DEVELOPMENT AND CHARACTERIZATION OF CELL-FREE AMINO ACID INCORPORATION SYSTEMS FROM CELLVIBRIO GILVUS

by

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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute
in candidacy for the degree of

MASTER OF SCIENCE

in

Biochemistry and Nutrition

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February, 1967

Blacksburg, Virginia

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III. INTRODUCTION

Although exciting developments on the characterization of subcellular systems from bacteria for protein biosynthesis have been achieved, most of them are based on the organism Escherichia coli, an intestinal bacterial species. Present work reports a subcellular system which was derived from Cellvibrio gilvus, an aerobic cellulolytic bacterium, and which was active with respect to amino acid incorporation into hot trichloroacetic acid (TCA) insoluble material. The development and investigation of the characteristics of this amino acid incorporation system from Cellvibrio gilvus will be an essential step for further studies on the elucidation of the mechanism for the biosynthesis of cellulases, which are extracellular enzymes produced by this bacterium.

It should be admitted here that the cell-free amino acid incorporating systems available to us are not ideal for the study of protein synthesis. To study some of the problem of regulation and control of protein synthesis, the formation of a particular protein will be necessary. In this sense bacterial preparations have, in general, been more successful in satisfying the requirement for the net synthesis of a specific protein. The advantage of choosing the synthesis of a particular enzyme (in present work, cellulase) for studying the general mechanism of protein biosynthesis may lie

in the fact that protein synthesis, even in very small amounts, can be readily detected by measuring the increase in enzyme activity.

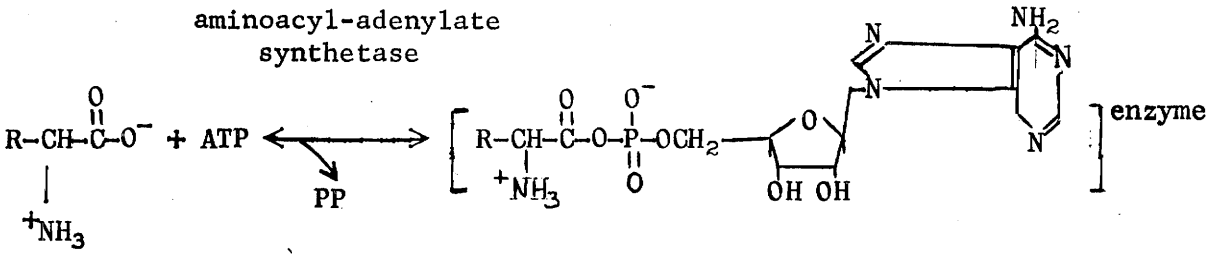
The importance and vital role of cellulolytic bacteria in nature can be found in a vast number of fields (24)--cellulose metabolism in lower plants, nutrition and physiology of ruminants, soil fertility, waste disposal, and the preparation of useful food products from normal waste materials. In Cellvibrio gilvus this inducible cellulase activity is the result of the synergistic activity of several cellulases whose specificity has been investigated by Storvick et al. (29). Accordingly investigations on the mechanism and location of the biosynthesis of these inducible, extracellular cellulases in a bacterium may be helpful in understanding the mechanism of cellulolytic microbial action in nature.

Cellvibrio gilvus was first isolated by Hulcher (24) from fresh bovine feces and given the species name of gilvus because of its pale yellow pigment. Since then studies have been made on the cellulase system of this bacterium (25, 26, 27, 28, 29, 30). The cellulase system of this Cellvibrio gilvus has been resolved into four distinct components by starch zone electrophoresis. The four components are known to cleave successively cellobiosyl or cellotriosyl moieties from the end of the cellulose chain.

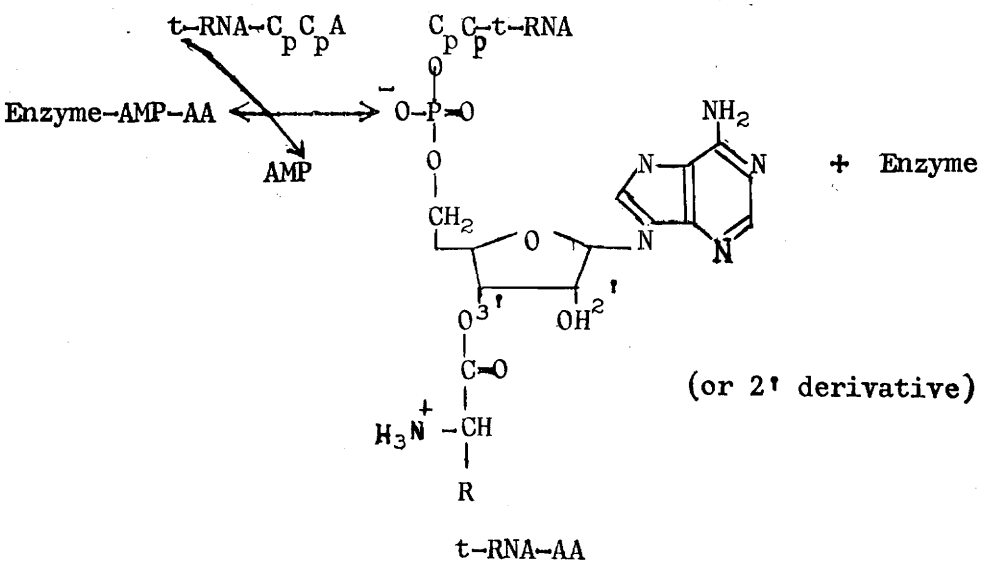
Since the pioneer work of Zamecnik and Hoagland and their coworkers (31, 32, 33, 34, 154) on the scheme of cell-free protein synthesis, an avalanche of work has been reported in this area.

Schematic sequential steps and the effect of some factors are outlined in Figure 1 and discussed in the next paragraph. The literature review and the discussion of this cell-free amino acid incorporation into enzyme protein will be reviewed according to the following sequence. The following abbreviations will be used: ATP (adenosine triphosphate), AMP (adenosine monophosphate), GTP (guanosine triphosphate), t-RNA (transfer ribonucleic acid), PP (inorganic pyrophosphate, AA (amino acid), t-RNA-AA (t-RNA charged with amino acid), m-RNA (messenger ribonucleic acid).

Stage I. Activation of amino acids



Stage II. Transfer of activated amino acids to t-RNA

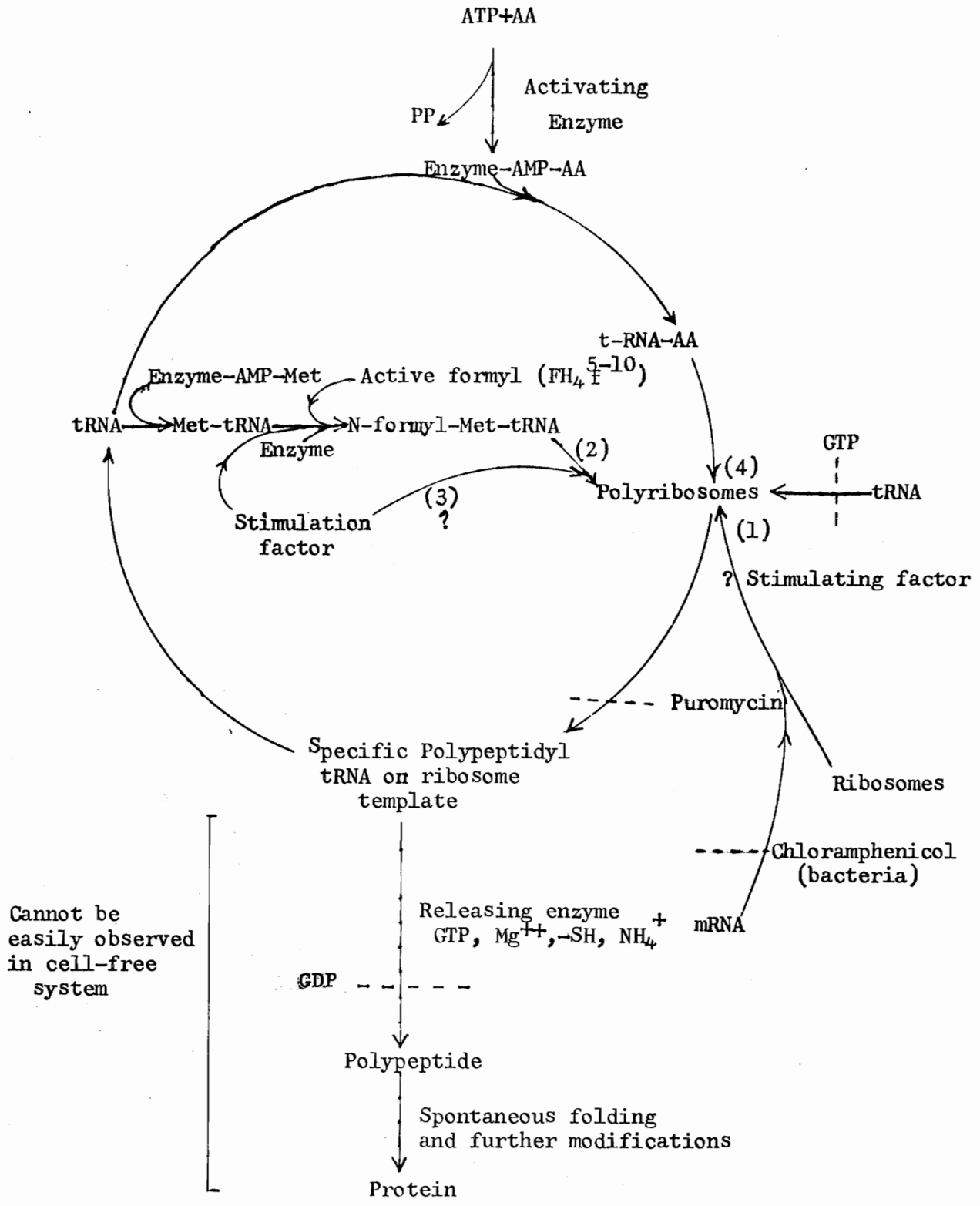


Stage III. Transfer of amino acids from t-RNA-AA to protein .

Stage IV. Release of polypeptide chain, folding, further modifications into well-defined protein as polypeptide chain grows.

Actually in cell-free systems itis difficult to observe Stage IV, but Stages I-III can be easily detected.

Figure 1. Schematic outline of the sequence of cell-free protein synthesis and the point of action of certain factors.



* Met-tRNA (methionyl-tRNA)

(1), (2), (3), and (4) show the order of reactions.

IV. REVIEW OF LITERATURE

A. Activation of amino acids:

Thermodynamic considerations of the reaction,

amino acids $\rightleftharpoons$ peptides

led to the prediction that amino acid incorporation into peptides requires coupling with an energy yielding reaction (34, 35). After many investigations on the requirement for energy coupling (36, 37, 38, 39, 40, 41, 42, 43, 44, 45), including the evidence that synthesis of enzymes in bacteria and in yeast depended on phosphorylation (46, 47), adenosine triphosphate (ATP) was shown to be required for protein synthesis in cell-free systems (48, 49, 50, 51). Enzymes, activating L-amino acids through a reaction with ATP and catalyzing the liberation of pyrophosphate and the formation of aminoacyl adenylate, were found in all kinds of animal and plant tissues and in microorganisms (52). Activation of all amino acids was observed in bacteria by Nisman et al. (54, 55). They reported that there were twenty enzymes, each one specific for one of the amino acids found in proteins. Hydroxamate assay and pyrophosphate-AA exchange method were chiefly used to study the amino acid activating enzymes (56). The specificity of these amino acid activating enzymes has been reported to be high but not absolute (56). Keller and Zamecnik (57) observed that components,

precipitating together when the 100,000 x g supernatant of disrupted cells was brought to pH 5, were required for amino acid incorporation. The presence of amino acid activating enzymes in this fraction was suggested by Hoagland et al. (58). In microorganisms, considerable amounts of activating enzymes were sometimes found, trapped in the cytoplasmic membrane fraction by bits of coagulated cytoplasm.

The activation mechanism of amino acids was first studied on microbial cells by DeMoss and Novelli (59). Discovering ^{32}PP-exchange with ATP and measuring individual contribution of each amino acid to the ^{32}PP-exchange in the following reaction,

$$AA + ATP^{32} \rightleftharpoons AA\text{-}AMP + {}^{32}PP$$

they concluded that each amino acid had its own specific activating enzyme. A second method to demonstrate and estimate the extent of the production of activated amino acids was a spectrophotometric measurement at an absorbancy 540 mμ due to the brown complex which was produced by the addition of acidified ferric chloride to hydroxamate, which was formed by the reaction of the activated amino acids with hydroxylamine, an efficient artificial acceptor of the acyl groups. Another method for measuring amino acid activation used radioactive substrates coupled with selective chromatography on ion exchange paper (61). The observation that some activation enzymes caused a rapid formation of hydroxamates although they catalyzed the

PP-exchange very poorly (62, 63) led to the suggestion that hydroxyamine reacted with t-RNA-AA rather than with E-AMP-AA.

Aminoacyl adenylate has an activated mixed acid-anhydride bond formed by condensation of the carboxyl grouping of the amino acid and 5'-phosphate of adenosine monophosphate. The structure of this aminoacyl adenylate complex has been proved by comparison of synthetic samples and material isolated from the purified activating preparation (60). The active centers of these activating enzymes were suggested to contain -SH (thiol) groups, for p-chloromercuribenzoate, monoiodoacetate, azide, and oxygen inhibit the enzymes, whereas reduced glutathione or mercaptoethanol (108) protect them.

B. Transfer of activated amino acids to t-RNA.

The enzyme bound nature of the intermediate, aminoacyl adenylate, has been established (64, 65, 66, 69, 68). The t-RNA was shown to accept the activated amino acid by Holley's (69) observation of a ribonuclease sensitive, amino acid-dependent exchange of AMP with ATP, and by the demonstration that activated amino acid is transferred to t-RNA (70). There is at least one specific t-RNA for each amino acid. Each t-RNA is a single polynucleotide chain composed of some eighty nucleotides (molecular weight ca. 25,000-30,000) found usually in the supernatant fraction of disrupted cells. The chain is believed to be folded upon itself, forming hair pin-shaped, double-stranded helical regions which alternate with single stranded areas.

At one end of the chain free 2' and 3' hydroxyl groups of the terminal nucleoside (adenosine) are exposed allowing one of these to accept the amino acid directly from the enzyme-bound aminoacyl adenylate. The terminal base sequence of nucleotides is: cytidylic acid, cytidylic acid and adenosine (abbreviated to t-RNA-$_{p}C_{p}C_{p}A$). Berg et al. (71) established that the same enzyme that activates the amino acid also catalyzes the transfer to t-RNA by observing that the ratio of methionine-activation to methionyl-t-RNA formation was constant throughout an extensive (100-fold) purification of the methionine activating enzyme from E. coli. Since the PP-ATP exchange was 100 times as active as the transfer to t-RNA, the transfer reaction was assumed to be the rate determining step. Both reactions were reversible. Ribonuclease inhibited the formation of AA-t-RNA by degrading the t-RNA.

C. Transfer of amino acid from aminoacyl transfer ribonucleic acid (AA-t-RNA) to protein:

It has been established that aminoacyl transfer RNA compounds transfer amino acids to the ribosomes, where they are integrated into growing polypeptides as shown first by Hoagland et al. with rat liver homogenates (70). Ribonucleoprotein particles, ribosomes, were first discovered in bacteria by Schachman, Pardee and Stanier (73). Ribosome particles were shown to be associated with cytoplasmic membrane in bacteria (158, 159). Ribosomes from various sources have a rather

constant chemical composition, i.e., about 40—50% RNA and 60-50% protein for 80 S ribosomes (yeast, 162, 163, 164; plant, 165, 166, 167; animal, 168, 169, 170, 171) and 60-65% RNA and 40-35% protein for 70 S ribosomes (bacteria, 13, 172). The protein contains large amounts of the basic amino acids and the dicarboxylic amino acids, half of which are in the amide form. Although the proteins have an overall basicity, this is outweighed by the acidity of RNA moiety, leaving ribosomes with a net negative charge. Therefore, aggregation or dissociation of ribosomes is dependent upon the concentration of magnesium ion (Mg^{++}) or polyamines such as spermidine, spermine or putrescine. The ribosomes of yeasts, plants and animals have a sedimentation coefficient of 80 S (121, 173), whereas those of bacteria have been found to possess a sedimentation coefficient of 70 S (173). These particles dissociate into several classes of subunits at low magnesium concentration. For example, 80 S particles may be reversibly dissociated into 60 S and 40 S subunits, and 70 S into 50 S and 30 S. Recently Hosokawa et al. (103) reported that smaller sub-particles 40 S and 23 S (and split proteins), which were derived from the dissociation of ribosome subunits (50 S and 30 S), were inactive in protein synthesis, upon mixing the split proteins with those sub-particles ribosomes were reconstituted, which were

active in in vitro polypeptide synthesis. This supported the hypothesis that at least part of the assembly of the ribosomes is an orderly, self-assembly process which is not directed by other pre-existing cell structures. In other words, information for the correct assembly must be contained in the structure of the sub-components.

This stage, the transference of amino acid from aminoacyl transfer RNA to ribosomal template, is an almost completely unknown one except for the requirement of guanosine triphosphate (GTP). The role of GTP is still not clear. It was reported that GTP is broken down to GDP and inorganic phosphate and that its energy might be coupled to further activation steps and/or to the release of protein (74). Recently it has been reported that the inhibition effect of deacylated t-RNA is competitively reversed by GTP and a soluble enzyme fraction and that GTP increases the amount of phenylalanine-t-RNA bound to the ribosomes in the presence of t-RNA. Transfer enzyme (76, 77) isolated from the soluble fraction of liver homogenate was reported to be involved in the transfer process.

Inhibitors of amino acid transfer have also been reported. Chloramphenicol (chloromycetin) is known to be a specific inhibitor for the bacterial ribosomal system (79). This seems to combine with the ribosomes preventing messenger-RNA binding or inactivating incoming messenger-RNA (m-RNA) so that aminoacyl-t-RNA has no binding site. Puromycin inhibits amino acid transfer in all cell-free

systems (80). During the growth of peptide chains on the ribosomal template, the t-RNA residue which is attached to carboxyl end of the C-terminal amino acid holds the growing chain onto the surface of the ribosomal template, presumably by base-complementary hydrogen bonding with the m-RNA template. Since puromycin structure is similar to that of aminoacyl t-RNA, the former may be able to replace the t-RNA residue, thereby releasing the polypeptide-t-RNA short of protein completion.

Actually the increase of soluble protein can be observed under the effect of puromycin. This is also evident for the stepwise polymerization of amino acids. Chloramphenicol seems to act at a stage before puromycin in systems from bacteria, for chloramphenicol prevents the release of peptides by puromycin when these two inhibitors are used together (161). Erdös and Ullman (81) reported that streptomycin inhibits the transfer of amino acids from t-RNA to protein in a sensitive strain but not in a resistant strain. Aminoacyl-tRNA's can be regarded as adaptors which will carry the activated amino acids to the m-RNA template and locate them at their correct positions by forming hydrogen bonds with the complementary bases on the m-RNA template according to the Crick's scheme (82, 155) for nucleic acid structure. Many ribosomes are united by a strand of m-RNA and the ribosomes pass in turn over the coding triplets, and the m-RNA is

released when its protein chain is completed (83). Initial support for this scheme began with the demonstration by Matthaei and Nirenberg (84) that E. coli cell-free systems, which were pre-treated with deoxyribonuclease (DNAase) to prevent endogeneous production of m-RNA, were highly (thousand-fold) stimulated by the addition of polyuridylic acid. Then Spyrides and Lipmann (85) showed that many ribosomes were bound to a single poly U chain and that only those ribosomes bound in clusters to poly U chains were active in peptide synthesis in poly U directed polyphenylalanine synthesis. This RNA strand could be broken by brief treatment with ribonuclease (RNAase), forming single ribosomes. The genetic coding sequences in m-RNA have been reported to be non-overlapping, triplet codes. It has been pointed out that several codons for leucine exist: CUU, CUC, UUA, UUG. Thus, the codes are degenerate. Not only are the codes degenerate, but also can be ambiguous in these cell-free systems. For example, UUU, the code word for phenylalanine, can also code for leucine. Magnesium ions, low temperature, amino glycoside antibiotics, and polyamines have been reported to produce mistakes in translation of the genetic code in sub-cellular bacterial systems (86, 156, 157). Other factors such as organic solvents (87), pH (88), and concentration of amino acids, or t-RNA (87, 88) also have influence on the specificity of polynucleotide-dependent amino acid incorporation into polypeptides. The translation mechanism of mammalian cells functions with higher fidelity in vitro than that of bacteria (104).

The discovery of N-formylmethionyl-t-RNA in E. coli protein by Marcker and Sanger (90) led to the postulation that N-formylmethionyl-t-RNA was the initiator of the polypeptide chain. This compound cannot be used for polypeptide chain elongation, as the amino group methionine is formylated, but can function in the initiation of polypeptide chains. Actually Adams and Cappecchi (3) observed the incorporation of formyl groups in N-formylmethionyl-t-RNA into at least two, if not all, of the several proteins coded by the phage R17 RNA, indicating the possibility that formylmethionyl-t-RNA initiated the synthesis of all of the R17 specific proteins. Similarly Webster and Lingrel (74) discovered N-formylmethionine masked protein and fragments in an in vitro protein synthesizing system from E. coli when RNA from a phage mutant was added. As a possible mechanism for initiation of protein synthesis, Nakamoto and Kolakofsky (91) suggested that if the ribosome-messenger association was sufficiently stable, proper alignment of aminoacyl-t-RNA's could take place on the ribosome, and polypeptide synthesis could occur without a modified aminoacyl-t-RNA, i.e., N-formylmethionyl-t-RNA, but as the affinity of the messenger RNA for the ribosome decreased, conditions must be altered in order to stabilize the chain-initiating complex with aminoacyl-t-RNA. For example, higher concentration of megnesium was required under such conditions. Actually they found that with low magnesium

concentrations and a high temperature, synthesis of polypeptides with synthetic messenger RNA was almost completely dependent on either N-formyl-methionyl-t-RNA or peptidyl-t-RNA, which might form a chain-initiating complex with messenger and ribosomes because of its resemblance to peptidyl-t-RNA. Existence of an initiation signal for protein synthesis and the requirement of factor(s) other than the transfer enzymes for the translation of this signal has been established, e.g., a factor is necessary for MS2 RNA and TMV-RNA (105), f_2 RNA (5), and two factors for MS2 RNA, Q β RNA, or TMV-RNA (89). Very recently a stimulation factor associated with DNA, which is present in cell extracts from E. coli has been reported to be nonspecific for a given RNA but active with T_4 m-RNA, and TMV-RNA (106). However, different postulations have been raised about the mode of action of the stimulation factor. Stanley et al. (89) observed in their cell-free system from E. coli that some synthetic polynucleotides (poly A, poly U) promote polypeptide synthesis equally well with crude or purified ribosomes, whereas natural m-RNA's are effective only in systems containing crude ribosomes, which may contain some factor(s) required for the translation of a natural messenger. They suggested that one of the possible functions of the factor(s) was the formation of the first peptide bond between formylmethionine, esterified to methionine-t-RNA_2, and the following amino acid, in light of the fact that factors exert their stimulatory effect in the presence of an

excess of transfer enzymes and that they are not involved in the activation or formylation of methionine or in the binding of methionyl-t-RNA to ribosomes. On the other hand, Eisenstadt and Brawerman (5,6) showing the need for a stimulating factor for f_2 RNA in the high-speed supernatant of E. coli, suggested that this stimulation factor could be concerned with the ribosome-viral RNA interaction and that ribosomes must undergo a reaction promoted by the factor in order to become capable of interacting with m-RNA. They postulated that the stimulation factor must be of the same species as the ribosomes and interact directly with the ribosomes. This interpretation is compatible with the experimental observation in the mixed-system of E. coli ribosomes plus Euglena gracilis supernatant, which was not responsive to f_2 RNA. If chain initiation by N-formylmethionine was a prerequisite for the protein synthesis, Euglena supernatant should have been capable of formylating methionine. However, they did not rule out the possible role of the stimulation factor in the formylation reaction of methionyl-t-RNA, for pre-incubation of the cell-free system with the factor would lead to formylation of methionine and would result in a more rapid response to viral RNA.

D. Release of finished polypeptide chain, folding, and further modifications into well-defined protein molecule:

During protein synthesis, the nascent polypeptide chain remains esterified to t-RNA on the surface of the ribosome (94, 95, 96, 97).

After chain completion, the residual t-RNA molecule must be cleaved off the carboxyl-group end of the chain by an energy requiring process, either still bound to the ribosomes or in the supernatants after release of the protein-t-RNA. There also have been some suggestions that nucleotide sequences which do not recognize a specific aminoacyl t-RNA, i.e., nonsense codons, act as chain terminating signals (95, 98). Studies on the release mechanism in terms of well-defined enzymes is very difficult, whereas it is easy to observe incorporation of amino acids into protein material (94). An ATP-generating system, Mg^{++}, GTP, NH_4^+, and the releasing enzyme (found in the soluble enzyme fraction isolated from the 100,000 x g supernatant) have been reported as necessary for chain release (160). Siekevitz (100) suggested the involvement of polyamines in this process. These completed polypeptides must fold into well-defined protein molecules in order to possess enzymic activity, serological characteristics, and other important physiological or biochemical properties. Folding generally results in the secondary and tertiary structure of the protein. Current theory attributes this folding implicitly to the amino acid sequence, occurring spontaneously after a sufficient amount of the polypeptide chain has been formed. Cellular environment such as pH, ionic strength, nature of the ions may also be important (101). In other words, enzymes contain amino acid sequences so uniquely constructed that the formation of active centers and three-dimensional conformations follow automatically and

reproducibly without additional genetic information (102). Sometimes association with prosthetic groups or other further transformation (for example transformation of zymogen into active enzyme) may occur.

EXPERIMENTAL

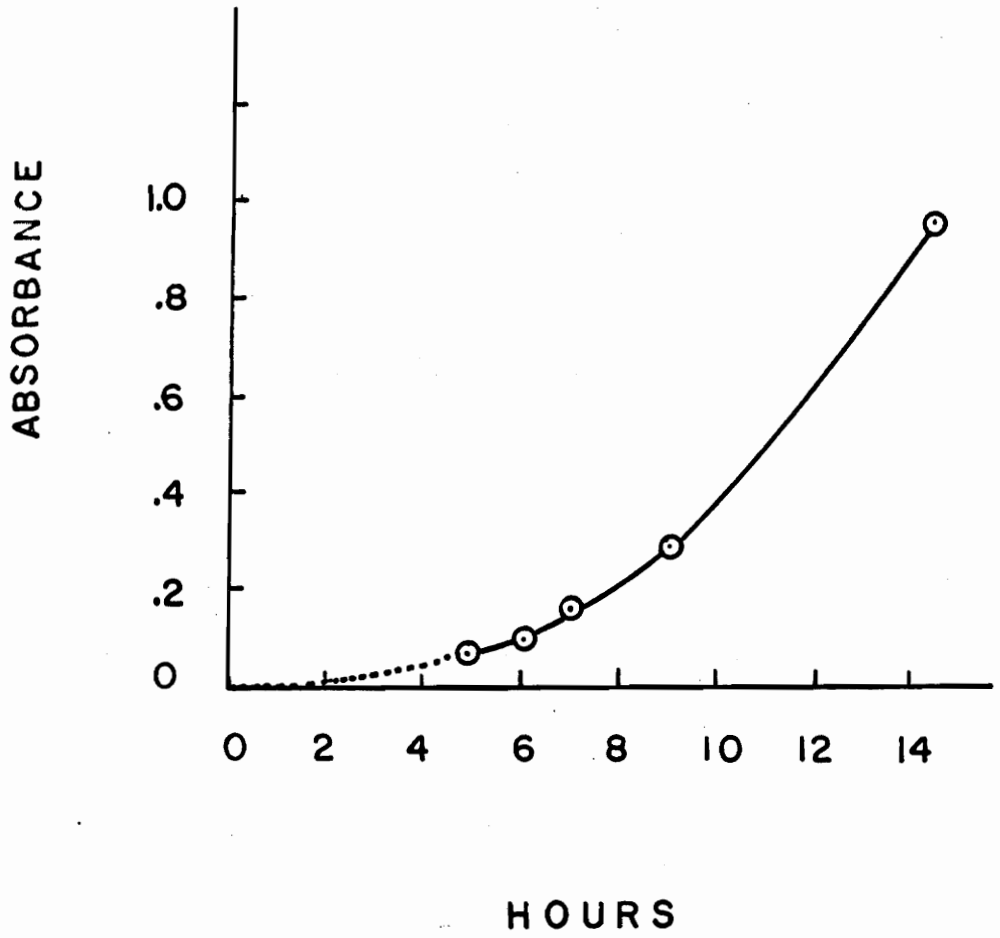
METHODS AND MATERIALS

Growth and harvest of cells:

The culture medium of Cellvibrio gilvus was an aqueous solution of 16 l, which had been autoclaved at 125° C for 90 minutes, containing 32 g glucose (purchased from J. T. Baker Chemical Company, Phillipsburg, N. J.), 16 g yeast extract (from Difco Laboratories, Detroit, Michigan), 80 g casein hydrolysate (from General Biochemicals, Chagrin Falls, Ohio), 4 g of potassium chloride (Baker analyzed reagent), sodium nitrate (Baker analyzed reagent) and ammonium chloride (Baker analyzed reagent), 8 g potassium hydrogen phosphate (Baker analyzed reagent), 35.2 ml of 1 N hydrochloric acid (Baker analyzed reagent), and 32 drops of antifoam ("Nalco", Nalco Chemical Company, Chicago, Ill.). The cells were grown in an incubator room at about 28° C with vigorous aeration for about 20 hours, at which time the cells were in their log phase of growth—an absorbancy of 0.2 at 540 mμ, measured by a Bausch and Lomb Spectronic No. 20 Colorimeter. The growth curve of the cells is shown in Figure 2. The cells were harvested in a cold room using a Sharples Super Centrifuge at 30,000 r.p.m. and a flow rate of 270 ml per minute. Collected cells were washed twice by suspension in 80 ml of cold standard buffer which is composed of 0.02 M Tris (tris-hydroxymethylaminomethane)-HCl (pH 7.8), 0.009 M magnesium acetate, 0.07 M potassium chloride, and 0.006 M mercaptoethanol, and centrifuged in a Sorvall Model RC-2 Centrifuge at 15,000 x g for 5 minutes. The yield of cells was about 20 g per 16 l.

Figure 2. Growth Curve of *Cellvibrio gilvus*.

Absorbance at 540 mμ was measured by a Bausch and Lomb Spectronic No. 20 Colorimeter. Composition of culture medium and method is reported in text.


ABSORBANCE
1.0
.8
.6
.4
.2
0
0
2
4
6
8
10
12
14
HOURS

Fractionation of cells:

All of the following manipulations were performed in the cold. The cells were disrupted by sonic oscillation for 45 seconds with a Raytheon Sonic Oscillator, Model DF 101 at maximum output current after resuspending the collected cells in standard buffer to a cell concentration of 0.3 g wet cells per ml. The suspension of broken cells was then centrifuged twice at 27,000 x g for 15 minutes. The supernatant was centrifuged in a Spinco Model L Ultracentrifuge at 100,340 x g for 60 minutes. The sedimented yellowish ribosomal pellet was washed with standard buffer and recentrifuged at 108,000 x g in the SW-39 L rotor for 60 minutes. This washed ribosomal pellet was suspended in standard buffer for the amino acid incorporation assay. The top two-thirds of the 100,340 x g supernatant was adjusted to pH 5 with 1N acetic acid using pH paper, and the precipitate was collected by centrifugation and dissolved in standard buffer, which was adjusted to pH 7.8 with 1N KOH. This fraction is referred to as "pH 5 fraction". Transfer-RNA was extracted from the 100,340 x g supernatant with phenol by the procedure of Nirenberg and Matthaei (1) (see Figure 3). The t-RNA preparation was negative to the biuret test and showed the characteristic adsorption spectrum of ribonucleic acid in a Beckman DB Spectrophotometer with a maximum absorbance at a wavelength of 260 mμ (Figure 4). The concentration of t-RNA was calculated by assuming $24A_{260}$ to be equivalent to a t-RNA concentration of 1 mg per ml (107). The cell fractionation procedure is summarized in Figure 3.

Figure 3. Schematic Representation of Cell Fractionation Procedure.

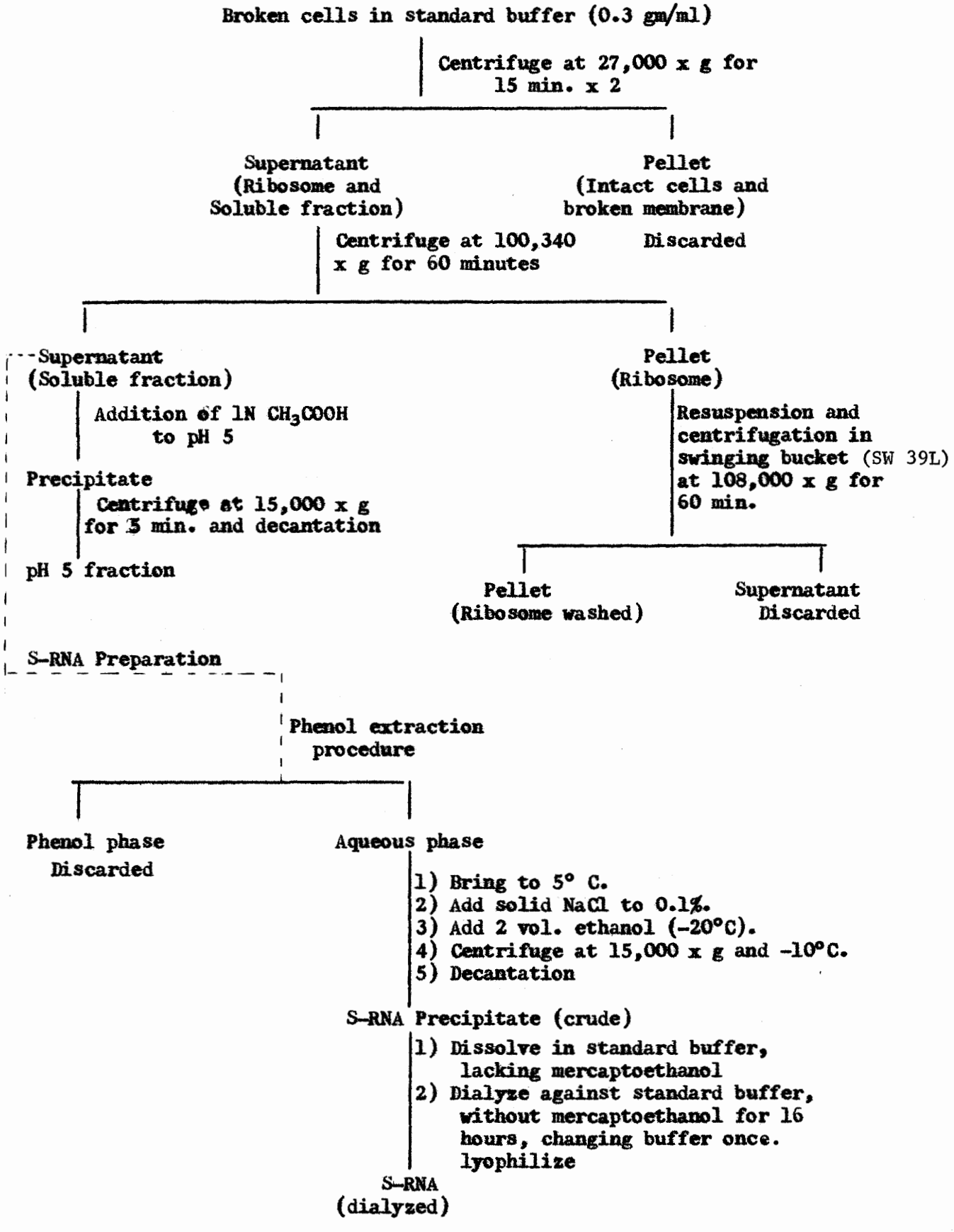
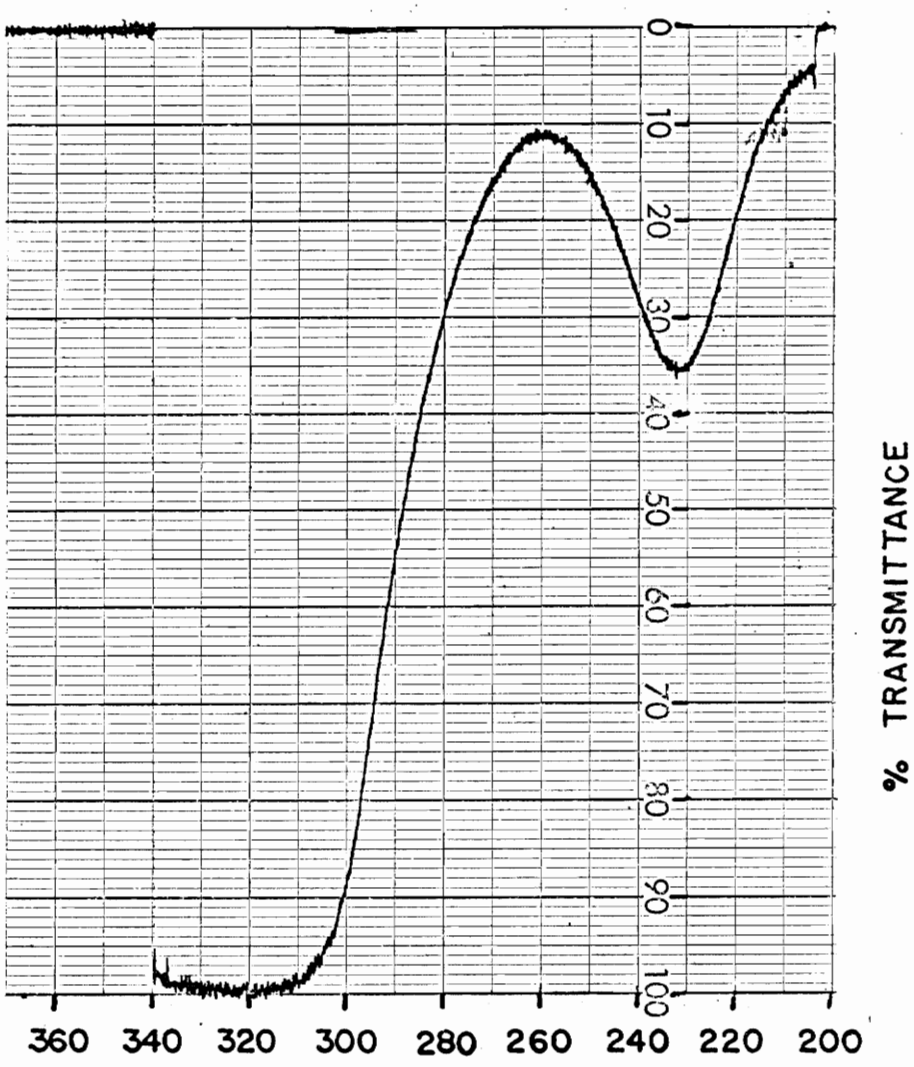

Broken cells in standard buffer (0.3 gm/ml)
Centrifuge at 27,000 x g for 15 min. x 2
Supernatant (Ribosome and Soluble fraction)
Pellet (Intact cells and broken membrane)
Discarded
Centrifuge at 100,340 x g for 60 minutes
Supernatant (Soluble fraction)
Addition of 1N CH_3COOH to pH 5
Precipitate
Centrifuge at 15,000 x g for 5 min. and decantation
pH 5 fraction
Pellet (Ribosome)
Resuspension and centrifugation in swinging bucket (SW 39L) at 108,000 x g for 60 min.
Pellet (Ribosome washed)
Supernatant Discarded
S-RNA Preparation
Phenol extraction procedure
Phenol phase Discarded
Aqueous phase
1) Bring to 5° C.
2) Add solid NaCl to 0.1%.
3) Add 2 vol. ethanol (-20°C).
4) Centrifuge at 15,000 x g and -10°C.
5) Decantation
S-RNA Precipitate (crude)
1) Dissolve in standard buffer, lacking mercaptoethanol
2) Dialyze against standard buffer, without mercaptoethanol for 16 hours, changing buffer once.
lyophilize
S-RNA (dialyzed)

Figure 4. Adsorption Spectrum of t-RNA Preparation, Isolated from Cellvibrio gilvus, in Beckman DB Spectrophotometer.

The t-RNA concentration used for this spectrum was 4 mg/ml.


0
10
20
30
40
50
60
70
80
90
100
% TRANSMITTANCE
360
340
320
300
280
260
240
220
200
WAVELENGTH, mμ

Amino Acid Incorporation:

Crystalline ATP (disodium salt), GTP (sodium salt), UTP (sodium salt), CTP (cytidine triphosphate, sodium salt) were purchased from P-L Biochemicals, Inc.; phosphoenolpyruvate (tricyclohexylammonium salt) and phosphoenolpyruvate kinase from C. F. Boehringer and Soehne Gmb H Mannheim; polyuridylic acid (3.9-7.1 S, ammonium salt) from Miles Chemical Company; DL-phenylalanine-1-^{14}C (0.10 mc/3.3 mg) from New England Nuclear Corporation; DL-leucine-1-^{14}C (54.6 mc/mM) from Volk Chemical Company; spermidine.3HCl, spermine.4HCl, and putrescine.2HCl from California Corporation for Biochemical Research; deoxyribonuclease (beef pancreas) and ribonuclease (beef pancreas) from Worthington Biochemical Corporation; chloramphenicol, a gift from Parke, Davis and Company, Detroit, Michigan; actinomycin D, a gift from Merck Institute, West Point, Pennsylvania; puromycin (dihydrochloride) from Nutritional Biochemicals Company.

The standard incorporation system is summarized in Table 1. The t-RNA, pH 5 enzyme, ribosome, polyuridylic acid (poly U) and ATP plus ATP-generating system were added in that order into glass tubes (13 x 100 mm). Incubation was started by the addition of the ^{14}C-amino acid. Tubes were incubated for 30 minutes at 30° C, and the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid (TCA) solution. The tubes were then heated in a boiling water bath for 15 minutes to solubilize any ^{14}C-aminoacyl-RNA. The

Table 1. Standard Incubation Mixture

1 mg. ribosomal protein	
1 mg. pH 5 enzyme protein	
100 µmoles NH_4Cl (ammonium chloride)	
0.1 µc ^{14}C-amino acid	Standard buffer
20 µmoles Tris-HCl (pH 7.8)	
9 µmoles Mg $(CH_3COO)_2$ (magnesium acetate)	
70 µmoles KCl (potassium chloride)	
6 µmoles CH_3CH_2SH (mercaptoethanol)	
2.3 µmoles ATP (adenosine triphosphate)	ATP plus ATP generating system (prepared fresh and brought to pH 7.8 with 1N KOH).
0.23 µmoles GTP (guanosine triphosphate)	
0.23 µmoles CTP (cytidine triphosphate)	
0.23 µmoles UTP (uridine triphosphate)	
5.6 µmoles PEP (phosphoenolpyruvate)	
320 µg pK (PEP kinase)	
0.4 mg. polyuridylic acid	*
0.6 mg. t-RNA	

Final volume was 1.0 ml.

*Added in case of phenylalanine incorporation test only.

precipitates collected after centrifugation and decantation were washed twice with 2 ml of cold 5% TCA solution, hot TCA solution, 96% ethanol, ethanol-chloroform-ether (2:1:1) and finally ether. The dried, washed precipitates were dissolved in concentrated formic acid, 0.3 ml per tube, plated onto aluminum planchets, dried and counted in a Nuclear Chicago gas flow counter, with a counting efficiency of 17.6%. Counts were corrected for background activity and converted to μμmoles of ^{14}C-amino acid incorporated per mg of ribosomal protein. Protein was determined according to the method of Folin-Ciocalteau using dialyzed bovine serum albumin as a standard and also using the nomograph (128) based on the extinction coefficients at wavelength 260 mμ and 280 mμ (143).

RESULTS AND DISCUSSION

Requirement for amino acid incorporation:

In Tables 2 and 3 are shown the requirements for the incorporation of ^{14}C-leucine and ^{14}C-phenylalanine, respectively, into hot trichloroacetic acid (TCA) insoluble material by the cell-free system from Cellvibrio gilvus. They show the absolute requirement for amino acid incorporation of ribosomes, pH 5 enzyme fraction, ATP plus ATP generating system and magnesium ions. These requirements are similar to those previously reported cell-free systems from various sources, i.e., bacteria (E. coli, 84, 117, 118, 119; Bacillus cereus, 108; Bacillus brevis, 115), yeast (Saccharomyces fragilis, 122; diploid of Saccharomyces fragilis and Saccharomyces dobzanskii, 111; Saccharomyces carlsbergensis, 124; Saccharomyces cerevisiae, 127), mammalian (rat liver, 125; rabbit reticulocyte, 113; rat brain, 20; rabbit brain, 217; higher plant (castor bean seedlings, 19; peanut and wheat embryo, 132; maize kernel, 133; spinach chloroplast, 134, tobacco leaves, 137; tobacco leaves chloroplast, 135, 136), other microorganisms (Euglena gracilis, 138; Crithidia oncopelti, 139). For optimum incorporation guanosine triphosphate (GTP) and ammonium chloride (NH_4Cl) were required. ^{14}C-phenylalanine incorporation was highly enhanced (thirteen-fold) by the addition of polyuridylic acid (poly U) in the presence of t-RNA from Cellvibrio gilvus. Addition of

Table 2

Requirements for ^{14}C-Leucine Incorporation by the Cell-free System Derived from Cellvibrio gilvus

Incorporation condition	^{14}C-Leucine Incorporation μμmoles/mg. ribosomal protein	% Incorporation
Complete system (30 min.)	31	100
Complete system (0 min.)	0.4	1.3
-ATP plus ATP generating system	4.0	13
-GTP	13.3	43
-NH_4Cl	24.8	80
-pH 5 enzyme fraction	1.9	6.1
-Ribosome	3	9.7

Composition of the reaction mixture is reported in Table 1. The reaction mixture was incubated for 30 minutes at 30° C.

Table 3

Requirements for ^{14}C-Phenylalanine Incorporation by the Cell-free System Derived from Gellvibrio gilvus

Incorporation condition	^{14}C-Phenylalanine Incorporation μμmoles/mg. ribosomal protein	% Incorporation
Complete system (30 min.)	359	100
-Poly U	27	7.5
-t-RNA	123	34.3
-Ribosome	86	24.0
-pH 5 enzyme fraction	62	17.3
-Mg^{++} (as acetate)	24	6.7

Composition of reaction mixture is reported in Table 1. The complete incorporation system contains 0.4 mg. polyuridylic acid and 0.6 mg. of t-RNA isolated from Cellvibrio gilvus. The reaction mixture was incubated for 30 minutes at 30° C.

t-RNA had no effect on ^{14}C-leucine incorporation (data not included), whereas it stimulated ^{14}C-phenylalanine incorporation in the presence of polyuridylic acid.

Effect of some antibiotics, deoxyribonuclease (DNAase) and ribonuclease (RNAase):

Table 4 contains the effects of several antibiotics, deoxyribonuclease (DNAase) and ribonuclease (RNAase) on the ^{14}C-leucine incorporation. Like all other cell-free systems [animal, (125, 140, 20, 113); plant, (134, 133, 132, 137); microorganisms, (84, 115, 118, 139, 108, 111, 113, 122)], ^{14}C -amino acid incorporation was almost completely inhibited by the addition of puromycin (97% inhibition by the addition of 0.1 mg/ml) and ribonuclease (99% inhibition by the addition of 0.88 mg/ml). Chloramphenicol, which is known to be inhibitory to cell-free systems from bacteria, (118, 115, 84, 108) but not on yeast, (111, 122, 127), other microorganisms, (138), plant, (132, 122, 142, 19) or animal, (113, 20, 125, 21), showed moderate (64%) inhibition, which was similar with E. coli cell-free systems (84). In some cell-free systems from plant origin (133, 134, 137) chloramphenicol was reported to be inhibitory. Spencer and Wildman (137) reported that amino acid incorporation into a cell-extract from tobacco leaves was strongly inhibited by the addition of chloramphenicol. They proved the absence of intact bacterial contamination in the cell-free system but did not check the contamination of bacterial cell-free systems

Table 4

The Effect of Some Antibiotics, Ribonuclease and Deoxyribonuclease on ^{14}C-Leucine Incorporation

Incorporation condition	^{14}C-Leucine Incorporation μμmoles/mg Ribosomal protein	% Inhibition
Complete system	31	0
+ Chloramphenicol (0.1 mg/ml)	11	64
+ Puromycin (0.1 mg/ml)	1	97
+ Actinomycin D (0.1 mg/ml)	30	3
+ Ribonuclease (0.88 mg/ml)	0.4	99
+ Deoxyribonuclease (0.02 mg/ml)	29	6

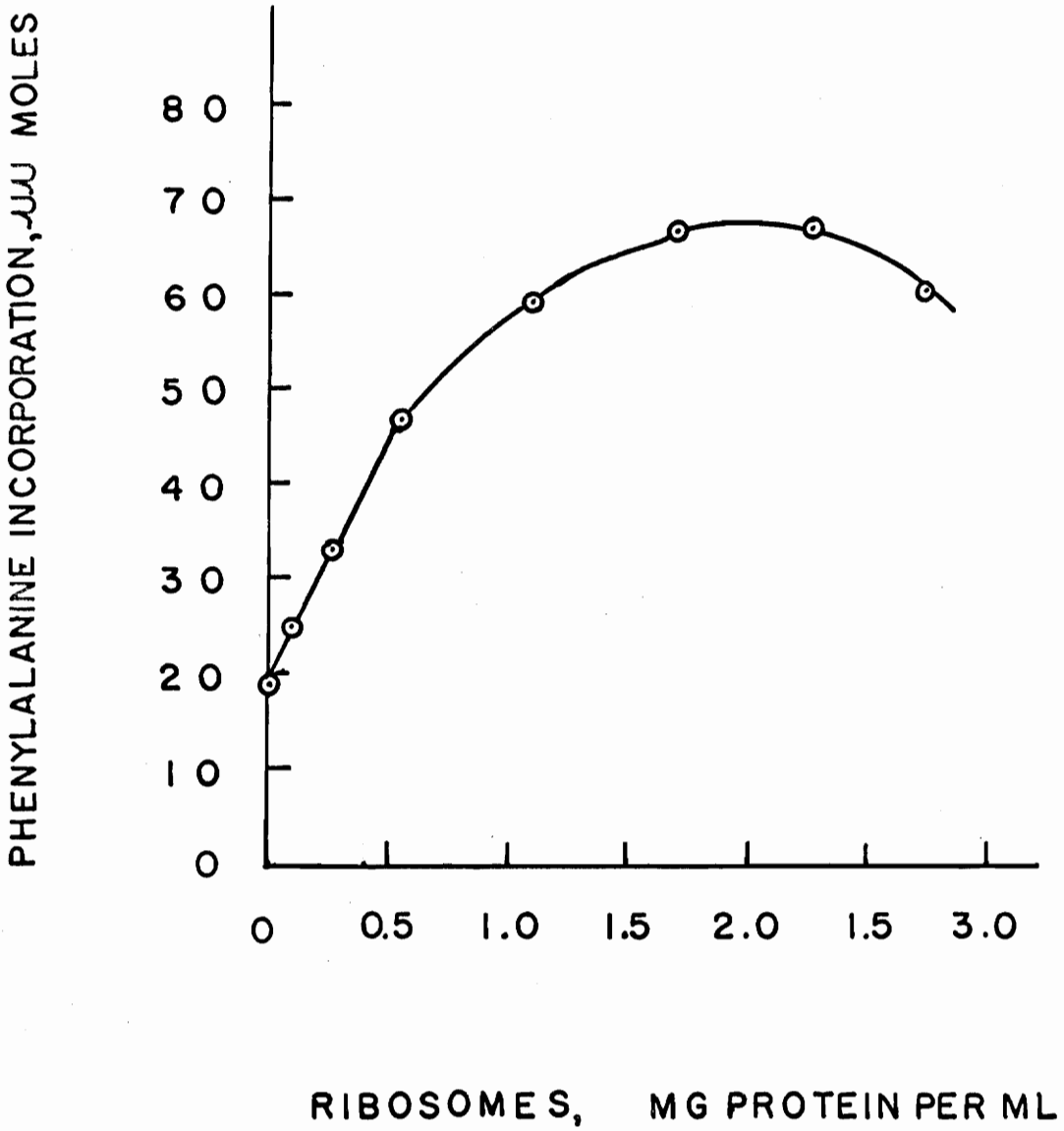
Indicated amounts were in addition to the reaction mixture of the complete system, whose composition is shown at Table 1. The reaction mixture was incubated for 30 minutes at 30° C.

which might have been produced from contaminated bacteria during the preparation procedures of the cell-free system of tobacco leaves itself. Therefore, it is uncertain whether chloramphenicol inhibition was due to bacterial contamination or to cell-free preparation of tobacco leaves itself. Rabson and Novelli (133) also reported chloramphenicol inhibition on their cell-free preparation from maize kernels, in which the chloramphenicol concentration required to inhibit the incorporation process was ten-twenty times as great as that required to inhibit protein synthesis in bacterial cell-free systems. Both deoxyribonuclease (DNAase) and low concentrations of actinomycin D have been known to have no significant effect on the cell-free systems cited above. However, high concentration of actinomycin D might cause mistakes in the translation of genetic code due to its polycationic character and similarity with polyamines and magnesium ions (109, 104).

Effect of ribosome concentration:

Figure 5 shows the effect of ribosomal protein concentration on polyuridylic acid (poly U) directed ^{14}C-phenylalanine incorporation. The incorporation was proportional to the amount of ribosomal protein concentration over the range of 0-0.6 mg. Above 0.6 mg of ribosomal protein concentration the linearity fell off and above 2 mg of ribosomal protein concentration an increase in the amount of ribosomal protein caused a decrease in ^{14}C-phenylalanine incorporation.

Figure 5. Effect of the ribosomal protein concentration on poly-uridylic acid-directed ^{14}C-phenylalanine incorporation. Reaction mixtures are reported in Table 1. Reaction mixtures were incubated for 30 minutes at 30° C.


PHENYLALANINE INCORPORATION, μμ MOLES
80
70
60
50
40
30
20
10
0
0
0.5
1.0
1.5
2.0
1.5
3.0
RIBOSOMES, MG PROTEIN PER ML

Non-linearity is probably due to the fact that polyuridylic acid becomes limiting. The inhibition effect at high concentration of ribosome may also be due to the fact that at high ribosomal concentration the concentration of ribonucleases associated with the ribosome may become significant (2).

In Figure 6 are presented the data for the effect of "pH 5 enzyme" (See Methods) on polyuridylic acid directed ^{14}C-phenylalanine incorporation. The ^{14}C-phenylalanine incorporation was linearly proportional to the amount of pH 5 enzyme protein in the range of 0-1.1 mg of protein. At higher concentrations of pH 5 enzyme, the stimulatory effect decreased but no inhibition was observed over the concentration range used in these experiments.

Effect of poly U and t-RNA concentration:

The ^{14}C-phenylalanine incorporation was increased linearly over the range of 0-0.3 mg of polyuridylic acid (Figure 7). A further increase of polyuridylic acid concentration caused a slight inhibition of incorporation. Recently So *et al.* (7) reported a similar inhibition of phenylalanine incorporation on the cell-free ribosomal system from *E. coli* by high polyuridylic acid concentrations. This inhibition was not due to a magnesium limitation because of the degree of inhibition by high polyuridylic acid concentrations was greater with increasing concentrations of magnesium ions. Jones *et al.* (8) and Martin and Ames (9) reported that bacterial ribosomes require

Figure 6. Effect of the Concentration of pH 5 Enzyme Protein on ^{14}C-Phenylalanine Incorporation.

Composition of reaction mixtures is shown in Table 1. Reaction mixtures were incubated for 30 minutes at 30° C.

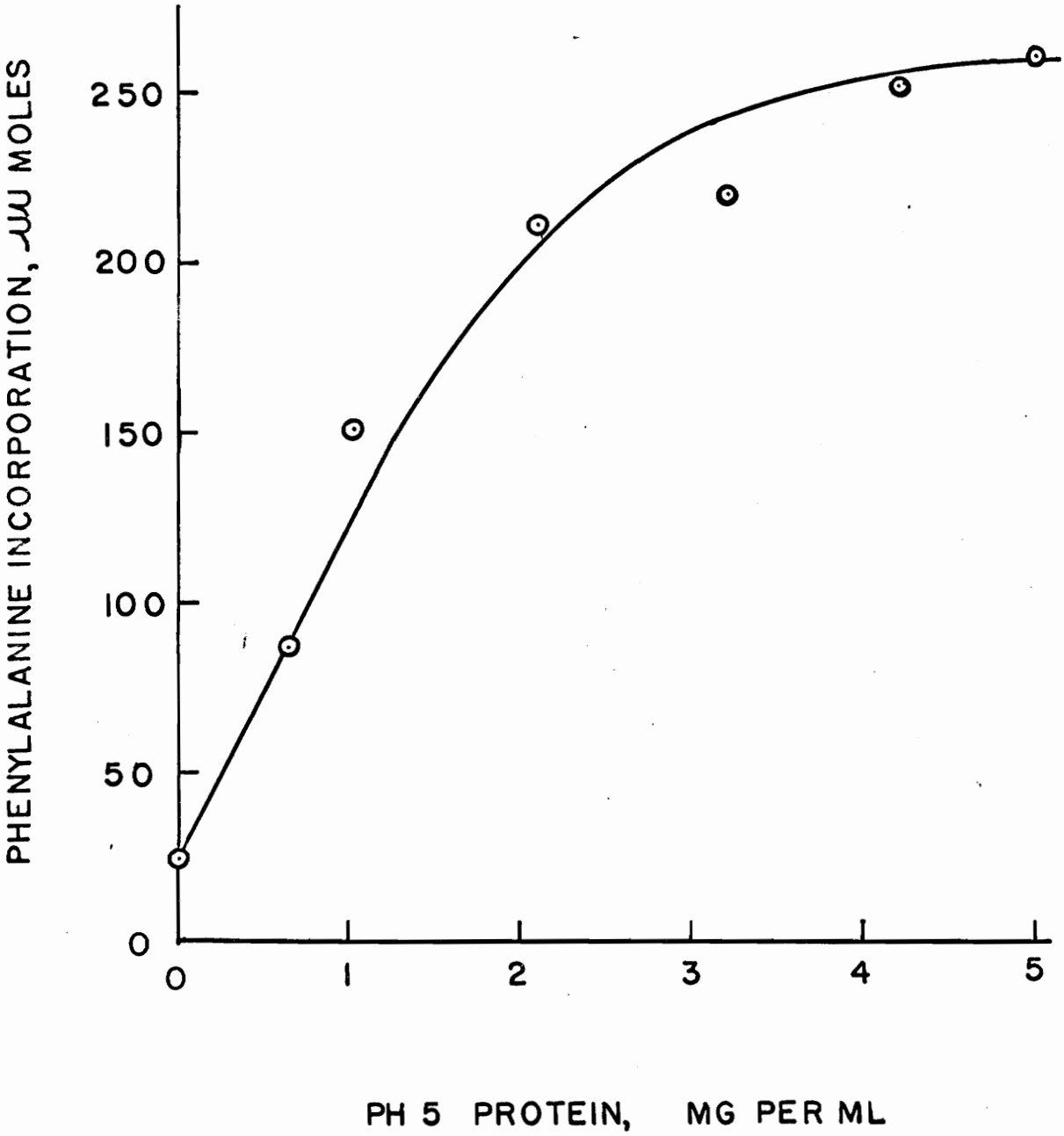
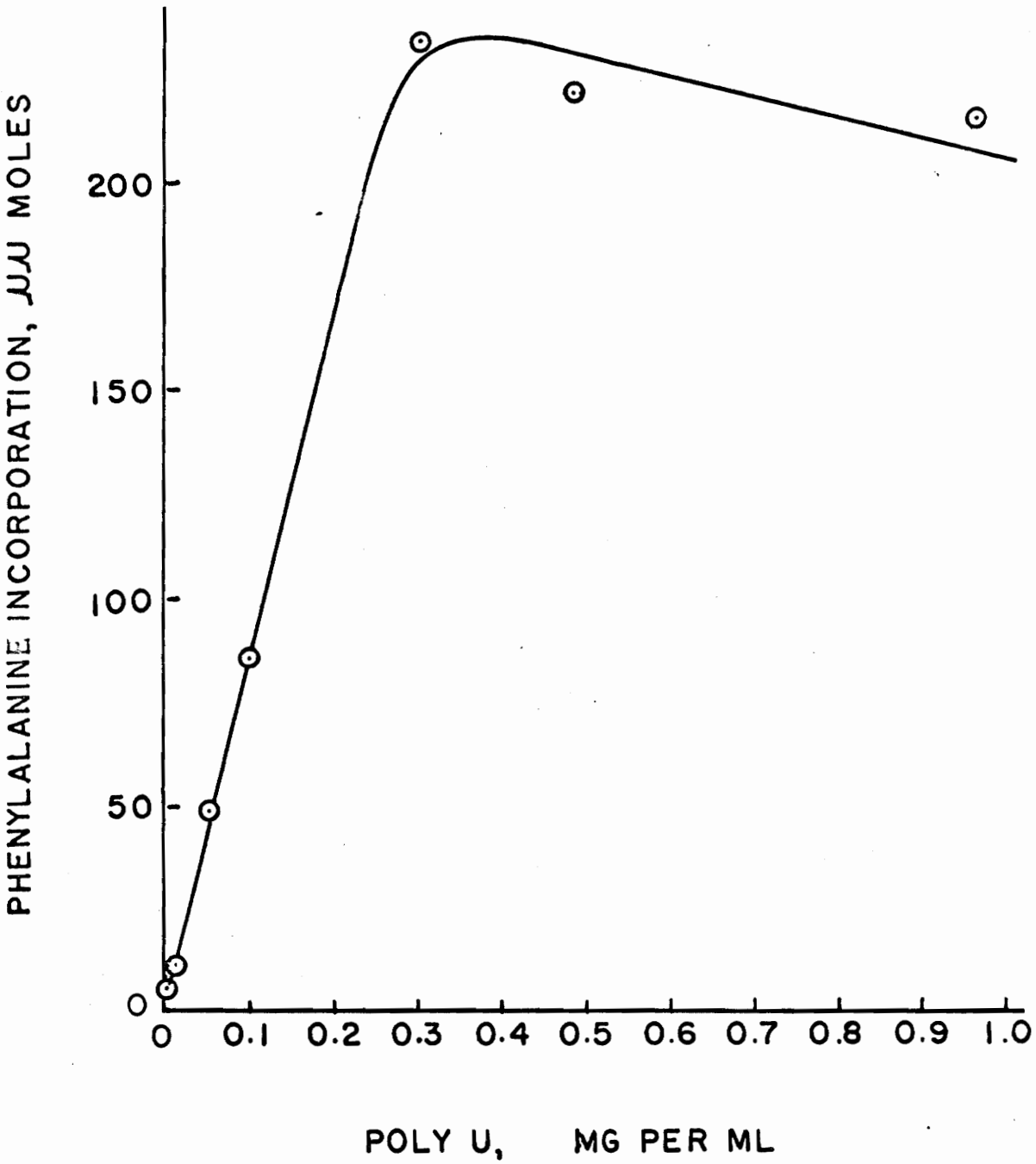

PHENYLALANINE INCORPORATION, μμ MOLES
250
200
150
100
50
0
0
1
2
3
4
5
PH 5 PROTEIN, MG PER ML

Figure 7. The Effect of Polyuridylic Acid (poly U) Concentration on ^{14}C-Phenylalanine Incorporation.

The composition of the reaction mixtures other than poly U, including t-RNA 0.6 mg. per ml. of reaction mixture, is reported in Table 1. Incubation temperature and time = for 30 minutes at 30° C.


PHENYLALANINE INCORPORATION, μμ MOLES
0
50
100
150
200
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
POLY U, MG PER ML

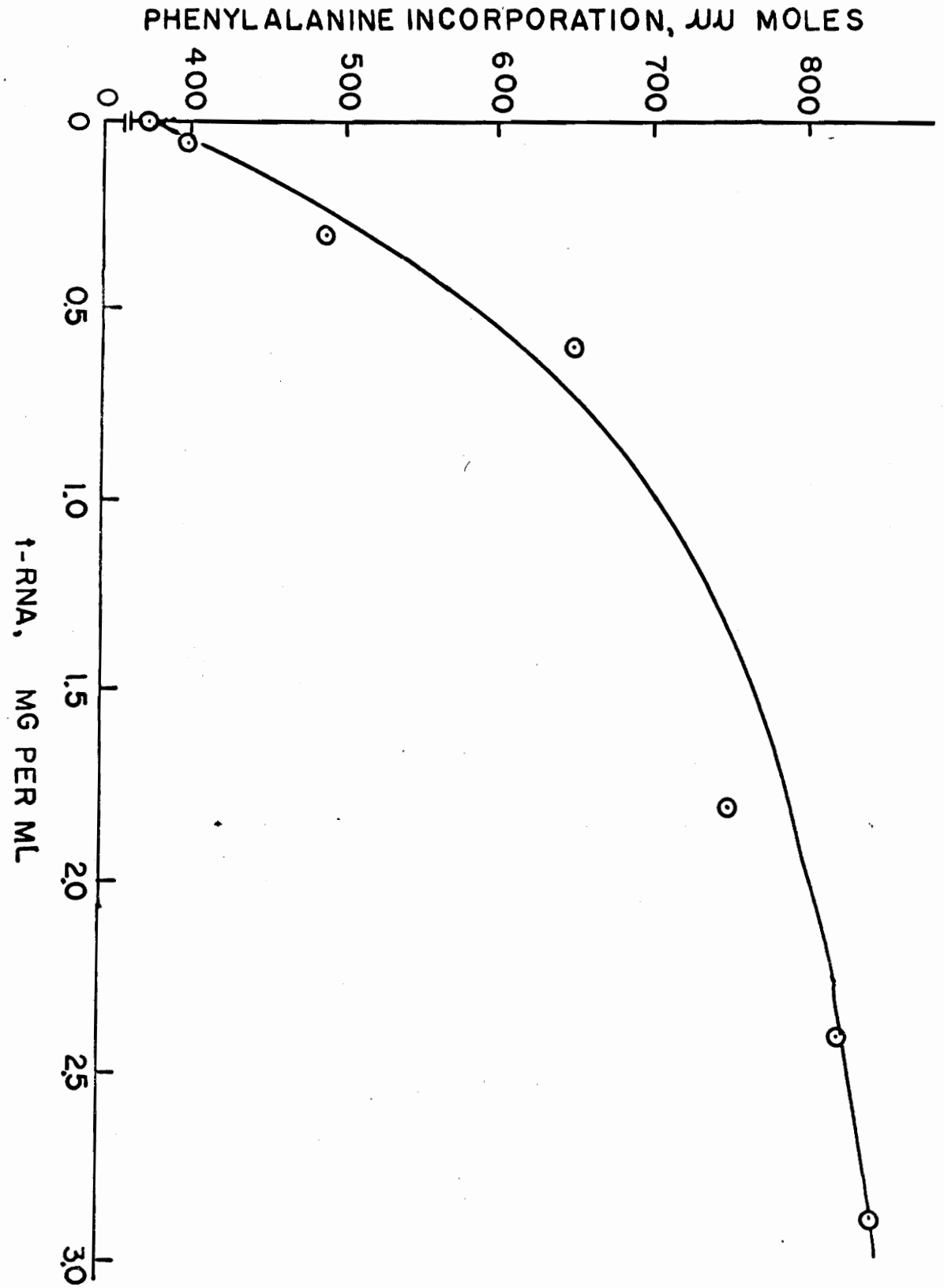
polyuridylic acid of chain length over 50 for significant phenylalanine incorporation and that incorporation is inhibited by the addition of short chain poly U. Poly U directed ^{14}C-phenylalanine incorporation (359 μμ moles per mg ribosomal protein) represented an eleven-fold increase over natural m-RNA directed phenylalanine incorporation (See Table 3). Presumably poly U forms polyribosomal complexes more readily with ribosomes than natural m-RNA under the conditions for amino acid incorporation. To investigate the reason for the low ^{14}C-leucine incorporation directed by natural m-RNA, the effect of MS2 RNA (which was kindly donated by R. L. Kaman, Department of Biochemistry and Nutrition, V.P.I.), on this incorporation system was checked. Addition of this MS2 RNA to the reaction mixture, up to a concentration of 1.2 mg per reaction mixture, had no effect on basal ^{14}C-leucine incorporation. This result could be explained by recent reports (5, 6, 89) using the cell-free E. coli ribosomal system that the high speed supernatant fraction of E. coli contains a factor which is required for polypeptide chain initiation in cell-free protein synthesis and that ribosomes must undergo a reaction promoted by the stimulation factor before interacting with natural m-RNA, whereas no such reaction is required for their interaction with poly U.

The stimulatory effect of t-RNA could be observed only in the presence of poly U (See Table 3), which is similar to other cell-free systems (23, 140, 108, 144). Recently Hung and Overby (23), in their

experiment with E. coli, reported that the ribosomal system prior to gel filtration contained endogeneous t-RNA and showed dependence on only m-RNA, whereas the preparation obtained from gel-filtration on a Sephadex G-100 column was dependent on both t-RNA and m-RNA. However, with our cell-free system, t-RNA had a stimulatory effect on poly U directed phenylalanine incorporation, even though the enhancement of incorporation with the increase of t-RNA concentration was only two-fold (Figure 8). Basal ^{14}C-leucine incorporation was not effected by the addition of t-RNA. This observation may possibly be ascribed to the presence of endogeneous t-RNA in saturating quantities as a contaminant in the cell-free system. Generally, lack of response or inhibition by any exogeneous components in cell-free systems has been explained by variation in pool size. For example, Bretthauer et al. (111) reported that in a non-preincubated system, t-RNA had no effect on phenylalanine incorporation and Marcus and Feeley (146) showed a stimulation effect of t-RNA in experiments using smaller amounts of t-RNA. Variation of added t-RNA requirement with each extract of Bacillus cereus was pointed out recently by Kobayashi and Halvorson (108). Decken and Campbell (145) reported an inhibitory effect of t-RNA on incorporation of leucine and phenylalanine in a cell-free system from rat liver. Inhibitory effect of excess t-RNA (124, 108, 127) and deacylated t-RNA (147, 148, 151) on various cell-free systems have often

Figure 8. Effect of t-RNA Concentration on ^{14}C-Phenylalanine Incorporation.

Composition of incubation mixtures without t-RNA, including polyuridylic acid 0.4 mg/ml of reaction mixture, is reported in Table 1. Incubation time and temperature: 30 minutes, 30° C.


PHENYLALANINE INCORPORATION, JJJ MOLES
0
400
500
600
700
800
0
0.5
1.0
1.5
2.0
2.5
3.0
t-RNA, MG PER ML

been reported. So and Davie (122) suggested that t-RNA, isolated from yeast and free of its terminal sequence -pCpCpA, inhibits incorporation by competing for the activating enzyme, the transfer enzyme, or the ribosomal site with t-RNA containing the intact terminal sequence and the inhibitory effect is eliminated after reconstitution of the terminal -pCpCpA sequence. Maeda and Imahori (127) with their cell-free system from Saccharomyces cerevisiae, demonstrated that the inhibitory effect was due to the presence of the entire t-RNA molecule. Kobayashi and Halvorson (108) suggested that uncharged t-RNA may inhibit amino acid incorporation, for omission of the 20 unlabeled amino acids in their cell-free system from Bacillus cereus reduced phenylalanine incorporation. Kaji and Kaji (149) assumed that deacylated phenylalanyl-acceptor t-RNA might attach at the binding site of phenylalanyl t-RNA. Hardesty et al. (151) reported that at the lower magnesium concentration binding to reticulocyte ribosomes required a soluble enzyme together with guanosine triphosphate (GTP). Very recently Nicholas and Conway (147) reported that when the rate of phenylalanine incorporation is limited by the concentration of GTP, deacylated t-RNA becomes very inhibitory and inhibition appears to be competitively reversed by GTP. In the present cell-free system from Cellvibrio gilvus, where GTP was supposed to exist in saturating amounts, it would be supposed that in poly U directed ^{14}C-phenylalanine incorporation de novo amino acid incorporation occurs far more actively than in the basal ^{14}C-leucine incorporation system. Thereby,

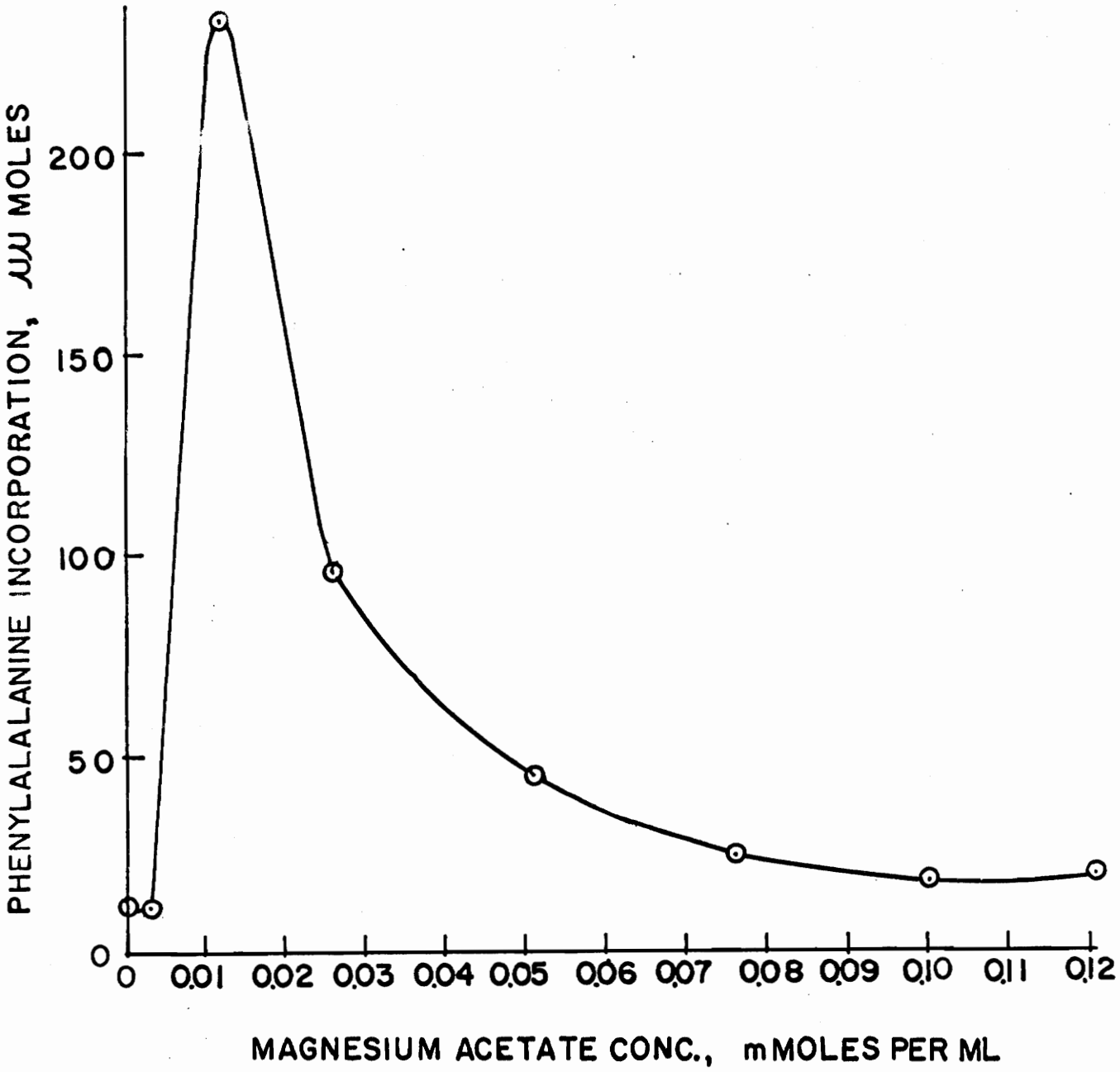
additional t-RNA brings about enhanced ^{14}C-phenylalanine incorporation, whereas ^{14}C-incorporation systems lack the stimulatory effect by the additional t-RNA. The low stimulatory effect of t-RNA on ^{14}C-phenylalanine incorporation may be due to the contaminated endogeneous t-RNA associated with the ribosomal and/or pH 5 enzyme preparation. In ^{14}C-leucine incorporation systems, the absence of the inhibitory effects of additional t-RNA may be due to the presence of saturating amounts of amino acids, (see paragraph, Effect of complementary amino acid mixtures)and GTP.

Effect of magnesium ion concentration:

All cell-free amino acid incorporation systems contain magnesium ions. The requirement of a critical concentration of magnesium ions for cell-free amino acid incorporation has been shown for many cell-free incorporation studies [bacteria, (108, 115, 118, 119, 152); plant, (19, 132, 137); yeast, (111, 122, 131); animal, (120, 128, 129, 130, 155); other microorganisms, (138, 139)]. The optimum concentration range of magnesium ion in those cell-free systems lies between 0.005 and 0.015 M, apparently depending upon the concentration of polyamines (111, 123, 143) and their endogeneous pool size. As shown in Figure 9, there was a narrow range of magnesium acetate concentration (0.01-0.015 M) for optimal amino acid incorporation with the Cellvibrio gilvus cell-free system.

Figure 9. Effect of Magnesium Acetate Concentration on ^{14}C-Phenylalanine Incorporation.

Standard buffer used all through this test contained 0.001 M of magnesium acetate and this magnesium acetate concentration in standard buffer was included in the indicated concentrations of magnesium acetate in this figure. Other compositions of the reaction mixtures are the same as reported in Table 1. The reaction mixture was incubated for 30 minutes at 30° C.


PHENYLALANINE INCORPORATION, μμ MOLES
200
150
100
50
0
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.11
0.12
MAGNESIUM ACETATE CONC., mMOLES PER ML

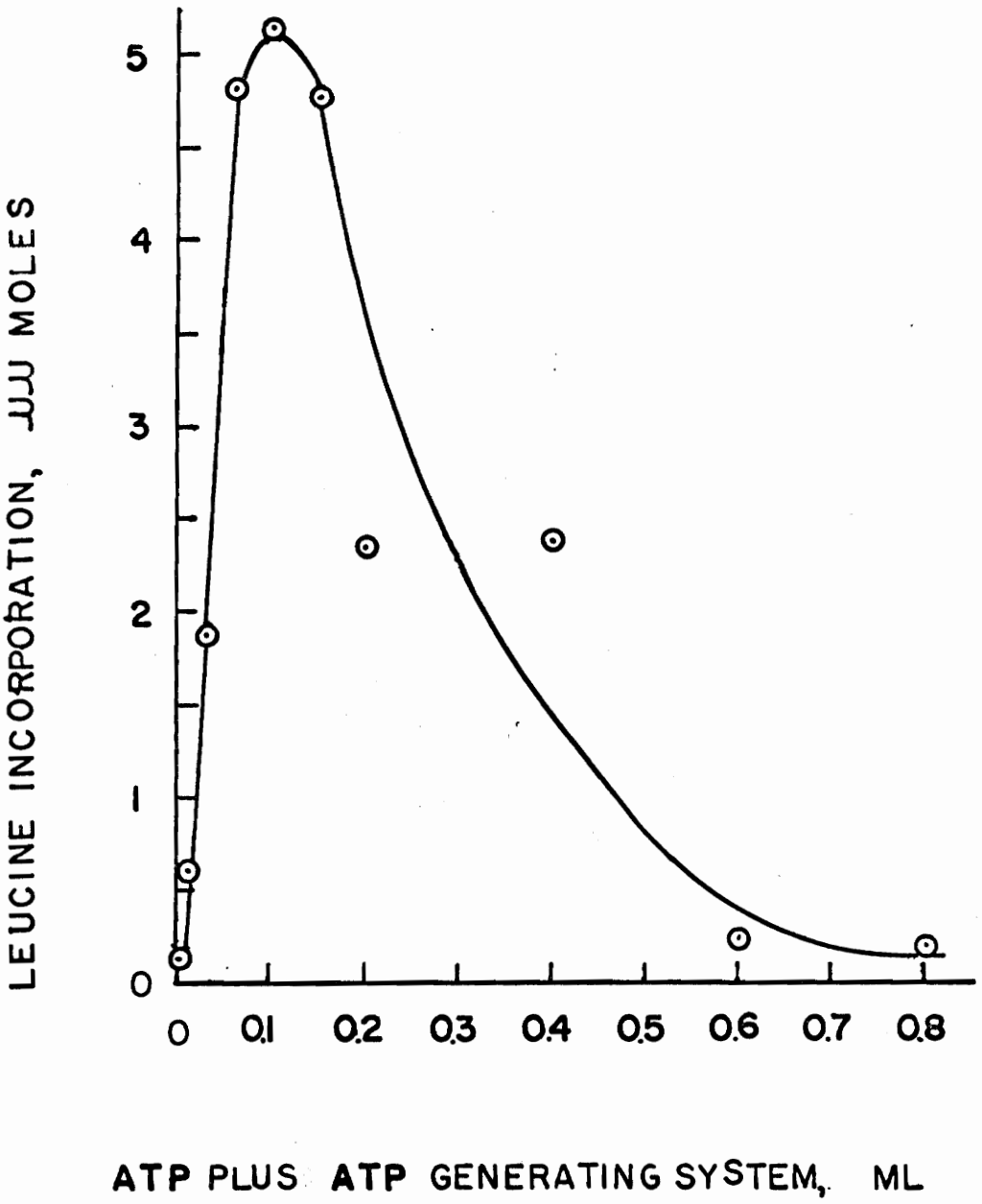
Bacterial ribosomes prepared in media containing a concentration of magnesium ions 10^{-2} M are predominantly in the 70 S form (143). Magnesium ions are reported not only to influence the association of the sub-units of the ribosomal particle (13, 143) but also effect the helical structure and conformation of the ribonucleic acids, thereby altering the interaction of t-RNA, m-RNA, and ribosomes (116).

Effect of the alteration of the amount of ATP plus ATP generating system:

Figure 10 shows the effect of the concentration of ATP plus ATP-generating system on ^{14}C-leucine incorporation. One ml of this ATP plus ATP-generating system contains 23 µmoles of ATP, 2.3 µmoles of GTP, 2.3 µmoles of CTP, 2.3 µmoles of UTP, 56 µmoles of phosphoenol-pyruvate (PEP), and 3.2 mg of phosphoenolpyruvate kinase (pK). The ATP-ATP generating system exhibited a sharp maximum for a value at 0.1 ml. Previously Allen and Schweet (113) reported that when the magnesium chloride level was kept at twice the ATP level, the inhibition effect of high ATP levels was much less. In the present cell-free system the ratio of optimum ATP concentration to the optimum concentration of magnesium acetate was 2.3×10^{-3} M: 10×10^{-3} M; i.e., 5:1. Uemura et al. (115) reported the optimum ratio on their cell-free system from Bacillus brevis to be 5×10^{-3} M: 5×10^{-3} M; i.e., 1:1. This ratio may be influenced by the endogeneous pool size of these components. Inhibition by high levels of ATP is generally attributed to the removal of Mg^{++} through formation of ATP complexes, thereby effecting RNA and ribosome structure (53).

Figure 10. Effect of ATP and ATP Generating System on ^{14}C-Leucine Incorporation.

One ml of the ATP-ATP generating system contained 23 μmoles of ATP, 2.3 μmoles of GTP, of CTP, of UTP, 56 μmoles of phosphoenolpyruvate (PEP), and 3.2 mg of phosphoenolpyruvate kinase (PK). Incubation mixtures other than this system are the same as those reported in Table 1. The reaction mixtures were incubated for 30 minutes at 30° C.


LEUCINE INCORPORATION, JJJ MOLES
0
1
2
3
4
5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ATP PLUS ATP GENERATING SYSTEM, ML

Effect of polyamines:

Various kinds of polyamines are known to be normal constituents of ribosomes and have been suggested to have a physiological role in maintaining the stability or function of ribosomes (13, 14, 15, 153). For example, E. coli ribosomes contain 1,3-diaminopropane, putrescine, and cadaverine (14); putrescine and spermidine (13); putrescine, spermidine, cadaverine and a trace of spermidine (114); rat liver ribosomes contain 1,3-diaminopropane, cadaverine, and spermine (14); ribosomes from rat and guinea pig livers and guinea pig and calf pancreas contain spermine (110). The stimulatory effect of low concentration of polyamines on various cell-free systems have been reported (E. coli, 94, 99; S. typhimurium, 17; Bacillus cereus, 108; yeast, 111, 158; rat liver, 93; Crithidia oncopelti, 139 (stimulation by spermidine and putrescine and inhibition by spermine)). On the other hand, high concentrations of polyamine inhibited cell-free systems (bacteria, 17; yeast, 111; rat liver, 93; mouse ascites leukemia cell, 112, 92). Martin and Ames (17) suggested that the polyamines, spermidine and putrescine stimulate the incorporation of amino acids into acid precipitable material by increasing the fraction of 100 S ribosomes, which were reported by Spyrides and Lipmann (85) to be primarily responsible for amino acid incorporation in E. coli. Other reports also suggested the stabilization of 100 S particles by polyamines (13, 14, 15, 18, 153). Recently Moller and Kim (18)

reported, in an experiment with a ribosomal system derived from Pseudomonas that at low magnesium ion concentrations, the addition of putrescine stimulated poly U primed protein synthesis and further addition of putrescine resulted in no additional stimulation, whereas putrescine inhibited leucine incorporation at low concentrations of magnesium ions, and this inhibition effect was prevented by increasing the magnesium concentration. In the cell-free system from Cellvibrio gilvus, in which 0.5 μmoles/ml each of spermidine, spermine, and putrescine were tested, all polyamines had a slight stimulatory effect on poly U directed phenylalanine incorporation and no significant effect on leucine incorporation under the magnesium ion concentration of 9 μmoles/ml (Table 5). The different effect of polyamines on endogeneous m-RNA directed ^{14}C-leucine and poly U directed ^{14}C-phenylalanine incorporation may be supposed to be due to mainly the difference in the degree of interaction among m-RNA, t-RNA, and ribosomes in two systems. Some previous reports can be cited as supporting this point of view (18, 62, 92, 99). For example, Mager et al. (99) found negligible stimulation in the cell-free systems from E. coli, where m-RNA was limiting. The slight stimulatory effect of polyamines on poly U directed ^{14}C-phenylalanine, where the concentrations of t-RNA and of poly U were not limiting, agrees with the observation of Ochoa and Weinstein (92) that high concentrations of poly U or t-RNA relieved spermine-induced inhibition. Therefore, high concentrations of polyamines may interact with m-RNA and t-RNA.

Table 5

Effect of Polyamines on ^{14}C-Leucine and ^{14}C-Phenylalanine Incorporation

Condition	^{14}C-phenylalanine Incorporation		^{14}C-leucine Incorporation	
	Incorporation μμmoles	% Stimulation	Incorporation μμmoles	% Stimulation
*Complete system	359	0	31	0
+ Spermidine	488	40	31	0
+ Spermine	668	90	35	10
+ Putrescine	460	30	25.1	-20

*Composition of complete system is reported in Table 1. In case of phenylalanine, complete system contained poly U (0.4 mg/ml) and t-RNA (0.6 mg/ml). The concentration of each polyamine added was 0.5 μmoles per one ml of reaction mixture. Reaction mixtures were incubated for 30 minutes at 30° C.

thereby inhibiting their normal functions (92). Felsenfeld (72) suggested a strong interaction between spermine and the phosphate backbone of various polynucleotides, both synthetic and natural. Ochoa and Weinstein (92) concluded from their experimental observation that if Felsenfeld's assumption is true, then the phosphate groups of RNA contained in microsomes were not as accessible to spermine as were the phosphate groups of poly U or t-RNA. Polyamine, like magnesium ions, may shield and neutralize the negative charges of phosphate backbone of RNA, thereby increasing the stability of codon bonds and enhancing protein synthesis. However, high concentrations of polyamine might cause excessive stability of the codon bond and thereby impede the sequential transcription of m-RNA (92). Polyamine functions were reported not to be replaced entirely by magnesium (18, 111).

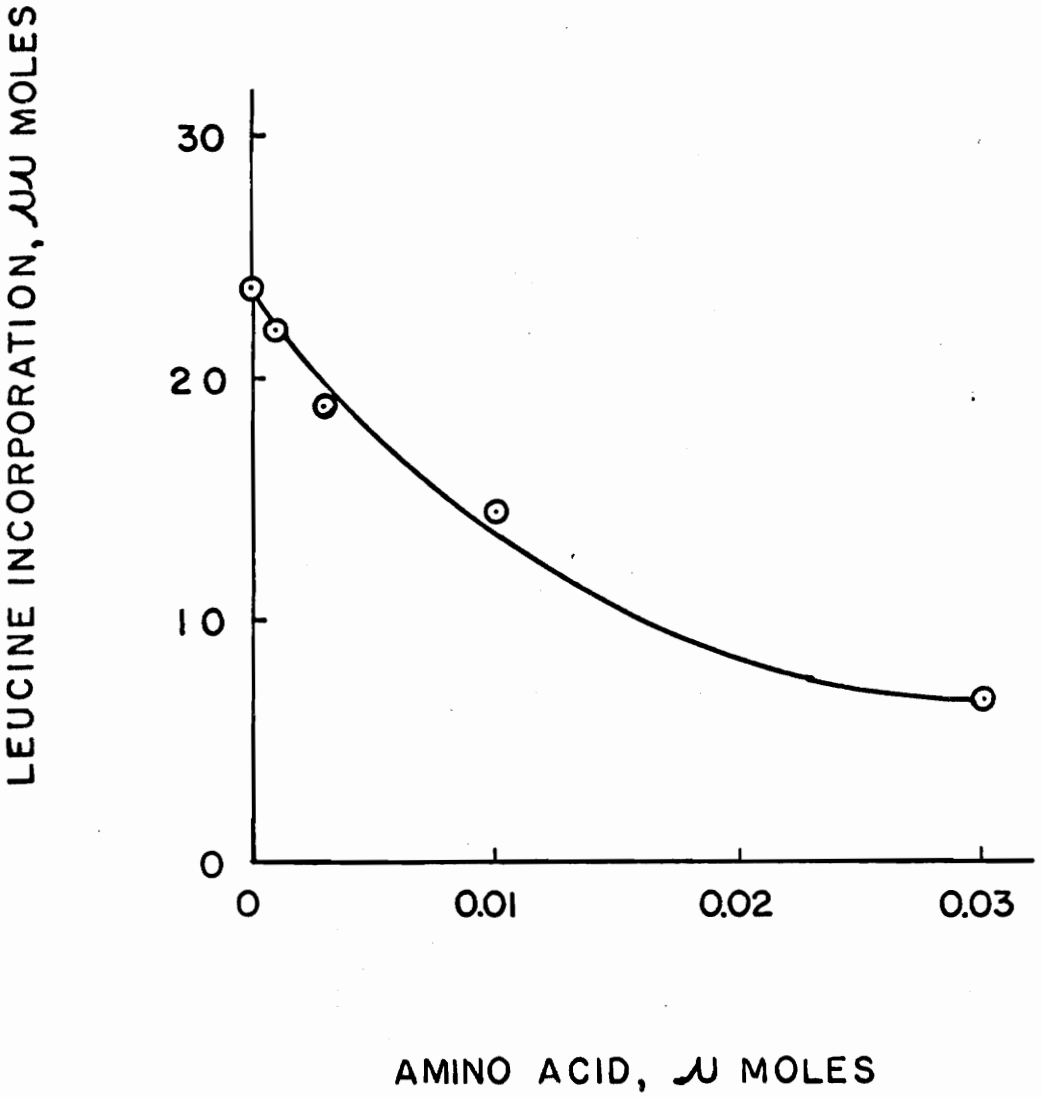
In contrast with our system, other cell-free systems showed different response with different polyamines to stimulation or inhibition effect. For example, Martin and Ames (17) with their cell-free systems, S. typhimurium observed the stimulation effect with a spermidine-putrescine combination but not with spermine, spermine plus putrescine, or spermidine plus spermine. Recently Chesters (139) reported an inhibition effect of spermine on the cell-free system from Crithidia oncopelti despite the stimulatory effect of the polyamines, putrescine and spermidine.

Effect of complementary amino acid mixtures (19 unlabeled amino acids):

The effect of 19 complementary-unlabeled amino acid mixtures (alanine, phenylalanine, aspartic acid, arginine, cysteine, glutamine, glutamic acid, glycine, histidine, cystine, isoleucine, lysine, methionine, proline, serine, threonine, tyrosine, tryptophan, and valine) on ^{14}C-leucine incorporation is reported in Figure 11. Additional exogeneous complementary amino acid mixtures inhibited ^{14}C-leucine incorporation, and as the concentration of the complementary amino acid mixtures increased, the inhibitory effect was increased. The depression effect by complementary amino acid mixtures on amino acid incorporation was previously reported by Barnett *et al.* (124) on cell-free system from yeast (*Saccharomyces carlsbergensis*) and by Allen and Schweet (113) on the cell-free system from rabbit reticulocyte at high levels of the concentration of the complementary amino acid mixtures. Similar failure to observe stimulation by complementary amino acid mixture has been reported for other cell-free systems from various origins [yeast, (111, 122, 127, 121); mammalian, (20); higher plants, (132, 137); *E. coli*, (118)], whereas some cell-free systems reported the stimulation effect of the complementary amino acid mixtures [bacteria, (108); plant, (19); insect, (120)]. Accordingly, the *Cellvibrio gilvus* cell-free system appears to contain each of the endogeneous amino acids in saturating amounts and a further addition of complementary amino acids inhibits ^{14}C-leucine incorporation. Whether the inhibition effect of additional

Figure 11. Effect of the Variation of the Concentration of Complementary ^{12}C-Amino Acid Mixtures on ^{14}C-Leucine Incorporation.

Indicated amino acid concentration represents μmoles of each of the nineteen complementary amino acids (see text) in the amino acid mixtures. Other incubation mixtures are the same as reported in Table 1. Incubation mixtures were incubated for 30 minutes at 30° C.


LEUCINE INCORPORATION, μμ MOLES
30
20
10
0
0
0.01
0.02
0.03
AMINO ACID, μ MOLES

complementary amino acid mixtures could be eliminated by the addition of t-RNA was not tested. Previously Kobayashi and Halvorson (108) suggested that the omission of the 20 unlabeled amino acids in poly U-directed phenylalanine incorporation may be due to the inhibition effect of uncharged t-RNA. In a cell-free system where active de novo protein synthesis occurs, the uncharged t-RNA liberated may be charged again by the saturating amounts of amino acids. However, as discussed before, GTP has also been reported to be a factor in preventing the inhibition of deacylated t-RNA. Therefore, several factors seem to be interrelated in a complicated pattern.

Effect of ammonium ion concentration:

Monovalent cations have been shown to be required for optimum incorporation in most cell-free amino acid incorporation systems of microbial origin (10, 11, 12, 52, 108). Lubin and Ennis (10), Spyrides (11), and Conway (12) reported that ammonium ion was more effective than the potassium ion or the sodium ion in stimulating amino acid incorporation on the cell-free system from E. coli. Spyrides (11) reported that ammonium ion was the most effective in inducing association of phenylalanyl t-RNA with the ribosomes in the presence of poly U, and potassium ion was found to be almost half as active as ammonium ion. In other cell-free systems, the stimulation effect of ammonium ion has been reported (Spinach chloroplast, 134; rat muscle, 16). Campbell et al. (20), however, reported they could not observe the

stimulation effect by ammonium ions, with their cell-free system from rat brain. Figure 12 shows the effect of ammonium ion concentration on ^{14}C-leucine incorporation. The optimum concentration of ammonium chloride was 0.1 M. The ^{14}C-leucine incorporation was greatly inhibited by high ammonium chloride concentration, for example, leucine incorporation at 1 M NH_4Cl was 16% of the optimum concentration. The inhibition effect of high ammonium ion concentration on ^{14}C-leucine incorporation is not clear. So et al. (7) reported that at optimum concentrations of magnesium ion and ammonium ion for phenylalanine incorporation, only a very small amount of leucine, and no isoleucine was incorporated, but at higher magnesium concentrations, phenylalanine incorporation was only slightly stimulated, whereas leucine incorporation was markedly stimulated by increasing the ammonium ion concentrations. Therefore, it seems likely that the inhibition effect of high concentrations of ammonium chloride might be related to the environmental effect on code assignment.

Effect of the variation of pH:

Figure 13 presents the effect of the variation of the pH of the incubation mixture on the ^{14}C-leucine incorporation. In the pH range 5.5-6.0 no increase in incorporation was observed. From around pH 6.0 the incorporation increases as pH increases, reaching maximum around pH 7.8. This finding was very similar with that for a cell-free ^{14}C-L-valine incorporating system from E. coli (84).

Figure 12. Effect of the Concentration of Ammonium Chloride on ^{14}C-Leucine Incorporation.

Common logarithm of ammonium chloride concentration in μmoles per ml of reaction mixture was taken in the representation of ammonium chloride concentration. Composition of reaction mixtures other than ammonium chloride are the same as represented in Table 1. Reaction mixtures were incubated for 30 minutes at 30° C.

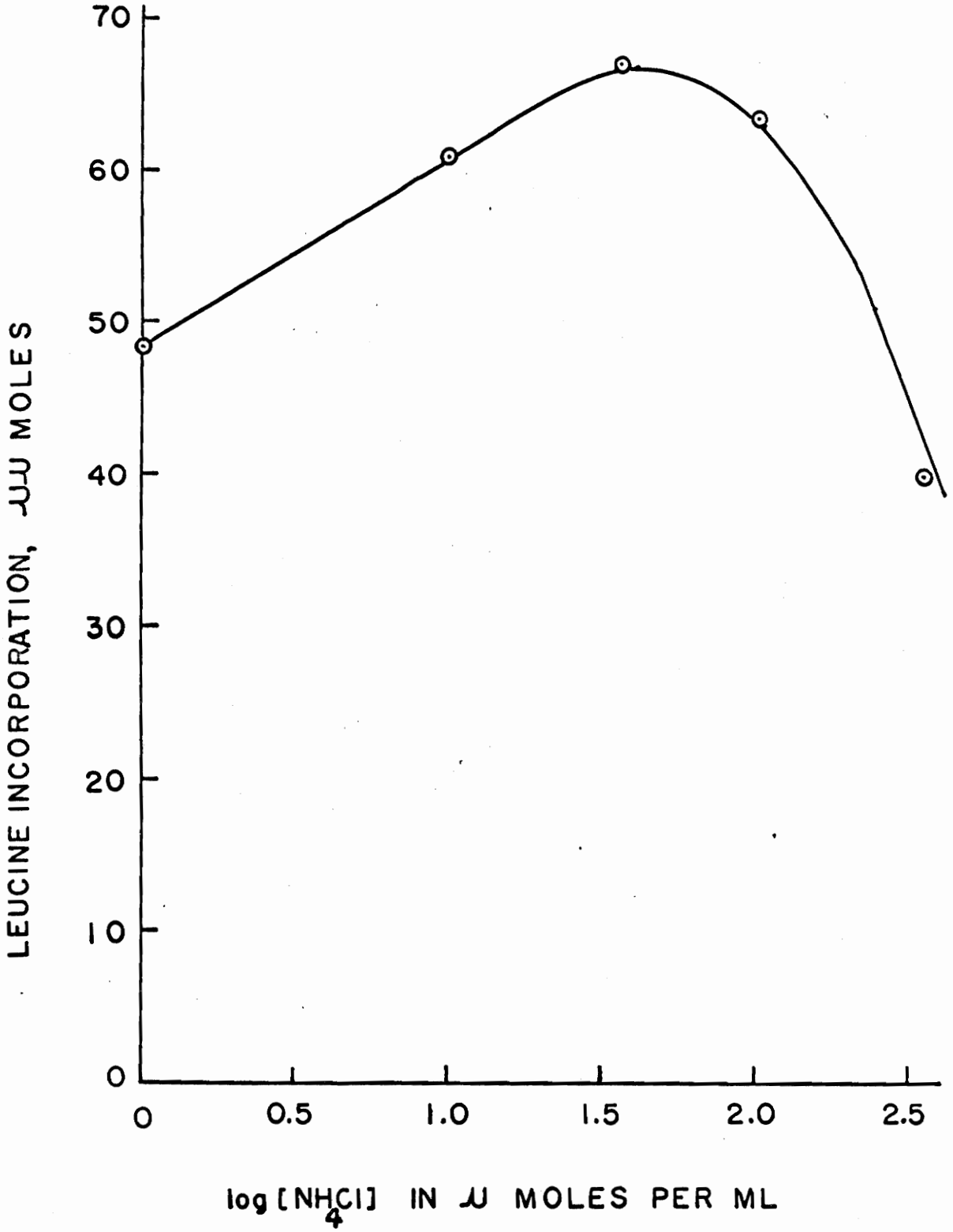
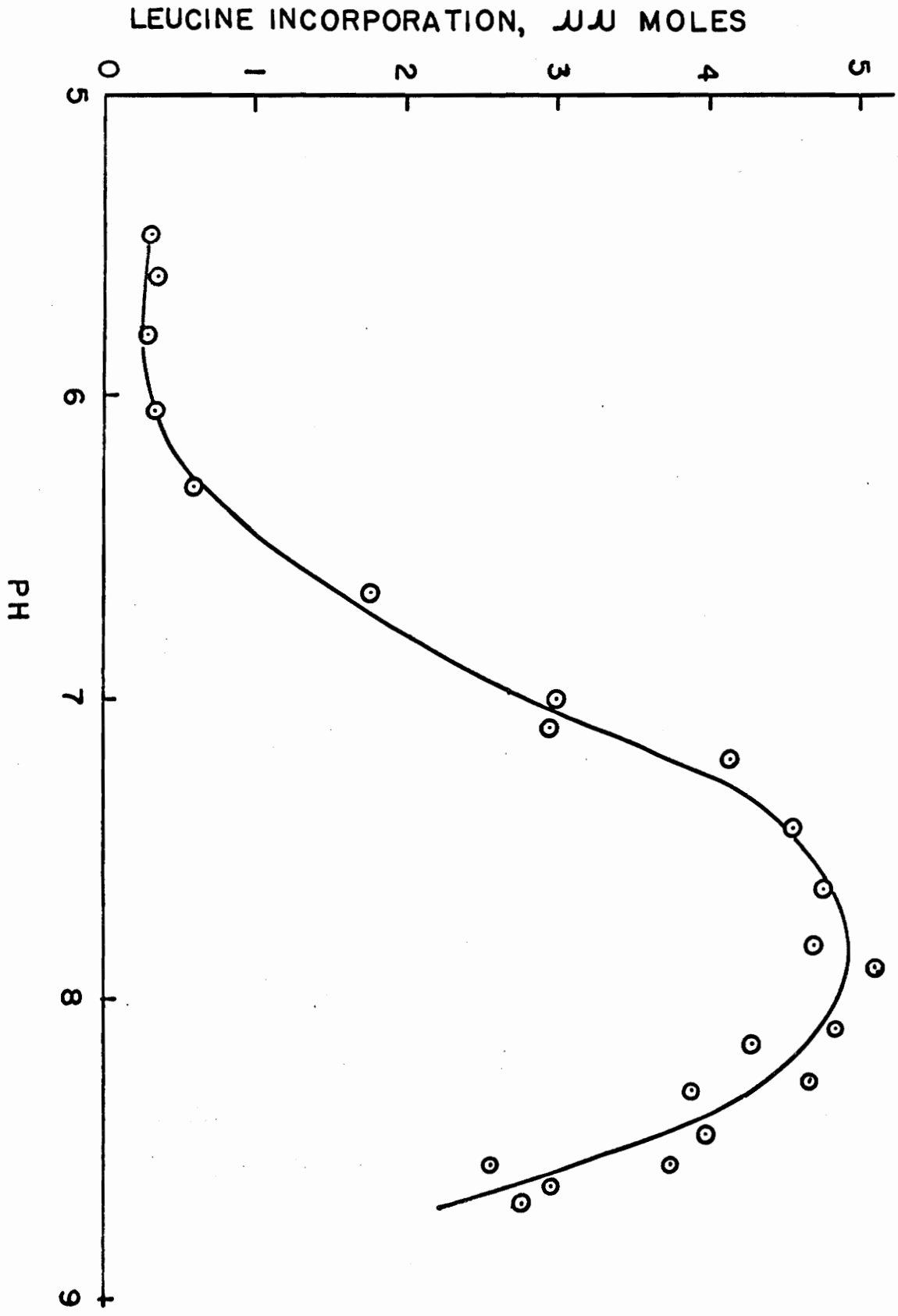

70
60
50
40
30
20
10
0
LEUCINE INCORPORATION, µµ MOLES
0
0.5
1.0
1.5
2.0
2.5
log [NH4Cl] IN µ MOLES PER ML

Figure 13. pH Effect on ^{14}C-Leucine Incorporation.

Composition of the reaction mixtures is shown in Table 1. To each incubation tube was added small portions of 1N KOH (or 1 N HCl) solution and the pH was measured by using Corning pH Meter Model 12. Reaction mixtures were incubated for 30 minutes at 30° C.


LEUCINE INCORPORATION, μμ MOLES
0
1
2
3
4
5
PH
5
6
7
8
9

Effect of the variation of incubation temperature:

The optimum incubation temperature for ^{14}C-leucine incorporation was 30° C (Figure 14). Above 30° C there was a rapid inhibition of incorporation. The optimum temperature of the Cellvibrio gilvus cell-free system was rather higher than the optimum temperature (about 25° C) of Saccharomyces carlsbergensis (124), a yeast. The incubation temperature of E. coli (118) and mammalian systems (113, 20) were usually run at 37° C.

Time course for ^{14}C-leucine incorporation:

Figure 15 shows the time course of ^{14}C-leucine incorporation. ^{14}C-Leucine incorporation increased linearly up to 20-25 minutes and plateaued at thirty minutes. The incubation temperature was 30° C. This time course is similar with the rat liver system (125) and a little faster than the E. coli system (118). The reason for the amino acid incorporation stopping after a certain incubation period (usually 30-60 minutes) has not been definitely established. It does not seem to be due to the inactivation of any stimulatory factors which have been reported to be present in the high-speed supernatant or any other soluble factors from certain species. This leveling off may be due to the inactivation of the ribosomal particles on longer incubation because the addition of supernatant to a reaction mixture containing crude extract during incubation had no effect on the

Figure 14. Effect of the Variation of Incubation Temperature on ^{14}C-leucine Incorporation.

Composition of the reaction mixture is shown in Table 1. Incubation time was 30 minutes.

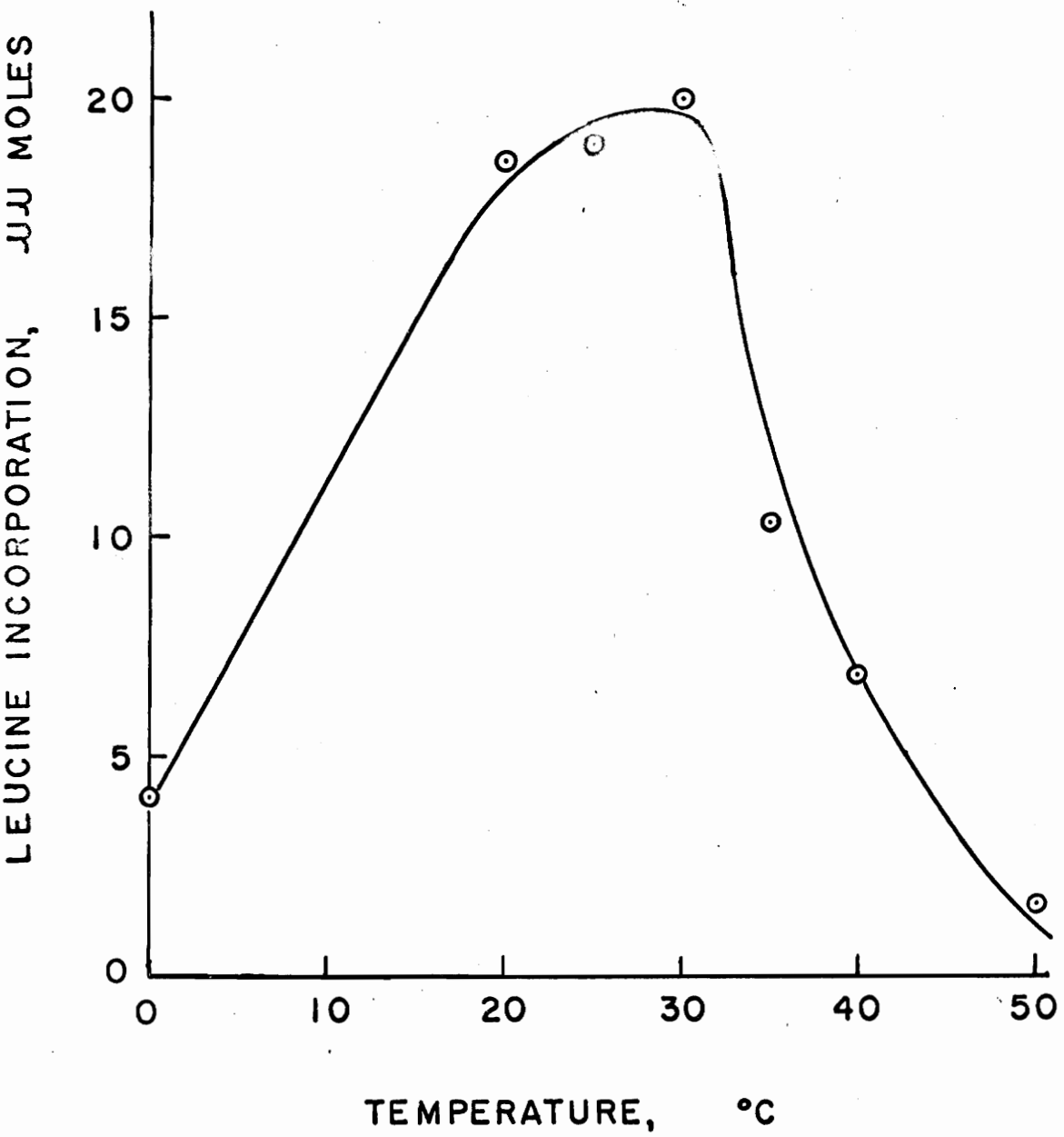
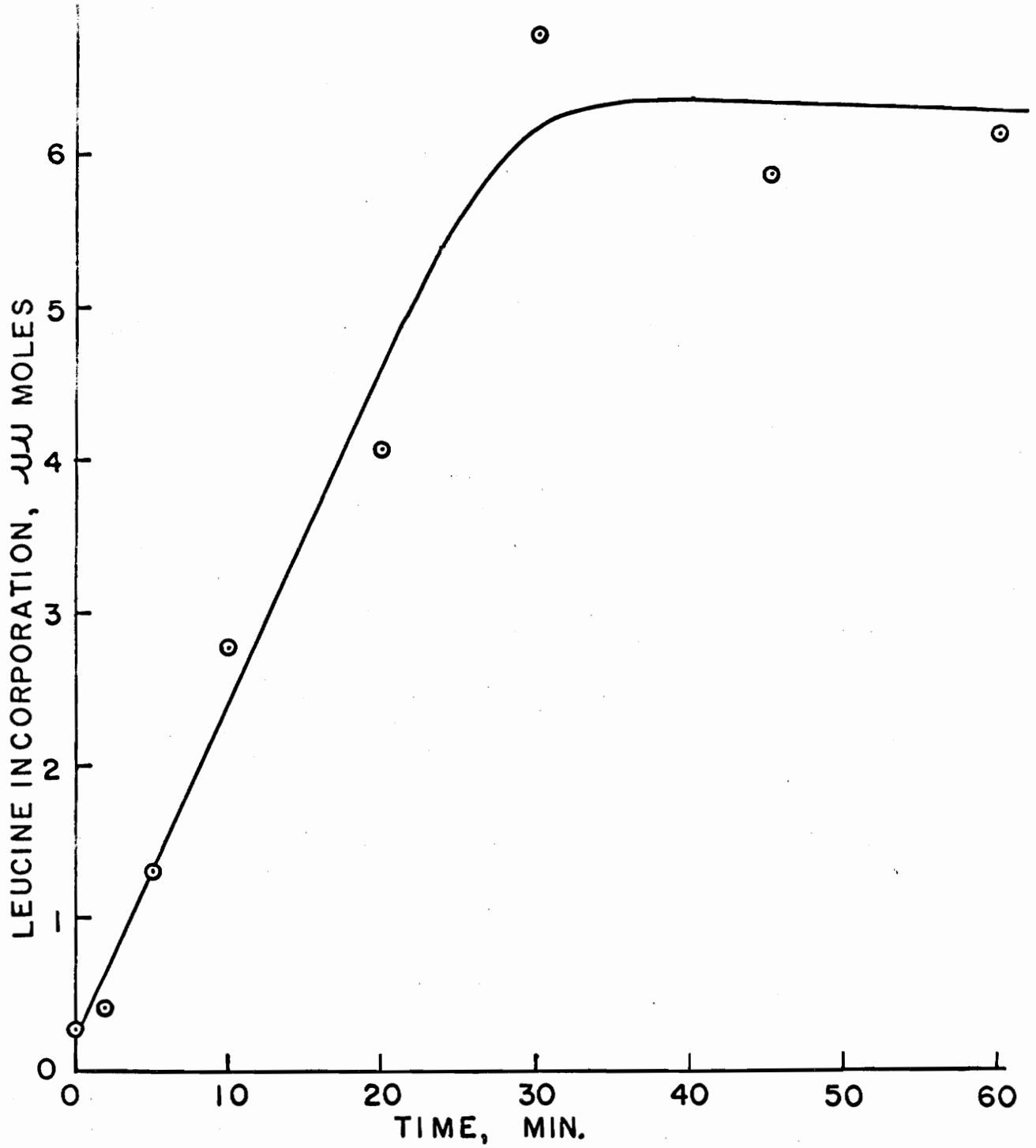

LEUCINE INCORPORATION, µµ MOLES
20
15
10
5
0
0
10
20
30
40
50
TEMPERATURE, °C

Figure 15. Time Course of ^{14}C-Leucine Incorporation

Composition of the incubation mixtures is shown in Table 1.

Incubation temperature was 30° C.


LEUCINE INCORPORATION, μμ MOLES
0
1
2
3
4
5
6
0
10
20
30
40
50
60
TIME, MIN.

incorporation according to several reports (maize kernel, 133; E. coli, 118; hemoglobin, 113). Tissiere et al. (118) assumed that the incorporation failure with longer incubation of the reaction mixtures was due to the failure to release completed chains. Rabson and Novelli (133) reached a conclusion similar to that of Tissiere et al. They suggested that particles may have a limited number of sites available for incorporation and perhaps the cessation of incorporation might be a consequence of filling the limited sites in the absence of an enzyme or factor to remove the newly-formed protein. Later, however, Allen and Schweet (113) disagreed with their idea on the grounds that no evidence for ribosome breakdown was found in their cell-free system from rabbit reticulocyte and that ribosomes with decreasing ability to incorporate amino acids could still release completed chains. They suggested that the depletion or loss of a ribosomal component in the process of amino acid incorporation would be the cause of the cessation of incorporation. However, it can be assumed that if m-RNA could be supplied continuously and the integrity of the polyribosomes could be maintained, the protein synthesis would continue for a prolonged period. Endogeneous ribonuclease may be the cause for the degradation of ribosomes. This cessation of incorporation can be one of the criteria for distinguishing between cell-free and in vivo systems. The limiting factor(s) in cell-free systems have not yet been clarified in detail.

The following observations can be cited as the evidence that amino acid incorporation is due to cell-free systems and not due to contaminated intact whole cells: (1) Time course of the incorporation reaction is different from bacterial growth curves (Figure 2), (2) Absolute requirement of exogeneous energy sources (ATP plus ATP generating systems), (3) Strong inhibition effect of RNAase, (4) Incorporation reaction is highly dependent on critical amounts of magnesium concentration, (5) Almost complete inhibition by puromycin. The optimum temperature (30° C) of cell-free systems coincided with the optimum temperature for bacterial growth. The cell-free system derived at the initiation point (O.D., 0.2) of log phase of bacterial growth was the most active, and the cell-free system derived at the plateau had far less incorporating activity.

VII. SUMMARY

A ribosomal system has been derived from Cellvibrio gilvus which is active with respect to amino acid incorporation into hot trichloroacetic acid-insoluble material. Optimal incorporation systems showed a requirement for ribosomes, pH 5 enzyme protein, ATP plus ATP-generating system, magnesium ion, ammonium ion, and GTP. ^{14}C-phenylalanine incorporation was stimulated by poly U in the presence of t-RNA which has been derived from this bacterium. The t-RNA had some activation effect on poly U-directed ^{14}C-phenylalanine incorporation, whereas no effect on natural m-RNA primed ^{14}C-leucine incorporation was observed. A complementary unlabeled amino acid mixture inhibited ^{14}C-leucine incorporation. ^{14}C-Leucine incorporation was greatly inhibited by RNAase and puromycin, moderately inhibited by chloramphenicol, but not effected significantly by DNAase and actinomycin D. The optimum pH and temperature for ^{14}C-leucine incorporation were 7.8 and 30° C, respectively. Polyamines (putrescine, spermine, and spermidine) had a slight stimulatory effect on poly U-directed ^{14}C-phenylalanine incorporation, but no effect on ^{14}C-leucine incorporation.

VIII. ACKNOWLEDGEMENT

My most sincere gratitude is expressed to the Department of Biochemistry and Nutrition for making it possible for me to fulfill my long-cherished desire to study under such favorable conditions as effective and helpful guidance and with sufficient equipment and materials. Now I have accomplished this work through the thoughtful and helpful arrangement, absolute support and aid of this department.

Appreciation is expressed to Dr. Lewis B. Barnett, my major professor, for his direct guidance to make this work a reality. Thanks is also due to Mr. Thomas O. Sitz for his experimental contribution to this work.

IX. BIBLIOGRAPHY

1. Nirenberg, Marshall W., and J. Heinrich Matthaei, Proc. Natl. Acad. Sci., U. S., 47, 1588 (1961).

2. Bretthauer, R. K., Nakao, Y., and Halvorson, H. O., Bacteriol. Proc. 102 (1964).

3. Adams, Jerry M., and Mario R. Capecchi, Proc. Natl. Acad. Sci. U.S. 55, 147 (1966).

4. Lamfrom, H., and Knopf, P., J. Mol. Biol. 9, 558 (1964).

5. Eisenstadt, Jerome M., and George Brawerman, Biochemistry 5, 2777 (1966).

6. Eisenstadt, Jerome M., and George Brawerman, Biochemistry 5, 2784 (1966).

7. So, Antero G., James W. Badley, and Earl W. Davie, Biochemistry 3, 1977 (1964).

8. Jones, O. W., E. Townsend, H. G. Sober, and L. G. Heppel, Biochemistry 3, 238 (1964).

9. Martin, R. G., and B. N. Ames, Proc. Natl. Acad. Sci., U. S. 47, 1415 (1961).

10. Lubin, M. J., and H. L. Ennis, Federation Proc. 22, 302 (1963).

11. Spyrides, G. J., Proc. Natl. Acad. Sci., U. S., 51, 1200 (1964).

12. Conway, T. W., Proc. Natl. Acad. Sci., U. S., 51, 1216 (1964).

13. Tisseres, A., J. D. Watson, D. Schlessinger, and B. R. Hollingworth, J. Mol. Biol. 1, 221 (1959).

14. Zillig, W., W. Krone, and M. Albers, Z. Physiol. Chem. 317, 131 (1958).

15. Colburn, J. L., B. H. Witherspoon and E. J. Herbst, Biochim. Biophys. Acta 49, 422 (1961).

16. Florini, J. R., Biochemistry 3, 209 (1964).

17. Martin, R. G., and B. N. Ames, Proc. Natl. Acad. Sci., U. S., 48, 2171 (1962).

18. Moller, Mary L., and Ki Han Kim, Biochem. Biophys. Res. Communs. 20, 46 (1965).

19. Parisi, B., and O. Aferri, Biochemistry 5, 1638 (1966).

20. Campbell, Mary K., Henry R. Mahler, Walter J. Moore, and Sujata Tewari, Biochemistry 5, 1174 (1966).

21. Stenzel, Kurt H., Ruth F. Aronson, and Albert L. Rubin, Biochemistry 5, 930 (1966).

22. Hoagland, M. B., E. Chargaff, and J. N. Davidson, The Nucleic Acids 3, 439 (1960).

23. Hung, P. P., L. Straube, and L. R. Overby, Biochem. Biophys. Res. Communs. 24, 656 (1966).

24. Hulcher, Frank Hope, Ph.D. Thesis, Department of Biochemistry and and Nutrition, Virginia Polytechnic Institute, Blacksburg, Va., U.S.A., January, 1957.

25. Hulcher, Frank H., and Kendall W. King, Bacteriol. J. 76, 571 (1958).

26. Hulcher, Frank H., and Kendall W. King, J. Bacteriol. 76, 565 (1958).

27. Storvick, Waldemar O., and Kendall W. King, J. Biol. Chem. 235, 303 (1960).

28. King, Kendall W., Advances in Enzymatic Hydrolysis of Cellulose and Related Materials, Pergamon Press, Oxford, London, New York, Paris, p. 159 (1965).

29. Storvick, W. O., F. E. Cole, and K. W. King, Biochemistry, 2, 1106 (1963).

30. Cole, Francis E., and K. W. King, Biochim. Biophys. Acta, 81, 122 (1964).

31. Hoagland, M. B., P. C. Zamecnik, and M. U. Stephenson, in, Symposium on Molecular Biology, E. R., Ed. 105 (Zirkle, Univ. of Chicago Press, Chicago, Ill., 1959).

32. Loftfield, R. B., Progress in Biophys. and Biophysical Chem., 8, 347 (1957).

33. Hoagland, M. B., in, The Nucleic Acids, III, Chapt. 37, (E. Chargoff and J. N. Davidson, Eds.) Academic Press, Inc., New York, N. Y.)

34. Lipmann, F., Advances in Enzymology 1, 99 (1941).

35. Linderstrom-Lang, K., Lane Medical Lectures, Stanford Univ., 6, 93 (1952).

36. Borsook, H., and J. W. Dubnoff, J. Biol. Chem. 132, 307 (1940).

37. Cohen, P. P., and R. W. McGilvery, J. Biol. Chem. 166, 261 (1946).

38. Cohen, P. P., and R. W. McGilvery, J. Biol. Chem. 169, 119 (1947).

39. Cohen, P. P., and R. W. McGilvery, J. Biol. Chem. 171, 121 (1947).

40. Johnston, R. B., and K. Bloch, J. Biol. Chem. 188, 221 (1951).

41. Webster, G. C., Arch. Biochem. Biophys. 47, 241 (1953).

42. Frantz, I. D., P. C. Zamecnik, J. W. Reese, and M. L. Stephenson, J. Biol. Chem. 174, 773 (1948).

43. Winnick, T., F. Friedberg, and D. M. Greenberg, Arch. Biochem. Biophys. 15, 160 (1947).

44. Melchior, J. B., M. Mellody, and I. M. Klotz, J. Biol. Chem. 174, 81 (1948).

45. Peterson, E. A., and D.M. Greenberg, J. Biol. Chem. 194, 359 (1952).

46. Monod, J., Ann. Inst. Pasteur 70, 318 (1944).

47. Spiegelman, S., Cold Spring Harbor Symp. 11, 256 (1946).

48. Peterson, E. A., and D. M. Greenberg, J. Biol. Chem. 194, 359 (1952).

49. Siekevitz, P., J. Biol. Chem. 195, 549 (1952).

50. Zamecnik, P. C., and E. B. Keller, J. Histochem. Cytochem. 2, 378 (1954).

51. Zamecnik, P. C., and E. B. Keller, J. Biol. Chem., 209, 337 (1954).

52. Novelli, G. D., and J. A. DeMoss, J. Cell. Comp. Physiol. Suppl. 1, 173 (1957).

53. Morris, A. G., and R. S. Sweet, Biochim. Biophys. Acta, 47, 415 (1961).

54. Nisman, B., E. H. Bergman, and P. Berg, Biochim. Biophys. Acta, 26, 639 (1957).

55. Nisman, B., Biochim. Biophys. Acta 32, 18 (1959).

56. Chantremne, H., The Biosynthesis of Protein, Pergamon Press, London, pp. 96-102 (1961).

57. Keller, E. B., and P. C. Zamecnik, J. Biol. Chem. 221, 45 (1956).

58. Hoagland, M. B., E. B. Keller, and P. C. Zamecnik, J. Biol. Chem. 218, 345 (1956).

59. DeMoss, J. A., and Novelli, G. D., Biochim. Biophys. Acta, 18, 592 (1955).

60. Wisman, Alan, Organization for Protein Biosynthesis, American Elsevier Publ. Co., Inc., New York, pp. 32-35 (1965).

61. Lotfield, R. B., and E. S. Eigner, J. Am. Chem. Soc., 81, 4753 (1959).

62. Novelli, G. D., Proc. Natl. Acad. Sci., U. S., 44, 86 (1958).

63. Annual Reviews of Microbiology, 14, 68 (1960). (Ann. Rev. Inc., Palo Alto, California, U.S.A.).

64. DeMoss, J. A., S. M. Genuth, and G. D. Novelli, Federation Proc. 15, 241 (1956).

65. Moldave, K., P. Castelfranco, and A. Meister, J. Biol. Chem. 234, 841 (1959).

66. McCorquodale, D. J., G. C. Müller, Arch. Biochem. Biophys. 77, 13 (1958).

67. Karasek, M. P., P. Castelfranco, P. R. Krishnaswamy, and A. Meister, J. Am. Chem. Soc. 80, 2335 (1958).

68. Kingdon, H. S., L. T. Webster, Jr., and E. W. Davie, Proc. Natl. Acad. Sci., U. S. 44, 757 (1958).

69. Holley, R. W., J. Am. Chem. Soc. 79, 658 (1957).

70. Hoagland, M. B., P. C. Zamecnik, and M. D. Stephenson, Biochim. Biophys. Acta 24, 215 (1957).

71. Berg, P. and E. J. Ofengand, Proc. Natl. Acad. Sci., U. S. 44, 78 (1958).

72. Felsenfeld, C., in Symposium Fundamental Cancer Research, M. D. Anderson Hospital and Tumor Institute, University of Texas Press, Austin, Texas, 1962, page 404.

73. Schachman, H. K., A. B. Pardee, and R. Y. Stainer, Arch. Biochem. Biophys. 38, 245 (1962).

74. Webster, G., and J. B. Lingrel, Protein Biosynthesis (R.J.C. Harris, ed.), Academic Press, New York, N. Y., 1961, page 301.

75. Webster G., and J. B. Lingrel, Biochem. Biophys. Res. Communs. 23, 1 (1966).

76. Nathans, D., G. Von Ehrenstein, R. Munro, and F. Lipmann, Federation Proc. 21, 127 (1962).

77. Fesenden, J. M., K. Moldave, Biochim. Biophys. Acta 1, 485 (1962).

78. Hulsman, W. C., and F. Lipmann, Biochim. Biophys. Acta 43, 129 (1960).

79. Lacks, F., F. Gros, J. Mol. Biol. 1, 301 (1959).

80. Yarmolinski, M. B., and G. L. de La Haba, Proc. Natl. Acad. Sci. U. S., 45, 1721 (1959).

81. Erdös, T., and A. Ullman, Natuer 183, 618 (1959).

82. Crick, F. H., Biochem. Soc. Symposium 14, 25 (1958).

83. Gooman, H. M. and A. Rich, Nature 199, 318 (1963).

84. Matthaei, J. H. and M. W. Nirenberg, Proc. Natl. Acad. Sci, U.S. 47, 1580 (1961).

85. Spyrides, G., and F. Lipmann, Proc. Natl. Acad. Sci., U. S., 48, 1977 (1962).

86. Weinstein, Bernard, Manuel Ochoa, Jr., S. Marvin Friedman, S. M. Friedman, and I. B. Weinstein, Proc. Natl. Acad. Sci., U. S., 52, 988 (1964).

87. So, A. G., and E. W. Davie, Biochemistry 4, 1973 (1965).

88. Grunberg-Manago, M., and J. Dondon, Biochem. Biophys. Res. Communs., 18, 517 (1965).

89. Stanley, Wendell M., Jr., Margarita Solas, Albert J. Wahba, and Severo Ochoa, Proc. Natl. Acad. Sci., U. S., 56, 290 (1966).

90. Marcker, K., and F. Sanger, J. Mol. Biol. 8, 835 (1964).

91. Nakamoto, Tokamusa, and Daniel Kolakofsky, Proc. Natl. Acad. Sci., U. S., 55, 607 (1966).

92. Ochoa, M., Jr., and I. B. Weinstein, Biochim. Biophys. Acta, 95, 176 (1965).

93. Hershko, A., S. Amoz, and J. Mager, Biochem. Biophys. Res. Communs., 5, 46 (1961).

94. Nathans, D., and F. Lipmann, Proc. Natl. Acad. Sci., U. S., 47, 497 (1961).

95. Gilbert, W., J. Mol. Biol. 6, 389 (1963).

96. Bretscher, M. S., J. Mol. Biol. 7, 446 (1963).

97. Bretscher, M. S., J. Mol. Biol. 12, 913 (1965).

98. Brenner, S., A. O. W. Stretten, and S. Kaplan, Nature 206, 994 (1965).

99. Mager, J., M. Benedict, and M. Artman, Biochim. Biophys. Acta, 62, 204 (1962).

100. Siekevitz, P., in Protein Biosynthesis, (R. J. Harris, Ed.), Academic Press, New York, N. Y., 1961, page 259.

101. Purutz, M. F., M. G. Rossmann, A. F. Cullis, H. Muirhead, G. Will, and A. C. North, Nature, 185, 416 (1960).

119. Lamborg, M. R., and P. C. Zamecnik, Biochim. Biophys. Acta 42, 206 (1960).

120. Fox, A. S., S. H. Kang, Federation Proc. 22, 303 (1963).

121. Peterman, M. L. (Ed.) The Physical and Chemical Properties of Ribosomes, Elsevier Publishing Co., 1964, pp. 122-125.

122. So, Antero G., and Earl W. Davie, Biochemistry 2, 132 (1963).

123. Martin, Robert J., and Bruce N. Ames, Proc. Natl. Acad. Sci. 48, 1271 (1962).

124. Barnett, L. B., G. Frens, and Koningsberger, Biochem. J. 84, 89 (1962).

125. Zamecnik, P. C., and E. B. Keller, J. Biol. Chem. 209, 337 (1954).

126. Takanami, M., Biochim. Biophys. Acta 39, 318 (1960).

127. Maeda, A., and K. Imahori, Biochim. Biophys. Acta 76, 543 (1963).

128. Adams, F., distributed by California Corporation for Biochemical Research, Los Angeles, Calif.

129. Hultin, T., H. A. Leon, and E. Cerasi, Exptl. Cell Res. 25, 660 (1961).

130. Kormer, A., Biochem. J. 81, 168 (1961).

131. Lucas, Jean M., A. H. W. M. Schuurs, and Melvin V. Simpson, Federation Proc. 22, 302 (1963).

132. Marcus, Abraham, and John Feeley, Biochemistry 240, 1675 (1966).

133. Rabson, Robert, and G. David Novelli, Proc. Natl. Acad. Sci., U. S., 46, 484 (1960).

134. Spencer, Donald, Arch. Biochem. Biophys. 111, 381 (1965).

135. Lamfron, H., and E. R. Glowacki, J. Mol. Biol. 5, 97 (1962).

136. Francki, R. I. B., N. K. Boardman, and S. G. Wildman, Biochemistry 4, 865 (1965).

137. Spencer, Donald, and S. G. Wildman, Biochemistry 3, 954 (1964).

138. Eisenstadt, Jerome, and George Brawerman, Biochim. Biophys. Acta 80, 463 (1964).

139. Chesters, J. K., Biochim. Biophys. Acta 114, 385 (1966).

140. Weinstein, I. Bernard, and Alan N. Schechter, Proc. Natl. Acad. Sci., U. S., 48, 1686 (1962).

141. Webster, G. C., Arch. Biochem. Biophys. 68, 602 (1957).

142. Rendi, R., and S. Ochoa, J. Biol. Chem. 237, 3711 (1962).

143. Warburg, O., and W. Christian, Biochem. Z. 310, 384 (1941).

144. Lengyel, Peter, Joseph F. Speyer, and Severo Ochoa, Proc. Natl. Acad. Sci., U. S., 47, 1936 (1961).

145. Decken, A. von der and P. N. Campbell, Biochem. J. 82, 448 (1962).

146. Marcus, A., and J. Feeley, Proc. Natl. Acad. Sci. 51, 1075 (1964).

147. Nicholas, Seeds W., and Thomas W. Conway, Biochem. Biophys. Res. Commums. 23, 111 (1966).

148. Nirenberg, M., and P. Leder, Science 145, 1399 (1964).

149. Kaji, H. and A. Kaji, Proc. Natl. Acad. Sci. 54, 213 (1965).

150. Nakamoto, T., T. W. Conway, J. E. Allende, G. Spyrides, and F. Lipmann, Cold Spring Harbor Symposium on Quantitative Biology, 28, 227 (1963).

151. Hardesty, B., R. Arlinghaus, J. Schaeffer, and R. Schett, Cold Spring Harbor Symposium on Quantitative Biology 28, 215 (1963).

152. Algranati, Israel D., and Peter Lengyel, J. Biol. Chem. 241, 1778 (1966).

153. Cohen, S. S., and J. Lichtenstein, J. Biol. Chem. 235, 2112 (1960).

154. Zamecnik, P. C., M. L. Stephenson, and L. I. Hecht, Proc. Natl. Acad. Sci., U. S. 44, 73 (1958).

155. Crick, F. H., Symp. Soc. Exptl. Biol. 12, 138 (1958).

156. Weinstein, I. Bernard, Manuel Ochoa, Jr., and S. Marvin Friedman, Biochem. Biophys. Res. Communs. 21, 339 (1965).

157. Weinstein, I. Bernard, Manuel Ochoa, Jr., and S. Marvin Friedman, Biochim. Biophys. Acta, 114, 593 (1965).

158. Connell, G. E., P. Lengyel, and R. C. Warner, Biochim. Biophys. Acta, 49, 422 (1961).

159. Godson, G. N., G. D. Hunter, and J. A. W. Butler, Biochem. J., 81, 59 (1961).

160. Heredia, Claudio F., and Harlyn O. Halvorson, Biochemistry, 5, 946 (1966).

161. Wiseman, Alan, Organization for Protein Biosynthesis, New York, American Elsevier Publishing Company, Inc., 1965.

162. Chao, F. C., and H. K. Schachman, Arch. Biochem. Biophys. 61, 220 (1956).

163. Ledgeberg, S., and J. M. Mitchison, Biochim. Biophys. Acta, 55, 104 (1962).

164. Yin, F. H., and R. M. Bock, Federation Proc. 19, 137 (1960).

165. Tsio, P. O.P., Ann. Rev. Plant Physiol. 13, 45 (1962).

166. Tsio, P. O. P., J. Bonner, and J. Vinograd, Biochim. Biophys. Acta, 30, 570 (1958).

167. Lyttleton, J. W., Biochem. J., 74, 82 (1960).

168. Edelman, I. S., P. O. P. Tsio, and J. Vinograd, Biochim. Biophys. Acta, 43, 393 (1960).

169. Ts'o, P. O. P. and A. Lubell, Arch. Biochem. Biophys., 86, 19 (1960).

170. Hamilton, M. G., L. F. Cavalieri, and M. L. Petermann, J. Biol. Chem. 237, 115 (1962).

171. Takanami, M., Biochim. Biophys. Acta 39, 152 (1960).

172. Petermann, M. D. (Ed.) The Physical and Chemical Properties of Ribosomes, Elsevier Publishing Co., New York, New York, 1964, p. 15.

173. Petermann, M. L. (Ed.) The Physical and Chemical Properties of Ribosomes, Elsevier Publishing Co., 1964, pp. 12-13.

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DEVELOPMENT AND CHARACTERIZATION OF CELL-FREE AMINO ACID INCORPORATION SYSTEMS FROM CELLVIBRIO GILVUS

ABSTRACT

Thong Sung Ko

A cell-free system which was active with respect to ^{14}C-amino acid incorporation into hot trichloroacetic acid insoluble material was derived from Cellvibrio gilvus and characteristics of the cell-free system of amino acid incorporation were investigated. This cell-free system for ^{14}C-amino acid incorporation was typical in its requirement for ribosomes, pH 5 enzyme proteins, a critical concentration of exogeneous source of adenosine triphosphate (ATP) plus ATP-generating system and of magnesium ions (0.01-0.015M in ^{14}C-phenylalanine incorporation) and in its inhibition by ribonuclease and puromycin. For optimum incorporation guanosine triphosphate (GTP) and optimum concentration of ammonium ions (100 mM in ^{14}C-leucine incorporation) were required. Moderate inhibition (64% inhibition in ^{14}C-leucine incorporation) by chloramphenicol, no significant effect by actinomycin D and deoxyribonuclease on ^{14}C-leucine incorporation was observed. Omission of poly U inhibited ^{14}C-phenylalanine incorporation about 93% in the presence of an exogeneous source of transfer ribonucleic acid (t-RNA). Addition of t-RNA doubled ^{14}C-phenylalanine incorporation, in the presence of polyuridylic acid, but had no effect on basal ^{14}C-leucine incorporation. At a concentration of 0.5 µmoles each of these polyamines (spermidine, spermine, and putrescine) per ml of reaction

mixtures, there was no noticeable effect on ^{14}C-leucine incorporation, whereas a slight stimulation effect was observed on ^{14}C-phenylalanine incorporation. Slight inhibition of ^{14}C-leucine incorporation by a mixture of complementary nineteen unlabeled amino acids was observed. Optimum pH and optimum temperature for this cell-free system of ^{14}C-leucine incorporation was about 7.8 and 30° C. ^{14}C-Leucine incorporation continued at undiminished rate for 30 minutes.