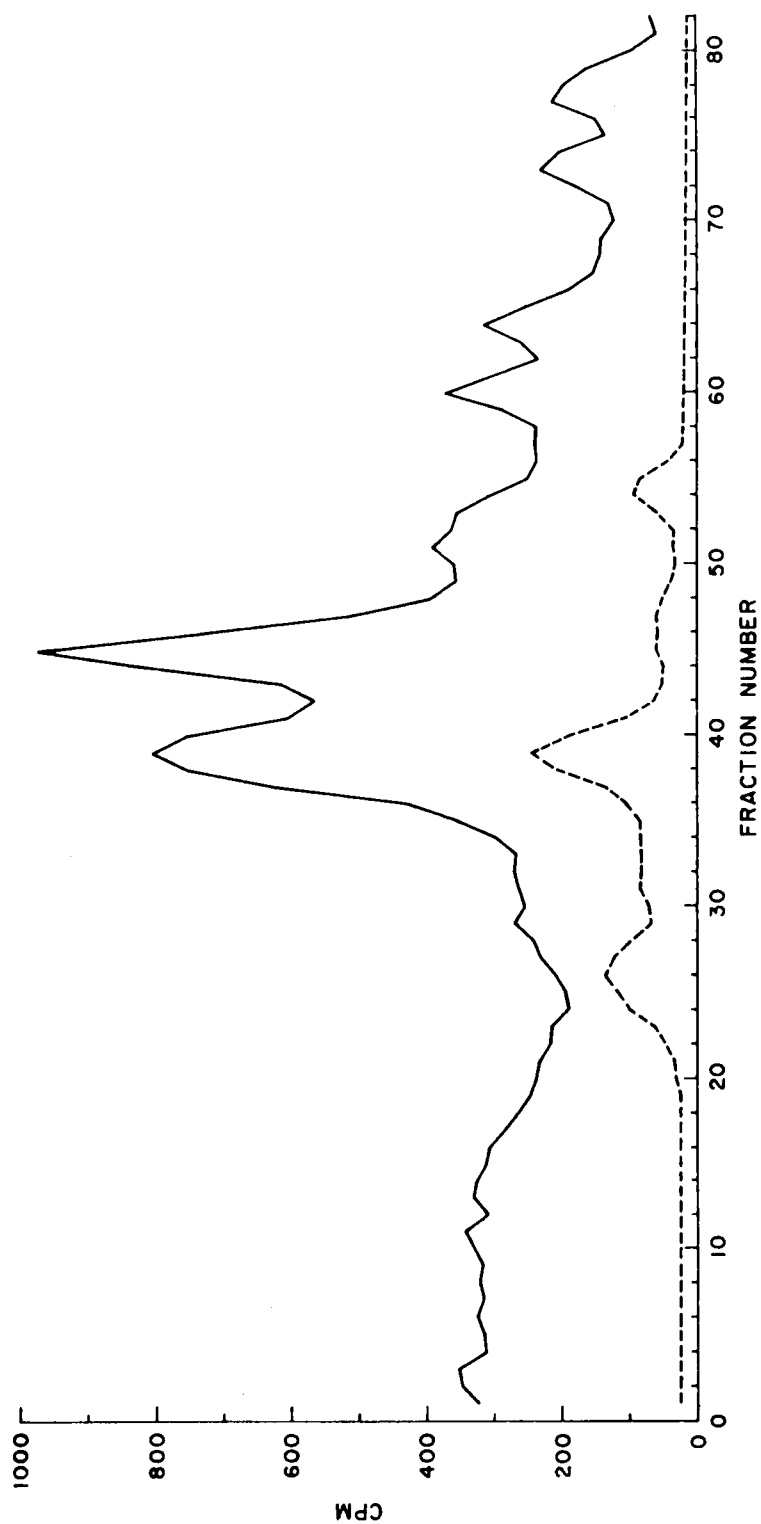


Figure 10. RPC-4 of Wheat Germ tRNA [^3H]Guanylated, [^{14}C]Asparaginy1.

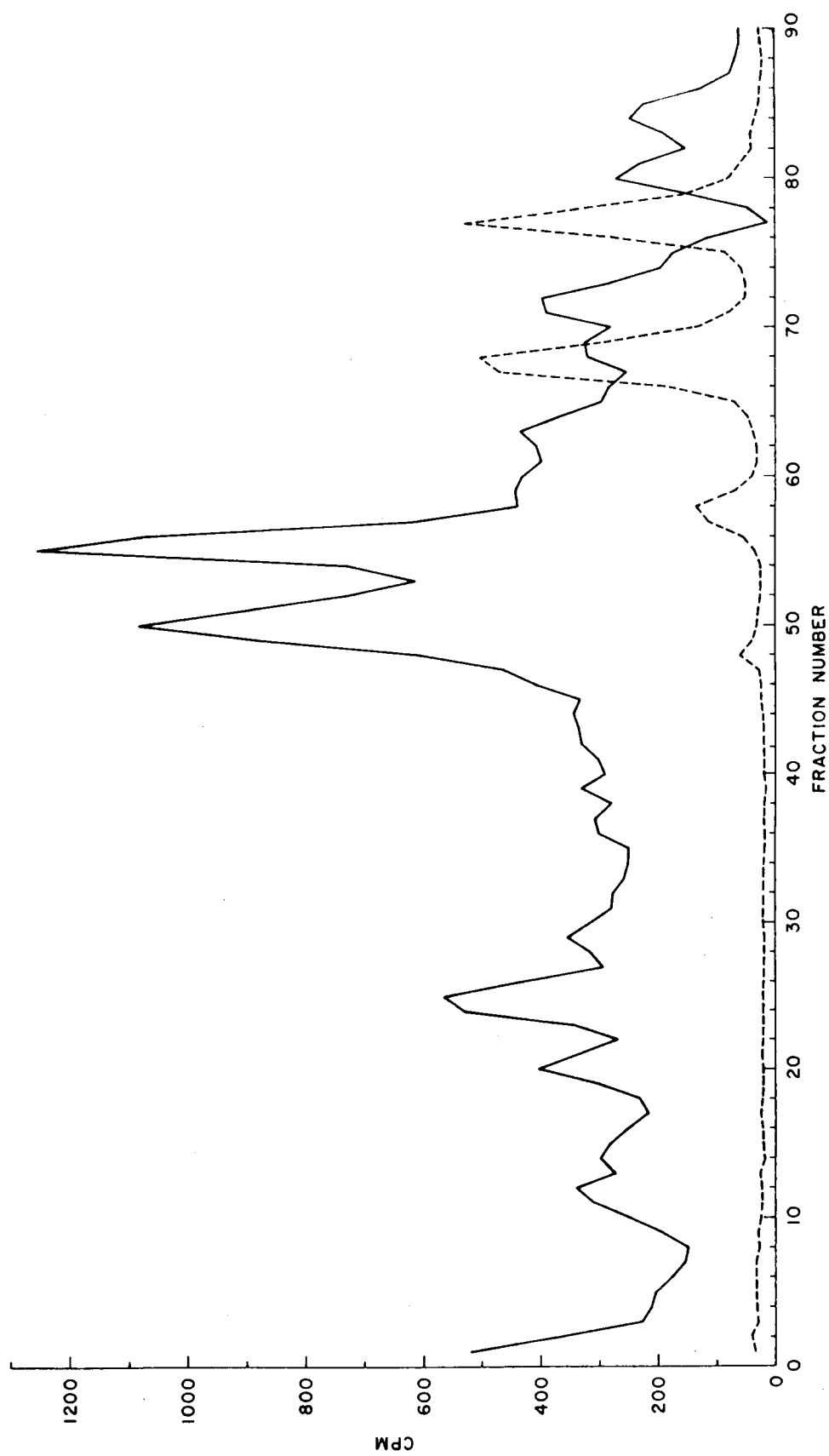


Figure 11. RPC-5 of Wheat Germ tRNA [^3H]Guanylated, [^{14}C]Tyrosyl.

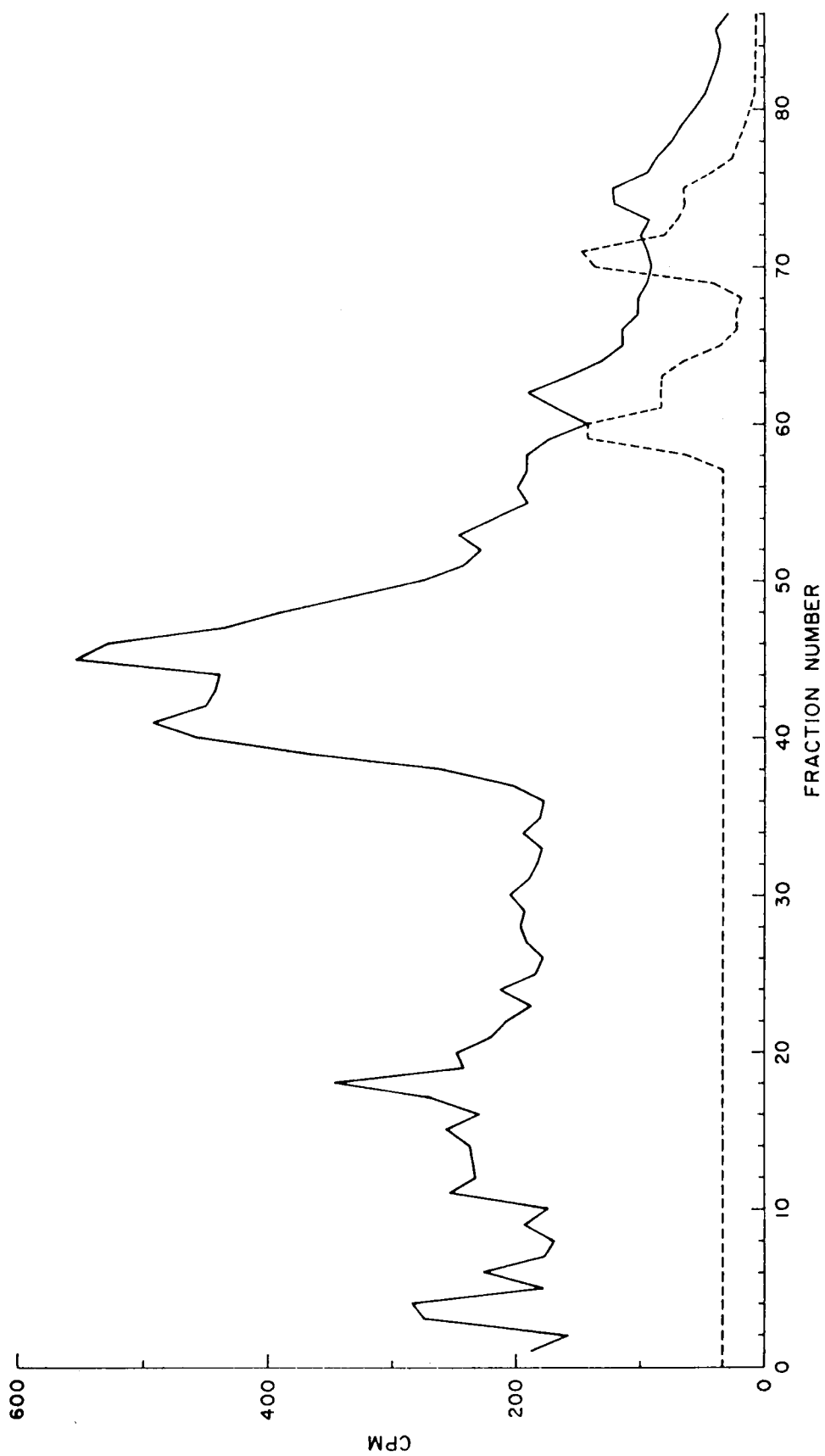


Figure 12. RPC-5 of Wheat Germ tRNA [^3H]Guanylated, [^{14}C]Histidyl.

Figure 13. Identification of Guanine Accepting tRNA in Wheat Germ.
(This figure is a combination of Figures 9-12 showing the identity of each peak.)

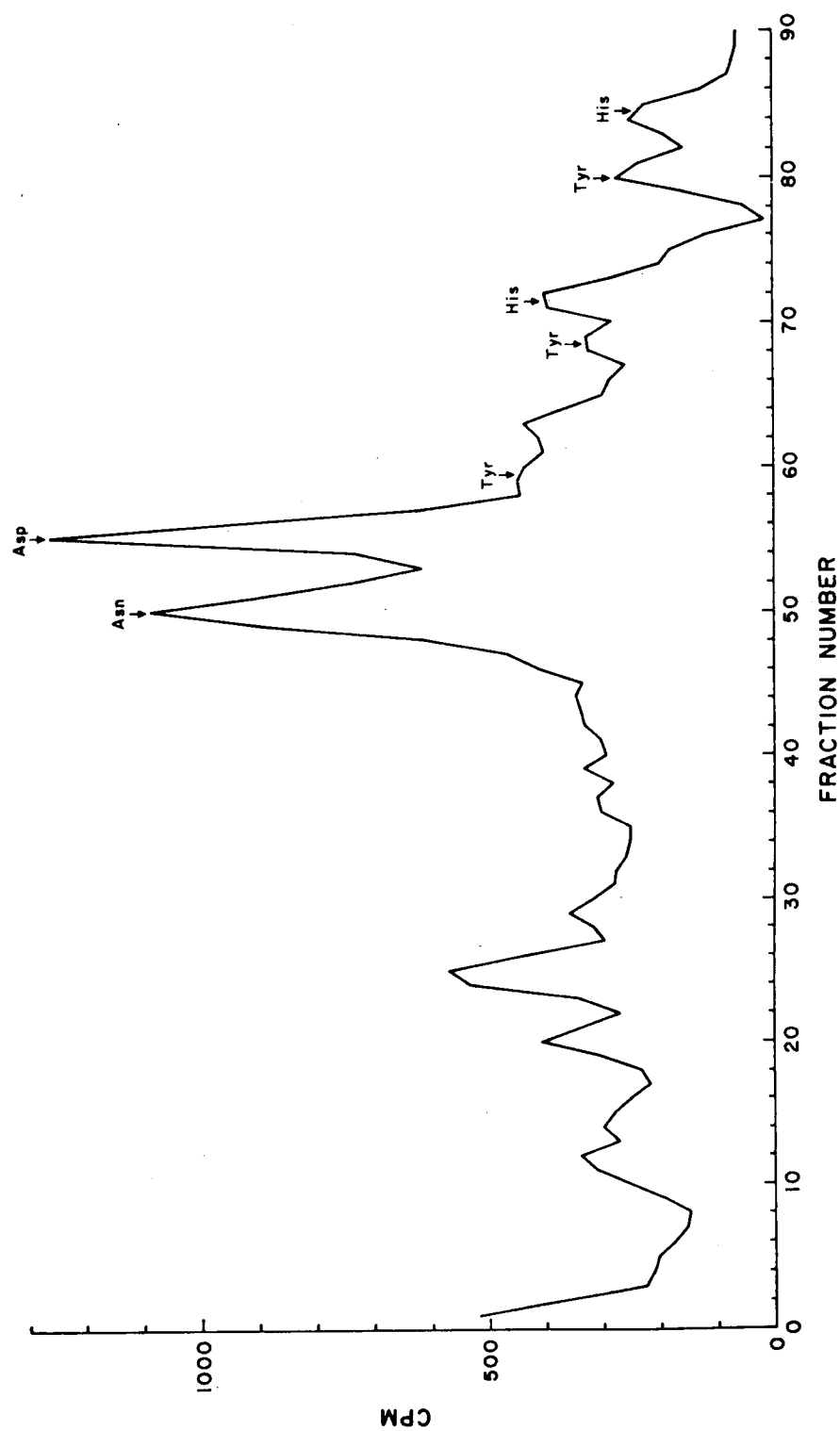


Figure 13

Chromatography on G-25 presumably removed cations from solution, although bound metals are probably still present. CsCl, NaCl, LiCl, KCl, NH_4Cl , and RbCl were tested for their ability to meet the cation requirement. The salts were tested at concentrations ranging from 0 to 0.28 M. NaCl was best at meeting the monovalent metal requirement of the enzyme. The results are shown in Table IV. The optimum concentration of NaCl was 0.114 M. Ranked in order of effectiveness in stimulating guanine incorporation were $\text{NaCl} > \text{LiCl} > \text{KCl} > \text{CsCl} > \text{NH}_4\text{Cl} > \text{RbCl}$. The results of the mammalian enzyme were $\text{Li}^+ > \text{K}^+ > \text{Cs}^+ > \text{NH}_4^+ > \text{Na}^+ > \text{Rb}^+$ (42).

TABLE IV
OPTIMUM MONOVALENT CATION REQUIREMENT FOR GUANYLATION

Monovalent Cation	Optimum Concentration in M	[^3H]Guanylation in cpm
Na^+	0.114	3,931
Li^+	0.08	3,816
K^+	0.08	3,597
Cs^+	0.07	2,449
Rb^+	0.08	2,277

Our initial attempts to further purify the S-200 stage enzyme met with failure. The S-200 fractions contained 0.5 M NaCl and 10% glycerol which had to be removed before the enzyme could be purified by the blue sepharose step. We tried to protect the enzyme during dialysis by addition of various combinations of Tris, pH 7.6, EDTA, mercaptoethanol,

0.5 M salt, and 10% glycerol without success. We also tried to use Sephadex G-25 and the desalting resin AG118-X instead of dialysis, but the enzyme still became inactive.

The rabbit enzyme does not require any divalent cations (42), but Mg^{++} does stimulate the *E. coli* enzyme (32). The wheat germ enzyme was tested for requirement of a divalent metal as follows: S-200 stage enzyme was incubated with the following salts, CaAc, BaAc, $SrCl_2$, $MgCl_2$, $ZnSO_4$, $FeSO_4$, $CuCl_2$, PbAc, $MnCl_2$, $CoCl_2$, $CdCl_2$, and NiAc, in the presence of 0.1 M KCl. At $1.0 \times 10^{-3}M$ and $1.0 \times 10^{-2}M$, the results in Table V show that Zn, Fe, Cd, Co, Ni, Pb, and Cu produced almost total inhibition of guanylation (86-100%) at both concentrations. At $10^{-3}M$, Ba, Sr, Ca, and Mg stimulated activity, Mn^{++} inhibited 86% at 10^{-2} but only 24% at $10^{-4}M$. Ba^{++} inhibited 18.2% at $10^{-2}M$ and stimulated by 25% at $10^{-3}M$. Ca^{++} , Zn^{++} , and Mn^{++} were also tested at $10^{-4}M$, but Zn^{++} and Mn^{++} were still inhibitory at this concentration. There was no difference in results when dialyzed enzyme was used.

The G-25 stage enzyme was dialyzed to determine the ability of Mg^{++} to replace the monovalent cation requirement. Mg^{++} can substitute for the monovalent cation as shown in Table VI. The monovalent cation cannot satisfy the divalent cation requirement. For the purified enzyme, a Mg^{++} concentration of $5 \times 10^{-3}M$ best meets the requirement for the S-200 stage dialyzed enzyme as shown in Fig. 14.

Guanylation of X-irradiated tRNA. Wheat germ tRNA was irradiated at 1,000, 5,000, 25,000, 50,000, and 75,000 Roentgens and the tRNA then assayed for guanine acceptance. The results are shown in Fig. 15. The

TABLE V
THE EFFECT OF DIVALENT METALS ON GUANYLATION

Metal	Source	Effect of Guanylation at Divalent Metal Concentrations		
		10^{-2}M	10^{-3}	10^{-4}M
Ba	BaAc	18 ↓	25 ↑	
Sr	SrCl_2	15.3↓	33.6↑	
Ca	CaAc	49 ↓	35.6↑	29.8↑
Mg	MgCl_2	46 ↓	39 ↑	
Mn	MnCl_2	86.6↓	32.4↓	24.2↓
Zn	ZnSO_3	98.5↓	96 ↓	55 ↓
Fu	FeSO_4	82.6↓	100 ↓	
Cd	CdCl_2	99.5↓	98 ↓	
Co	CoCl_2	95.7↓	86.5↓	
Ni	NiAc	91.9↓	83.5↓	
Pb	PbAc	99.5↓	98 ↓	
Cu	CuCl_2	98.5↓	90.6↓	

↑ = Percentage of activation.

↓ = Percentage of inhibition.

TABLE VI
COMPARISON OF MONOVALENT AND DIVALENT CATIONS
AS REQUIREMENTS FOR GUANYLATION

Treatment of Dialyzed Enzyme	[³ H]Guanine cpm/A ₂₆₀
<u>G-25</u>	
untreated dialyzed enzyme	79
untreated dialyzed enzyme + 0.1 M KCl	3,548
untreated dialyzed enzyme + 0.005 M Mg ⁺⁺	2,068
untreated dialyzed enzyme + KCl + Mg ⁺⁺	2,925
<u>S-200</u>	
untreated dialyzed enzyme + 0.1 M KCl	5,777
untreated dialyzed enzyme + KCl + 10 ⁻³ M Mg ⁺⁺	12,511
dialyzed enzyme	353
dialyzed enzyme + Mg ⁺⁺	9,576.59
undialyzed S-200 + KCl	15,816

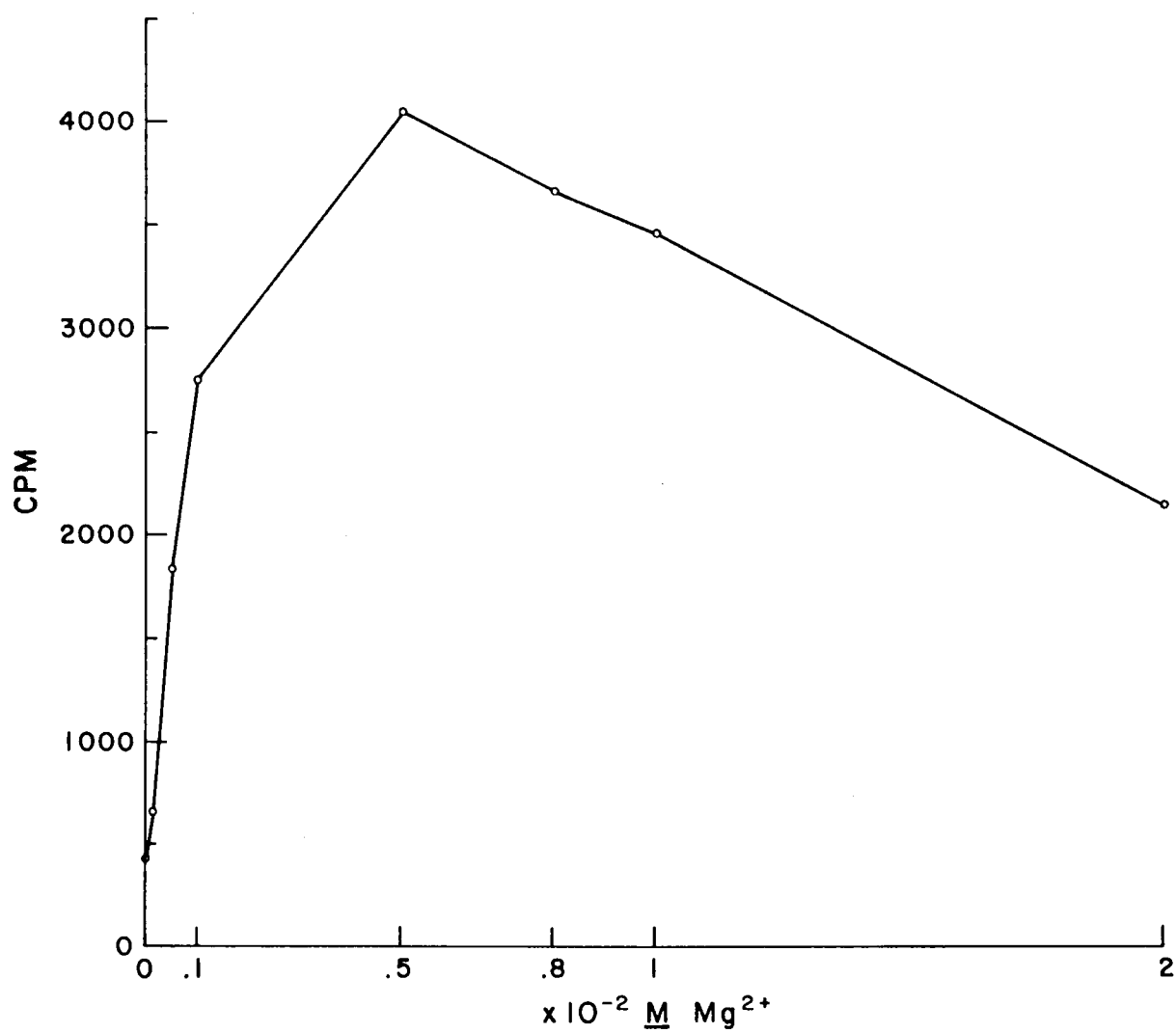


Figure 14. Mg^{2+} Requirement for Guanylation.

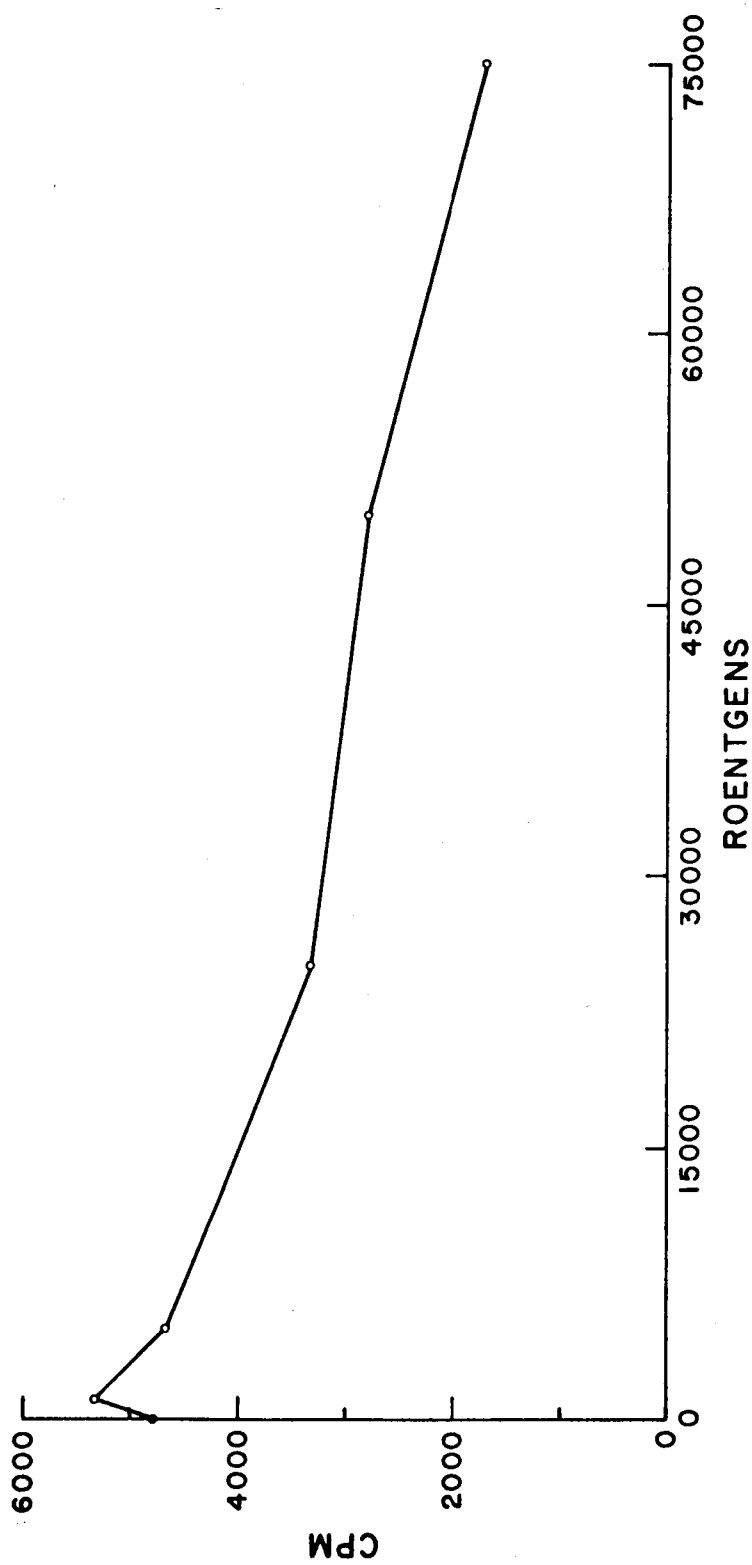


Figure 15. The Effect of X-Irradiated tRNA Substrate on Guanylation.

higher doses produced a linear decay, with a dose of 60,000 Roentgens yielding 50% inactivation. Curiously, 1,000 Roentgens increased guanylation by approximately 15% over the unirradiated sample. The unexpected increase in guanylation at 1,000 Roentgens necessitated a study of the effects of low doses. Wheat germ transfer RNA was irradiated at 500, 1,000, 1,500, 2,000, 3,000, 4,000, and 5,000 Roentgens and the tRNAs assayed in triplicate for guanine acceptance. The results shown as line A in Fig. 16 indicate that the stimulation of guanylation was not only reproducible but a saw-tooth pattern was observed. Depressions occur at 500 and 3,000 Roentgens while increases occur at 2,000 and 5,000 Roentgens. The irradiated tRNAs were reassayed 24 hrs later and the results, again based on triplicates, are shown in Fig. 16 as line B. The two experiments are almost identical showing the increases and decreases of the saw tooth effect at the same doses. The experiment was repeated with other samples of wheat germ tRNA and also with yeast tRNA with similar results.

The initial experiment was performed by irradiating each sample separately. To determine if the method of irradiation might be producing the saw-tooth curve, the transfer RNA sample was irradiated using the split dose method outlined in the Methods section. New preparations of tRNA and S-200 stage enzyme were used. The results, based on quadruplicate samples, are shown in Fig. 17, and the saw-tooth pattern is still observed. The peaks and depressions have shifted slightly, probably due to differences in measurement of the split dose under the X-ray beam.

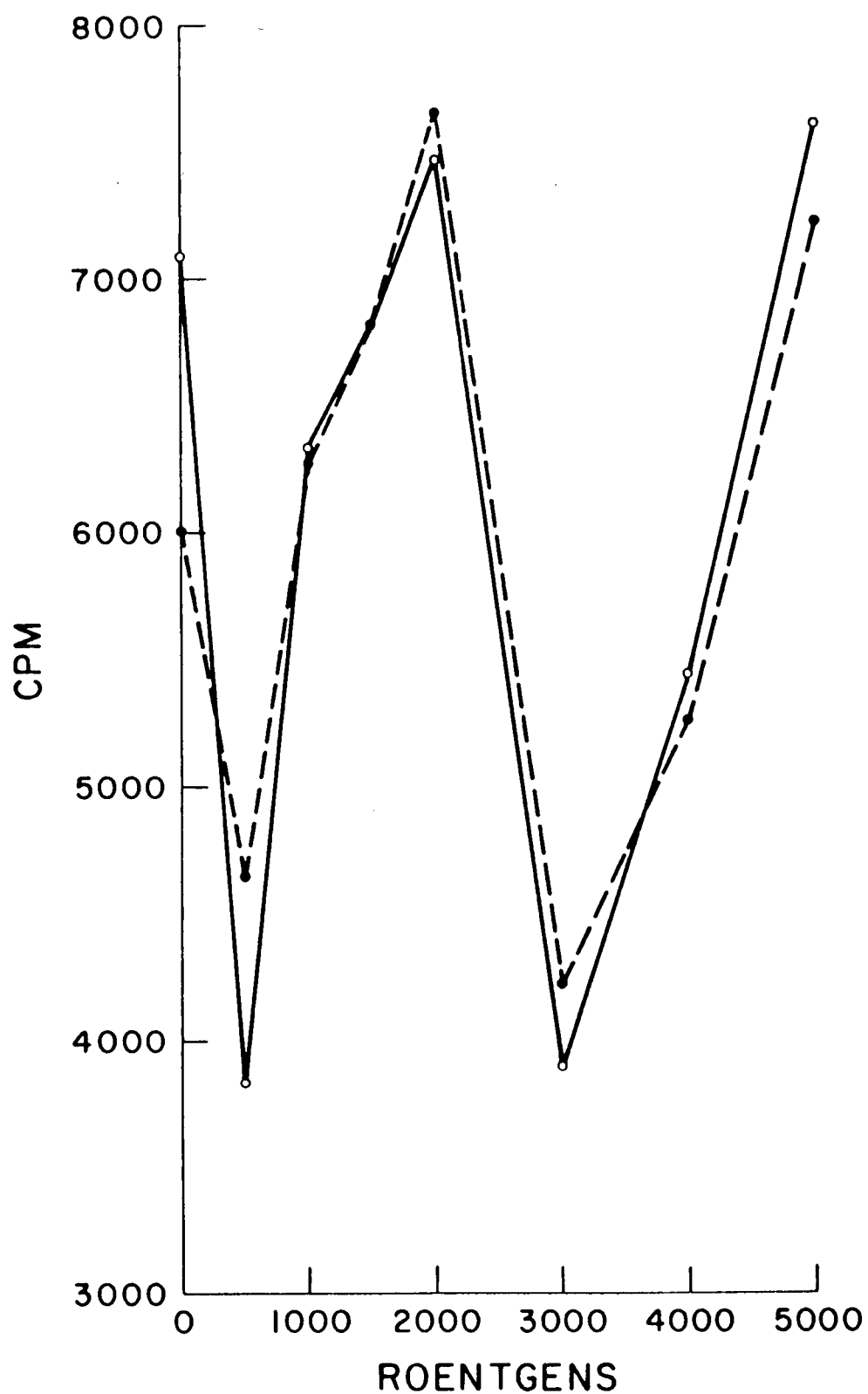


Figure 16. Low Dose Modification of Guanylation by X-Rays: the Saw-Tooth Effect. (●---● A, o—o B)

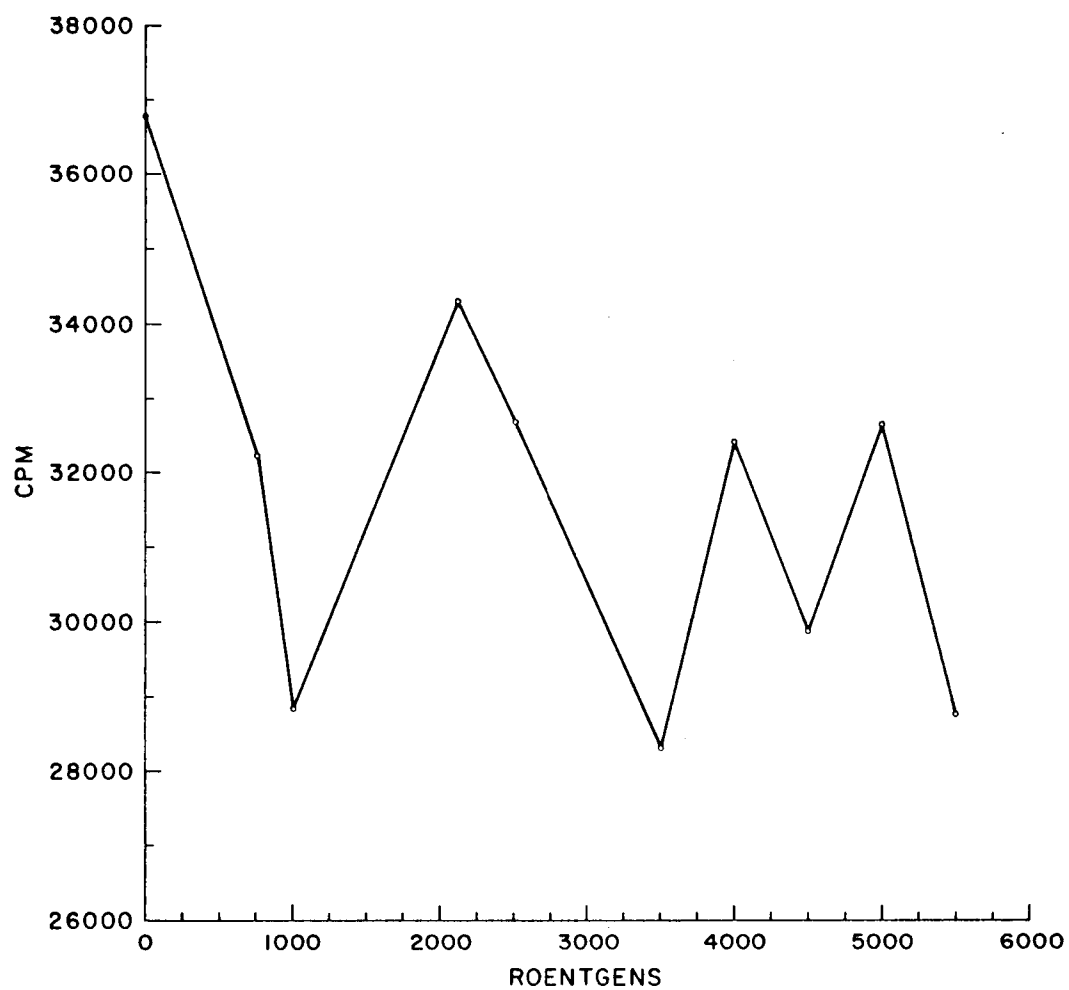


Figure 17. The Effect of X-Irradiated tRNA on Guanylation—Split Dose Exposure.

The effects of the irradiation were assumed to be due to indirect effects since the tRNA was irradiated in dilute aqueous solution. Wheat germ tRNA was irradiated at a concentration of 30 A₂₆₀ units/ml in the presence of other solutes to determine if they affected the saw-toothed pattern. Group I of the tRNA samples to be irradiated was a control containing wheat germ tRNA 30 A₂₆₀ units/ml (5mM Tris pH 7.6, mM EtSH). The solution in Group II contained 30 A₂₆₀ units of tRNA/ml, 1.5 mg/ml of bovine serum albumin, 5 mM Tris, pH 7.6, and 5 mM EtSH. Group III had 30 A₂₆₀ units of tRNA/ml, 5 mM Tris, pH 7.6, and 0.1 M EtSH. Group IV had 30 A₂₆₀ units of tRNA/ml, 50 mM Tris, Ph 7.6, and 5 mM EtSH. Each group was irradiated at 0, 1,000, 2,000, 3,000, and 4,000 Roentgens. Guanylation was then carried out in triplicate for each dose using 100 µl per assay tube of S-200 enzyme. The results are shown in Fig. 18. The control sample showed that peaks occurred at 1,000 and 3,000 Roentgens with depressions at 2,000 and 4,000 Roentgens. In Group II, which contained bovine serum albumin, the depression at 2,000 Roentgens was diminished and the 4,000 Roentgen depression was absent. Group III which had the 0.1 M EtSH shifted the entire graph down, with peaks at 1,000 and 2,000, and depression at 2,000 and 4,000. EtSH was more effective at shifting the graph down at the higher doses than at 1,000 and 2,000 Roentgens. There was a greater depression observed at the 2,000 Roentgen dose in Group IV which contained the 50 mM Tris, pH 7.6.

A possible explanation for the stimulation of guanylation was that tRNA species not normally guanylated were converted to a guanylatable form by irradiation. [³H]guanylated wheat germ tRNA was prepared from

Figure 18. Modification by Radioprotectants. (Wheat Germ tRNA was irradiated in the presence of different radioprotectants in a standard solution of 5mM Tris and 5mM EtSH. The results, based on triplicates are shown for: ---- standard solution, o—o 1:1 dilution with Bouine Serum Albumin, —•— 50mM Tris, and — — 100 mM EtSH.)

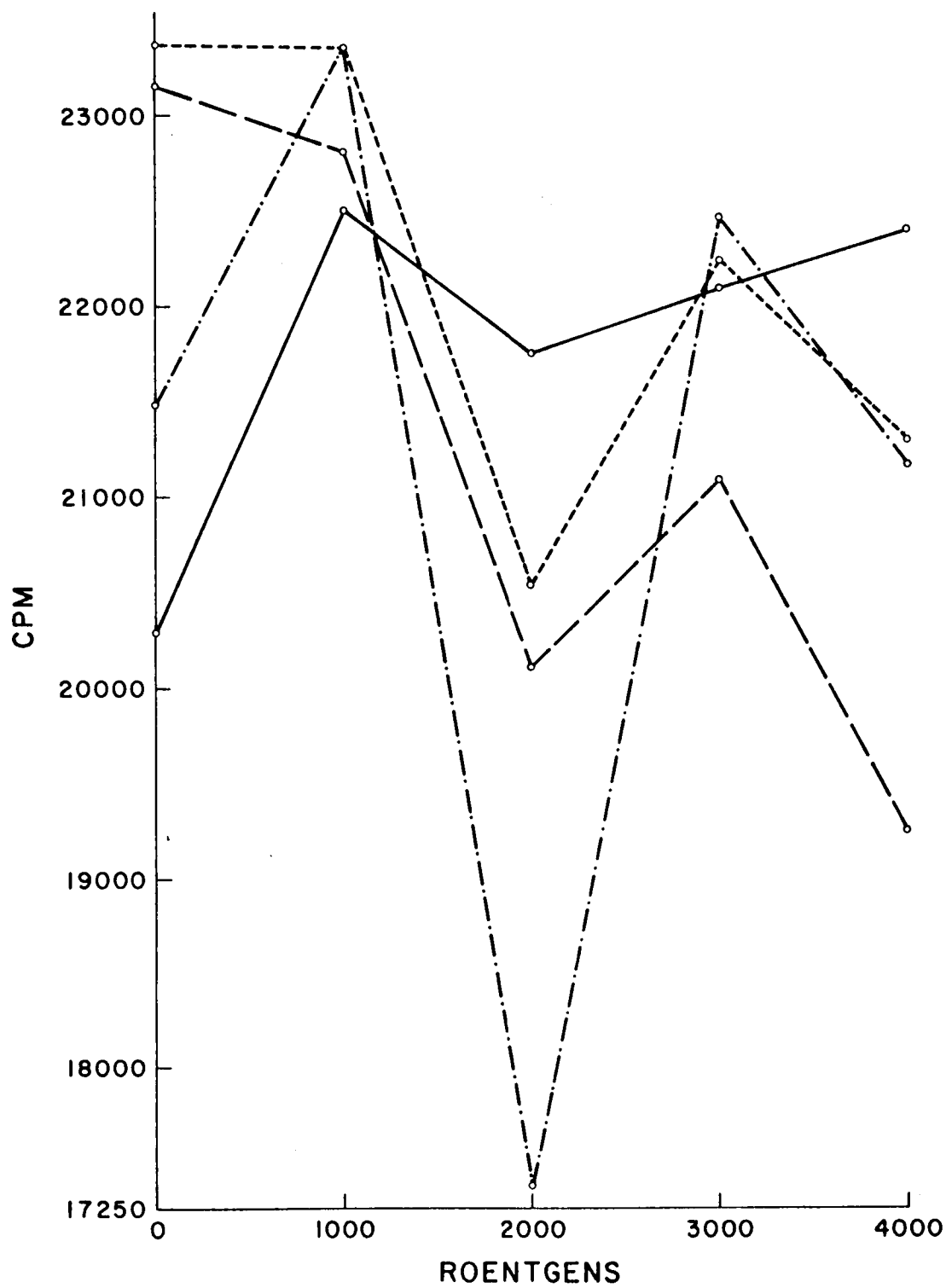


Figure 18

unirradiated tRNA and tRNA irradiated at 2,000 Roentgen, and the samples analyzed by chromatograph on RPC-5. The results shown in Figs. 19 and 20 indicate that no additional peaks were present in the irradiated sample. The increases then must occur within those tRNAs already capable of being guanylated.

It was of interest to see if the capacity of wheat germ tRNAs to be charged by their cognate amino acids was similarly affected by irradiation. The capacity of tRNA^(Asp) and tRNA^(Asn) to accept their cognate amino acid after irradiation at doses that produced the saw-tooth pattern for guanylation was tested. Unlike guanylation, the aminoacylations exhibited a linear decrease. There was an 11% loss of acceptance at a dose of 5,000 Roentgens.

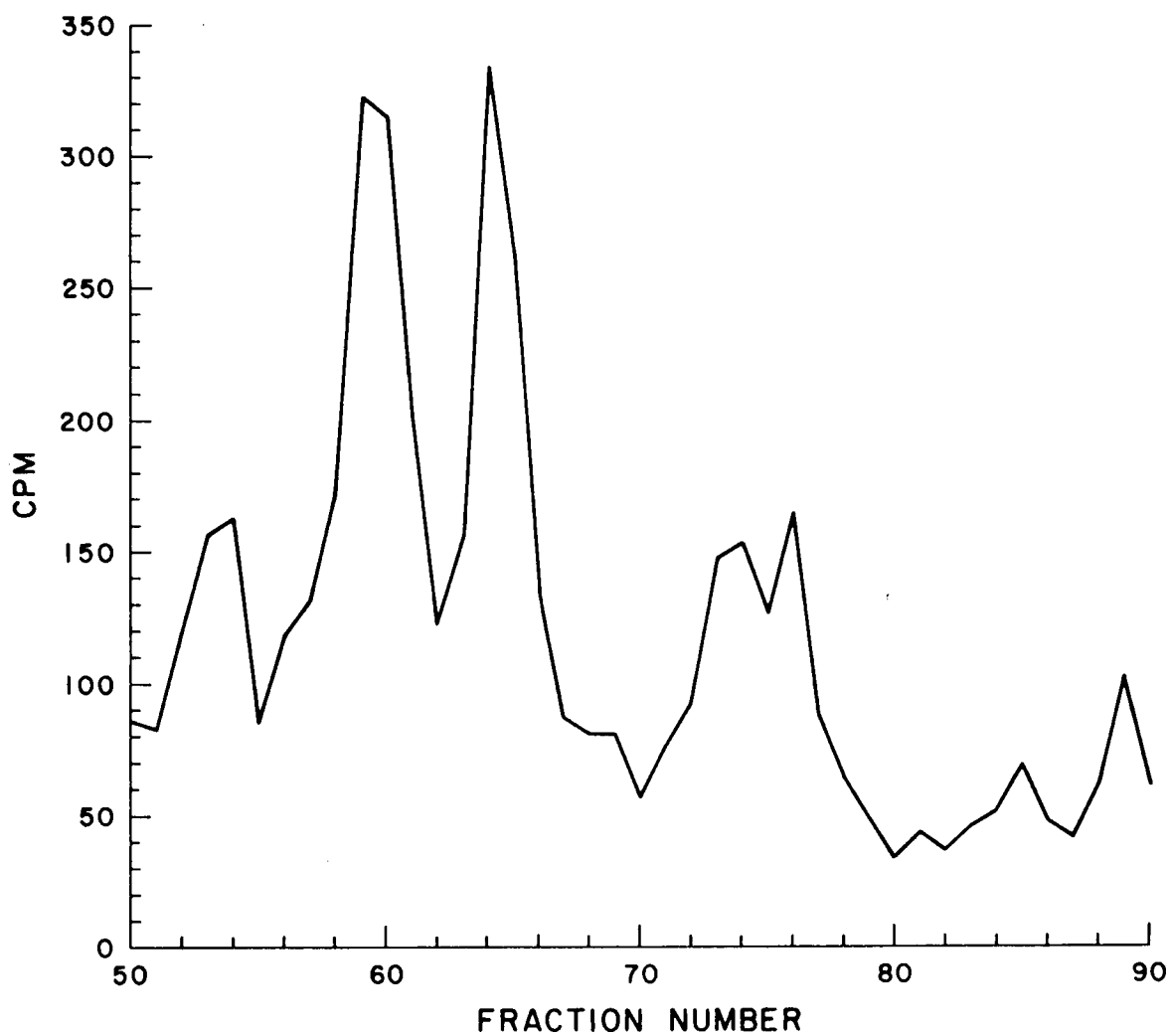


Figure 19. RPC-5 Chromatography of Unirradiated Wheat Germ tRNA.

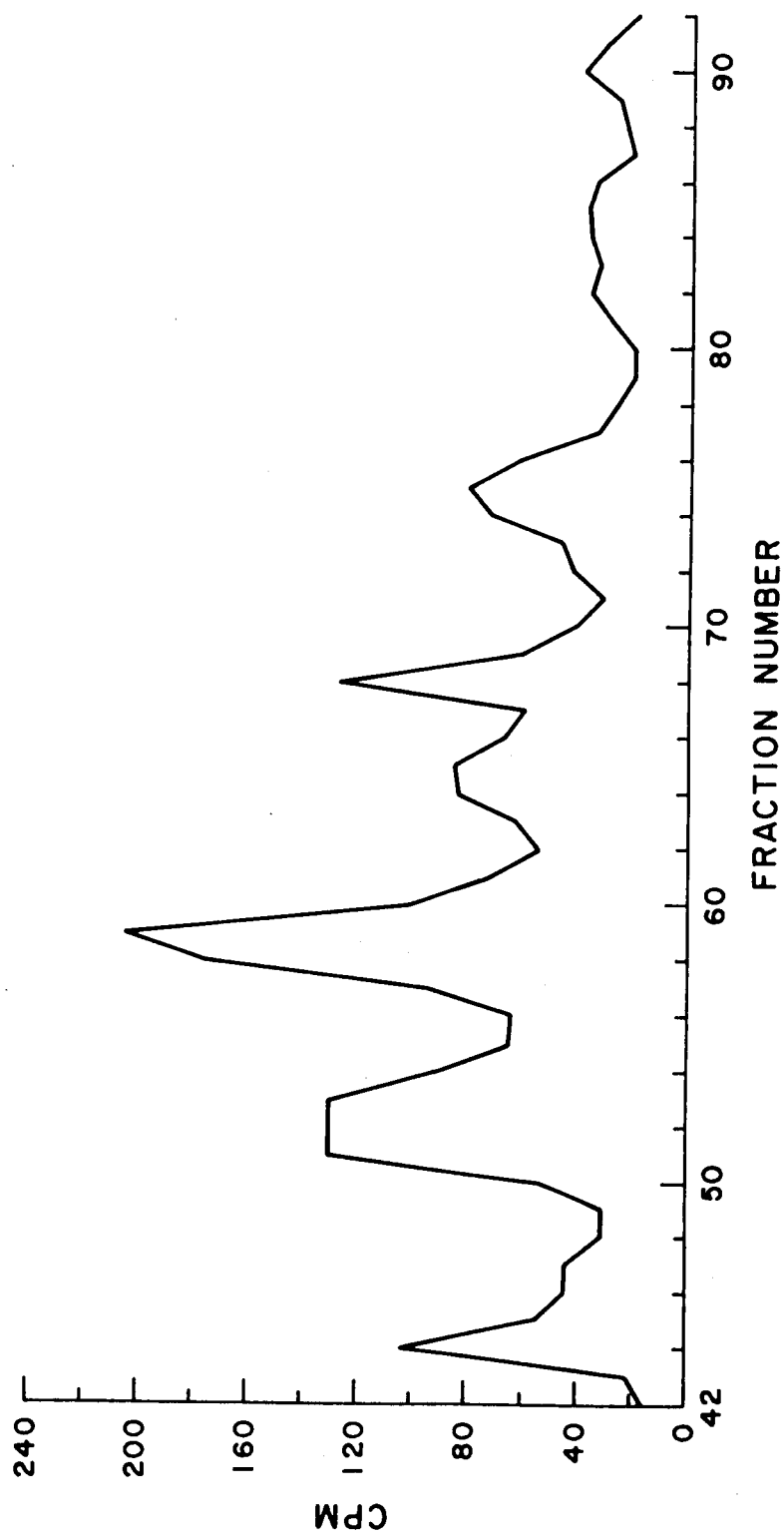


Figure 20. RPC-5 Chromatography of 2000R X-Irradiated Wheat Germ tRNA.

V. DISCUSSION

This paper reports the purification of guanine insertase to homogeneity from wheat germ. It is the first report of guanylation in plants. Previous studies have been done with rabbit reticulocytes (2), *E. coli* (3), Ehrlich ascites cells (5), and rabbit erythrocytes (1) from which guanine insertase has recently been purified to homogeneity. The specific activity of the purified wheat germ enzyme (2,029 pmoles guanine incorporated per hr per mg of protein) is comparable to that published for the rabbit erythrocyte enzyme, 2,633 pmoles (1). A study on the *E. coli* enzyme, in which the degree of purification is not stated, reports a specific activity of 50,000 (32).

The wheat germ enzyme has a higher K_m for guanine of $1.05 \times 10^{-6}M$ than the rabbit enzyme with a K_m of $1.5 \times 10^{-7}M$ (1).

The major wheat germ tRNAs that are guanylated by the enzyme are tRNA^(Asp)_{II} and tRNA^(Asn)_{II}. The major rabbit reticulocytes tRNAs to be guanylated by the rabbit enzyme are tRNA^(His)_{III} and tRNA^(Asn)_{II} (49). Wheat germ tRNA^(His) is guanylated to a lesser extent than tRNA^(Asp) and tRNA^(Asn).

The mass of the wheat germ enzyme, 140,000 daltons, is larger than the 104,000 daltons reported for the rabbit erythrocyte enzyme. Both require a monovalent cation. The optimum concentrations of one cation, K^+ , are about equal—0.107 M for the rabbit enzyme (42) and 0.08 M for the wheat germ enzyme. The optimum pH for both is also similar—7.6 for wheat germ and 7.8 for the rabbit enzyme (42).

The purification and recovery of the wheat germ enzyme are comparable to the mammalian enzyme. The wheat germ system is less expensive to study when one compares the cost of rabbits along with their maintenance. The entire purification procedure from crude extract to homogeneity can be accomplished in about 2-1/2 to 3 days.

One significant difference between the two enzymes is the demonstration of a divalent metal cation requirement best filled by Mg^{++} as a cofactor for wheat germ enzyme. Mg^{++} is required for stability and is probably bound to the enzyme. The monovalent cation maintains the proper ionic strength.

One possible function of Q in the anticodon is to prevent misrecognition of the first or second codon position which occurs at high Mg^{++} concentrations. The optimal Mg^{++} concentration for *E. coli* (32) and Erhlich ascites tumor (40) guanine insertase is $1.0 \times 10^{-2}M$. Miscoding has been reported at $10^{-2}M$ Mg^{++} (21). The miscoding studies were done at a potassium concentration of $5 \times 10^{-2}M$, but the potassium concentration of some tissues can be 10 times higher. Perhaps miscoding studies done at higher K^+ levels would demonstrate miscoding at a lower concentration of Mg^{++} . If Q does function to prevent miscoding due to Mg^{++} , then levels of tRNA(Q+) and its enzymes should respond to Mg^{++} concentration. The production of tryptophane (50) and asparagine (51) in plants is influenced by Mg^{++} levels. Magnesium content in wheat is twice as high in grain than in straw and also varies between winter and summer wheat (51).

Five-methyl-2-thiouridine is found in the wobble position of some tRNAs which recognize the XA_G^A codon and may serve a function analogous

to Q. The sulfurtransferase catalyzing this modification requires a divalent metal (52). Comparison of studies on Q (6), sulfurtransferase (52), and magnesium (53) indicate similar differences among organs with liver showing the highest concentration. These coincidences further indicate that Q may function to prevent miscoding due to Mg^{++} .

Guanylation is more sensitive to low doses of X-rays than aminoacylation and ribosome binding. The 50% inactivation of the capacity of tRNA to be guanylated occurs at 60,000 Roentgens (or 57,000 rads); whereas, capacity to bind to polyribosomes is inactivated at 50,000 rads (54). Interpretation of the data on the effect of dose vs. guanine acceptance is complicated by the saw-tooth effect shown in Fig. 16 (p. 44). The results are attributable to indirect effects caused by the radiolysis of water by the X-rays since the saw-tooth effect observed when tRNA was irradiated in distilled water was modified when compounds that react with radicals were present in solution along with the tRNA. Tris is a radioprotectant of phage DNA in aqueous solutions (55); however, Fig. 18 (p. 47) shows that Tris increases inactivation of guanine acceptance, particularly at 2,000 Roentgens. Perhaps Tris forms radicals which react with the modified bases present in tRNA.

The molecular basis for the saw-tooth effect remains obscure. Several possibilities should be considered. One possible explanation is that the stimulation of guanylation is caused by the insertion of guanine at sites other than the first position of the anticodon. The damage caused by irradiation might modify other sites on the molecule so

that they become guanylated. Sequence analysis of guanylated irradiated tRNA would confirm or refute this possibility even though this explanation is unlikely. RPC-5 elution profiles of guanylated irradiated tRNA (Figs. 19 and 20, pp. 51-52) show no new guanylated peaks due to irradiation. Even after irradiation at a dose that stimulates only those tRNA families recognizing the XAC^{U} codon are guanylated. Modifications allowing guanylation at other sites should also show up in tRNA other than those presently capable of undergoing this modification.

Another possibility accounting for the saw-tooth effect would be the ability of guanine insertase to act in repair. Unlike "dark repair," this repair would proceed without requirement for ATP (42). The enzyme would recognize and excise damaged Q base or guanine and replace it with intact guanine. This would also account for the ability of the enzyme to turn over guanine. Uracil DNA glycosidase (56), 3-methyl adenine DNA glycosidase (57), and trans-N-deoxyribolase I (58) are repair enzymes which function by N-glycosidase action. Trans-N-deoxyribolase I from *lactobacillus helveticus* is capable of replacing a purine for a purine (59). *E. coli* endonuclease II, another enzyme with N-glycosidase properties, can recognize and repair radiation-damaged DNA (59).

The rabbit reticulocyte guanine insertase exchanges guanine for Q base when used on *E. coli* tRNA (3) but does not exchange Q for guanine in yeast tRNA or rabbit liver tRNA (4)—only guanine for guanine. Results showing inhibition of the wheat germ guanine insertase by Memphis factor (shown to be Q base) indicates the ability of the enzyme to recognize Q. The results of this paper demonstrate that the wheat germ guanine

insertase enzyme replaces guanine with guanine. It is possible that the radiation damage changes the conformation of the tRNA to allow Q to be exchanged for guanine.

The fourth explanation is that chemical changes occurred in the tRNA causing conformational changes which favor enzyme recognition of the guanine acceptor tRNAs. In examining each of the explanations, it must be recalled that the enzyme reacts with 7 isoacceptors in wheat germ to produce the guanylated products.

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