

AMINOACYLATION KINETICS AND SPECIFICITY
FOR VIRAL GENOMIC RNAS

by

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LIST OF ABBREVIATIONS

A ₂₈₀	- absorbance at 280 nm
AMV	- alfalfa mosaic virus
BBMV	- broad bean mottle virus
BMV	- brome mosaic virus
BPMV	- bean pod mottle virus
BSMV	- barley stripe mosaic virus
CCMV	- cowpea chlorotic mottle virus
CcTMV	- cowpea strain of tobacco mosaic virus
CM	- carboxymethyl
CMV	- cucumber mosaic virus
(c.p.m.)	- counts per minute
CYMV	- cacao yellow mosaic virus
DBAE	- diboryl aminoethyl
DEPC	- diethyl pyrocarbonate
EF-1	- elongation factor one
EF-Tu	- elongation factor Tu
EMV	- eggplant mosaic virus
GTAMV	- green tomato atypical mosaic virus
HEPES	- N-2-hydroxymethyl piperzaine-N-2-ethanesulfonic acid
HTS	- histidine tRNA synthetase

NT - tRNA nucleotidyltransferase
OMV - okra mosaic virus
PEMV - pea enation mosaic virus
PMSF - phenylmethyl-sulphonyl fluoride
POPOP - 1,4 bis 2-(5-phenyloxazolyl)-benzene
PPO - 2,5 diphenyloxazole
PSV - peanut stunt virus
PVP - polyvinylpyrrolidone
SBMV - southern bean mosaic virus
STNV - satellite tobacco necrosis virus
TMV - tobacco mosaic virus
TSV - tobacco streak virus
TYMV - turnip yellow mosaic virus
U.C.R. - University of California at Riverside
VTS - valine tRNA synthetase
WCMV - white clover mosaic virus
WCuMV - wild cucumber mosaic virus

INTRODUCTION

The 3' end of genomic RNA from some plant viruses is known to have a tRNA-like amino acid acceptor activity. The observed activity is like tRNA aminoacylation in that an aminoacyl tRNA synthetase, a specific amino acid and ATP are required for the aminoacylation and the amino acid is esterified to the 3' OH of the terminal AMP of the viral RNA. This viral RNA is much larger than tRNA and contains genetic information which codes for viral proteins. The function of the amino acid acceptor activity in the viral life cycle is unknown. Many of the viruses possessing this activity are important agricultural pests. Therefore, the understanding of this property of these viruses may be economically important.

Quantitative information about the quality and specificity of viral RNA aminoacylation is almost absent from the literature. Methods were developed for accurate measurements of the maximum mole percent of aminoacylation and aminoacylation rate of viral genomic RNA. In order to extend the current understanding of the taxonomy of viral RNA amino acid acceptor activity, the RNAs of nine viruses representing eight taxonomic groups were tested for amino acid and

aminoacyl tRNA synthetase source specificity.

Since the quality of viral RNA as a substrate of aminoacyl tRNA synthetases is largely unknown, there is some question as to whether viral RNA is likely to be aminoacylated in vivo. In order to determine if the aminoacylation of viral RNA is in fact quantitatively comparable to the normal aminoacylation of tRNA, the kinetic parameters of the aminoacylation of tRNA and turnip yellow mosaic virus RNA were determined and compared in the presence of aminoacyl tRNA synthetases from four unrelated sources.

LITERATURE REVIEW

Aminoacylation of Viral RNA - In 1965 it was first reported that the turnip yellow mosaic virus (TYMV)¹ single stranded RNA genome could be enzymatically aminoacylated with valine (1). This report also indicated that other amino acids could be incorporated into TYMV-RNA but this was never substantiated. The next report, in 1970, showed that TYMV-RNA could be aminoacylated with valine by a crude Escherichia coli (E. coli) aminoacyl-tRNA synthetase preparation, with 0.8 mole valine esterified per mole of RNA (2). Most of the valine was shown to be bound to the 1.9×10^6 dalton genomic RNA.

Using purified enzyme preparations, it was found that TYMV-RNA was also a substrate for E. coli tRNA nucleotidyl-transferase, an enzyme which adds the C-C-A_{OH} sequence to the 3' end of tRNA (3). Only the AMP residue is added to native TYMV-RNA. The presence of this 3' terminal AMP is a prerequisite for the aminoacylation of TYMV-RNA, or of any tRNA, by an aminoacyl tRNA synthetase. It was found by the same authors that the native 3' terminal sequence of TYMV-RNA is A-C-C-A_{OH} and that the two cytidine residues

¹ The abbreviations used in this text are defined in the List of Abbreviations, page vii.

also can be added back to TYMV-RNA by tRNA nucleotidyl transferase subsequent to their nucleolytic cleavage from TYMV-RNA (4). In addition to the E. coli valine tRNA synthetase, TYMV-RNA has been aminoacylated with wheat, bean and yeast valine tRNA synthetases (5).

TYMV is a member of the tymovirus group, members of which normally contain their genomes as a single large infectious RNA. The large (2×10^6 daltons) RNAs from four other members of this group; eggplant mosaic virus (EMV), cacao yellow mosaic virus (CYMV), wild cucumber mosaic virus (WCuMV) and okra mosaic virus (OMV); have been aminoacylated with valine by E. coli valyl-tRNA synthetase. Of these, only OMV-RNA, like TYMV-RNA (6), lacks the 3' terminal adenosine necessary for aminoacylation.

These other viruses differ from TYMV in that their normal virions are associated with light particles which contain only 4S RNAs (7). These 4S RNAs appear to be tRNAs, as they can be aminoacylated with a variety of amino acids. One member of this group, EMV, appears to have in addition, a lysine specific tRNA bound non-covalently to its large RNA (7).

The single stranded genomic RNA of several members of the bromovirus group - brome mosaic virus (BMV), cowpea

chlorotic mottle virus (CCMV) and broad bean mottle virus (BBMV) - have been shown to be specifically esterified to tyrosine in the presence of plant (wheat and bean) tyrosine tRNA synthetases (5,8). The typical RNA genome of this group is divided into three strands, all of which are active messenger RNAs and all of which accept tyrosine (9). The mole percent aminoacylation of this group, per mole of each RNA specie, is about 60-90%.

Cucumber mosaic virus (CMV) RNA also accepts tyrosine from plant tyrosine tRNA synthetases (5). This virus is a member of the cucumovirus group which is most closely related to the bromovirus group.

Tobacco mosaic virus, a member of the tobamovirus group, has an RNA genome that specifically accepts histidine (30-40 mole %) from yeast, wheat and rat histidine tRNA synthetases (10,11,12). Addition of 3' terminal nucleotides is not required for this reaction (10). It has been reported by Sela (13) that TMV-RNA, following fragmentation by crude tobacco enzymes, also contains serine and methionine specific amino acid accepting activities. This interesting result has not, however, been confirmed. The cowpea strain of TMV (CcTMV) has been shown

to be specifically aminoacylated with valine by a yeast valine tRNA synthetase (14). Although this virus is not closely related to other strains of TMV which accept histidine, it is nevertheless in the tobamovirus group (15).

The RNA of bean pod mottle virus (BPMV), a comovirus, has been reported to accept tyrosine after internal cleavage of the RNA (16). This finding was presented as a preliminary report not accompanied by data but it has some bearing on the data given above for TMV-RNA and on the situation for certain animal virus RNAs.

The RNA of mengovirus, an animal picornavirus, has been aminoacylated with histidine by a mouse liver histidine tRNA synthetase (17). This RNA has poly A at its 3' terminus and requires enzymatic internal cleavage to expose the aminoacyl acceptor portion of the RNA. The serine specific aminoacylation of the RNA of encephalomyocarditis virus, another picornavirus also has been reported².

There have been a few reports of viral RNAs which do not aminoacylate under the usual conditions. The RNAs from alfalfa mosaic virus (AMV) (18), tobacco streak virus (TSV) (18), satellite tobacco necrosis virus (STNV) (11), poliovirus (11) and pea enation mosaic virus (PEMV) (19) do not

² Stebbing, N. and Lindley, A. (1976) (Personal communication to I. Sela. Science 194, 528.)

appear to be capable of enzymatic aminoacylation. The findings presented in this section are summarized in Table I.

Relationship of Aminoacylation to Infectivity - As yet, a role for genomic RNA aminoacylation in the plant virus life cycle has not been found. There are, however, indications that proper aminoacylation is important to the infectivity of plant viral RNA. In 1966, in spite of the fact that the aminoacylation capacity of TMV had not yet been found, it was reported that the enzymatic removal of four residues from the 3' end of TMV-RNA abolished the infectivity of that RNA (20). If less than four residues were removed, TMV-RNA retained its infectivity and its capacity to be "repaired" by tRNA nucleotidyltransferase (10). When BMV-RNA is aminoacylated with N-acetyl tyrosine, instead of tyrosine, 75% of the infectivity of this RNA is lost (21). The N-acetyl tyrosine-RNA ester linkage is very stable and would presumably block the proper aminoacylation of BMV-RNA with tyrosine.

Experiments have been done to explore the possibility that the aminoacylation of viral RNA affects its ability to be translated into proteins. It has been shown that valyl-TYMV-RNA can donate esterified valine to peptide synthesis in an in vitro protein synthesizing system from E. coli (22).

TABLE I
VIRUSES WITH
AMINOACYLATABLE GENOMIC RNA

<u>VIRUS</u>	<u>AMINO ACID</u>	<u>GROUP</u>	<u>REFERENCES</u>
Broad Bean Mottle	tyrosine	BROMO	(5)
Brome Mosaic	"	"	(12)
Cowpea Chlorotic Mottle	"	"	(5)
Cucumber Mosaic	tyrosine	CUCUMO	(5)
Peanut Stunt ³	"	"	---
Tobacco Mosaic	histidine	TOBAMO	(11)
Cowpea Strain	valine	"	(14)
Turnip Yellow Mosaic	valine	TYMO	(2)
Cacao Yellow Mosaic	"	"	(6)
Eggplant Mosaic	"	"	(6)
Okra Mosaic	"	"	(6)
Wild Cucumber Mosaic	"	"	(6)
Bean Pod Mottle	tyrosine	COMO	(16)
Encephalomyocarditis ²	serine	PICORNA	---
Mengovirus	histidine	"	(17)

³Determined by the author (see Results)

²Stebbing loc. cit.

Valyl-TYMV-RNA is, however, only 1% as efficient as E. coli valyl-tRNA in this respect. Tyrosylated BMV-RNA appears unable to donate tyrosine to peptide synthesis in a wheat germ in vitro protein synthesizing system (23). Using the same system to assay the messenger function of BMV-RNA, it was found that prior chemical degradation of the 3' end of the BMV-RNA did not affect its efficiency of translation (24). It should be noted that wheat is a natural host of BMV and that native BMV-RNA can be tyrosylated with wheat tyrosine tRNA synthetase.

Experiments have also been done to see if the aminoacylation of viral RNA plays a role in viral replication. TMV, TYMV and BMV-RNAs can form a complex with protein biosynthesis elongation factor one (EF-1) from wheat germ only if the RNA is first aminoacylated (25,26). GTP is released from EF-1 on binding tyrosyl-BMV-RNA. Aminoacylated TMV and TYMV-RNA also bind elongation factor Tu (EF-Tu) from E. coli. It is known that the RNA phage QB is replicated by an enzyme complex containing EF-Tu (27). It was thought, therefore, that plant viral replicases might also employ host EF-1 and that the binding of the viral RNA to the replication complex might be facilitated by aminoacylation of the viral RNA and binding of the esterified 3' end to the

EF-1 (28). While Q β replicase is quite active in replicating TYMV-RNA, aminoacylation of the RNA has no effect on the efficiency of replication in this system, thus casting some doubt on this model (26). Further studies using the TYMV specific replicase isolated from infected plant tissue, show that aminoacylation has no effect on the binding of TYMV-RNA to the replicase (28). It should be noted that the results of these experiments do not entirely preclude the possibility of a role for the aminoacylation of viral RNA in translation or in replication in vivo.

Viral RNAs capable of aminoacylation are known to be recognized and acted on by several enzymes other than aminoacyl tRNA synthetases and tRNA nucleotidyltransferases. One of these, nuclease P from E. coli, is a specific endonuclease which cleaves 4 S sized tRNA molecules from larger tRNA precursors as a step in tRNA processing (29). This enzyme cleaves a 4.5 S sized fragment from the 3' end of whole TYMV-RNA. This 4.5 S RNA can be aminoacylated with valine. N-Acylaminoacyl-tRNA hydrolase, an enzyme which hydrolyzes the ester bond of N-acyl derivatives of amino acids to tRNAs, is also active on N-acylvalyl-TYMV-RNA (30). Another tRNA enzyme, the one from E. coli which converts a specific uridine residue in the tRNA "T" loop to ribothymidine, uracil

tRNA methyltransferase, stoichiometrically (1:1) incorporates a methyl group into TYMV-RNA⁴.

Shortly after treatment with interferon, mouse L cells produce a factor which specifically deacylates histidiny1 TMV-RNA (31). This factor does not deacylate aminoacyl tRNA from mouse cells. This factor may be part of the interferon-induced defense system against viruses such as mengovirus which infects mice.

Structure and Sequence - For most plant virus RNAs the site of aminoacylation (if present) is at the 3' terminus of the genome (2,9,11). Additionally, the amino acid acceptor site has been found at the 3' end of the encapsidated messenger RNA for coat protein isolated from TYMV, TMV and BMV (9,14,32). An aminoacylatable 5 S, 3' terminal fragment of TYMV, isolated by partial T₁ ribonuclease digestion, has been examined (33). This fragment (159 residues), and the previously mentioned fragment (111 residues) isolated following hydrolysis of TYMV-RNA by nuclease P, have been sequenced in two laboratories (33,34). The 3' terminal 108 residues appear to constitute the valine tRNA-like TYMV-RNA. The remainder of the 159 residue fragment exactly matches

⁴ Dudock, B. Personal Communication.

the base sequence expected for the C terminus of the viral coat protein messenger RNA (33). The sequence of the tRNA-like region can be folded into secondary structures bearing some resemblance to tRNA. The proposed structures are considerably larger and of different conformation than the known standard tRNA secondary structure (35). Curiously, no minor bases have been reported in the 108 nucleotide TYMV-tRNA structure whereas standard tRNAs contain many minor bases. The proposed structures do bear strong resemblances, however, to valine tRNAs in the anticodon stem and loop regions.

Three other aminoacylatable viral RNAs have been partially sequenced at the 3' terminus. The sequence of the 59 nucleotides at the 3' end of EMV-RNA bears a general resemblance to the corresponding sequence in TYMV-RNA (36). The sequences of TMV (71 residues) and GTAMV (74 residues), which is a strain of TMV, resemble each other but are not similar to that of TYMV-RNA (37,38). None of these RNAs have been reported to contain minor bases.

Kinetics of Aminoacylation - Very little has been published on the kinetics of viral RNA aminoacylation. The rate of TMV-RNA aminoacylation by rat liver histidine tRNA synthetase has been determined as a function of KCl concen-

tration (13). As a control, the homologous rat histidine tRNA was also used. The rate of TMV aminoacylation was found to be ten times greater than that of the tRNA in the absence of KCl. At 140 mM KCl, however, the rate of tRNA aminoacylation was nearly ten times more than that of the TMV-RNA. The aminoacylation rates of RNA from various strains of TMV have been compared at constant KCl concentrations but little difference was noted (15).

Kohl et al. compared the aminoacylation activities of RNAs from TYMV, CMV, TMV, BMV, BBMV, and CCMV (5). Crude aminoacyl tRNA synthetases from E. coli, yeast, bean and wheat were used. The results of these experiments were presented as rates but in many cases the values given represented maximum or near maximum mole percents of aminoacylation, thus making internal comparisons of the data difficult. Furthermore, the possibility that tRNA nucleotidyltransferase might be limiting the TYMV-RNA reactions was not considered. Nevertheless, the results of these experiments may be used in roughly evaluating the specificity of the above mentioned four synthetases for the six viral RNAs. The positive results of these experiments were noted previously.

Only one Michaelis constant (K_m) of aminoacylation has been reported for a viral RNA (12). The experiment conducted

to measure this K_m , in which BMV-RNA was used as the RNA substrate, was not designed to measure initial rates. Therefore, the K_m value reported must be considered erroneous.

The kinetics and specificity of homologous and heterologous native tRNA aminoacylation have been extensively studied. The results of these studies are reviewed elsewhere (39,40,41). Amino-acyl tRNA synthetases consist of from 1 to 4 subunits (42). The reaction mechanisms of these enzymes are considered quite complex as three substrates (ATP, amino acid and tRNA) and three products (aminoacyl tRNA, pyrophosphate and AMP) are involved in the reaction. Multisubunit synthetases sometimes exhibit non-Michaelis-Menten kinetics due to allosteric and/or multiple active substrate binding sites on the enzyme complex (43). The binding of one substrate may affect the affinity of the synthetase for another (42,43).

EXPERIMENTAL PROCEDURES

Materials

RNA - Yeast unfractionated tRNA was purchased from Plenum Scientific Research Inc. (Hackensack, New Jersey). E. coli tRNA^{Val}₁ was a gift from Dr. Brian Reid of the University of California at Riverside (U.C.R.) and is considered to be 100% pure⁵. TYMV and TMV-RNAs were supplied by Dr. Paul Desjardins of U.C.R. as part of his collaboration in this research project. This viral RNA was extracted from whole virions with special precautions taken to avoid nuclease degradation of the RNA. E. coli unfractionated tRNA was obtained from Sigma (St. Louis, Missouri).

Purified Virus - The following pure viruses were donated by Dr. S. Tolin of this university: CMV, BBMV, southern bean mosaic virus (SBMV), peanut stunt virus (PSV) and alfalfa mosaic virus (AMV). White clover mosaic virus (WCMV) was a gift of Mr. John Patton of this university. Barley stripe mosaic virus (BSMV) was donated by Dr. Myron Brakke of the University of Nebraska.

Sources of Enzymes - The MRE-600 strain of E. coli was supplied by Dr. Barbara Bachmann of the E. coli Genetic

⁵ Personal communication from Dr. Brian Reid, U.C.R.

Stock Center at Yale University. Fleishman's (Standard Brands, Inc., New York, N.Y.) dry baker's yeast (Saccharomyces cerevisiae) was used as inoculum for yeast culture. Bean sprouts (Phaseolus vulgaris) were obtained from Jack Rabbit brand light red kidney beans (Wickes Corporation, Saginaw, Michigan). The post-mitochondrial rat liver supernatant fraction was a gift of Dr. C. J. Ackerman of this department.

Radiolabeled Amino Acids - ^{14}C valine, specific activity (S.A.) 225-280 Ci/mole, was obtained from ICN Pharmaceuticals (Irvine, California) or Amersham-Searle, Inc. (Arlington Heights, Illinois). ^3H valine, S.A. 31-37 Ci/mole, was obtained from Amersham-Searle, Inc. A ^{14}C mixed L-amino acid preparation, containing alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine in the proportions present in an algal hydrolysate, S.A. 50 Ci/mole for each amino acid, was purchased from ICN Pharmaceuticals. ^3H histidine, S.A. 11 Ci/mole, and ^3H tyrosine, S.A. 52 Ci/mole, were obtained from New England Nuclear (Boston, Massachusetts). A ^3H mixed L-amino acid preparation, including the same amino acids as the ^{14}C mixture listed above, was also pur-

chased from New England Nuclear. The specific activities of the amino acids in this mixture varied from 2 to 80 Ci/mole.

Growth Media - Bacto-tryptone and yeast extract were obtained from DIFCO Laboratories (Detroit, Michigan). Grandma's brand unsulphured molasses (Duffy-Mott Co. Inc., New York, N.Y.) was used in the yeast culture medium.

Chromatography Materials - Microcrystalline DEAE-cellulose (DE-52) and carboxymethyl (CM) cellulose (CM-23) were purchased from Whatman, Inc. (Maidstone, England). G-25 Sephadex was obtained from Pharmacia Inc. (Piscataway, New Jersey) and silicic acid (100 mesh) from Mallinckrodt Chemical Works (St. Louis, Missouri). Di-boryl amino ethyl cellulose (DBAE-cellulose) was purchased from Collaborative Research Inc. (Waltham, Massachusetts).

Gel Electrophoresis Reagents - Acrylamide, methylene bisacrylamide, N,N,N',N', tetramethylethylenediamine (TEMED) and agarose were obtained from Bio-Rad Laboratories (Richmond, California). Ultra-pure grade urea was purchased from Schwartz-Mann (Orangeburg, New York).

Miscellaneous - N-2-Hydroxymethyl piperazine-N-2-ethanesulfonic acid (HEPES) and Na₂ATP were obtained from Calbiochem (La Jolla, California). "Scintillar" grade 2,5-diphenyloxazole (PPO) and toluene were obtained from Mallin-

krodt. 1,4 bis 2-(5-phenyloxazolyl) -benzene (POPOP) was purchased from Sigma Chemical Co. (St. Louis, Missouri). Sequanal grade N-methyl morpholine was purchased from Pierce Chemical Co. Whatman 3 MM filter paper was used for the filter disc assays. All other reagents were purchased from Sigma or Baker Chemical Co. (Phillipsbury, New Jersey) and were of reagent grade or better.

Enzyme Assays

Standard Aminoacylation Assay Reaction Components - The standard aminoacylation assay was used to determine either the rate of an aminoacylation reaction or the maximum mole percent of aminoacylation. Variations of this assay were used for identifying which of 15 amino acids was being esterified to RNA, for assays of chromatography column effluents, and for tRNA nucleotidyl transferase assays.

The concentrations of amino acids in the standard assay reaction were: Valine-20 μM , histidine-4 μM , tyrosine-20 μM or 10 μM . The valine concentration exceeded the K_m for valine of the yeast (12 μM) (44) and bean (<5 μM)⁶ valine tRNA synthetases. The K_m of the E. coli valine tRNA synthetase for valine (120 μM) (45) was higher than the concentration

⁶ Crude determination by the author.

of valine used in the assay. The K_m of the bean tyrosine tRNA synthetase for tyrosine is about $20 \mu M^7$ and the K_m of the yeast histidine tRNA synthetase for histidine is about $2 \mu M$ (46). In general, the amino acid concentration was kept low enough to allow sensitive (high specific activity) economically feasible assays. Specific activities of the amino acids as used in the assays ranged from 50 to 280 Ci/mole for ^{14}C amino acids and from 0.5 to 16 Ci/mole for 3H amino acids.

The concentration of ATP in the assay was 4 mM which exceeds all known K_m s of aminoacyl tRNA synthetases for ATP (K_m s are approximately $10^{-4} M$) (47). Ten mM $MgCl_2$ was included in the assay to give a 2.5:1, Mg^{++} :ATP ratio which is optimum for the yeast valine tRNA synthetase (44). The reaction was buffered by 50 mM TRIS-Cl at pH 7.5 (25°). This buffer was found by the author to be superior to HEPES or phosphate for aminoacylation reactions catalyzed by yeast valine tRNA synthetase. The reaction mixture also contained 0.5 mM dithiothreitol (DTT). The RNA concentration in the assay varied from 0.005 to 100 μM , and enzyme preparations were added to a concentration of 0.0005 to 4 mg/ml. Enzyme was added in the solvent 30-50% glycerol, 10-15 mM TRIS-Cl,

⁷Crude determination by the author.

pH 7.5, and 1-3 mM 2-mercaptoethanol. This solution constituted one-sixth of the volume of the assay. All assay reagents were stored at -30°C .

Standard Aminoacylation Reaction Protocol - All reagents were thawed then kept on ice immediately prior to performing the assays. The reactions were carried out in 1 x 7.5 cm glass test tubes at a total volume of 60 μl . Amino acid, ATP, MgCl_2 , DTT and TRIS-Cl were added in 35 μl from a stock solution. The RNA was added in 15 μl of H_2O solution. To initiate the reaction, 10 μl of enzyme solution was added. This was immediately followed by vortex mixing and immersion of the tube in a 37°C water bath. Aliquots were pipetted at appropriate times onto 1/2 or 5/8 inch filter paper squares mounted on pins. The squares were then immersed in 10% trichloroacetic acid (TCA) at 0°C and washed with stirring once in 10% TCA for seven minutes, twice in 5% TCA for five minutes, once in ethanol for five minutes and once in diethyl ether for five minutes. The squares were dried and put in scintillation vials containing 5 ml of 0.5% PPO and 0.01% POPOP in toluene.

The assay vials were counted in a Beckman LS-133 scintillation spectrometer for 20-100 minutes. The background level was about 30 counts per minute (c.p.m.). The fluor was replaced when background rose above 40 c.p.m. Experi-

mental background levels were determined by substituting rRNA for viral RNA or tRNA. The efficiency of counting was determined by fully aminoacylating a known quantity of E. coli tRNA ^{14}C Val₁, with valine of known specific activity, measuring the c.p.m. esterified and dividing the c.p.m. by the theoretical decompositions per min value. The more direct method of determining the specific activity by applying labeled amino acid directly to the paper squares and then determining the c.p.m. proved unreliable. The efficiency of counting was 16 percent for ^3H and 66 percent for ^{14}C .

Mole Percent of Aminoacylation - For the determination of the maximum mole percent aminoacylation, additional details were observed. Sufficient RNA was added to yield 500 or more c.p.m. when fully aminoacylated. A sufficient quantity of enzyme was added to drive the reaction to within 90 percent of completion in five minutes. After 10 to 15 minutes of incubation, 50 μl of reaction mixture was pipetted onto a 5/8" paper square, washed and counted.

The procedure for the ^{14}C mixed amino acid assay was the same except that 15 amino acids were added. The purpose of this assay was to determine the levels of RNA aminoacylation and the identities of the amino acids esterified. Each amino acid was present at 50 Ci/mole and at the following concentrations:

<u>L-Amino Acid</u>	<u>μM</u>	<u>Amino Acid</u>	<u>μM</u>
Gly	3.3	Phe	6.7
Ala	6.7	Tyr	3.3
Ser	3.3	Asp	6.7
Thr	4.2	Glu	10.4
Pro	4.2	Lys	5.0
Val	6.7	Arg	5.8
Leu	11.7	His	1.25
Ile	4.2		

To determine the identity of the esterified amino acid, 1 μl of a 100 mM solution of a selected ¹²C amino acid was added to the assay, thus reducing the S.A. of that amino acid at least 100 fold.

A different procedure was used for the more sensitive ³H mixed amino acid assay. The commercial ³H amino acid mixture was diluted with ¹H amino acids to a uniform specific activity of either 0.2 or 1 Ci/mmmole. ATP, MgCl₂, DTT and TRIS-Cl, at standard assay concentrations, were added with the amino acids in 10 μl per assay. The concentrations of amino acids in the reaction were:

<u>L-Amino Acid</u>	<u>μM</u>	<u>L-Amino Acid</u>	<u>μM</u>
Ala	16	Lys	12
Arg	14	Phe	16
Asp	16	Pro	10
Glu	25	Ser	8
Gly	8	Thr	10
His	3	Tyr	8
Ile	10	Val	16
Leu	28		

RNA and enzyme were added separately in 5 or 10 μl to give a final assay volume of 25 μl. After 10 or 15 minutes incuba-

tion at 37°C, 20 µl of reaction mixture was placed on a 1/2" paper square which was then washed and counted. The small total volume of this assay allowed the use of higher concentrations of amino acids without compromising the high specific activity. The 1 Ci/mmole ³H mixed amino acid assay can reliably detect the aminoacylation of as little as one pmole of RNA.

Rate Determinations - For precise rate determinations, the following details were observed. The increase in aminoacylated RNA between 0.5 and 1.5 minutes after reaction initiation was measured by assaying 25 µl aliquots. The short time regimen minimized the possibility of thermal degradation of ATP and enzyme, while 0.5 minute was sufficient time to allow the reaction mixture to come to 37°C. The reactions were designed not to exceed 40% of completion at 1.5 minutes in order to minimize non-linearity of the rate.

tRNA nucleotidyltransferase (NT) was assayed by coupling the adenosylation of native TYMV-RNA with its subsequent aminoacylation. The conditions and procedure for this assay were the same as those for the standard aminoacylation rate assay with two exceptions. A three-fold excess of valine tRNA synthetase was added to the reaction mixture prior to adding the NT. The reaction mixture was preincubated at 37°C for 1 minute prior to adding

the NT to allow any TYMV-RNA possessing a 3' terminal AMP residue to be aminoacylated. Under these conditions, further aminoacylation of the TYMV-RNA is dependent on added tRNA nucleotidyltransferase.

Assays of chromatography column effluents were carried out in 25 μ l, which included 5 or 10 μ l of column effluent. Standard assay reagent concentrations were employed. High RNA concentrations and short incubation times were used so that the level of aminoacylation attained in the reaction was usually proportional to the rate of aminoacylation.

Electrophoresis

Agarose-Urea Gel Electrophoresis of RNA - To determine the size distribution of viral RNAs, electrophoresis was carried out on denaturing agarose-urea gels. The gels were prepared and run essentially as described by Woo et al. (48). A mixture containing 6 M urea, 2% agarose and 25 mM Na citrate, pH 3.5, was refluxed until the pH reached approximately 7.0 and poured into standard gel tubes to a depth of 6 cm. The tubes were placed in a gel electrophoresis tank containing 25 mM Na citrate, pH 3.5, in the upper and lower reservoirs. Two to 80 μ g of RNA sample in 6 M urea, 15% sucrose and 20 mM Na citrate, pH 3.5, were heated five minutes at 60°C and layered on each gel. Electrophoresis was carried out for two to four hours at 2.5 m

amps per gel. The gels were stained with 0.2% O-toluidine blue in 10% acetic acid and destained by diffusion.

Protein polyacrylamide gel electrophoresis was performed as described by Davis et al. (49). The gels were prepared by Joyce Foster of this department.

Enzyme Preparation

General Procedures - This section contains a description of standard procedures used in the preparation of several enzymes (Fig. 1).

Protein content of solutions was determined from the 280 nm absorbance (A_{280}) using an $E_{280}^{0.1\%}$ of 1.0. If nucleic acid was also present in the solution, concentrations were determined as described by Warburg and Christian (50). Ultraviolet absorption spectra of protein solutions were performed on a Beckman Acta III spectrophotometer.

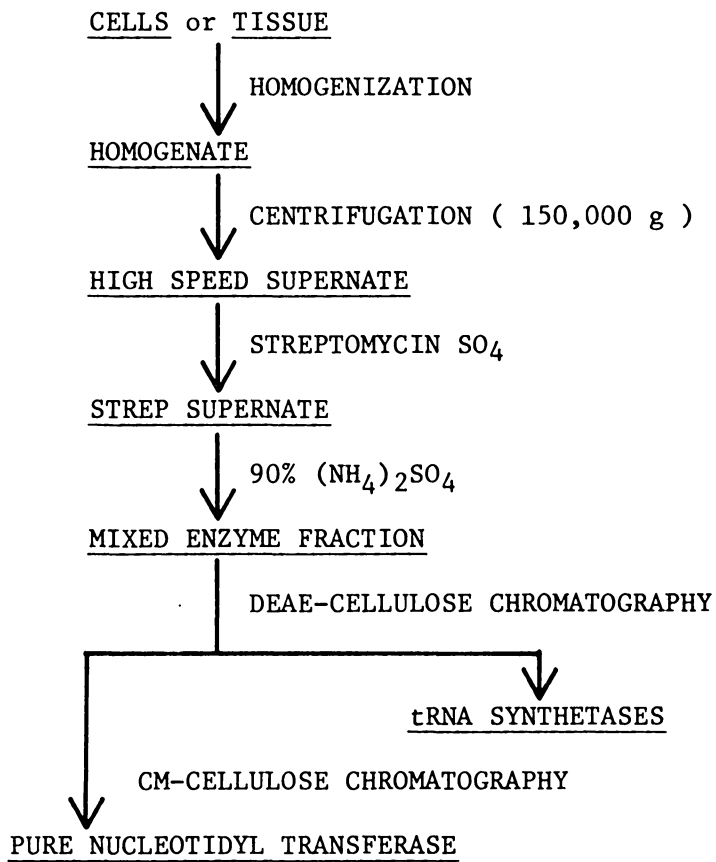
Homogenates of cells or tissues were subjected to low and high speed centrifugations to obtain a soluble high speed supernatant enzyme fraction. Low speed centrifugation was performed in a Sorvall SS-34 rotor at 12,000 x g for 15 min. The supernatant was centrifuged at 150,000 x g for 1 hr in a Beckman TY-65 rotor. The pellet was discarded or used as a source of rRNA. Three to five mM $MgCl_2$ was present in the homogenate to facilitate pelleting of the ribosomes.

The RNA in the enzyme preparations (high speed super-

FIGURE 1

General Enzyme Preparation Scheme

GENERAL ENZYME PREPARATION SCHEME



natant) was precipitated out with streptomycin SO_4 by adding one-sixth volume of 10% streptomycin SO_4 dropwise over 15 min at 0°C . The solution was stirred for 10 min and centrifuged for 10 min at $12,000 \times g$ in a Sorvall SS-34 rotor. The pellet was discarded and the supernatant was dialyzed for three hours at 0° against 20 mM TRIS-Cl, pH 7.5, with 3 mM 2-mercaptoethanol. An additional precipitate which formed during dialysis was pelleted out as described above. Enzyme fractions treated in this way contained less than 1% nucleic acid.

Ammonium-sulfate precipitation of proteins was performed by adding fine granular $(\text{NH}_4)_2\text{SO}_4$ over 15 min, with stirring, to 85 or 90% saturation (at 0°C). The proteins were pelleted by centrifugation at $27,000 \times g$ for 20 min in a Sorvall SS-34 rotor and frozen at -30°C until needed. Before further purification or use in assays, the pellets were dissolved in a small volume of 10 mM TRIS-Cl, (pH 7.5 at 25°C), 3 mM 2-mercaptoethanol, or the appropriate chromatography buffer, and dialyzed against the same buffer for four to six hours.

Further purification of the enzymes was accomplished by DEAE-cellulose chromatography. A 2.5 cm diameter column was packed to a height of 35 cm in a starting buffer containing 10 or 20 mM TRIS-Cl (pH 7.5, 25°), and 2 mM 2-mercaptoethanol. The sample, 200 to 600 mg of protein, was

dissolved in 20 to 70 ml of the starting buffer and loaded onto the column. After washing the column with starting buffer, the column was eluted with a 0.6 to 1.1 linear KCl concentration gradient. The gradient began at 0-50 mM and ended at 250-500 mM KCl. The column was run at 1-2° with a flow rate of 2-3.3 ml/min and 5 to 8 ml fractions were collected. The A₂₈₀ of all fractions was determined and ultraviolet absorption spectra were taken of some fractions to monitor the purity of the proteins. In most cases, 15 to 26 of the fractions were assayed for aminoacyl tRNA synthetase activity as described previously. DEAE-cellulose chromatography of crude aminoacyl tRNA synthetase preparations served three purposes: to remove any traces of tRNA not eliminated by the streptomycin SO₄ treatment; to diminish the possibility that enzymes, such as nucleases, which degrade aminoacyl tRNA synthetase substrates might be present in the reaction mixture; and to allow the concentration of high levels of aminoacyl tRNA synthetase activity into small volumes.

All enzyme preparations were stored unfrozen at -30° in a 30-50% glycerol solution as noted on page 19. This eliminated enzyme degradation due to repetitive freezing and thawing. Many of the enzyme preparations were dialyzed against a buffered solution of 60-70% glycerol for 6-10

hours at 0°C. This produced about a three-fold concentration in addition to introducing sufficient glycerol to prevent freezing.

Preparation of E coli Enzymes - E. coli cells were grown to late log phase (absorbance at 600 nm of 1.5) in six liters of the following medium: 10 g/l Bacto-Tryptone, 1 g/l yeast extract, 2.5 g/l K₂HPO₄, pH 7, 12 g/l glucose, 0.16 g/l Na citrate, 0.1 g/l MgCl₂, 1 mg/l FeSO₄·7H₂O, 0.3 mg/l MnSO₄·H₂O, 0.4 mg/l CuSO₄·5H₂O, 0.4 mg/l ZnSO₄·7H₂O. The medium plus 200 ml late log phase inoculum was incubated at 37° with aeration and stirring in a Virtis 40-100 fermentor for 1.5-2.0 hours. The culture was cooled to 0°C with ice and cells were pelleted in a Sharples T-1 centrifuge. The cell pellet was stored at -70°C.

To prepare an E. coli homogenate, 10 g of cells were suspended in 20 ml of 10 mM TRIS-Cl (pH 7.5 at 25°C), 5 mM MgCl₂, 1 mM EDTA and 1.5 mM 2-mercaptoethanol. The suspension was sonicated on ice at 180 watts in four 2 min bursts with a Branson W185 sonifier. The sonicate was allowed to cool for 2 minutes between bursts.

The sonicate was centrifuged and streptomycin SO₄ treated as described previously and chromatographed on DEAE-cellulose. The aminoacyl tRNA synthetase fraction consisted of proteins eluting from the column between 0.1 and 0.25 M

KCl (2). This fraction was concentrated by 90% $(\text{NH}_4)_2\text{SO}_4$ precipitation and stored in 50% glycerol at -30°C .

Preparation of Yeast Enzymes - Yeast cells were grown to late log phase (absorbance at 600 nm of 1.25) in the following medium: 10 g/l molasses, 2 g/l K_2HPO_4 , 2 g/l acetic acid, 2 g/l Bactotryptone, 0.2 g/l yeast extract, 2 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.2 g/l MgCl_2 , 1 mg/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mg/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.4 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.4 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Eight liters of medium were incubated with 500 ml of yeast inoculum (absorbance at 600 nm of 1.25) at 30°C with aeration and stirring in the Virtis fermentor for 5.5 hours. A pH of 4.5 was maintained by the addition of 10 N NaOH. The culture was then cooled to 0°C with ice and the cells were pelleted in the Sharples centrifuge. The pellet was stored at -70°C .

To prepare a homogenate one volume of thawed yeast cell pellet was suspended in two volumes of E. coli sonication buffer. The cells were broken by two passes through a French pressure cell (Amer. Inst. Co. 5-598A) operating at 20,000 p.s.i. After the first pass, one hundredth volume of 0.1 M phenylmethyl-sulphonyl fluoride (PMSF) in n-propanol was mixed into the homogenate. PMSF is an irreversible specific inhibitor of serine proteases (52). The soluble enzyme fraction was obtained by centrifugation.

tRNA nucleotidyltransferase (NT) was obtained from the high speed supernatant with the following procedure (51). Fractional $(\text{NH}_4)_2\text{SO}_4$ precipitation of the supernatant was performed at 47.5% and 65% saturation (at 0°C). The proteins precipitating at 65% $(\text{NH}_4)_2\text{SO}_4$ were dialyzed and streptomycin SO_4 treated as described above. The soluble proteins were fractionated on DEAE-cellulose equilibrated with 20 mM TRIS-Cl (pH 7.5 at 25°C). All fractions containing protein not retained (breakthrough peak) were pooled and chromatographed on CM-cellulose (Fig. 2). Effluent fractions were assayed for NT and active fractions were pooled and concentrated by glycerol. All of the above steps were carried out at $0-4^\circ$.

Valine and histidine tRNA synthetases (VTS and HTS, respectively) were partially purified from the yeast high speed supernate. Initial purification was accomplished by streptomycin SO_4 treatment followed by 85% $(\text{NH}_4)_2\text{SO}_4$ precipitation. The VTS and HTS were then purified by DEAE-cellulose chromatography (Fig. 3). VTS and HTS fractions were pooled and concentrated by glycerol. All of the above purification steps were carried out at $0-4^\circ$.

Preparation of Bean Enzymes - Dry beans were washed in 1% NaClO for 1 minute, rinsed several times with distilled

FIGURE 2

Elution Profile of NT from CM-Cellulose

Proteins were eluted (flow rate = 0.5 ml/min) from the column (0.8 x 26 cm) with a 0 to 100 mM KCl gradient. The conductivity, A_{280} , and NT activity of the effluent was monitored.

○—○ NT activity (^{14}C val c.p.m.), □—□ conductivity.

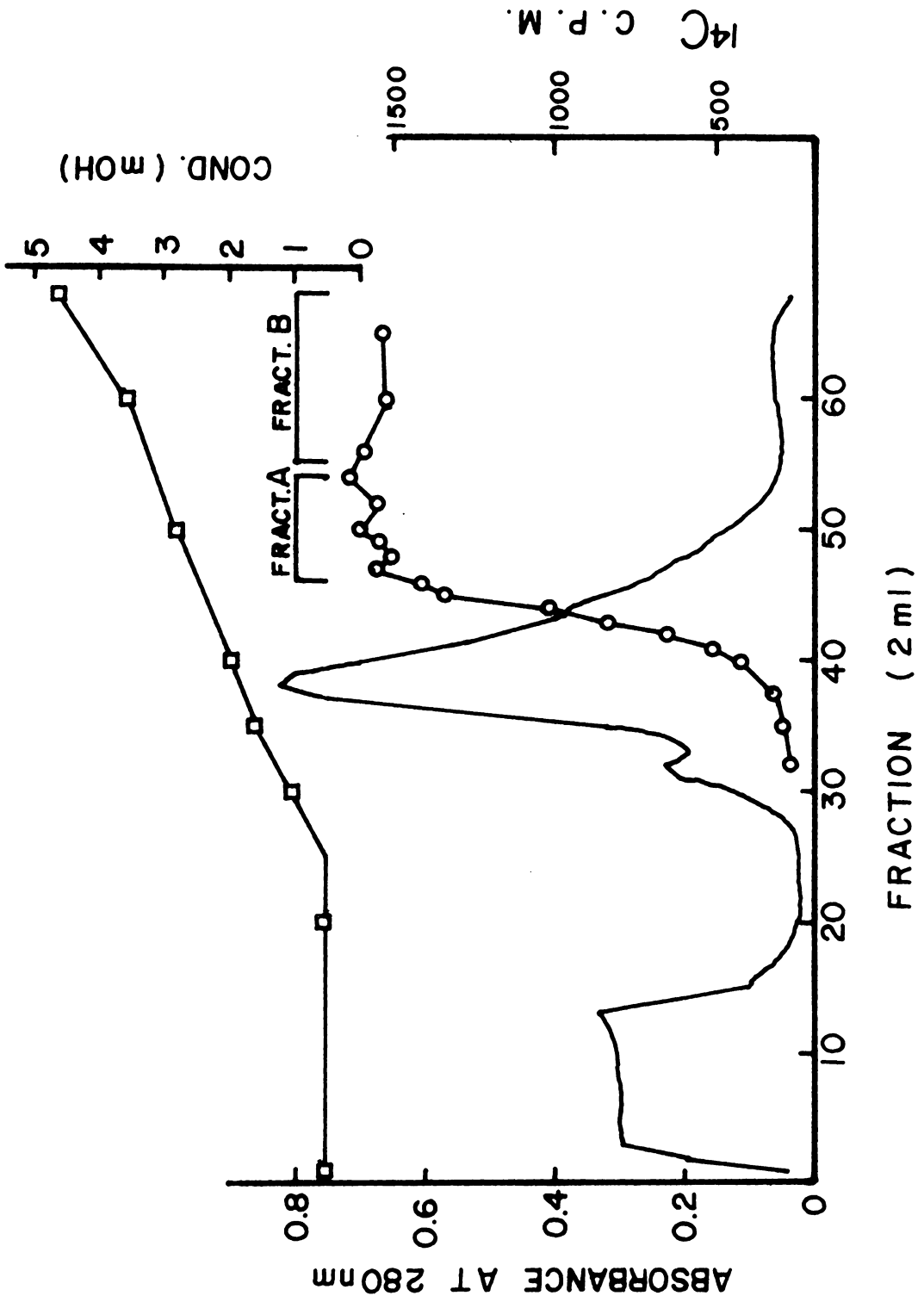
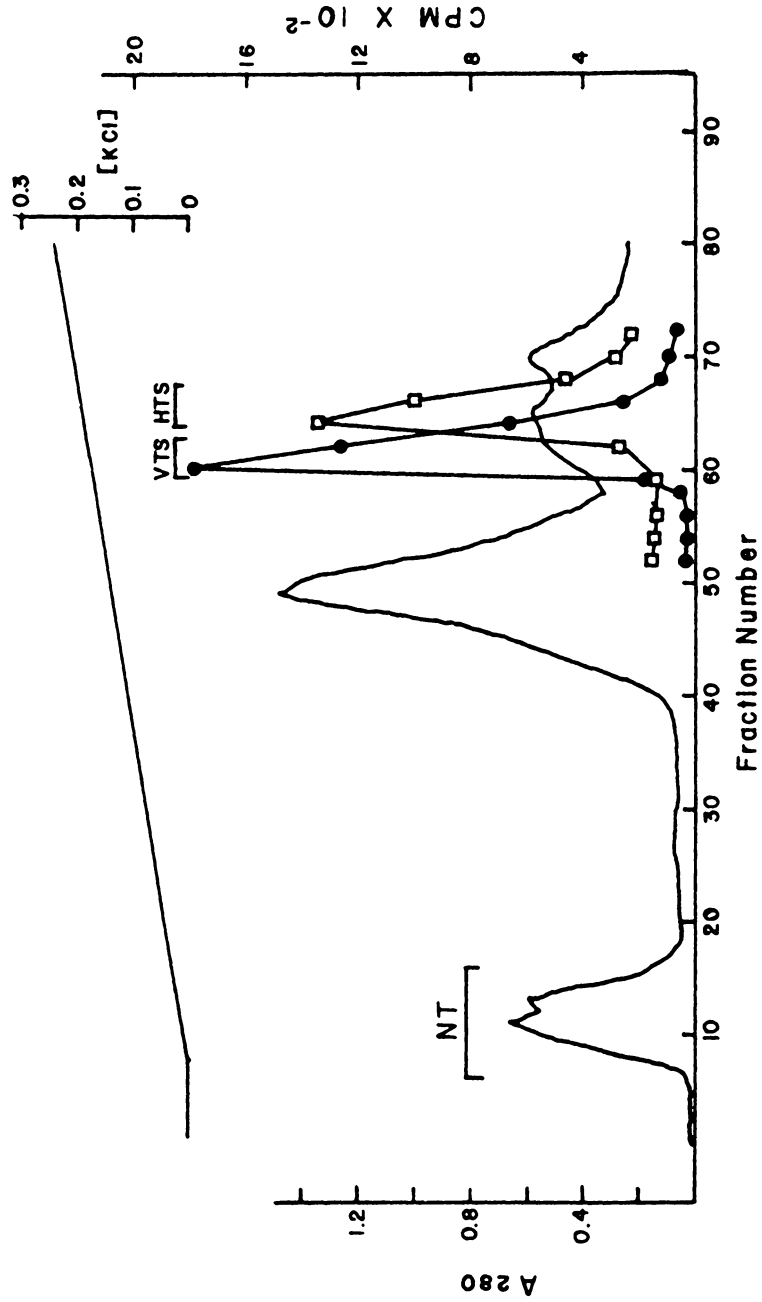


FIGURE 3

Elution Profile of Yeast VTS and HTS

from DEAE-Cellulose

Proteins were eluted from the column with 0 to 250 mM KCl gradient in 20 mM TRIS-Cl (pH 7.5 at 20°). VTS activity, HTS activity (double label assay) and A₂₈₀ of the effluent were monitored. ●—● VTS activity (¹⁴C Val c.p.m.), □—□ HTS activity (³H His c.p.m.).



H₂O and then left in distilled water overnight in a wide-mouth jar closed with two layers of cheese cloth. The water was poured off and the beans were rinsed twice daily with distilled water for five days. They were then spread on wet paper towels under clear plastic for one or two days. Unsprouted or molded beans were discarded. When the primary leaves were green but unfolded (6 to 8 days old) maximum levels of VTS activity were present⁸. Cotyledons and seedcoats of harvested beans were discarded and the remaining tissue rinsed in distilled water at 0° to 4°C (53).

Grinding buffer contained 50 mM TRIS-Cl, pH 7.5 (at 25°C), 10 mM MgCl₂, 9 mM 2-mercaptoethanol and 10% glycerol and was used at 1.5 ml per gm of tissue. The sprouts were minced and then ground with cold buffer for 15 seconds at half speed in a Sorvall omni-mixer in the 80 ml cup. 1 gm

⁸Low speed supernatants were prepared from the bean sprouts and were assayed for VTS activity after dialysis against 20 mM TRIS-Cl, (pH 7.5 at 25°), 3 mM 2-mercaptoethanol for 45 minutes.

<u>Day of Harvest</u>	<u>VTS S.A. (units/mg protein)</u>	<u>Leaf Color</u>	<u>Leaf State</u>
4	0.034	yellow	folded
5	0.045	yellow	folded
6	0.100	green	folded
7	0.066	green	unfolded

polyvinylpyrrolidone per 10 g of tissue and 0.1 ml of 0.1 M PMSF in n-propanol per 10 ml of buffer were added to the homogenate to reduce oxidized polyphenol and protease levels which otherwise destroyed VTS activity (54). Grinding was continued for 30 seconds at full speed. The homogenate was squeezed through four layers of medium cheese cloth and clarified by low and high speed centrifugation as described above. The supernatant was treated with streptomycin SO₄ and the proteins were precipitated with 90% (NH₄)₂SO₄. A VTS fraction was purified from this mixed enzyme fraction by DEAE-cellulose chromatography. The VTS eluted from the column at 0.2 M KCl in 20 mM TRIS, 3 mM 2-mercaptoethanol, and 5% glycerol.

Rat Liver Enzyme Preparation - The rat liver post-mitochondrial supernatant was received in a 10 mM TRIS-PO₄, pH 7.4, 0.05 mM EDTA, 0.25 M sucrose buffer. The supernatant was made 3 mM in 2-mercaptoethanol and 1 mM in PMSF then subjected to high speed centrifugation. The supernatant was treated with streptomycin SO₄ and the proteins were pelleted with 90% (NH₄)₂SO₄. The pellet was resuspended and dialyzed against 10 mM TRIS-Cl, pH 7.5 (at 25°), plus 3 mM 2-mercaptoethanol, and glycerol was added to 30%. No further purifications of this mixed enzyme fraction were performed.

RNA Preparation

Spectral Methods - Ultraviolet spectra were taken of all RNA samples on an Acta III spectrophotometer (Beckman). The RNA was considered to be protein-free if the A_{260}/A_{280} ratio exceeded 1.75 and the absorption maximum was 256 to 258 m μ . The extinction coefficients for the RNAs used are as follows: E. coli tRNA^{Val}₁, 1.6 nmole⁹ per A_{260} unit¹⁰; all other tRNA, 1.8 nmole per A_{260} unit (55); TYMV-RNA, 23.3 A_{260} unit per mg (56); all other RNA, 20 A_{260} unit per mg.

Phenol Extraction of RNA - The phenol mixture used to extract RNA from protein consisted of 89.5% phenol, 10% n-cresol and 0.5% 8-hydroxyquinoline (57). To prevent degradation of the RNA by nucleases all glassware used for the extraction and subsequent handling of the RNA was heated to 180° for several hours. Diethyl pyrocarbonate (DEPC), an alkylating agent and nuclease inhibitor, was added at 10 to 20 μ l per ml of crude RNA sample before extraction (58). RNA was extracted twice with the phenol mixture, once or twice with a 1:1 mixture of phenol mixture and chloroform containing 1% isoamyl alcohol, once with chloroform plus

⁹Personal communication from Brian Reid, U.C.R.

¹⁰An A_{260} unit is the quantity of RNA which yields an A_{260} of one when dissolved in 1 ml of solution.

1% isoamyl alcohol and five times with diethyl ether. Extraction involved emulsification of the aqueous RNA solution with an equal volume of organic phase followed by centrifugation (if necessary) to separate the phases and removal of the RNA solution for further extraction. Residual ether was removed by evaporation with a stream of air at room temperature. 0.1 volume 1 M KCl and 2 volumes of ethanol were added to precipitate the RNA from solution. The mixture was allowed to stand overnight at -30° before the precipitate was recovered by centrifugation (10 min at $8,000 \times g$ with Sorvall SS-34 rotor).

For the extraction of RNA from SBMV, BSMV, PSV and AMV, a modified procedure was used. These RNAs were mixed with two volumes of the following buffer: 0.1 M Na acetate, pH 5.0, 0.2% sodium dodecyl sulfate (SDS), and 0.2% DEPC. For SBMV and BSMV extraction 1 mM EDTA was also present in the buffer. The RNA was extracted as above except that a volume of chloroform was mixed into the phenolic emulsion prior to separation of the RNA solution from the organic phase. KCl was not added prior to ethanol precipitation. To remove from extracted RNA any residual Na acetate which might lower the pH of the assays, the ethanol pellets were washed once in a solution of 3.3 mM TRIS-Cl or HEPES, pH 7.5

(at 25⁰), 33 mM KCl and 66% ethanol. The pellets were then air dried and dissolved in distilled water. The use of EDTA in the extraction buffer resulted in a 2 1/2 fold higher yield of RNA from SBMV. To maximize RNA yield, the organic phases of all RNA extractions were extracted a second time with water or buffer. First and second extractions were combined before ethanol precipitation.

Adenosylation of TYMV-RNA - TYMV-RNA used in kinetic experiments was adenosylated or pre-repaired as outlined below. The reaction had 50 mM TRIS-Cl, pH 7.5 at 25⁰C, 0.5 mM DTT, 10 mM MgCl₂, 4 mM ATP, 15% glycerol (by volume), 3.6 mg/ml native TYMV-RNA and 0.063 mg/ml yeast NT in a total volume of 2 ml. The mixture was incubated 4 minutes at 37⁰, and the reaction was terminated by adding 10 µl of DEPC, vortexing, and cooling on ice. The RNA was purified from the reaction mixture by phenol extraction followed by ethanol precipitation, resolution and chromatography on G-25 Sephadex to remove residual adenosine phosphates and salts. The elution of adenosine phosphates (ATP, ADP and AMP) from the G-25 column was monitored by their characteristic ultraviolet spectrum ($A_{260}/280 = 5$). Alternatively, the RNA was repurified on a silicic acid G-25 column (3). Two cm of silicic acid were layered on top of a 40 cm tall G-25 column

(1.5 cm diameter). The column was equilibrated with 0.1 M Na acetate, pH 4.5. Before loading the RNA solution onto the column, 4.5 ml of 0.1 M Na acetate pH 6.4, 0.2 M NaCl were loaded on the column. RNA was then loaded and washed in with 0.5 ml of the same buffer and eluted with 0.1 M Na acetate pH 4.5. Under these conditions, proteins are bound to the silicic acid while RNA passes through. It was necessary to warm the column to 40-50° to elute the TYMV-RNA as it appeared to form a gel in the G-25. The RNA was precipitated with ethanol and redissolved as described previously.

16 S plus 23 S E. coli rRNA was extracted from E. coli ribosomal pellets by the above phenol method. The rRNA was purified by velocity sedimentation on 5-20% sucrose density gradients. The sucrose was buffered with 0.15 M NaCl, 15 mM Na citrate pH 7.0 and 10 mM EDTA and the gradients were run 11 hrs at 25,000 rpm in a Beckman SW27 rotor at 20°C in the Spinco L2-65B centrifuge.

Preparation of Yeast tRNA Val - A limited quantity of yeast tRNA Val was purified by DBAE-cellulose chromatography according to the methods of McCutchan et al. (59). Under the proper conditions, DBAE-cellulose will specifically bind tRNA possessing a free 3' OH group as a result of the affinity of vicinal hydroxyl groups for the borate anion.

Aminoacyl tRNA will not bind to the column.

Prior to use, free amino groups on the DBAE-cellulose were acetylated with acetic anhydride as described by McCutchan et al. (59). The DBAE-cellulose column used had a bed volume of 4 ml and a height of 10 cm. As a preliminary step, un-aminoacylated mixed tRNA was chromatographed on the column to remove any tRNA which normally would not bind to the column. The tRNA which bound to the DBAE-cellulose was subsequently aminoacylated in the following 0.85 ml reaction mixture: 50 mM TRIS-Cl, pH 7.5 (at 25°), 10 mM MgCl₂, 4 mM ATP, 20 μm ³H valine S.A. 100 Ci/mole, 0.22 mg/ml yeast VTS, 1.3 mg/ml yeast mixed tRNA. The RNA was repurified from the reaction mixtures by G-25 silicic acid chromatography as described previously.

This tRNA was fractionated on the DBAE-cellulose column under tRNA binding conditions. RNA not bound to the column (enriched in valyl-tRNA) was concentrated by ethanol precipitation and resolution. Valyl-tRNA was further purified by two more passes on the column as above followed by deaminoacylation and rechromatography on the column (tRNA^{Val} retained). The yield of valyl-tRNA was about 50% per cycle. This procedure yielded 8 μg of 60% pure yeast tRNA^{Val} from 2 mg of unfractionated tRNA (6% tRNA^{Val}). The relatively

low yield and incomplete purity of the tRNA^{Val} may be due to the dubious quality of the DBAE-cellulose. The results presented here are similar to those obtained by the manufacturers of this product (DBAE-cellulose lot no. 733-33).

Kinetic Analysis

V_{max} and K_m Determination - The aminoacylation rate assay described previously was used to determine Michaelis-Menten kinetic parameters by observing the effect of RNA concentration on the aminoacylation rate. The active concentrations of RNA in the assays were determined as follows: the mole percent aminoacylation of the RNA at the end of the measured reaction was divided by two; the quotient was subtracted from the maximum mole percent of aminoacylation of the particular RNA; the fractional form of this difference was multiplied by the initial molar concentration of RNA as determined from its optical density and the molar extinction coefficient. The final value obtained is a good approximation of the average concentration of RNA substrate during the reaction.

Each kinetic experiment usually consisted of rates determined in duplicate at four different RNA concentrations, at least one of which was less than the observed K_m. The inverse of each mean and the standard deviation was calcu-

lated for the rate of each RNA concentration and plotted against the inverse of RNA substrate concentration (Lineweaver-Burk plot). The slope and intercepts of the Lineweaver-Burk plots were determined by least squares fit with the concentrations weighted 4:3:2:1 highest to lowest, respectively. These calculations were performed with a SR-51 II (Texas Instruments) calculator. In some of the kinetic determinations, as noted in the results, the reaction mixtures were incubated for 1 minute at 37° and cooled prior to adding the enzyme. This treatment increased the rate at RNA concentrations below 100 nM to allow linear Lineweaver-Burk plots. The function of the pre-incubation in the reaction is unknown; this effect was not observed at higher RNA concentrations.

RESULTS

Enzyme Preparations

E. coli mixed enzyme fraction (the 0.1 to 0.25 M KCl DEAE-cellulose fraction) comprised 60% of the total proteins in the E. coli soluble enzyme fraction and contained NT activity and a mixture of aminoacyl tRNA synthetase activities. The VTS specific activity in the preparation was 11.5 units¹¹ per mg protein with E. coli tRNA^{Val}₁ as the RNA substrate. In the ³H mixed amino acid assay this fraction catalyzed the aminoacylation of 44 mole% of unfractionated E. coli tRNA. As only 5% of the aminoacylation was with valine this result indicates the presence of many aminoacyl tRNA synthetase activities in the preparation. The E. coli mixed enzyme fraction retained about 50% of its original VTS activity after 1.5 years. This fraction also was able to catalyze the adenosylation (repair) of TYMV-RNA (assay concentration - 0.8 μ M) at a rate that indicated the presence of 0.04 units of NT per mg of protein.

Yeast Enzymes - The yeast VTS DEAE-cellulose fraction (Fig. 3) had an initial specific activity of 8 units/mg protein using unfractionated yeast tRNA (assay concentration - 2.5 mM) as the substrate. This enzyme had a storage half-

¹¹ nmoles of amino acid esterified per minute.

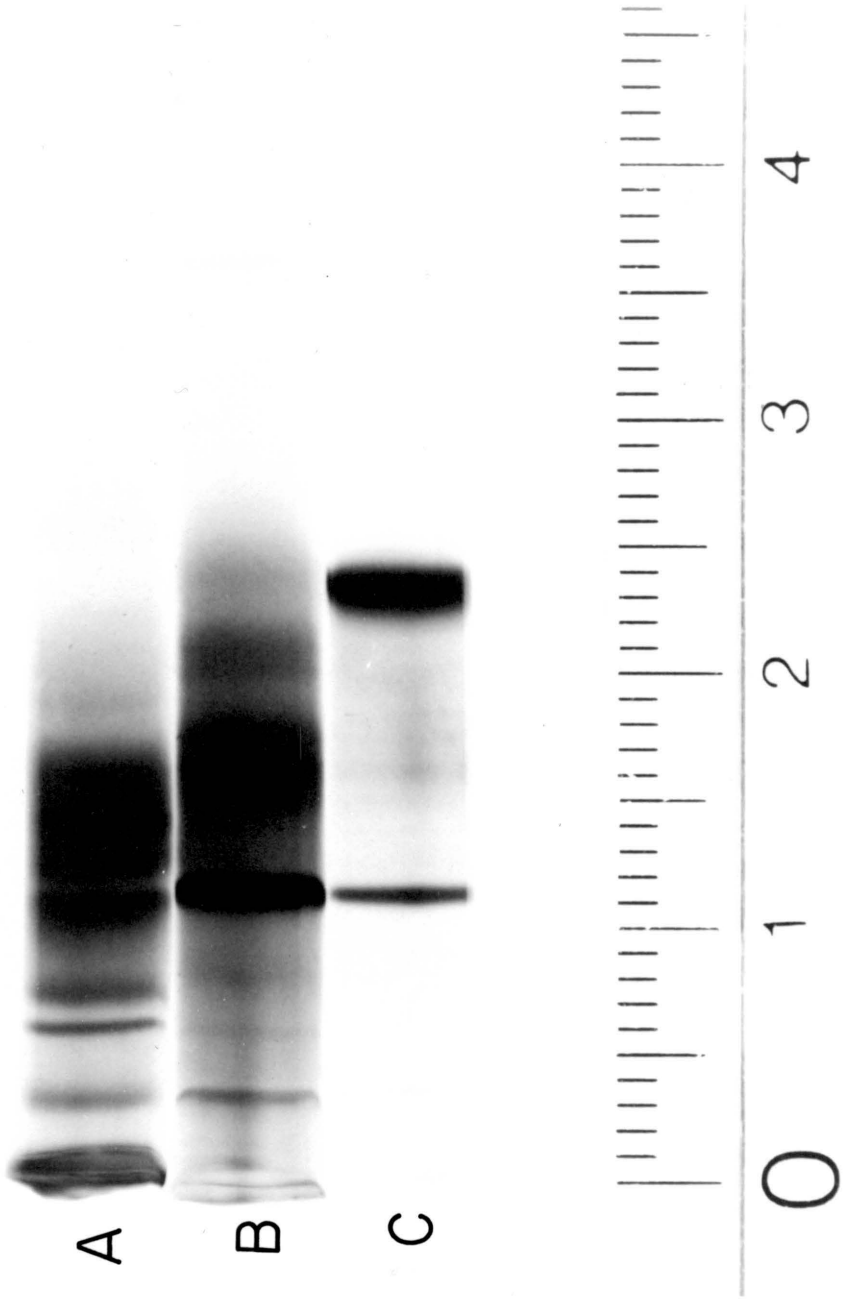
life of about three months. Gel electrophoresis of yeast VTS showed that most of the contaminating proteins were removed by DEAE-cellulose chromatography (Fig. 4). The yeast HTS DEAE-cellulose fraction (Fig. 3) had a specific activity of 1.2 units/mg protein when using TMV-RNA (assay concentration - 1.5 μ M) as the substrate.

The yeast mixed enzyme fraction was used in experiments requiring the presence of all aminoacyl tRNA synthetase activities. This fraction contained a VTS specific activity of about 1 unit per mg of protein and a NT specific activity of about 0.2 units per mg of protein. In either the ^3H or the ^{14}C mixed amino acid assay the yeast mixed enzyme fraction catalyzed the aminoacylation of 14 to 18 mole% of unfractionated yeast tRNA. There is no obvious explanation for this low level of aminoacylation. Part of this deficit is due to the absence of glutamine, asparagine, tryptophan, cysteine and methionine from the reaction mixture. It is unlikely, however, that tRNAs specific for these amino acids constitute a large part of the unfractionated tRNA. Other possible explanations are: the tRNA preparation contains inactive RNA; some of the aminoacyl tRNA synthetase activities are not present in the mixed enzyme fraction; or, the conditions of the mixed amino acid assay do not allow the expression of some of the

FIGURE 4

Purification of Yeast VTS

Polyacrylamide gels of yeast proteins and bovine serum albumin (BSA). A, yeast mixed enzyme fraction (50 μ g). B, yeast VTS DEAE cellulose fraction (50 μ g). C, yeast VTS DEAE cellulose fraction (10 μ g) plus BSA (5 μ g). Electrophoresis is from left to right.



aminoacyl tRNA synthetase activities. The deficiency is not due to slow aminoacylation since plateau levels of aminoacylation were observed.

The ability of yeast mixed enzyme fraction to esterify three selected amino acids to RNA was tested¹². The mixed assay system was reasonably efficient in esterifying all three amino acids as compared to efficiency of the standard aminoacylation assays. These limited data indicate that the assay conditions used are versatile and that the yeast mixed enzyme fraction contains a variety of aminoacyl tRNA synthetases. Whether other untested synthetase activities are absent is not known.

¹²Aminoacylation mole percents were determined from standard and from mixed amino acid assays:

<u>RNA</u>	<u>Amino Acid</u>	<u>Mole % Aminoacylation</u>	
		<u>Standard Assay</u>	<u>Mixed Assay</u>
yeast tRNA	¹⁴ C valine	6.5	5
yeast tRNA	¹⁴ C tyrosine	1.8	1.8
TMV-RNA	¹⁴ C histidine	40	58
TYMV-RNA	³ H valine	87	48

It was important to fully purify the yeast NT, since contaminating nucleases would have degraded TYMV-RNA during preparative 3' adenosylation. The NT purified from yeast had a specific activity of 53 units/mg protein when assayed with 0.65 μ M TYMV-RNA as the substrate. This represents about a 200-fold purification following the two chromatographic steps. NT fraction A from the CM-cellulose column (Figure 2) appeared nearly pure when examined by gel electrophoresis (Figure 5). This pure NT was tested for contaminating nucleases by incubating it with TYMV-RNA and rRNA under the conditions used for repairing TYMV-RNA. Incubated RNA was analyzed on agarose-urea gels, and no significant evidence of nucleolytic hydrolysis was found (Figure 6). The same result was obtained when yeast VTS was tested in this way using rRNA as the substrate.

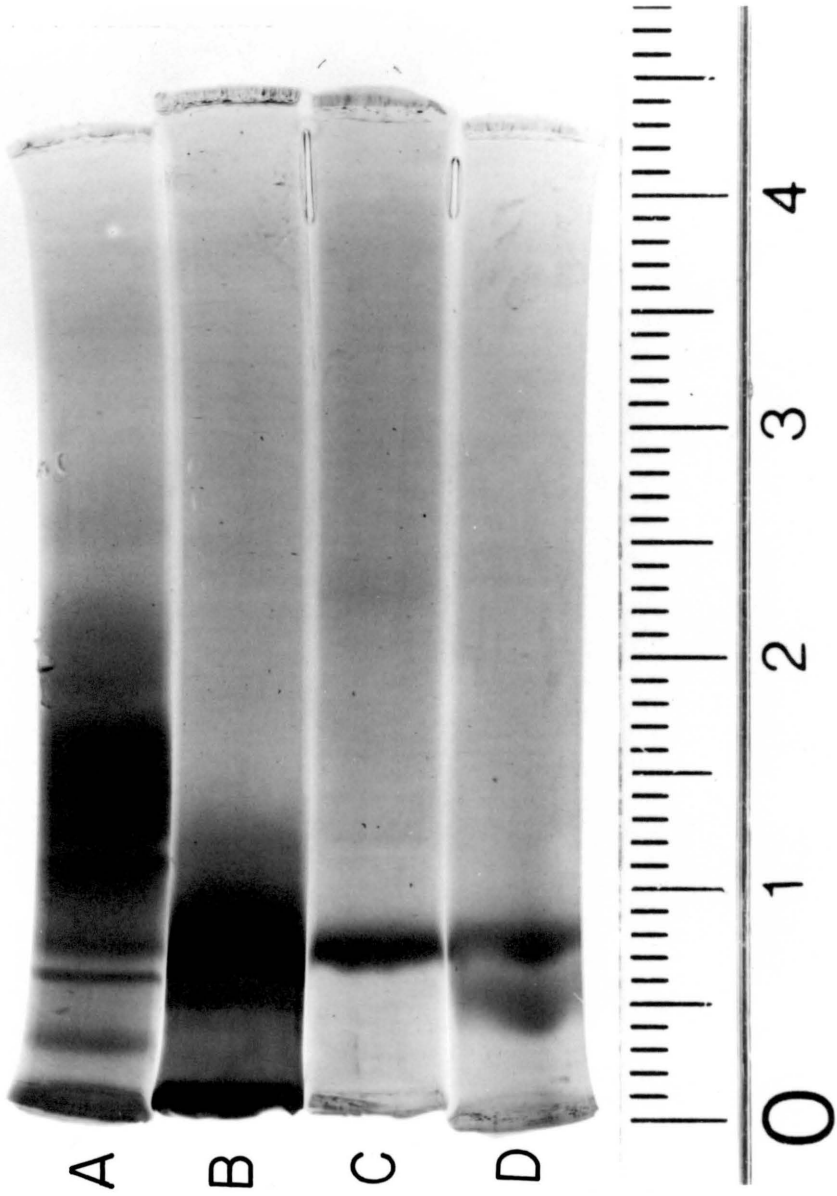
Bean Enzymes - The bean VTS fraction from DEAE-cellulose chromatography had a specific activity of 0.3 units/mg protein with unfractionated yeast tRNA (assay concentration 2.3 μ M) as the RNA substrate and had a half-life in storage of about 3 weeks. An attempt was made to purify tyrosine tRNA synthetase from bean on DEAE-cellulose but most of the activity was lost on the column. The tyrosine tRNA synthetase specific activity of crude (mixed enzyme fraction) prep-

FIGURE 5

Purification of Yeast NT

Polyacrylamide gels of yeast proteins.

A, yeast mixed enzyme fraction (50 μ g). B, yeast NT DEAE-cellulose fraction (50 μ g; see Figure 3). C, yeast NT CM-cellulose fraction A (5 μ g; see Figure 2). D, yeast NT CM-cellulose fraction B (10 μ g; see Figure 2). The specific activity of fraction A is 53 units/mg protein and that of fraction B is 25 units/mg protein. The difference in these specific activities is consistent with the relative amount of a contaminating protein in fraction B (upper band gel D). Electrophoresis is from left to right.



arations was about 0.15 units/mg protein, with a half-life of one to three months. The bean mixed enzyme fraction aminoacylated unfractionated yeast tRNA to 12% in the ^3H mixed amino acid assay. A NT activity of 0.03 units/mg protein was detected in the bean mixed enzyme fraction with TYMV-RNA (assay concentration 0.87 μm) as the RNA substrate.

Rat Enzymes - The VTS activity of the rat liver mixed enzyme fraction was 0.30 units/mg protein using yeast unfractionated tRNA (assay concentration 2.3 μM) as the substrate. The VTS activity was stable over 2 months. The NT activity of the rat enzyme preparation was 0.18 units/mg protein with a TYMV-RNA concentration of 0.45 μM .

Enzymes in General - All enzymes were tested for endogenous incorporation of amino acids into TCA precipitable material. No significant incorporation was found for any of the enzyme preparations subjected to streptomycin SO_4 treatment. This shows that active tRNAs were not present in these enzyme preparations. As noted previously, the yeast NT and VTS were found to be nuclease free. These two enzymes aminoacylated native TYMV-RNA to a maximum of 87 mole %. VTS fractions from E. coli, bean and rat also aminoacylated TYMV-RNA to 87%. This correspondence indicates that these other enzyme preparations are also func-

tionally nuclease free, as the RNA remained sufficiently free of nucleolytic hydrolysis to allow maximum aminoacylation. This conclusion is based in part on the observation that tRNA is highly susceptible to inactivation by nucleolytic hydrolysis of the 3' terminus (62).

Characterization of Viral RNA - RNA extracted from purified virus was analyzed by agarose-urea gel electrophoresis (Figures 6,7). The RNAs from TMV, TYMV, BSMV, CMV, and PSV show little degradation. A portion of the RNAs from BBMV, AMV, and SBMV is heterogeneous low molecular weight material, probably the result of degradation. Molecular weights of the viral RNAs were estimated from semilog plots of electrophoretic mobility (60) (Table II). Moderate degradation of these few viral RNAs does not necessarily inactivate their amino acid acceptor activity. For example, BBMV-RNA was partly degraded but was capable of aminoacylation to 65% with tyrosine (Table III) which is the same value as reported by Kohl et al. (5). All viral RNAs were protein-free as determined by ultraviolet spectroscopy.

Aminoacylation of TYMV-RNA

It had previously been determined that both VTS and NT are required for aminoacylation of TYMV-RNA with valine (3). Consequently, these two enzymes were purified from yeast.

TABLE II
 MOLECULAR WEIGHTS OF VIRAL RNAS¹³

<u>SMBV</u>	<u>BSMV</u>	<u>WCMV</u>	<u>BBMV</u>	<u>PSV</u>	<u>CMV</u> ¹⁴	<u>AMV</u>	<u>TMV</u> ¹⁴
1.35	1.75	1.75	0.91	0.93	0.91	1.15	2.0
0.67	1.12	1.18	0.54	0.83	0.68	0.79	
0.54	0.97	0.57	0.27	0.59	0.33	0.63	
				0.29		0.28	

¹³MW x 10⁶ daltons; data from Figure 7

¹⁴These RNAs were used as size standards (61)

FIGURE 6

Test of Yeast NT and VTS for

Ribonuclease Activity

Agarose-urea gel electrophoresis of RNA incubated for 4 min at 37° with yeast VTS or NT as described in Procedures. A, native (untreated) TYMV-RNA. B and C, prerepaired TYMV-RNA. D, 23S and 16S rRNA incubated with NT. E, 23S and 16S rRNA (heavily loaded) incubated with VTS (0.19 mg/ml). Electrophoresis is from left to right.



A)



B)



C)



D)



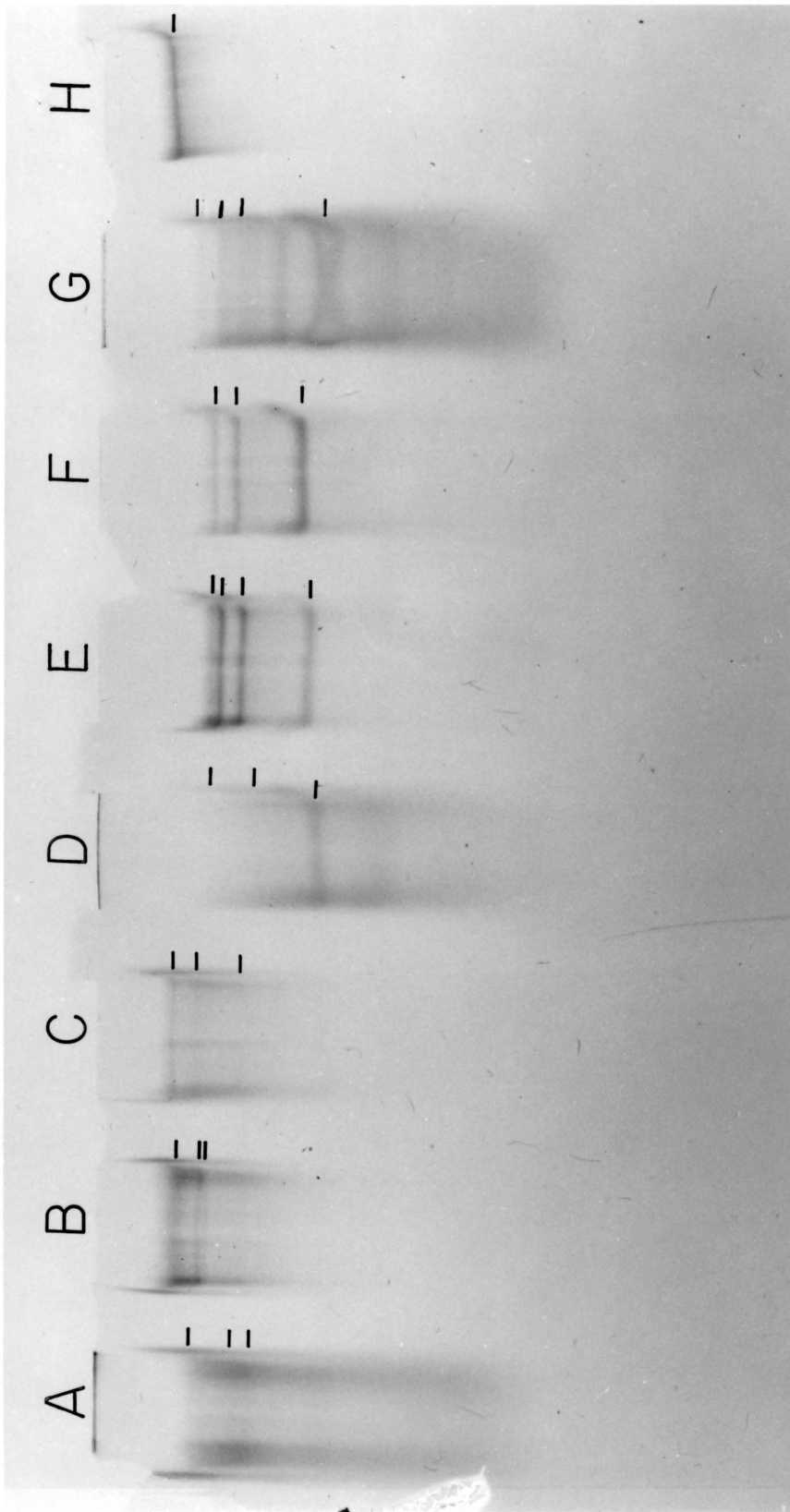
E)



FIGURE 7

Agarose-urea Gel Electrophoresis
of Viral RNAs

Electrophoresis was performed as described in Procedures except that a slab gel (0.15 x 8 x 15 cm) was used in place of tubes. A, SBMV-RNA (35 μ g). B, BSMV-RNA (11 μ g). C, WCMV-RNA (6 μ g). D, BBMV-RNA (15 μ g). E, PSV-RNA (10 μ g). F, CMV(S)-RNA (10 μ g). G, AMV-RNA (35 μ g). H, TMV-RNA (4 μ g). The molecular weights of RNA bands indicated (-) are listed in Table II. Electrophoresis is from top to bottom.

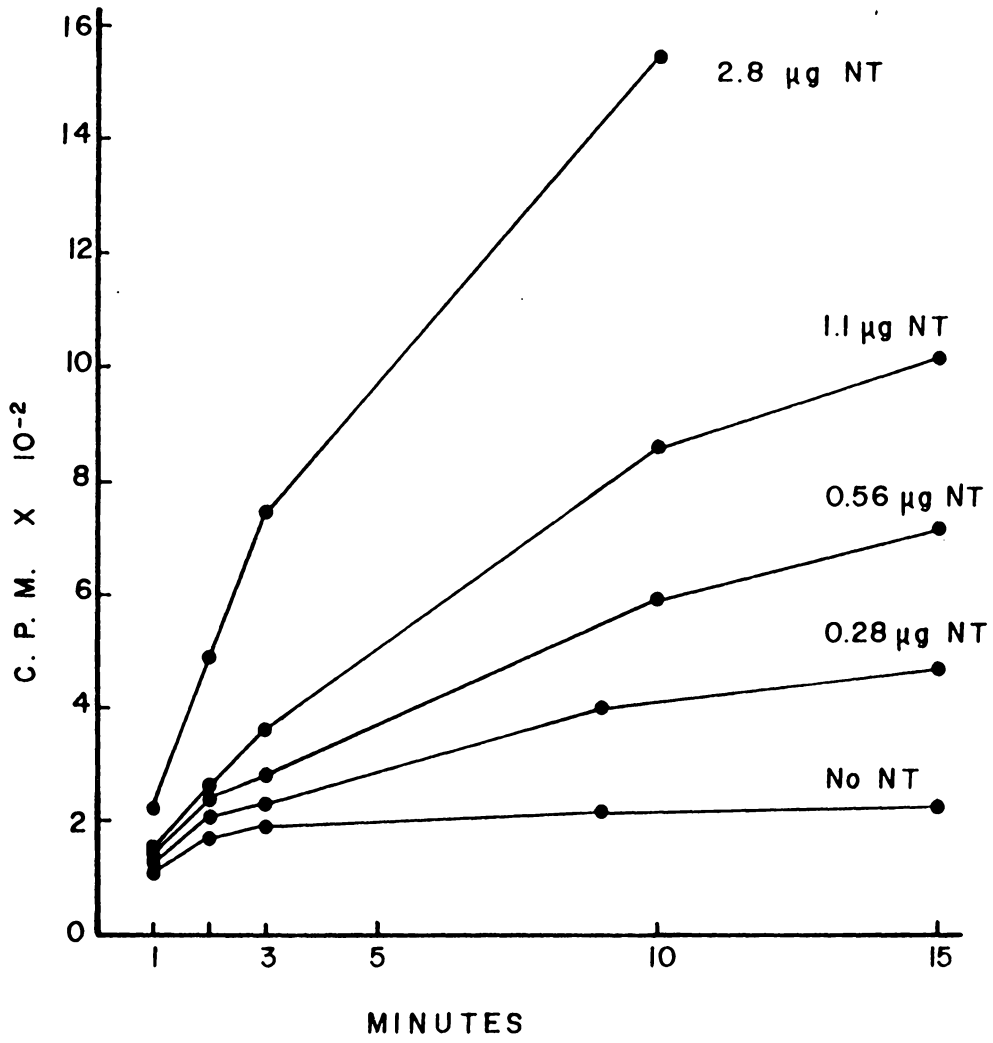


It was discovered that a small percentage of native TYMV-RNA is aminoacylated in the absence of added NT (Figure 8). Using yeast VTS DEAE-cellulose fraction it was found that the native (no added NT) aminoacylation capacity of TYMV-RNA is 8 mole %. This shows that native TYMV-RNA contains 8 mole percent of 3' AMP. When CM-cellulose purified NT was added to the above assay, TYMV-RNA could be aminoacylated up to 87 mole %. Figure 8 shows that the rate of aminoacylation of native TYMV-RNA is a function of the NT activity present in the reaction mixture. Therefore, for accurate VTS activity determinations, the native TYMV-RNA is best used when it has previously been repaired (3' adenosylated) prior to the VTS reaction. Figure 9 shows that the overall progress of VTS catalyzed aminoacylation of prerepaired TYMV-RNA is similar to that for unfractionated yeast tRNA. The initial rate of prerepaired TYMV-RNA aminoacylation is greater than any initial rate where 3' repair and aminoacylation are occurring simultaneously (Figures 8 and 9). This highest rate, however, is only about half of the rate observed for the aminoacylation of yeast unfractionated tRNA. As in Figure 9, aminoacylation rather typically became non-linear after the reaction was 40 to 50% complete. To avoid the complications introduced

FIGURE 8

Effect of NT Level on the
Aminoacylation of TYMV-RNA

A yeast VTS fraction (0.56 mg/ml) was assayed in the presence of 1.4 mg/ml TYMV-RNA and various amounts of NT (DEAE-cellulose fraction, Fig 3) under standard assay conditions (except incubated at 32°). The total assay volume was 120 μ l and 20 μ l aliquots were assayed at the times indicated. The mole percent of aminoacylation was 12% at 15 min in the absence of NT and 80% at 10 min in the presence of 2.8 μ g NT. Indicated c.p.m. is corrected for background (40 c.p.m.).



by preincubating the VTS assay as was done for the data in Figure 9, TYMV-RNA was preparatively prerepaired and repurified as described in Procedures. This RNA was capable of aminoacylation to 85 mole % in the standard aminoacylation assay in the presence or absence of added NT and was used in all other kinetic experiments presented here.

Specificity of Aminoacylation of Viral RNAs

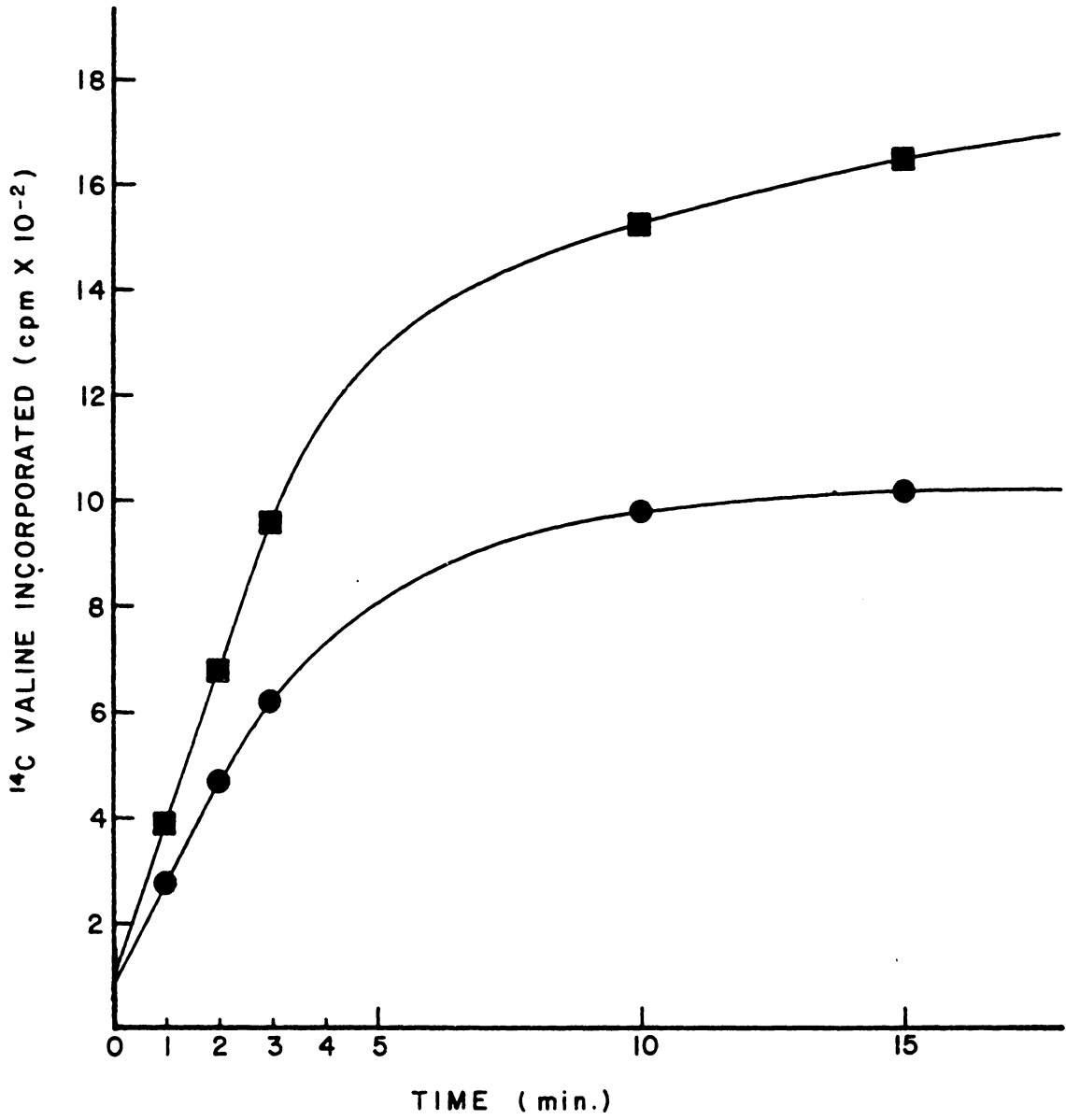
Nine viral RNAs were tested for amino acid acceptor activity in the presence of VTS from 3 sources (Table III). Four viral RNAs listed, TYMV, TMV, CMV, and BBMV, have been reported to be amino acid acceptors as noted in the Literature Review. The amino acid specificity, enzyme specificity, and the mole % of aminoacylation for these four are in good agreement with published values (2,5,18). It is notable that TYMV-RNA can be aminoacylated by VTS from three unrelated sources whereas the other viral RNAs are specific for the aminoacyl tRNA synthetase from a single source. This uniquely broad specificity exhibited by TYMV-RNA was considered an appropriate subject for more detailed study, the results of which are reported in a subsequent section.

The remaining five viral RNAs listed have not previously been reported to be capable of aminoacylation. They were chosen as representatives of five different viral taxonomic

FIGURE 9

Aminoacylation of Yeast tRNA and
TYMV-RNA with Valine by Yeast VTS

Conditions are the same as in Figure 8 except that the TYMV reaction mixture was incubated for 5 min with 2.8 μ g NT before adding more ATP (0.3 μ moles) and the VTS (0.56 mg/ml). TYMV-RNA (0.86 mg/ml) (●—●) was aminoacylated to 80 mole % at 15 min. Yeast unfractionated tRNA (0.375 mg/ml) (■—■) was aminoacylated to 4.8 mole % at 15 min.



groups as indicated in Table III. Only RNA from PSV, a member of the same group as CMV, showed competence as an amino acid acceptor. Like CMV-RNA, PSV-RNA specifically accepts tyrosine and is specific for the bean tyrosine tRNA synthetase. Considering the deficiencies noted for the mixed amino acid assay, negative results for RNAs from AMV, SBMV, BSMV and WCMV are not conclusive.

Kinetic Analysis of TYMV-RNA Aminoacylation

To quantitate the recognition of TYMV-RNA by VTS from E. coli, yeast, bean and rat, in terms of kinetic parameters, the rates of TYMV-RNA aminoacylation were observed as a function of RNA concentration. In order to compare the aminoacylation kinetics of TYMV-RNA with those of prokaryotic and eukaryotic tRNA, control experiments were performed in which E. coli tRNA and yeast tRNA were substituted for TYMV-RNA. With the exception of the K_m of yeast VTS for its homologous tRNA ^{Val}₁ (44) the kinetic parameters reported here are not in the literature. Figures 10 through 20 present the data gathered in these experiments in the form of double reciprocal (Lineweaver-Burk) plots. The kinetic parameters obtained are summarized in Table IV.

Typical Michaelis-Menten kinetics were observed for the E. coli VTS for all three RNA substrates (Figures 10-12).

TABLE III
MOLE % AMINOACYLATION OF VIRUS RNAS¹⁵

<u>Virus</u>	<u>Group</u>	<u>Amino Acid</u>	<u>Source of Synthetase</u>		
			<u>E. Coli</u>	<u>Yeast</u>	<u>Bean</u>
TYMV	TYMO	Val	87	87	87
TMV	TOBAMO	His	0	40	0
CMV	CUCUMO	Tyr	0	0	60
BBMV	BROMO	Tyr	0	0	65
PSV	CUCUMO	Tyr	0	0	22
WCMV	POTEX		-	0	0
AMV	MONOTYPIC		-	0	0
SBMV	MONOTYPIC		-	0	0
BSMV	HORDEI		-	0	0

¹⁵Based on the following molecular weights: TYMV - 1.9×10^6 (61), TMV - 2.0×10^6 (61), CMV - 1.0×10^6 (average) (5), BBMV - 0.75×10^6 (average) (5), PSV - 1.0×10^6 (estimated average).

TABLE IV
COMPARISON OF AMINOACYLATION KINETICS

<u>Source of VTS</u>	<u>Source of tRNA</u>					
	<u>TYMV-RNA</u>		<u>E. coli tRNA</u>		<u>Yeast tRNA</u> ¹⁹	
	V_{\max}^{16}	K_m	V_{\max}	K_m	V_{\max}	K_m
<u>E. coli</u>	16	1100	100	350	15	3800
Yeast	50	7	30 ¹⁷	330	100	17
Bean	100	150	No Reaction		60	1300
Rat	50	150	15 ¹⁷	NM ¹⁸	100 ¹⁷	NM ¹⁸

¹⁶ V_{\max} values (arbitrary units) were normalized with the highest value observed as 100.

¹⁷ Maximum observed velocity (not V_{\max})

¹⁸ Non-Michaelis-Menten kinetics

¹⁹ Unfractionated tRNA except for yeast VTS reaction

The K_m for E. coli tRNA is about three times lower than the K_m for TYMV-RNA and ten times lower than the K_m for yeast tRNA. The V_{max} of E. coli VTS with E. coli tRNA is considerably higher than that found for TYMV-RNA and yeast tRNA. As might be expected, the homologous E. coli tRNA is clearly the most competent substrate, in terms of V_{max} and K_m , for the E. coli VTS. TYMV-RNA appears to have a higher affinity for the E. coli VTS than does yeast tRNA as indicated by its three-fold lower K_m .

The yeast VTS was found to have strikingly low K_m s for TYMV-RNA ($K_m = 7$ nM) and yeast tRNA^{Val} ($K_m = 17$ nM) (Figs 13,15). The K_m for TYMV-RNA is the second lowest K_m reported for any tRNA, aminoacyl tRNA synthetase combination (64). The K_m found for the yeast tRNA^{Val}, also considered to be very low, is within the range reported by Ebel et al. (44). The V_{max} of yeast VTS for TYMV-RNA is lower than, but comparable to, the V_{max} for yeast tRNA^{Val}. E. coli tRNA^{Val₁} is inferior to both TYMV-RNA and yeast tRNA^{Val} as a substrate for yeast VTS. Also, E. coli tRNA^{Val₁} appears to exhibit substrate inhibition with yeast VTS at concentrations above 500 nM (Fig. 14).

TYMV-RNA was found to be clearly superior to yeast tRNA, in terms of K_m and V_{max} , when acting as a substrate

FIGURE 10

Kinetics of E. coli VTS with
Respect to TYMV-RNA Concentration

The double reciprocal plot was derived from rate determinations as described in Procedures. The $1/V$ scale is normalized for protein concentration (in mg). The error bars represent the standard deviation range.

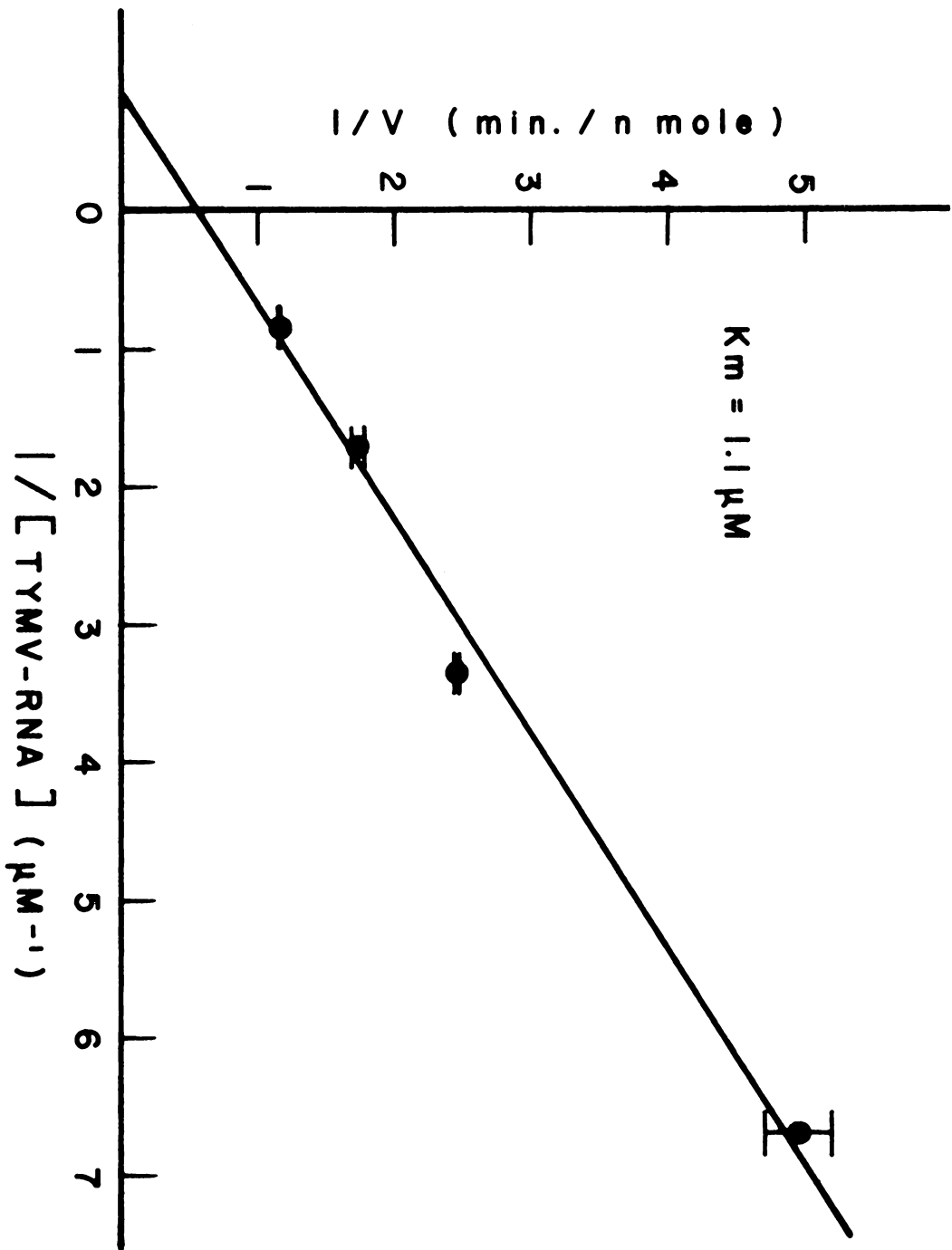


FIGURE 11

Kinetics of E. coli VTS with

Respect to E. coli tRNA Concentration

Plotted as in Figure 10, E. coli tRNA^{Val}₁

was used.

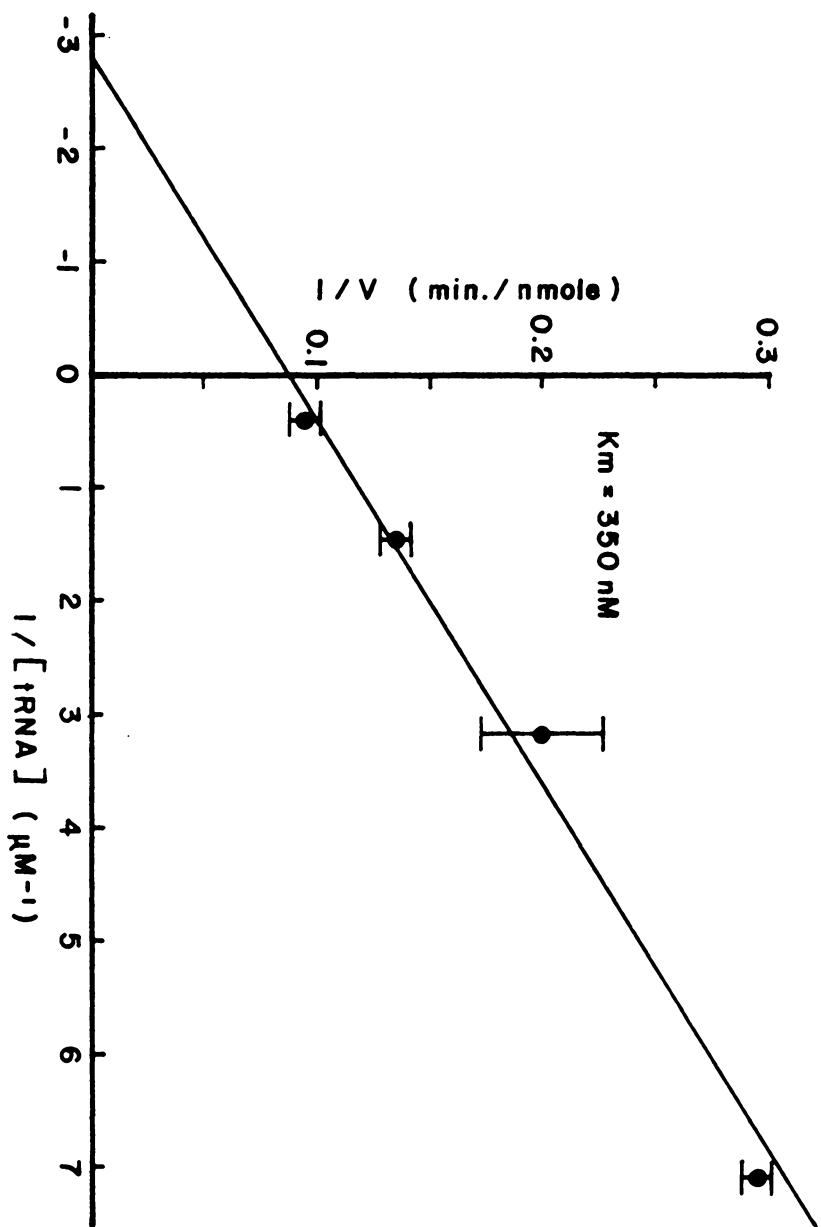


FIGURE 12

Kinetics of E. coli VTS with Respect
to Yeast tRNA Concentration

Plotted as in Figure 10. Yeast unfrac-
tionated tRNA was used.

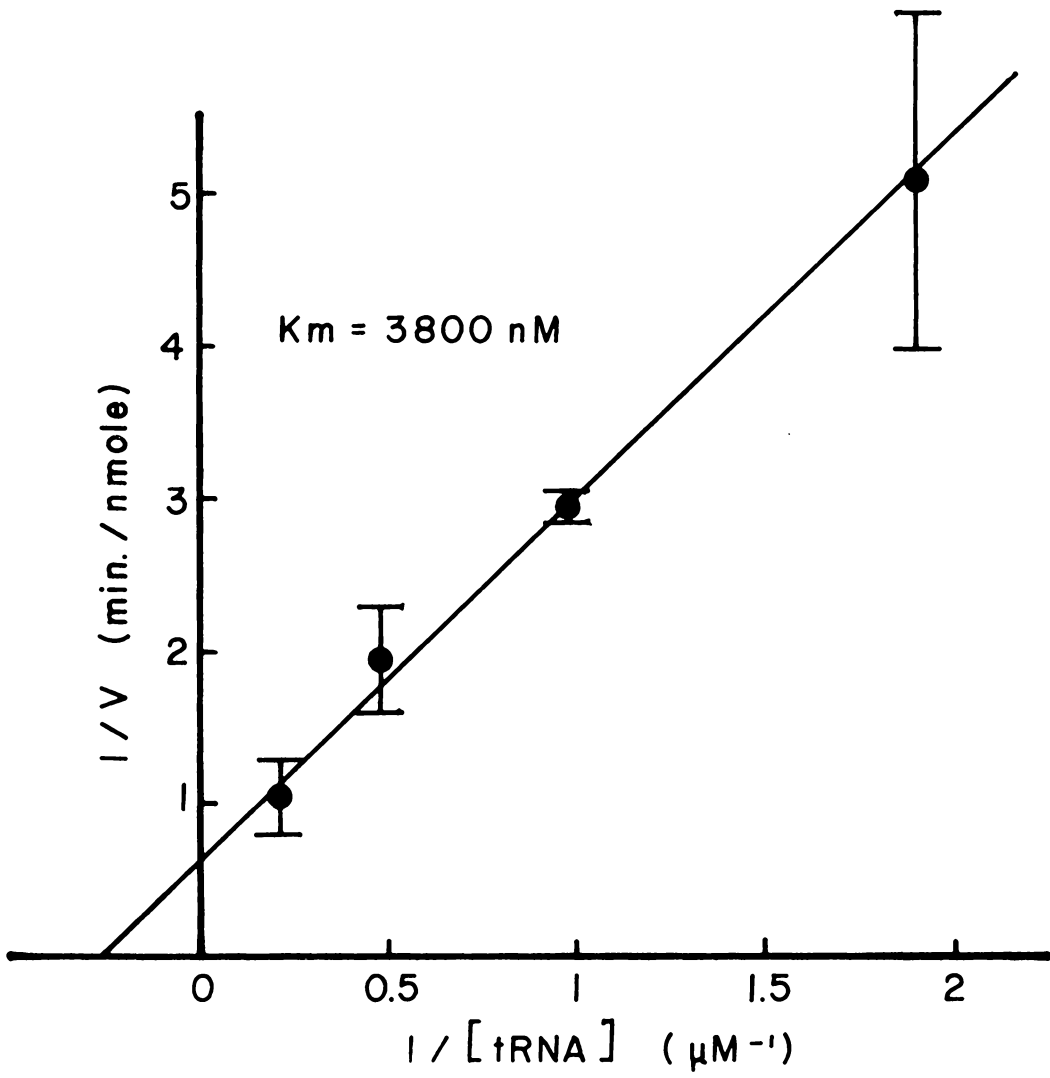


FIGURE 13

Kinetics of Yeast VTS with
Respect to TYMV-RNA Concentration

Plotted as in Figure 10. Reaction mixture was preincubated for 1 min at 37° with 0.008 mg/ml yeast NT CM-cellulose fraction A to insure repair.

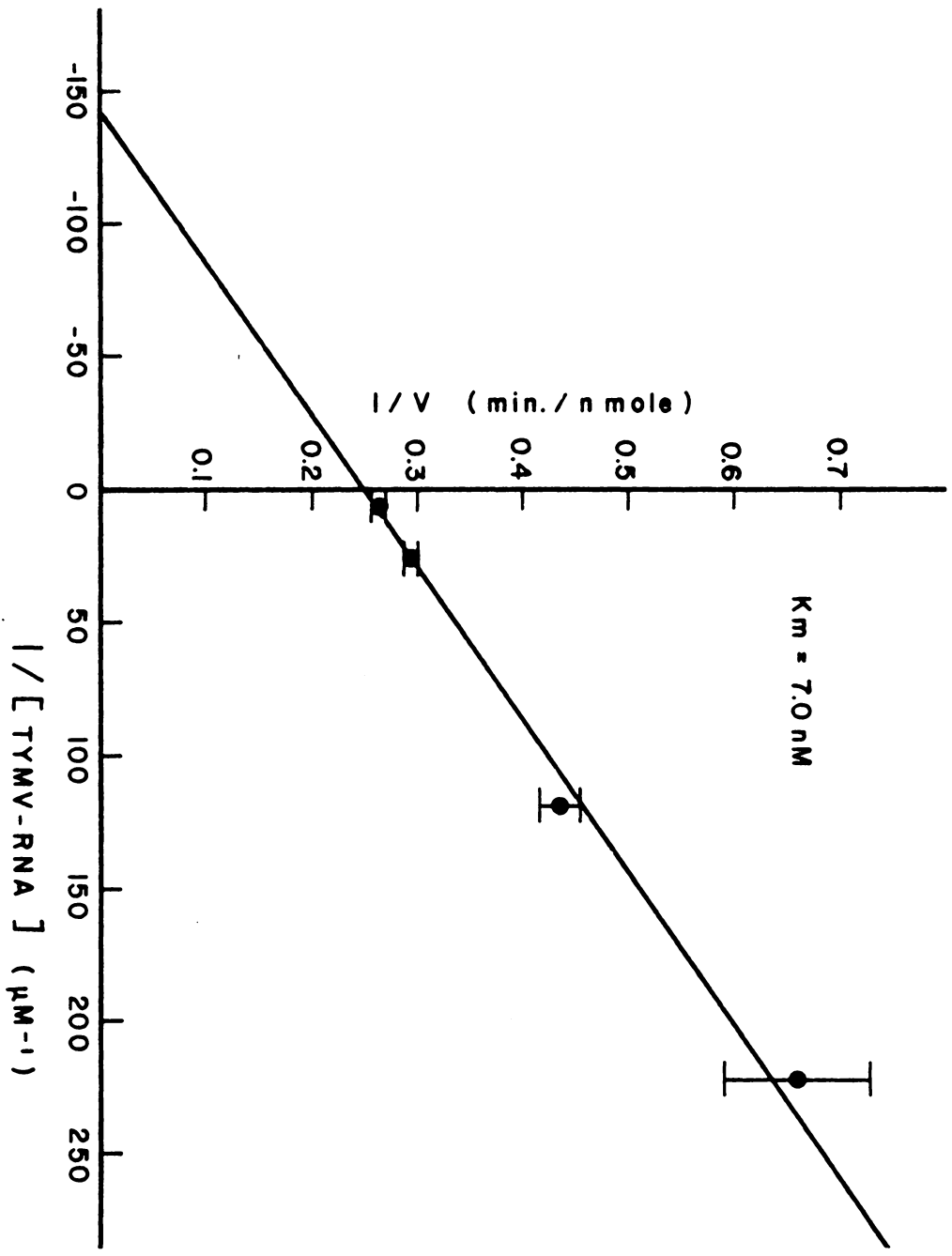


FIGURE 14

Kinetics of Yeast VTS with

Respect to E. coli tRNA Concentration

Plotted as in Figure 10 except that data points represent single rate determinations and the slope and intercepts were estimated.

E. coli tRNA₁^{Val} was used.

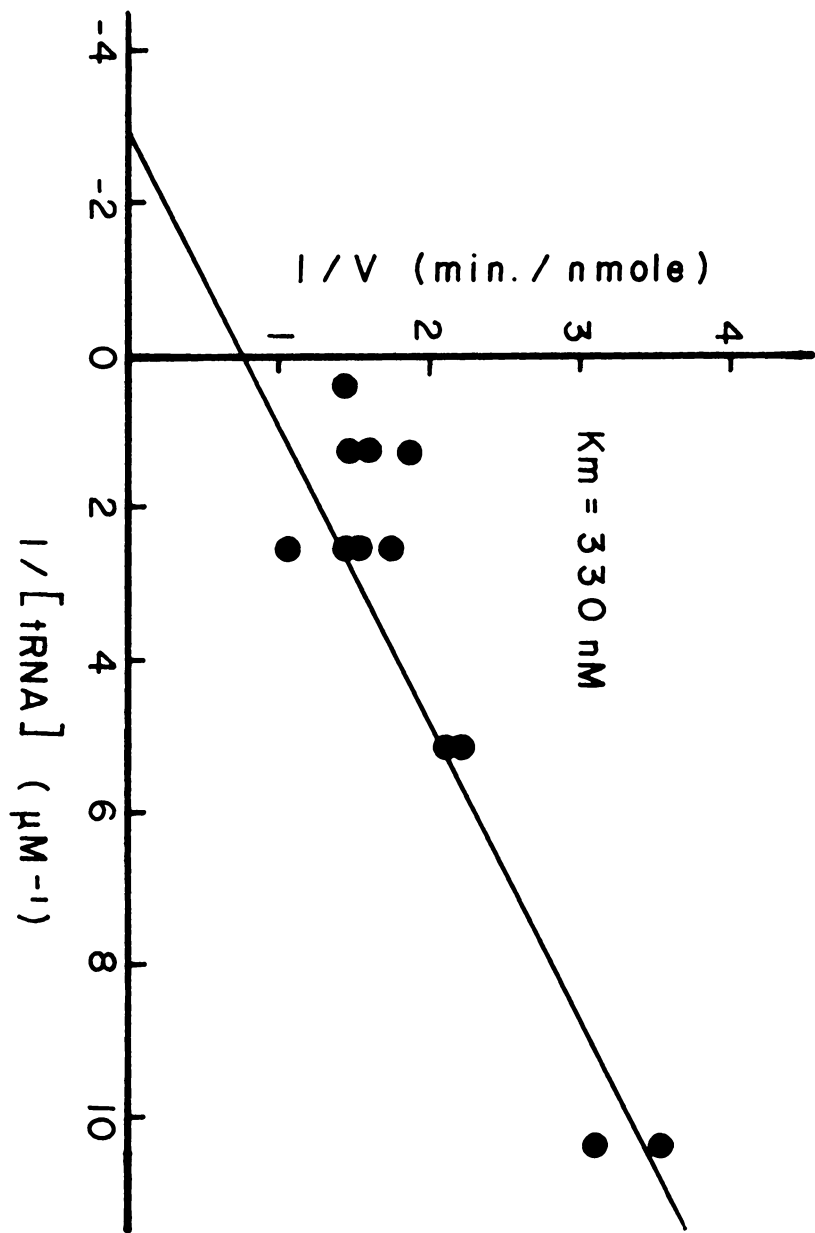


FIGURE 15

Kinetics of Yeast VTS with

Respect to Yeast tRNA Concentration

Plotted as in Figure 10. Assay mixture was preincubated 1 min at 37°. Yeast tRNA^{Val} was used.

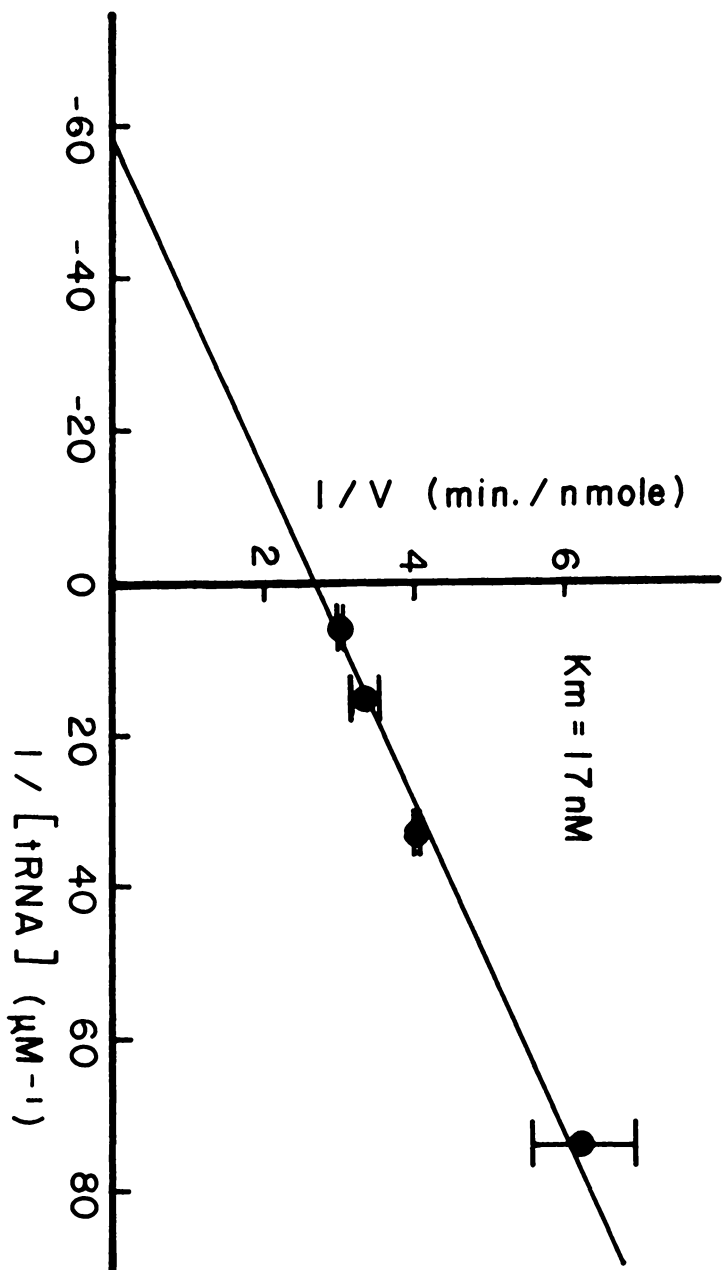


FIGURE 16

Kinetics of Bean VTS with

Respect to TYMV-RNA Concentration

Plotted as in Figure 10. Assay mixture
was preincubated 1 min at 37^o.

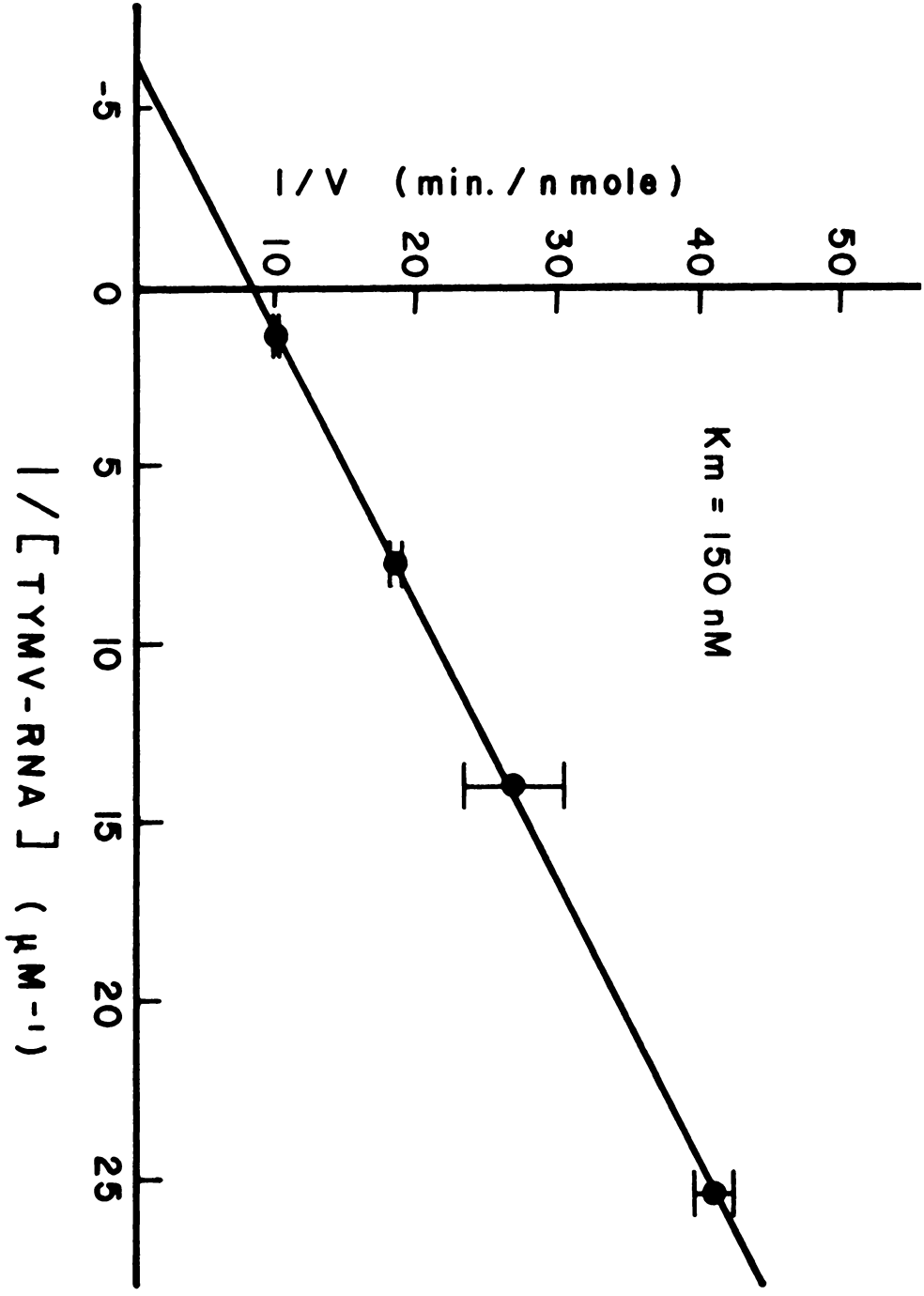


FIGURE 17

Kinetics of Bean VTS with

Respect to Yeast tRNA concentration

Plotted as in Figure 10. Assay mixture was preincubated 1 min at 37⁰. Yeast unfractionated tRNA was used.

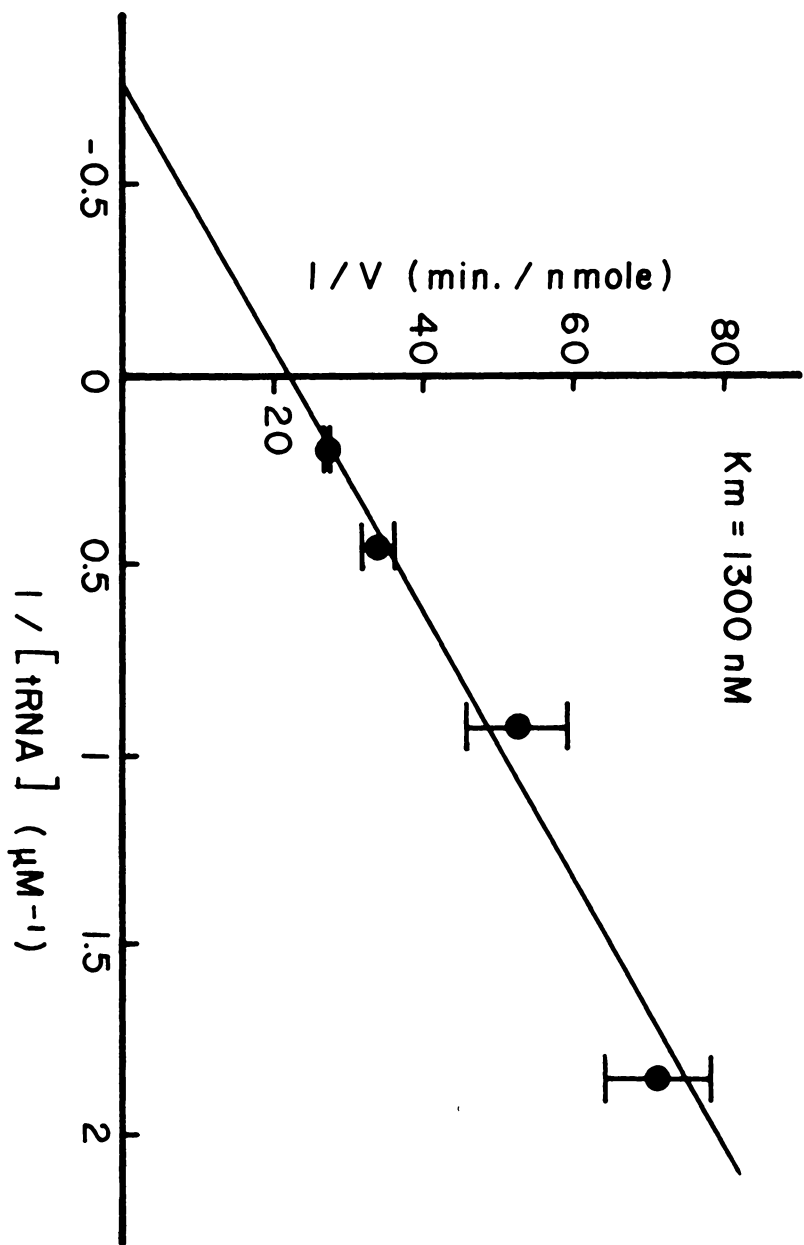


FIGURE 18

Kinetics of Rat VTS with

Respect to TYMV-RNA Concentration

Plotted as in Figure 10.

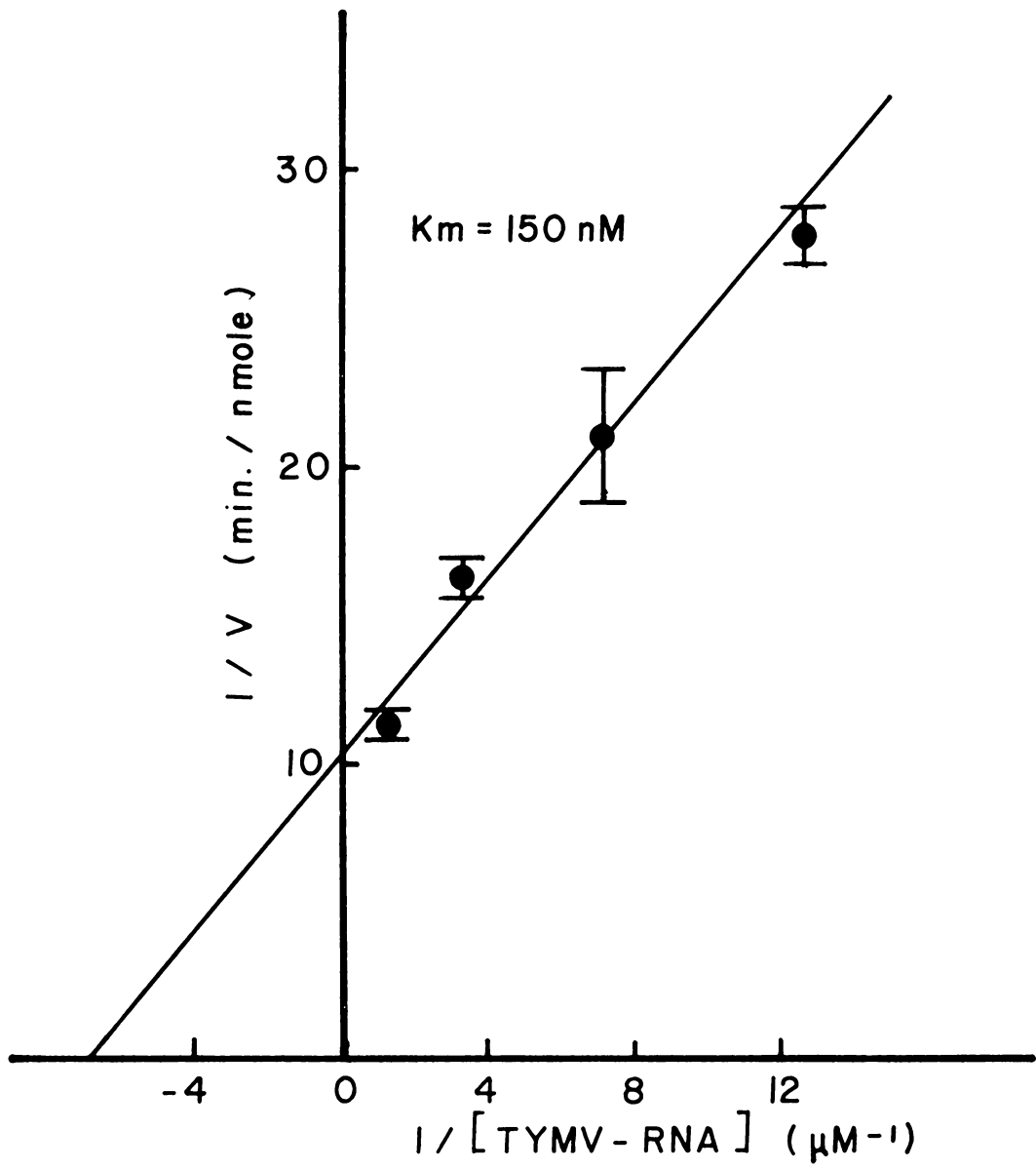


FIGURE 19

Kinetics of Rat VTS with Respect

to E. coli tRNA Concentration

Plotted as in Figure 14 except intercepts
were not determined. E. coli tRNA₁^{Val} was
used.

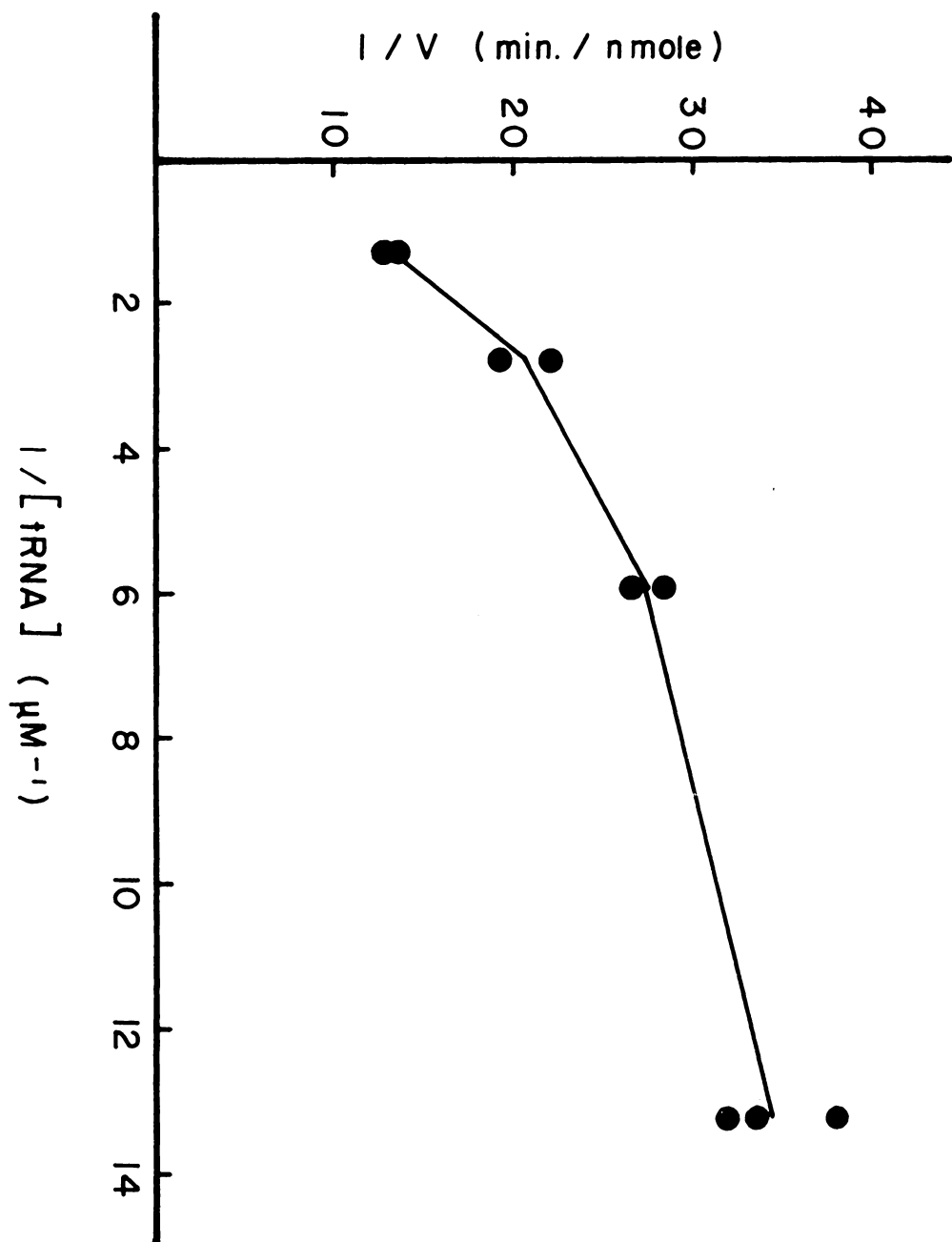
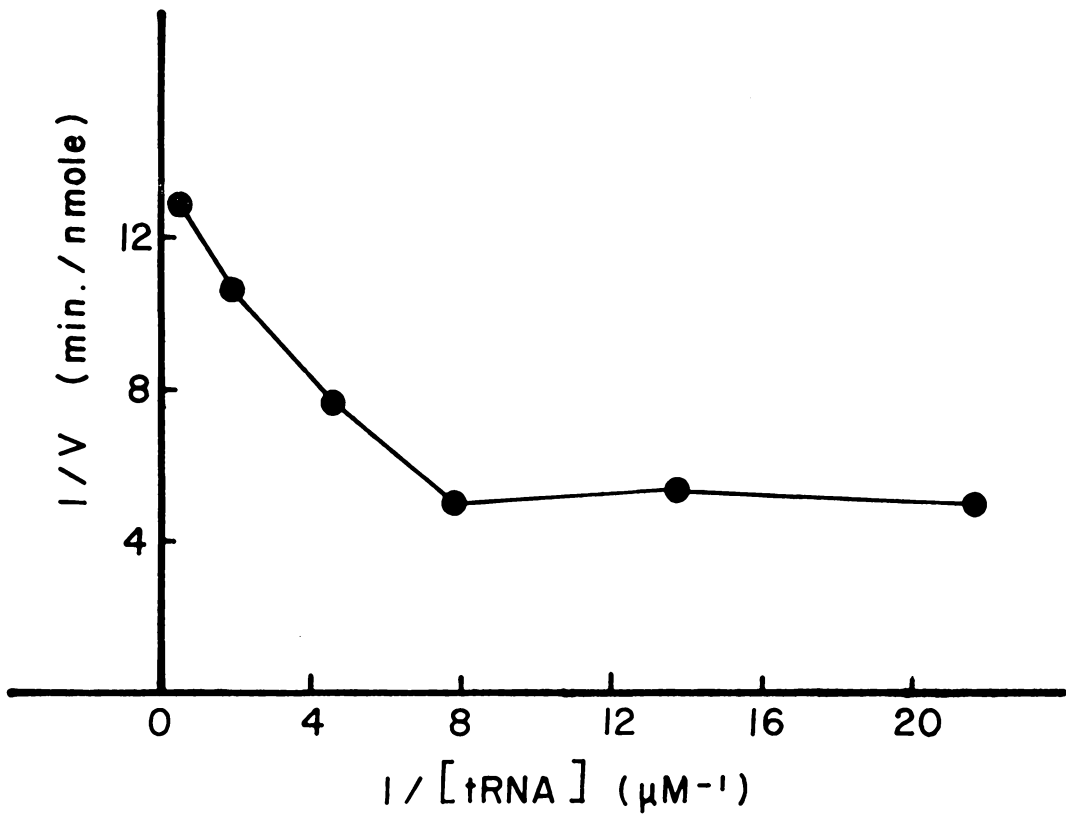


FIGURE 20

Kinetics of Rat VTS with Respect
to Yeast tRNA Concentration

Plotted as in Figure 14 except that data points represent single rate determinations and slope and intercepts were not determined.



for bean VTS (Figures 16 and 17). E. coli tRNA₁^{Val} was not aminoacylated with valine in the presence of bean VTS.

The rat liver VTS exhibited complex kinetics with respect to RNA concentration. The plot of rat liver VTS kinetics with yeast tRNA as the substrate, however, suggests strong substrate inhibition, even at low concentrations of tRNA (Figure 20). Nevertheless, yeast tRNA, at 120 to 50 nM, is aminoacylated faster than is TYMV-RNA or E. coli tRNA. The curving plot representing the kinetics of E. coli tRNA aminoacylation by rat liver VTS (Fig. 19) cannot be explained by a simple kinetic model. The apparent change in K_m and V_{max} over the range of tRNA concentrations might be consistent with the presence of two or more kinetically distinct, active aminoacylation sites in the system. The plot of TYMV-RNA aminoacylation kinetics with the rat VTS is nearly linear (Figure 18) but bears some resemblance to the curving E. coli tRNA₁^{Val} plot (Figure 19).

As seen in Table IV the interaction of TYMV-RNA with VTS from diverse sources is characterized by broad specificity and a high degree of kinetic competence as compared to eukaryotic and prokaryotic tRNAs. For each VTS, TYMV is a better substrate than at least one of the normal tRNAs.

Ionic Strength Effects on Aminoacylation - It has been

observed previously that KCl strongly inhibits the aminoacylation of TYMV-RNA by E. coli VTS and of TMV-RNA by rat liver VTS (5,13). To characterize further the effect of KCl on TYMV-RNA aminoacylation, experiments were performed in which KCl was added to standard assay mixtures containing relatively high concentrations of TYMV-RNA or tRNA and various valine tRNA synthetases (Table V). KCl inhibited all reactions except homologous yeast tRNA aminoacylation and TYMV-RNA aminoacylation by rat VTS. The E. coli homologous reaction was only slightly inhibited by KCl. Thus, it appears that KCl increases the specificity of E. coli and yeast VTS for the homologous tRNA substrate. The KCl stimulation of TYMV-RNA aminoacylation by rat VTS contrasts with the inhibition reported for TMV-RNA in a similar experiment (20). Kohl et al. (5) reported that KCl (40 mM) inhibited the E. coli VTS rate of TYMV-RNA aminoacylation to a much greater extent than it inhibited the bean VTS rate of TYMV-RNA aminoacylation. The fact that initial rates were not consistently measured by Kohl et al. probably accounts for these results, which differ sharply from those presented here.

In a more detailed study of the effect of KCl on aminoacylation by yeast VTS, the ionic strength of the standard assay (about 115 mM) was lowered by replacing the 50 mM TRIS

TABLE V
EFFECT OF KCl ON THE VTS RATE²⁰

VTS Source	RNA Source					
	TYMV μM^{21}		E.coli ²² μM^{21}		Yeast ²³ μM^{21}	
<u>E. coli</u>	-5	(0.34)	-1.5	(0.8)	-2	(2.3)
Yeast	-4	(0.17)	-7	(0.4)	+1.4	(1.2)
Bean	-4	(0.17)	-		-3.5	(2.3)
Rat	+1.6	(0.17)	-		-	

²⁰3 μl of 1 M KCl was added to standard assay mixtures to a final KCl concentration of 47.5 mM (ionic strength of 157 mM). Rates are expressed as the fold decrease (-) or increase (+) as compared to standard assay controls (ionic strength of 115 mM).

²¹Active RNA concentration (see Procedures)

²²E. coli tRNA ^{Val}₁

²³Unfractionated yeast tRNA

with 10 mM HEPES (pH 7.4 at 25^o). The rates of aminoacylation of TYMV-RNA and of yeast tRNA were observed as a function of KCl concentration using this otherwise low (80 mM) ionic strength assay (Figure 21). Surprisingly, yeast tRNA aminoacylation by yeast VTS was inhibited by KCl. This change in KCl sensitivity by yeast VTS is very possibly explainable by the substitution of TRIS by HEPES. Aminoacylation of TYMV-RNA by yeast VTS was found to be almost completely inhibited by 100 mM KCl.

Competitive Inhibition of TYMV-RNA Aminoacylation by

TYMV-RNA-C-C_{OH} - To obtain information about the binding of TYMV-RNA to yeast VTS, TYMV-RNA-C-C_{OH} (unrepaired TYMV-RNA) was used as an inhibitor in the kinetic analysis of TYMV-RNA aminoacylation (Fig 22). The double reciprocal plots obtained in the presence and absence of RNA-C-C_{OH} show that the molecule is indeed an effective inhibitor of the TYMV-RNA aminoacylation rate and intersection of the lines near the ordinate shows the inhibition to be competitive. The K_I (32 nM) of the TYMV-RNA-C-C_{OH} is equivalent to its dissociation constant and is significantly higher than the K_m (7 nM) of TYMV-RNA-C-C-A_{OH}, which is equal to or lower than its dissociation constant (40). This indicates that the terminal AMP of TYMV-RNA contributes to the binding of the RNA by the

FIGURE 21

KCl Inhibition of the Yeast VTS Rate of
TYMV-RNA and Yeast tRNA Aminoacylation

Standard assay conditions were used with 10 mM HEPES (pH 7.4 at 25^o) replacing TRIS-Cl (50 mM) as the buffer. Rates were determined in the presence of 0 (ionic strength 80 mM), 50 (ionic strength 130 mM) or 100 mM KCl (ionic strength 180 mM). \square — \square yeast unfractionated tRNA (1.2 μ M), \bullet — \bullet prerepaired TYMV-RNA (0.11 μ M)

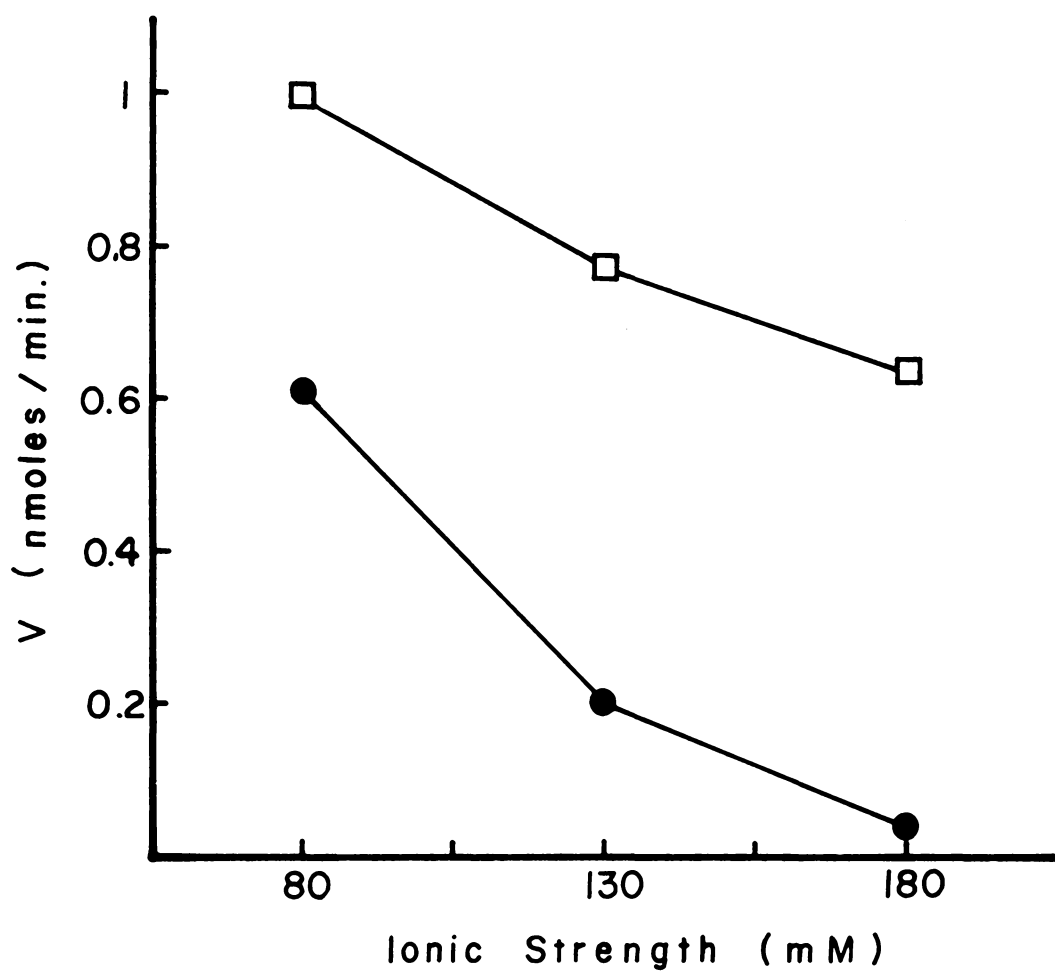
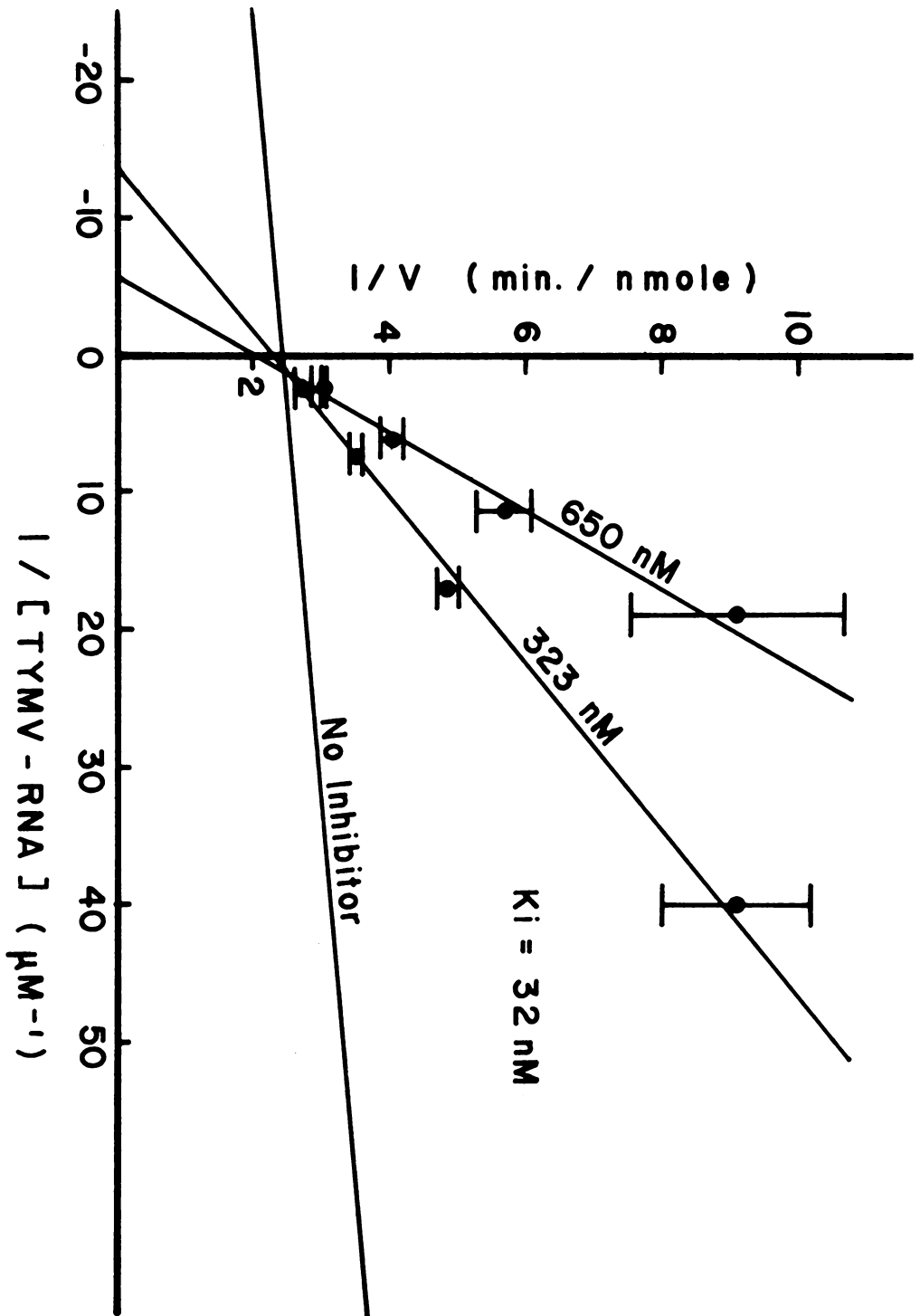


FIGURE 22

Inhibition of TYMV-RNA Aminoacylation

By TYMV-RNA-C-C_{OH}

Plotted as in Figure 10. The No
Inhibitor plot is from Figure 13 (normalized).
Native and prerepaired TYMV-RNA were mixed to
obtain the concentrations of inhibitor (TYMV-RNA-
C-C_{OH}) and substrate (TYMV-RNA-C-C-A_{OH}) indi-
cated on the figure. The native TYMV-RNA was
assumed to contain 8 mole % C-C-A_{OH} and 79 mole
% C-C_{OH}. The K_1 (32 nM) is the mean (S.D. =
5 nM) of the K_1 s derived for each plot.



enzyme active site. In contrast to this finding, Ebel et al. (39) determined that the terminal AMP of yeast tRNA^{Val} did not affect binding of this RNA to yeast VTS.

DISCUSSION

Although many papers have been published which describe aspects of the aminoacylation of viral genomic RNA, there is almost no information available on the kinetic nature of the reaction. It has recently been found that the structures of the amino acid accepting RNAs of plant viruses bear little resemblance to usual tRNAs. This difference raises the question of whether or not viral tRNA functions are in fact comparable to tRNA as substrates of aminoacyl tRNA synthetases. As a result, a kinetic analysis of viral RNA aminoacylation has become increasingly important as an approach to answering this question.

In general, researchers in this field have not used the methodology necessary for accurate characterization of the aminoacylation of viral RNA. Methods were developed here to determine initial rates of aminoacylation for use with high molecular weight viral RNAs being acted on by a variety of aminoacyl tRNA synthetases. The methods proved to be reliable over a wide range of enzyme and RNA concentrations and were sensitive enough to determine extremely low K_m values. The development of a preparative TYMV-RNA "prerepair" method was quite important since this product

not only eliminated serious problems (e.g., NT limited rates) in determining VTS rates but it also allowed the unique application of TYMV-RNA-C-C_{OH} as an inhibitory kinetic probe. For comparative studies, bacterial, fungal, animal and plant aminoacyl tRNA synthetases were prepared. These preparations were tRNA free, functionally ribonuclease free and catalytically active, which allowed accurate mole percents of aminoacylation and rate determinations. The presence of ribonuclease would have resulted in a steady decrease in active RNA concentration during assays and the presence of endogenous active tRNAs would have resulted in anomalously high levels of aminoacylation. Some problems arose as a result of the instability in storage of some of the enzymes. This was compensated for by using increasing amounts of enzyme and normalizing rates where indicated. The molecular weight distribution of TYMV-RNA was slightly lowered during preparative prerepair but the aminoacylation capacity of this RNA was nearly identical to that of native RNA (Figure 6). The heterogeneous size distribution of native RNA is characteristic of TYMV²⁴.

The results of the mole percent of aminoacylation determinations for nine viral RNAs using three enzyme sources

²⁴Personal communication from J. M. Kaper.

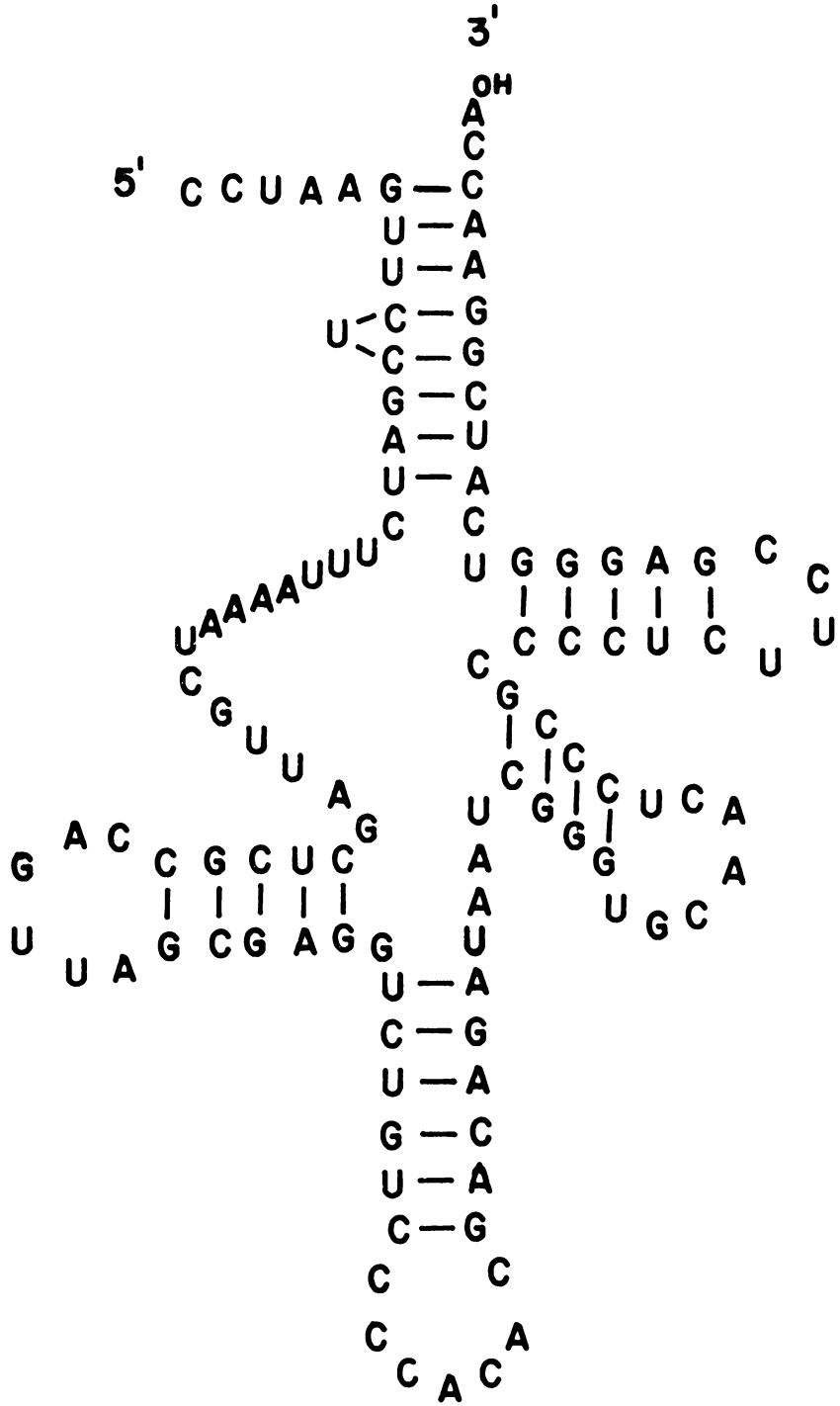
(Table III) indicate that the specificity of viral RNA aminoacylation is a useful taxonomic trait.

The discovery reported here that PSV-RNA accepts tyrosine in the presence of bean tyrosine tRNA synthetase establishes the Cucumoviruses as the third viral group to exhibit intra-group specificity with regard to both the amino acid and the aminoacyl tRNA synthetase source required for aminoacylation. The other two groups are the Tymoviruses and the Bromoviruses (Table I). A fourth group, the Tobamoviruses, does not exhibit this group specificity for a single amino acid. It may be significant that these are rod-shaped viruses whereas the other three groups (Bromo- Cucumo- and Tymoviruses) are icosahedral viruses. No other rod-shaped group of viruses has been reported to have members whose RNA is capable of aminoacylation, including those tested here - BSMV and WCMV (Table III).

TYMV-RNA was chosen for kinetic studies because of its broad specificity for aminoacyl tRNA synthetases as determined by the author and others (5). The nucleotide sequence of the amino acid accepting portion of TYMV-RNA (34) (3' terminal 108 residues) has been examined in order to predict the most likely secondary structure of this tRNA-like fragment (Figure 23). The structure bears little similarity to yeast or

FIGURE 23
Secondary Structure of the
3' End of TYMV-RNA

A secondary structure optimizing method was used to predict the most likely helical structure for the 3' 108 nucleotides of TYMV-RNA, the tRNA-like region. Parameters optimized included helix length, G+C content, and thermodynamic stability, and the most stable combination of helices ($\Delta G = -44$ kcal) forms this structure. The structure was predicted by Miss Laura Swell under the direction of Dr. D. R. Lightfoot.



E. coli tRNAs. It contains an extra loop and a long unpaired stretch not present in valine tRNA, lacks most of the "critical" bases (35) (including all minor bases) common to all tRNAs and is 31 bases larger than yeast tRNA^{Val}₁ or E. coli tRNA^{Val}₁. The TYMV-RNA structure is most similar to yeast tRNA^{Val}₁ in the anticodon region (49-63 bases from the 3' end) differing by only one base pair in the stem and 3 base substitutions in the loop. It is remarkable that, in spite of these large structural differences from usual valine tRNAs, TYMV-RNA is so competent and so versatile as a substrate in the aminoacylation reaction (Table IV). The high rates and low K_m s determined for TYMV-RNA indicate that it may reach high levels of aminoacylation in vivo and thus support the possibility of an important physiological role for the tRNA-like function.

While TYMV-RNA interacted well with aminoacyl tRNA synthetases from all sources, some of the heterologous tRNA-synthetase interactions with usual tRNAs were non-Michaelian (Figures 14,19,20). This distinction probably bears on the existence of allosteric or of multiple active sites on the enzymes (43,65). The data for unfractionated yeast tRNA seen in Figure 20 may be interpreted as the result of non-cognate tRNA with relatively high K_i s interacting

at a second site on the VTS (65). TYMV-RNA on the other hand, is large and has unusual base composition and structure relative to tRNAs and therefore may sterically hinder the binding of a second RNA molecule to a multi-component aminoacyl tRNA synthetase. Thus, the TYMV-RNA-synthetase interaction may tend to inhibit the expression of complex kinetics. This phenomenon may also be explained by the absence of recognition sites on the TYMV-RNA which are required for productive interaction with allosteric sites on the enzyme.

It is possible that the extraordinarily low K_m (7 nM) of yeast VTS for TYMV-RNA is lower than that for yeast tRNA^{Val} (17 nM) as a result of the higher V_{max} of the homologous reaction. If the rate of catalysis is k_{+2}^{25} , and it is large compared to the rate of substrate dissociation, k_{-1} , the two fold higher V_{max} of yeast VTS for yeast tRNA^{Val} could result in as much as a two-fold increase in the K_m with no changes in the dissociation constant K_S (k_{-1}/k_{+1}). The data presented here are consistent with this interpretation (Table IV).

$$^{25} K_m = \frac{k_{+2} + k_{-1}}{k_{+1}}$$

It follows from this observation that the dissociation constants for TYMV-RNA and for yeast tRNA^{Val} may be nearly equal. A way of testing this possibility was attempted using the unrepaired TYMV-RNA-C-C_{OH}. If one assumes that the terminal AMP of TYMV-RNA has no function in binding the RNA to the yeast VTS, as was found to be the case for yeast tRNA₁^{Val} (39), the K_i (competitive inhibition constant) of TYMV-RNA-C-C_{OH} should equal the K_S of TYMV-RNA-C-C-A_{OH} (40). The inhibition experiment in Figure 21 was performed to determine the K_i of TYMV-RNA-C-C_{OH}, and presumably K_S of TYMV-RNA-C-C-A_{OH}, and the rate constants (k₊₁, k₋₁ and k₊₂). Surprisingly, the K_i (32 nM) was significantly higher than the K_m (7 nM) showing that for TYMV-RNA the 3' terminal AMP does function in binding to yeast VTS, and the above assumption is incorrect.

The effect of KCl on aminoacylation (Table V and Figure 21) shows that the rate of RNA aminoacylation is strongly dependent on small changes in the ionic strength of the reaction mixture. As discussed in Results, the magnitudes and directions of the rate changes depend on the particular VTS and RNA in the reaction. The KCl dependent changes in rate could be explained by a change in either the RNA conformation, the VTS conformation, or ion mediated interactions

between the RNA and the VTS. The data in Table V suggest that the aminoacylation of homologous tRNA is favored at high ionic strength but the data are not sufficiently detailed to suggest the mechanism of this effect. The range of ionic strength (115 to 157 mM) used in the KCl experiments (Table V) is about the same as what is generally considered to be the range of physiological ionic strengths. The marked change noted in the KCl response of homologous yeast aminoacylation when HEPES buffer was substituted for TRIS-Cl (Figure 21) indicates that caution should be used in applying these in vitro results to the in vivo environment.

Infectious TYMV-RNA is transcribed in vesicles associated with the outer chloroplast membranes of infected plant cells and is encapsidated as it is released into the cytoplasm (66,67). As shown here, most of the encapsidated RNA is incapable of aminoacylation (unrepaired) which indicates that it is unlikely that the aminoacylation function plays a role in infectious RNA (plus strand) synthesis or encapsidation. The aminoacylation function may, however, be involved in other steps of the viral infection cycle, e.g. negative RNA strand synthesis or protein synthesis, which occur outside the TYMV induced replication vesicles. The

results presented here show that it is likely that TYMV-RNA will be repaired and aminoacylated where active NT and VTS are present, as in the cytoplasm. Further progress in determining the function of the TYMV-RNA valine acceptor activity will require work with other enzymes involved in the TYMV infection cycle and in vivo studies.

The fact that TYMV-RNA is an excellent VTS substrate in spite of its unusual structural features gives it particular importance as a substrate which can be used effectively to elucidate the mechanisms of the tRNA recognition and catalysis by aminoacyl tRNA synthetases and other tRNA enzymes. Comparative studies using TYMV-RNA and valine tRNAs are likely to identify specific binding sites and structural features of tRNA required for enzyme recognition and catalysis which otherwise would be difficult to determine.

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AMINOACYLATION KINETICS AND SPECIFICITY
FOR VIRAL GENOMIC RNAS

by

Robin Clark

(ABSTRACT)

The esterification of amino acids to the tRNA-like structures of viral genomic RNA was studied as catalyzed by amino acyl tRNA synthetases from E. coli, yeast, bean and rat. Peanut stunt virus RNA was esterified with tyrosine in the presence of bean tyrosyl-tRNA synthetase to a maximum of 22 mole percent (average MW = 1×10^6). The rate of TYMV-RNA aminoacylation was extensively studied. In the presence of enzymes from E. coli, yeast and bean, the rate of aminoacylation was inhibited 4 fold by 47.5 mM KCl. In the presence of rat enzyme a 1.5 fold increase in rate was observed. In identical studies using tRNA, added KCl generally favored aminoacylation of tRNA by the homologous enzyme but disfavored heterologous reactions.

The kinetic parameters (K_m and V_{max}) of TYMV-RNA aminoacylation in the presence of valyl-tRNA synthetases

from the four sources were determined and compared to results obtained for yeast and E. coli tRNA controls. TYMV-RNA was found to be a uniquely competent and versatile substrate as compared to tRNA. The K_m of yeast valyl-tRNA synthetase for TYMV-RNA (7 nM) is the second lowest K_m reported for any aminoacyl tRNA synthetase. Kinetic studies using TYMV-RNA with or without the 3' terminal AMP indicate that this AMP is involved in the binding of TYMV-RNA to yeast valyl-tRNA synthetase.