Studies on the mechanism of action of chloramphenicol
by Edward Joseph Morgan

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
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Abstract:
Inhibition of protein synthesis by the antibiotic chloramphenicol has been viewed in the past as involving inhibition of peptide chain elongation. This view has been supported by chloramphenicol's inhibition of the puromycin reaction and inhibition only of peptides larger than the tripeptide in the polyadenylic and" polyuridylic acid directed systems. Evidence is hereby presented to demonstrate that in a natural mRNA directed in vitro protein synthesizing system, inhibition of protein synthesis by chloramphenicol is at the level of initiation.

S-30 assisted nascent 14C-labeled material was inhibited by 75% when MS2 mRNA directed protein synthesis was incubated with a concentration of 2.5 µg./ml. of chloramphenicol, whereas completed in vitro chains were inhibited by only 25%. Total acid precipitable counts were inhibited by 50%. Inhibition of nascent material would not be expected to be greater than inhibition of completed chains if peptide elongation were affected. A model of peptide chain initiation would, however, be expected to inhibit the nascent material greater than the completed chains.

Tryptic peptide fingerprints were made of both the ribosomal bound and released MS2 mRNA directed 14c-labeled in vitro MS2 coat protein synthesized in the presence of 2.5 µg./ml. of chloramphenicol. Chloramphenicol was found only to alter the level of completed chains and nascent material, leaving the distribution and number of tryptic peptides unaffected. This suggests that chloramphenicol's mechanism of action was neither peptide chain termination, premature release nor random initiation. Furthermore, since inhibition of peptide chain extension should not have produced a greater inhibition of nascent material than of completed material, the results suggest inhibition of initiation.

A high salt ribosomal wash, known to contain the recognized bacterial initiation factors, has been shown to stimulate protein synthesis in the polycytidylic acid directed system. An in vitro MS2 mRNA directed system was developed which was fully dependent upon addition of these crude initiation factors. This system utilized DEAE washed ribosomes, crude initiation factors and a fractionated S-100 supernatant, among other things. A concentration of 0.9 µg./ml. of chloramphenicol inhibited total acid precipitable counts by 50%.

Incorporation of 14C-amino acids independent of crude initiation factors was not inhibited at a concentration of 10 µg./ml. of chloramphenicol. These results suggest that chloramphenicol's mechanism of action at low concentrations in a natural messenger RNA directed system is at the level of initiation, namely at the level of action of the recognized bacterial initiation factors.

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STUDIES ON THE MECHANISM OF ACTION OF CHLORAMPHENICOL

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Chemistry

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"To make a great dream come true, the first requirement is a great capacity to dream; the second is persistence — a faith in the dream."

Hans Selye, M.D., Ph.D.

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ABSTRACT

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1. INTRODUCTION

(a) Receptors and drug action

The most fundamental aspect of pharmacodynamics is that which deals with the mechanism of drug action. The knowledge of molecular mechanisms of antimicrobial drugs has increased exponentially during the past several years. This advance has paralleled, and was in large part a by-product of, increased understanding of the replication of genetic information and its translation into the synthesis of specific proteins.

As early as 1868, even before Langley coined the term "receptive substance", he suggested that drug/cell interactions, and hence the actions and effects of drugs, were probably governed by the law of mass action. This view was extensively developed by A. J. Clark in the 1920's, and it remains the keystone of most theories of drug action. Consequently, theories of drug action are quite similar to theories of enzyme action and are essentially identical when a drug serves as an antimetabolite or enzyme inhibitor. The cellular component directly involved in the initial action of a drug is usually termed its receptor. Receptor sites are similar to the active sites of enzymes. Carboxyl, amino, sulfhydryl, phosphate and similar reactive groups are thought to be utilized in a spatially oriented pattern complementary to that of the drugs with which they react. The binding of drug to receptor is thought

to be accomplished mainly by ionic and other rather weak van der Waals type forces, although occasionally a firm covalent bond is formed.

(b) The modes of action of antimicrobial drugs

Antimicrobial agents may be conveniently grouped according to their molecular mechanism of action.

1. Agents that impede replication of genetic material. Nalidixic acid falls within this category and is an effective agent against bacteria, primarily those which are Gram negative. It is structurally related to the purines, and has been shown to block DNA synthesis in susceptible organisms (McNall, 1960).

2. Agents that impair translation of genetic information. Such drugs inhibit either protein synthesis (the tetracyclines and erythromycin) or induce formation of defective protein molecules (kanamycin and neomycin). The former are bacteriostatic and the latter bacteriocidal.

3. Agents that impair transcription of genetic material. Puromycin is a drug of this class. The drug structurally resembles transfer RNA, and forms a peptide bond with the growing peptide chain causing premature release of the growing chain.

4. Agents that alter structure and function of the cell wall. The penicillins are most representative of this group, inhibiting cross linking (transpeptidation) of the glycopeptide cell wall (Tipper & Schrominger, 1965).

5. Agents that restrict function of the cell membrane. Polymyxin B and colistin (Polymyxin E) are drugs of this class. Both act as cationic detergents with an affinity for phosphate radicals, thereby altering the osmotic barrier function of the cell membrane (Newton, 1954).

For the molecular biologist, drugs that inhibit the transcription and translation of genetic information have been vital tools in the stepwise dissection of the mechanisms of genetic expression. One of these drugs, chloramphencol, is commonly used as an inhibitor of protein biosynthesis. Although discovered in 1943, its molecular mechanism of action has remained obscure.

In clinical use, these same inhibitors of gene action have been potent weapons against microbial infections and neoplastic processes. Associated with the potency of these drugs are serious and life threatening side effects. The iatrogenic leukemias of antineoplastic agents and chloramphenicol induced aplastic anemias are all too common in clincial practice.

(c) Antimicrobial drugs in protein synthesis

The amount of knowledge concerning protein biosynthesis has exploded into a vast series of delicate reactions and subreactions since the notion in the early 1940's by Brachet and Casperson that RNA might be involved in protein synthesis (Brachet, 1942). Requirements for specific RNA species, ribosomes, supernatant factors, ribosomal factors

and energy sources have been demonstrated, and further refinements of these mechanisms are certain to emerge.

It is a simplistic notion to assume that a particular agent inhibits protein synthesis at "initiation", "elongation", or "termination". The definitions of these steps as well as the actual molecular mechanisms overlap, and little is said of the detailed inhibitory reaction itself. Indeed, what was considered initiation in 1970 was shown to be a part of elongation in 1971 (Thach & Thach, 1971). Accordingly, a detailed review of protein biosynthesis is in order.

Protein synthesis may be conveniently categorized into three groups of processes as defined below:

1. Initiation. The assembly of the molecular biological subunits up to but not including translocation or formation of the first peptide bond.

2. Elongation. The formation of the first peptide bond, and all peptide bonds thereafter, as directed by a specific messenger RNA ($_m$RNA).

3. Termination and release. The steps taking place after the completion of the final peptide bond, resulting in a polypeptide chain released from the protein synthesizing subunits.

It was well established by 1963 (Watson, 1963) that protein biosynthesis utilized the genetic message coded by $_m$RNA, and with the help of specific transfer RNA's, the $_m$RNA formed a complex with

ribosomes allowing the genetic message to be translated. Since then, the individual steps within this process have been examined, and further requirements have been shown.

Initiation

Initiation is defined as those steps occurring up to, but not including the formation of the first peptide bond. Furthermore, initiation does not include any movement of the ribosome relative to the $_{m}$RNA (Thach & Thach, 1971).

Protein synthesis occurs in the form of an acid precipitable polypeptide when a mixture of ribosomes washed with a high salt buffer, a synthetic $_{m}$RNA, GTP as an energy source, specific divalent cations, supernatant factors, and the appropriate ^{14}C amino acids are incubated at 37^{o}C (Nathans, Notani, Schwarz & Zinder, 1962). Such a system will not, however, allow for translation of natural $_{m}$RNA's unless factors recovered in the high salt ribosomal wash are added (Brawerman & Eisenstadt, 1966; Stanley, Salas, Wahba & Ochoa, 1966). The ribosomal wash contains initiation factors as defined by their role in the initiation of protein synthesis. They have been extensively purified (Revel, Lelong, Brawerman & Gros, 1968; Revel, Herzberg, Becarevic & Gross, 1968), and a summary of their properties appears in Table I.

In order to adequately discuss the role of initiation factors in protein synthesis, it is helpful to refer to a visual model. It is

recognized that such a model suffers from all the limitations of any attempt to graphically display a complex biological process (Fig. 1).

The first step in initiation is the formation of complex (I), comprised of a 30s ribosomal subunit, f-met-tRNA, initiation factors f_1 and f_2, an appropriate initiation codon (in this case AUG), GTP, and the requisite divalent cations (Thach & Thach, 1971).

Although Ohta, Sarkar & Thach (1967) and Lengyel & Soll (1969) have argued that f-met-tRNA binds to the "A" (acceptor) site, Thach & Thach (1971) have argued that the ultimate functional binding of the former is to an "unaccommodated P" site, or more succinctly, to the "pre P" site (P for peptidyl), although it may have passed through an "A" site. The "pre P" site is the same as the "P" site, however the amino acid portion of the aa-tRNA complex is not orientated in a spatially correct manner to the peptidyl transferase. When the tRNA is in the "P" site, it is complex (III). Refer to Fig. 1.

Once complex (I) is formed, a 50s ribosomal subunit joins to form complex (II), a rather short-lived intermediate (Thach & Thach, 1971). Upon the formation of complex (II), f_1 dissociates to recycle again. Complex (II) immediately proceeds to complex (III), during which time the f-met-tRNA is "accommodated" (sterically shifted) into the "P" site from the "pre P" site. GTP is hydrolyzed as the energy source for this step and f_2 is split off (Hershey & Thach, 1967; Kolafofsky, Dewey, Hershey & Thach, 1968).

Factor f_3 is also classified as an initiation factor (Revel, Lelong, Brawerman & Gros, 1968), however its exact function remains in question. Several groups (Ravel, Shorey, Garner, Dawkins & Shive, 1969; Kaempfer, 1971) have implicated f_3 as a specific recognition protein in natural $_m$RNA initiation. Dubnoff & Maitra (1969) have reported that in addition to stimulating a natural $_m$RNA system, f_3 causes the dissociation of free 70s ribosomes into 50s and 30s subunits. In addition, they demonstrated that f_3 is also required for complex (I) formation. Perhaps the most convincing model of action of f_3 comes from Kaempfer (1971) who has shown that factor f_3 prevents the formation of 70s ribosomal particles from 50s and 30s subunits. Kaempfer's work also has shown an inverse relationship between the rate of protein synthesis and the concentration of free 70s ribosomes. He concludes that f_3 serves to regulate the rate of initiation of protein synthesis. When the rate of protein synthesis is high, the concentration of f_3 decreases, due to its use in complex (I) formation. Consequently, the 50s and 30s subunits released during peptide termination form 70s ribosomes which are inactive in peptide initiation. The rate of protein synthesis may then drop, the concentration of free f_3 increases, dissociating the 70s to free 50s and 30s subunits which can again initiate protein synthesis.

In any case, with the formation of complex (III), the initiation process ends. In this state the "P" site is filled with f-met-tRNA, and the adjacent "A" site is empty. The process of elongation now ensues.

Many antibiotics exert their effect by inhibiting the initiation process. The tetracyclines and lincosinamides abolish bacterial protein synthesis by inhibiting the binding of aa-tRNA to ribosomes. The tetracyclines bind specifically to the 30s ribosomal subunit and presumably block complex (I) formation (Suarez & Nathans, 1965). Lincomycin binds specifically to the 50s subunit, and perhaps blocks complex (II) formation (Chang, Sih & Weisblum, 1966).

Although not classed as a drug, GMP-PCP (5'-guanylmethelene-diphosphonate) can block formation of complex (III) (Thach & Thach, 1971). This complex requires the hydrolysis of GTP to GDP and inorganic phosphate for its formation. GMP-PCP will bind in place of GTP, but since it cannot be hydrolyzed, the complex is competitively inhibited.

Elongation

Immediately after the formation of complex (III), elongation proceeds with the binding of transfer factor Tu, GTP, and the second aa-tRNA (in the illustration it is val-tRNA) to form complex (IV) (Thach & Thach, 1971). In this state, the aa-tRNA is bound to the "pre A" site (analagous to the "pre P" site). Tu, in a manner analagous to f_2, catalyzes the hydrolysis of GTP thus "accommodating" the new aa-tRNA and forming complex (V).

With the two aa-tRNA molecules in correct steric apposition to the peptidyl transferase enzyme, peptide bond formation occurs. The

result of this, complex (VI), retains the f-met-val-tRNA in the "A" site, and a free tRNA in the "P" site.

Complex (VI) is now in a position to undergo translocation. Although several intermediates may be involved, the hydrolysis of GTP catalyzed by elongation factor G causes the movement of the $_{m}$RNA three nucleotides in the 5' direction (Watson, 1964). The next triplet in sequence is then made available for recognition by the "pre A" site.

Complex (VII) is formed with the simultaneous release of free tRNA from the "P" site, and the "P" site now contains f-met-val-tRNA. The complex is now in a state analagous to that of complex (III), and a new specific aa-tRNA may bind to the now vacant "pre A" site. Elongation now proceeds in a cyclic fashion for each successive codon until a termination codon is encountered.

A whole host of agents have been shown to block peptide elongation. GMP-PCP will also block formation of complexes (V) and (VII), as both require hydrolysis of GTP for their formation. Fuscidic acid also inhibits the transition from complex (VI) to (VII) by inhibiting G factor (Tanaka, Kinoshita & Masukawa, 1968). Puromycin can react with any complex (III)-like monosome, forming an aa-puromycin molecule which dissociates from the complex prematurely terminating protein synthesis (Morris, 1961). The aminoglycoside antibiotics, streptomycin, kanamycin, and neomycin produce, among other things, specific misreadings in the genetic code during elongation (Davies, Gilbert & Gorini,

1964). After attaching to the ribosome, these drugs appear to permit incorporation of one or more incorrect amino acids into a growing peptide chain, resulting in synthesis of defective proteins.

Termination

It was found by the study of specific $_m$RNA mutants that certain codons caused termination of peptide synthesis. When codons UAA (ochre), UAG (amber), and UGA (umber) were read by the ribosomal complex, protein synthesis was terminated by release of the peptide from the ribosomal bound tRNA (Garen, 1968; Last, Stanley, Salas, et al., 1967).

Factors necessary for termination in natural messenger RNA systems have been reported. Factor R_1 is specific for termination codons UAA or UAG, and Factor R_2 for codons UAA or UGA (Capecchi, 1967; Caskey, Tomkins, Scolnick, Caryk & Nirenberg, 1968; Tomkins & Nirenberg, 1968). The absence of these release factors causes inhibition of protein synthesis by preventing release of the completed peptide chain. Upon release of the growing peptide chain, the ribosomal complex dissociates, producing free 30s and 50s ribosomal subunits, as well as free $_m$RNA. The 30s ribosomal subunit may now again initiate protein synthesis, or form transient 70s units (Kaempfer, 1971).

(d) Chloramphenicol in protein synthesis

Chloramphenicol's mode of action has eluded scores of investigators since its discovery in a soil sample obtained from Venezuela in 1943. Originally excreted by Streptomyces venezuelae, chloramphenicol earned the distinction of being the first commercially synthesized drug (Controulis, 1949). Its ability to inhibit protein synthesis in bacteria (Wisseman, Hann, Hoops & Smadel, 1962) led to its common use in molecular biology. For this reason it is highly desirable to know its precise molecular mechanism of action.

Chloramphenicol is an incredibly effective drug against Gram positive and negative bacteria, blue-green algae and certain viruses (Kozinsky, 1963), while remaining quite ineffective against mammalian cells (Vazquez, 1966). It is a related member of the ephedra series of alkaloids, with an accepted chemical name of D(-)threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol, and was first commercially synthesized by Long & Troutman in 1949. Its structure is shown in Fig. 2.

The first mode of action experiment implicated its inhibitory action on bacterial esterases (Smith, Worrel & Swanson, 1949), but it was left to Gale and Paine in 1951 to give the first clue that chloramphenicol inhibited protein metabolism. In a now classic experiment, Hahn and Wisseman in 1951 showed that chloramphenicol inhibited the

formation of adaptive enzymes. Since that time, it has been generally accepted that chloramphenicol inhibits protein synthesis.

Structural studies on the drug show that the dichloroacetamido, hydroxyls and propanol groups are necessary for biologic activity (Brock, 1961; Smith & Hinman, 1953; Wooley, 1950). The nitro group need only be a polarizing group for activity (Brock, 1961). The amide bond and hydroxyls are thought to be the essential constituents for biological activity, since alteration of these moieties reduces the biological activity to zero (Brock, 1961). It is of further interest that, of the four isomers possible, only the D(-)threo has biological activity (Hahn & Wisseman, 1951).

Since 1951, experiments have centered on finding the exact molecular mechanism of action. It is now known that chloramphenicol does not inhibit amino acid activation (DeMoss & Novelli, 1955), does not inhibit formation of the amino acyl tRNA complex (Lacks & Gros, 1959), and does not have any effect on DNA or RNA synthesis (Gale, 1962). Furthermore, chloramphenicol does not inhibit the binding of tRNA to ribosomes (Cannon, Krug & Gilbert, 1963; Jardetzky & Julian, 1964) and it does not prevent the release of completed protein (Das, Goldstein & Kanner, 1966).

Binding studies have shown that chloramphenicol binds weakly but specifically to the 50s ribosomal subunit (Vazquez, 1966a, 1964), and such binding occurs immediately upon the incubation of ribosomes

and chloramphenicol. The binding requires no energy and is reversible (Lehninger, 1961). Such studies show a chloramphenicol-ribosome binding ratio of 1:1 at chloramphenicol concentrations below 50 μg./ml. (Wolfe & Hahn, 1965), and a binding ratio of 2:1 at concentrations exceeding 200 μg./ml. (Das, Goldstein & Kanner, 1965). The implication of two different binding configurations is not clear.

It would appear obvious from the literature that chloramphenicol inhibition is due to interference with protein synthesis. However, extreme care must be taken in the interpretation of such data. Chloramphenicol has been shown to be a potent inhibitor of glucose transfer in teichoic acid biosynthesis, powerfully inhibiting cell wall synthesis in concentrations of chloramphenicol of 500 μg./ml. (Stow, 1971). Due to the differential permeability of chloramphenicol among microorganisms, it is difficult to extrapolate and compare the many in vivo effects of chloramphenicol.

The in vivo concentrations of chloramphenicol required for 50% inhibition range from 0.38 μg./ml. for Hemophilus influenzae to over 100 μg./ml. for Pseudomonas. Escherichia coli alone varies between 3.1 and 100+ μg./ml., with a median of 6.2 μg./ml. (Kagan, 1970). Indeed, such a wide range of effective chloramphenicol concentrations in E. coli might reflect subtle differences in mechanisms of inhibition of protein synthesis, as well as differences in drug permeability.

In vitro studies using purified ribosomes again show a wide range in the effectiveness of chloramphenicol. Using 60% inhibition of total ^{14}C acid precipitable counts as a reference, a poly A system requires 67 µg./ml. of chloramphenicol (Julian, 1965), whereas an MS2 $_m$RNA-directed system requires only ~2 µg./ml. of chloramphenicol (see "Experimental Results").

2. EXPERIMENTAL RATIONALE

MS2 virus is an icosahedral shaped bacteriophage of 25 millimicrons diameter, a molecular weight of about 3.5 million daltons, and a sedimentation constant of 81 Svedburg units (Strauss & Sinsheimer, 1963). It has an information content in its single stranded RNA core sufficient to code for three proteins: the coat protein, a maturation factor, and an RNA dependent RNA polymerase (Capecchi, 1966).

When purified MS2 RNA is placed into an in vitro protein synthesizing system, over ten times as much coat protein is produced as the other two proteins (Ward, 1968; Kolakofsky, Dewey, Hershey & Thach, 1968). If the amino acid histidine is left out of the incubation mixture, only coat protein should be produced (Nathans, Notani, Schwarz & Zinder, 1962).

Such a system allows the in vitro production of a unique protein with a known amino acid sequence (Weber & Konigsberg, 1967; Lin, Tsung & Fraenkel-Conrat, 1967). Furthermore, it has been shown that the in vitro product is essentially the same as the native coat protein molecule (Nathans, Notani, Schwarz & Zinder, 1962; Nathans, Oeschger, Eggen & Shimura, 1966). The amino acid sequence of MS2 coat protein is shown in Fig. 3. A convenient yet highly reproducible and exact method for assaying the MS2 in vitro coat protein may be gleaned from the techniques developed by Katz, Dreyer & Anfinsen in 1959.

Fingerprinting involves the reproducible oxidation and enzymatic digestion of a protein to smaller peptides. The protein is first oxidized with peroxyformic acid, which ruptures disulfide bonds and usually increases the solubility in aqueous solvents. Hydrolysis with trypsin is next employed, which cleaves the protein at the carboxyl ends of lysine and arginine. This procedure produces a distinct set of smaller peptides with a distribution, size, and properties characteristic of the original coat protein.

These tryptic peptides, eleven in the case of MS2 coat protein, are displayed as a two-dimensional map, the fingerprint. The peptides resulting from the hydrolysis of the coat protein are first separated by high-voltage electrophoresis on a sheet of filter paper, and secondly by ascending chromatography in a direction 90° to that used during electrophoresis. The result is a two-dimensional display of eleven spots which have been separated by size and charge, and made visible by spraying with ninhydrin, a reagent which reacts with amino groups producing a visible color.

A fingerprint can also be made of the in vitro MS2 coat protein by initially labeling the in vitro protein with ^{14}C-amino acids. The protein is then fingerprinted. To visualize the ^{14}C-tryptic peptides, a sheet of unexposed X-ray film is exposed to the fingerprint for about a week, and the X-ray film developed. Each radioactive tryptic peptide will produce a radio-opaque spot on the X-ray film. In this case, only ten

spots should occur as peptide number eleven will not have any ^{14}C-amino acid associated with it, if ^{14}C-lysine and ^{14}C-arginine are used as the only radioactive labels.

The translation at any point in time of the coat protein cistron of the MS2 virus natural $_m$RNA may be envisioned as involving a population of ribosome/messenger complexes in all stages of translation. Consequently, every complex will have incorporated an N-terminal amino acid, but only those complexes in the final stages of polypeptide synthesis will have incorporated a C-terminal amino acid. If chloramphenicol (CAP) were to block a step in elongation, we would expect a population of ribosome/messenger complexes frozen in the various stages of elongation. Hence, we would not expect any decrease in the ribosomal ^{14}C nascent peptides as compared to a normally occurring protein synthesizing system. Furthermore, as elongation was inhibited on a $_m$RNA strand, an increase in the quantity of the N-terminal peptides might be expected as newly initiated ribosomes were allowed to synthesize peptides before being inhibited in some stage of elongation.

Nathans (1965) examined the effect of CAP on the released ^{14}C-labeled MS2 *in vitro* peptides. His methodology utilized a fingerprint of a tryptic digest of the non-ribosomal bound ^{14}C-labeled MS2 *in vitro* peptides. He found no increase in the tryptic N-terminal peptides over the C-terminal peptides when such peptides were synthesized in the presence of 3.5 μg./ml. of CAP. This was in contrast to the released

peptides formed in the presence of puromycin, which showed an increase in the number of N-terminal peptides. These results were in accord with an elongation inhibition model for CAP, as one would expect the released peptides to be whole and possess an equal number of N- and C-terminal peptides. His work also tended to rule out CAP induced premature release, for in this case we should expect results similar to those seen with the released peptides synthesized in the presence of puromycin. However, he did not examine CAP's effect on the ribosomal bound ^{14}C-nascent peptides. It is here that the true test for an elongation inhibition model exists, since the peptides resulting from inhibition would likely be trapped on the messenger-ribosome complex. It was thus decided to compare the ribosomal bound to released products using Nathan's procedures.

3. MATERIALS AND METHODS

(a) Bacterial and bacteriophage strains

Escherichia coli Q-13 was obtained from General Biochemicals in late log phase, unwashed. The bacteria were delivered frozen in 40-g. chunks and stored at -10°C until used. Cat. #150070. E. coli Q-13 was also obtained from Dr. G. Streisinger of the University of Oregon, Eugene. Such bacteria were used as a source of ribosomes, S-30 extracts and as a virus host. Escherichia coli A-19 was obtained from Dr. J. Clark of the University of Illinois, Urbana, and used for S-30 extracts and as a virus host. Escherichia coli K-12 was used as a virus host. Escherichia coli B was used as a host differentiating phage T_4 and MS2. Escherichia coli MRE-600 was obtained from Dr. J. Davies of the University of Wisconsin, Madison. It was grown on media LS and used for the preparation of initiation factors and washed ribosomes.

Bacteriophage MS2 was obtained as a lyophylized powder from the American Type Culture Association, Rockville, Maryland. Cat. #15597-B. It was used for the preparation of virus $_m$RNA and coat protein.

(b) Bacterial and viral growth media

All weights refer to grams per liter of single distilled water. All media were autoclaved for 30 minutes at 15 to 20# of steam.

TYS^{+} Medium

10 g. Bacto-tryptone

1 g. Bacto-yeast extract

8 g. sodium chloride

After autoclaving, the following (each autoclaved separately) were added using sterile technique:

10 ml. of 10% glucose

2 ml. of 1 M calcium chloride

1 ml. of thiamine hydrochloride (10 mg./ml.)

(TYS medium less the glucose, calcium and thiamine was designated TYS^{-}. Upon the addition of these solutions, TYS^{+}.)

Top Layer

TYS^{+} medium with 4 gm./liter Bacto-agar

Agar Plates and Slants

TYS^{-} medium with 15 g./liter Bacto-agar

Serial Dilution Solution

TYS^{-} medium

LS Medium

4.0 g. nutrient broth

4.0 g. NaCl

0.5 g. glucose

0.5 g. yeast extract

1.0 g. KH_2PO_4

0.5 g. $CaCl_2$

0.5 g. $MgCl_2$

(c) Buffers

All buffers were made with doubly-distilled water, the second distillation of tap distilled water occurring in a Corning model AG-2 distillation apparatus. Periodic samples of this water revealed a minimum specific resistance of 800 $K\Omega cm^{-1}$.

All pH adjustments were made with distilled hydrochloric acid. Such acid was the middle third distillate of a 1:2 dilution of distilled water with reagent hydrochloric acid. Measurements of pH were made on a Corning model 12 pH meter, after prior standardization using pHydrion tablets.

Bentonite, a colloidal aluminum silicate clay, was prepared by the method of Dunn & Hitchborn (1965). Such washed bentonite suspensions have been noted for their adsorbant properties in removing ribonucleases from aqueous solutions. Two drops of bentonite suspension per liter of buffer was added and the resulting dispersion allowed to stand at 2°C for at least two hours. The buffer was then filtered through Millipore HAWP00010 50-mm. nitrocellulose filters into a sterile ribonuclease-free reagent bottle using an autoclaved filtration apparatus.

All buffers were stored at 2°C unless otherwise indicated.

Buffer A_1f_2

0.02 M Tris

0.01 M $Mg(OAc)_2$

0.01 M β-mercaptoethanol (added just before use)

The buffer was adjusted to pH 7.4 at room temperature with distilled hydrochloric acid, then bentonite treated and filtered.

Buffer A_sF_2

0.05 M Tris

0.001 M dithiothreitol (added just prior to use)

NH_4SO_4 (enzyme grade, added to saturation at 2°C)

The buffer was adjusted to pH 7.4 at 2°C with distilled hydrochloric acid then bentonite treated and filtered. NH_4SO_4 was then added to saturation at 2°C.

Buffer B_1f_2

1.0 M NH_4Cl

0.02 M Tris

0.002 M $Mg(OAc)_2$

0.002 M dithiothreitol (added just prior to use)

The buffer was adjusted to pH 7.8 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer CBN-1

Pyridine: 50 parts

Acetic acid: 15 parts

n-butanol: 75 parts

glass distilled water: 60 parts

(All parts by volume)

Stored at room temperature.

Buffer EBN-1

Pyridine: 1.2%

Acetic acid: 1.3%

n-butanol: 2.5%

Glass distilled water: 95%

(All percentages by volume)

Stored at room temperature.

Buffer Hi-IFEM

0.002 M $MgCl_2$

1.0 M NH_4Cl

0.01 M Tris

0.001 M dithiothreitol (added before filtering)

The buffer was adjusted to pH 7.5 with distilled hydrochloric acid then bentonite treated and filtered.

Buffer IFEM-1

0.002 M $MgCl_2$

0.025 M NH_4Cl

0.01 M Tris

0.001 M dithiothreitol (added just prior to use)

The buffer was adjusted to pH 7.5 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer IFEM-2

0.002 M $MgCl_2$

0.100 M NH_4Cl

0.010 M Tris

0.001 M dithiothreitol (added just prior to use)

The buffer was adjusted to pH 7.5 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer IFEM-3

0.002 M $MgCl_2$

0.35 M NH_4Cl

0.010 M Tris

0.001 M dithiothreitol (added just prior to use)

The buffer was adjusted to pH 7.5 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer LMSN

0.08 M NH_4Cl

0.0085 M $Mg(OAc)_2$

0.01 M Tris

0.01 M β-mercaptoethanol (added just prior to use)

The buffer was adjusted to pH 8.1 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer LMSN-16

0.08 M NH_4Cl

0.016 M $Mg(OAc)_2$

0.01 M Tris

0.01 M β-mercaptoethanol (added just prior to use)

The buffer was adjusted to pH 7.8 at 2°C with distilled hydrochloric acid then bentonite filtered.

Buffer MSN

0.08 M NH_4Cl

0.085 M $Mg(OAc)_2$

0.01 M Tris

0.01 M β-mercaptoethanol (added just prior to use)

The buffer was adjusted to pH 8.1 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer N

0.08 M NH_4Cl

0.011 M $Mg(OAc)_2$

0.1 M Tris

0.01 M β-mercaptoethanol (added just prior to use)

The buffer was adjusted to pH 8.1 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer OA

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer OB

0.5 M NH_4Cl

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer OC

0.25 M NH_4Cl

0.01 M $MgCl_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer OD

0.5 M NH_4Cl

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

Buffer pH 5'

0.01 M Tris

0.05 M KCl

0.011 M $Mg(OAc)_2$

The buffer was adjusted to pH 5.2 at 2°C with distilled hydrochloric acid then bentonite treated and filtered.

(d) Reagents and chemicals

Alumina

Levigated Abrasive Grain, obtained from the Norton Company, Cat. #220461.

Ammonium Sulfate

Special enzyme grade, obtained from Schwarz/Mann Chemicals, Cat. #1946. Used for salting out initiation factors.

Ammonium Sulfate

Reagent grade, obtained from the Baker-Adamnson Chemicals. Used for salting out virus.

Arginine ^{14}C

Specific activity 306 mCi/mM. Obtained from Amersham-Searle Co., Cat. #CFB 62.

Chloramphenicol

Obtained as a gift from Parke, Davis and Company.

Lysine ^{14}C

Specific activity 312 mCi/mM. Obtained from Amersham-Searle Co., Cat. # CFB 69.

Millipore Filters

HAWP nitrocellulose filters. Obtained from the Millipore Corporation.

Trypsin

TPCK treated trypsin, "B" grade, essentially free of chymotrypsin. Obtained from Calbiochem., Cat. #64852.

(e) Ribonuclease Removal

Ribonuclease (RNAase) was removed from glassware by either: a) heating in a dry oven at 240°C for 10 hours; or b) autoclaving for 20 minutes at 15# pressure. RNAase was removed from plastic ware by

either: a) autoclaving for 20 minutes (autoclavable plastic; or b) treatment with 15% hydrogen peroxide for 15 minutes.

RNAase was removed from buffer solutions by adding 2 drops per liter of a standard bentonite suspension (Dunn & Hitchborn, 1965) after the buffer was prepared. The treated buffer was filtered through a Millipore filtration apparatus equipped with a HAWP 047 Millipore filter, and collected in a sterile Pyrex bottle using sterile filtration.

(f) Growth of phage MS2 on Escherichia coli

The following procedure produced 15 liters of MS2 virus lysate at a titer of approximately 10^{11} virus per ml., and used essentially the method of Clark (1965). The initial virus source was a lyophylized powder of MS2 obtained from the American Type Culture Association.

A loop of E. coli (or other f^+ host) was inoculated from a fresh slant into two ml. of TYS^+ broth. This culture was then grown at 37°C to early log phase, about 10^8 bacteria per ml., or until particles of growth could be seen with swirling. One drop of the rehydrated American Type Culture Association MS2 virus was then added and the incubation continued at 37°C for six to twelve hours. The titer at the end of this period was always a minimum of 10^{11} virus per ml. This two-ml. lysate of MS2 virus was next used to infect 100 ml. of early log phase E. coli (in TYS^+) at a multiplicity of infection (m.o.i.) of 8, yielding a 100-ml. lysate at the same titer of 10^{11} virus per ml.

Twelve two-liter flasks, each containing one and one-half liters of TYS^{+} broth, were allowed to incubate at 37°C for six to ten hours. Any flasks showing cloudy contaminants were discarded. Sufficient E. coli (from a stock stationary phase solution) was added to the remaining sterile flasks to yield a concentration of about 10^{6} bacteria per ml. The bacteria were then allowed to grow to early log phase, at which time they were infected with an aliquot from the 100 ml. MS2 lysate at a m.o.i. of 8. The flasks were swirled in a Gyrotary Shaker (New Brunswick Scientific Company) at 37°C for six to eight hours, after which time they were placed in the cold room for use about eight hours later. The final titer in all cases was higher than 10^{11} virus per ml.

The lysate was next spun in the GSA rotor of the Servall centrifuge for 30 minutes at 5,000 RPM (4080 xg). The supernatant was poured into a chilled 15-liter carboy and more lysate was added to the centrifuge bottles, collecting the cell debris in a single pellet. Both the carboy and centrifuge bottles were thoroughly washed and rinsed with distilled water prior to use, but were not made ribonuclease free.

After centrifuging the entire 15 liters, 311 g. of crystalline reagent grade ammonium sulfate were added per liter of lysate supernatant and the solution stirred with a motorized Teflon propellor. The resulting solution was chilled in the cold room for eight to ten hours.

The precipitate was resuspended by stirring and siphoned into GSA rotor bottles. The phage paste was collected by centrifugation at 5,000 RPM (4080 xg) for 30 minutes. Nine ml. of glass distilled water, adjusted to pH 9 with solid NaOH, were added to each GSA bottle. The bottles were then tilted to allow the water to contact the paste and left for eight to ten hours in the cold room at 2°C.

The suspension was then collected into a single GSA bottle, rinsing the remaining bottles with pH 9 distilled water. Fifty micrograms of DNAase I was then added and the enzyme allowed to work until the paste was free of strands of DNA, usually about ten minutes later.

The DNAase digest was centrifuged for 20 minutes at 13,000 xg in the Beckman Model L-2 centrifuge and the precipitate discarded. The supernatant was next centrifuged for 20 minutes at 26,000 xg and the precipitate again discarded. The phage was now pelleted by centrifugation of the supernatant in the Ti-50 rotor for three hours at 60,000 xg in ribonuclease free centrifuge tubes.

One ml. of pH 9 distilled water was added to the brownish pellet and allowed to dissolve overnight. The solution was clarified by centrifugation at 10,000 xg for 15 minutes in the Ti-50 rotor. The resulting phage solution was then stored at -90°C in the Revco Cold Chest in one-ml. portions. Such aliquots were suitable for extraction of the active MS2 RNA over a year later.

The virus suspension so produced was slightly yellow and opalescent. It was extracted directly for highly purified MS2 RNA. For the extraction of highly purified MS2 coat protein, it was repelleted twice at 60,000 xg for three hours to obtain a clear pellet.

The virus suspension had the ultraviolet spectrum as shown in Fig. 4 and an extinction coefficient of one optical density unit per mg. per ml. These physical constants agreed with those of Strauss & Sinsheimer (1963). Sedimentation velocity profiles obtained in the Beckman Model E analytical ultracentrifuge yielded a single peak of 80 Svedberg units. Electron micrographs made by the author of the purified virus showed polyhedral bodies of approximately 30 mμ which were photographically identical to electron micrographs taken of the MS2 virus by Strauss & Sinsheimer (1963). Fig. 5 depicts a flow chart on the growth and purification of MS2 virus.

(g) Extraction of messenger RNA from phage MS2

The following procedure utilizes the frozen phage suspension. The RNA product obtained is fully capable of directing *in vitro* protein synthesis in combination with *E. coli* ribosomes and supernatant factors.

One ml. of a thawed phage suspension was added to a ribonuclease free 12-ml. conical centrifuge tube. One ml. of glass distilled water saturated phenol (redistilled reagent grade) and 0.1 ml. of 2 M

ammonium carbonate (in glass distilled water) were added simultaneously. The suspension was gently mixed with a ribonuclease free Pasteur pipette for ten minutes at room temperature, taking care to avoid frothing. The mixture at this point had a cloudy light brown color.

The solution was then centrifuged at full speed in the clinical centrifuge at 2°C for 15 minutes. The top aqueous layer containing the RNA was removed with a ribonuclease free Pasteur pipette and placed into a second chilled ribonuclease free conical centrifuge tube. This aqueous extract usually turned cloudy white within a few minutes.

One ml. of cold glass distilled water was then added to the phenol phase for an additional extraction of RNA. The solution was mixed and centrifuged as above and the top aqueous layer added to the first aqueous extract. The coat protein containing phenol phase was saved for further purification.

All traces of phenol were removed from the aqueous layer by extracting six times with reagent grade ether in the cold room. The centrifuge tube was filled to within one centimeter of the top with ether and extracted by mixing with a Pasteur pipette. The conical centrifuge tube was centrifuged at top speed in the clinical centrifuge for 15 minutes and the ether layer aspirated and discarded. At the completion of the sixth extraction the bottom aqueous phase was clear.

All traces of ether were removed by gently blowing a jet of nitrogen over the solution (in an ice bath) until all odor of ether was gone.

Six drops of cold 3 M sodium acetate (in glass distilled water) were then added and the solution mixed. The final flocculation of RNA was achieved by the addition of two volumes of cold reagent grade 100% ethanol and mixing. If the precipitate remained dispersed, 3 M sodium acetate was added dropwise until the solution began to flocculate. In most cases no extra sodium acetate was required.

The flocculated RNA solution was allowed to sit for 40 minutes, followed by centrifugation in the clinical centrifuge for 20 minutes at top speed in the cold room. The ethanol was decanted, and the paste lyophylized to a crusty white dryness. Two hundred fifty to five hundred microliters of cold glass distilled water was added to the dry RNA and allowed to dissolve overnight.

The following day the ultraviolet spectrum of the product was obtained to determine the concentration of RNA. An extinction coefficient of 25 optical density units per mg. of RNA per ml. of water was used, and the RNA bottled in one-mg. portions. Such aliquots were stored at -90°C in the Revco Cold Chest until used. Activity has been found to decrease approximately 50% over a twelve-month period, as measured by its stimulation of ^{14}C-amino acid incorporation. Fig. 6 depicts a flow diagram of the extraction and purification of MS2 RNA.

(h) Extraction of phage MS2 coat protein

This procedure used a phage suspension which had been further purified by pelleting the virus twice at 93,000 xg for 3 hours. The pellet then appeared clear and gave a single sharp peak of 80s in the Beckman Model E analytical ultracentrifuge.

Ten volumes of cold redistilled reagent grade acetone were added to the phenol phase produced by an RNA extraction of the virus. The mixture was allowed to sit for 20 minutes at 2°C followed by centrifugation at top speed in the clinical centrifuge for 45 minutes. The resulting supernatant was aspirated and discarded while the pellet was washed twice with 5 ml. of cold distilled acetone by resuspension and centrifugation for 20 minutes at top speed in the same centrifuge.

The washed protein pellet was suspended in one ml. of 0.5 M sodium hydroxide and dialyzed at 2°C against one liter of 0.5 M sodium hydroxide using a forced flow dialyzer. When performic acid oxidation was planned, the protein was dialyzed against distilled water at 2°C to remove all traces of sodium hydroxide.

Using the extinction coefficient of one optical density unit per mg. per ml. of water (Strauss & Sinsheimer, 1962), the protein was lyopilized to dryness and stored in 1.0-mg. portions in the Revco Cold Chest at -90°C.

(i) Preparation of S-30 extracts

The following method utilized Escherichia coli Q-13 which is a nitrogen mustard induced mutant deficient in cellular ribonucleases, thus permitting the preparation of S-30 extracts low in these ribonucleases. The extraction has also been successfully used on E. coli MRE-600 and K-12. The method follows that of Clark (1965).

Twenty grams of frozen E. coli cells were placed in a large mortar maintained at 2°C, and to it added 10 g. of alumina. Grinding was begun without allowing the cells to thaw, and more alumina added as necessary to keep the cell paste covered. After five minutes of grinding, 5 ml. of cold buffer N was added and grinding continued for another five minutes. A total of 40 ml. of buffer N and 30 g. of alumina were added during the grinding procedure.

The ground cells were transferred to a ribonuclease free 250-ml. beaker, the cells allowed to thaw completely and 20 µg. of DNAase I added from a stock solution of one mg. DNAase per ml. of glass distilled water. The paste was then gently stirred with a glass rod until the DNAase had completed its action, usually within five minutes.

The cell paste was transferred to clean 30-ml. polycarbonate centrifuge tubes and centrifuged at 2°C for 10 minutes at 10,000 xg in the Beckman Model L type 30 rotor. The supernatant was then transferred to clean 30-ml. polycarbonate tubes and spun for 30 minutes at 30,000 xg at 2°C in the same rotor, discarding the precipitate. The top

three-fourths of this supernatant (the S-30 fraction) was removed with a ribonuclease free syringe and transferred to a ribonuclease free 250-ml. beaker. The bottom one-fourth of the supernatant and precipitate was discarded.

In some cases a French Pressure Cell was used to lyse the cells (see "Methods: Preparation of crude initiation factors"). The ensuing steps, however, were identical to those following the alumina grinding.

The S-30 fraction was subsequently pre-incubated under conditions which permitted protein synthesis to occur and allowed any free endogenous $_m$RNA and tRNA to be discharged from the ribosomes. To each 16.0 ml. of the S-30 extract the following were added:

2.0 ml. of 0.12 M PEP (in buffer N or LMSN)

1.0 ml. of 0.05 M ATP (in buffer N or LMSN)

0.6 ml. of 0.01 M GTP (in buffer N or LMSN)

0.8 ml. of a 10^{-3} M solution of each of the 20 L amino acids in buffer N

30 EU of pyruvate kinase

The solution was mixed gently and incubated at 37°C for 40 minuntes. Dialysis then ensued in cellulose tubing which had been pretreated by autoclaving for 15 minutes in 10^{-3} M NaEDTA and 1% $NaHCO_3$ followed by extensive rinses with distilled water.

The S-30 fraction was dialyzed for 12 hours at 2°C against three equally spaced changes of two liters of buffer N (or LMSN). Upon

completion of dialysis the solution was transferred to chilled 30-ml. polycarbonate centrifuge tubes and clarified at 20,000 xg for 10 minutes at 2°C in the Beckman Model L type 30 rotor. The top three-fourths of the solution was then placed into ribonuclease free vials, 1.0 ml. per vial, and stored in the Revco Cold Chest at -90°C.

The stability of preparations so prepared showed an activity loss of less than 50% after 14 months of storage when assayed against an MS2 RNA directed in vitro system.

(j) Ribosome preparation

All reagents, glassware, centrifuge tubes, and materials to be used in the preparation were made sterile and RNAase free by the appropriate treatment (see "Materials and Methods: Ribonuclease removal"). The procedure followed that of Stanley, Salas, Wahba & Ochoa (1966).

Two hundred grams of frozen Escherichia coli strain Q-13 or MRE-600 were ground in three portions on a large mortar and pestle until the cells were slightly thawed. Approximately 70 gm. of alumina were added to each portion and the mixture ground to a thick paste. Approximately 35 ml. of buffer N were added to each portion and the mixture ground to a slurry. This procedure yielded approximately 350 ml. total volume.

The ground homogenate was then placed in centrifuge tubes and centrifuged 40 minutes at 15,000 RPM (39,000 xg) in the SS-34 rotor of the Sorvall RC2-B centrifuge to remove the alumina and cell debris. The

supernatant fraction was removed and 0.6 mg. of deoxyribunuclease I (Worthington Biochemicals) was added and stirred into this fraction. The supernatant fraction was kept at 4°C for 90 minutes to allow complete digestion of the DNA. The DNAase treated fraction was then centrifuged in the 30 rotor of the Beckman Model L-2 ultracentrifuge at 18,000 RPM (30,000 xg) for two hours. The supernatant fraction was removed and transferred to tubes suitable for the 50-Ti rotor, then centrifuged for 4 hours at 50,000 RPM (170,000 xg) in the Model L-2 ultracentrifuge. The top 90% of the supernatant fraction was removed with a syringe and saved as S-100. Five milliliters of buffer OC^+ were added to each tube and the pellet allowed to dissolve overnight with occasional stirring of the pellet surface with a stirring rod.

The tubes containing the dissolved pellet were then filled with buffer OC^+ and centrifuged for 15 minutes at 25,000 RPM (42,000 xg) in the type 50-Ti rotor to remove extraneous debris. The supernatant fraction was then layered on a diethylaminoethyl (DEAE)-cellulose column previously prepared (see "Materials and Methods: DEAE column preparation") and the column washed with 2 liters of buffer OC^+. The ribosomes were eluted from the column with buffer OD^+ and concentrated by centrifugation at 50,000 RPM for 4 hours in the 50-Ti rotor of the Model L-2.

The ribosomes were dissolved in a small amount of buffer BC. The concentration of ribosomes was determined by measuring their absorption at 260 mμ which then permitted the concentration to be

adjusted to either 25 or 50 mg./ml. The resulting solution was then bottled in small aliquots (0.25 to 0.5 ml.) and kept frozen at -90°C prior to use.

(k) DEAE Column preparation — purified ribosomes

Two hundred grams of DEAE cellulose (Calbiochem) were washed with 2 liters of 0.1 N sodium hydroxide by suction filtration, washed to neutrality with distilled water, washed with 2 liters 95% ethanol, and rewashed with distilled water. The resin was then suspended in distilled water and autoclaved for 20 minutes. The resin was poured into a Type KS-100 Pharmacia column (5.0 x 100 cm.) and packed under 10 psi nitrogen pressure. The column was then washed with 4 liters of buffer OC^+. The final dimensions of the resin were 61 x 5 cm.

(l) Preparation of pH 5' fraction

A column was prepared by autoclaving 50 g. of Sephadex G-50 coarse in buffer pH 5' for one-half hour. After cooling to room temperature, the "fines" were aspirated off. The resin was resuspended followed by aspiration of the "fines" two additional times. Finally, the resin was suspended in buffer pH 5' and poured into a 2 cm. x 17 cm. Pyrex column equipped with an extra course porosity sintered glass disc onto which the resin settled. A stopcock governed the flow rate. The column was then washed with 100 ml. of buffer pH 5' and yielded a final resin size of 2 cm. x 12 cm.

The pH 5' fraction was prepared by loading the column with 1.0 to 2.0 ml. of the S-100 fraction and eluting with buffer pH 5'. The milky white precipitate was collected in a ribonuclease free 15-ml. conical centrifuge tube, usually about 6.0 ml. The precipitate was then centrifuged at top speed in the clinical centrifuge for 15 minutes. The supernatant was discarded and the precipitate washed twice with buffer pH 5' by resuspension and centrifugation. The precipitate was finally dissolv' in 0.25 to 1.0 ml. of buffer LMSN-16 and used as the pH 5' fraction. In cases where some sediment remained after dissolving in buffer LMSN-16, it was removed by centrifugation at top speed in the clinical centrifuge for 10 minutes.

(m) Preparation of crude initiation factors

Several methods were tried in the purification of crude initiation factors. The polyethylene glycol/dextran separation reported by Herzberg, Lelong & Revel (1969) proved to be both time consuming and yielded a low recovery. As a result, the procedures developed by Wahba, Chae, Iwasaki, Maxumder, Miller, et al. (1969) were utilized. This method involved washing a ribosomal pellet with a high ionic strength buffer, and recovering the crude factors by an ammonium sulfate precipitation.

Initiation factors were prepared from Escherichia coli MRE-600 which had been grown on LS medium to early log phase. The bacteria were then washed twice with 0.2 M NH_4Cl and once with buffer N. They

were then divided into 25-g. portions and stored at -90°C in the Revco Cold Chest.

To 25 g. of freshly thawed and packed E. coli MRE-600 were added 25 ml. of buffer A_1f_2. The mixture was stirred and immediately passed through the French Pressure Cell at a pressure of between 10,000 and 25,000 psi. The lysate was collected into a ribonuclease free 250-ml. chilled beaker. Two hundred micrograms of DNAase was then added and the enzyme allowed to complete its action, usually within five minutes. The suspension was then poured into Beckman 30-ml. polycarbonate centrifuge tubes and spun at 18,000 RPM (30,000 xg) for 30 minutes in the Model L-2 preparative ultracentrifuge at 2°C. The pellet was discarded and the upper three-fourths of the supernatant was spun again for 4 hours at 50,000 RPM (93,000 xg) in the Ti-50 rotor using 10-ml. polycarbonate screw cap centrifuge tubes. The top two-thirds of this supernatant was carefully removed and bottled in 1.0-ml. portions, designated IFS-100 (the S-100 fraction of an initation factor preparation). The remaining one-third of the supernatant was discarded.

To the pellet was added approximately twice the volume of buffer B_1f_2. The pellet was then gently but completely stirred, and then allowed to soak overnight.

Upon completion of this ribosomal high salt wash, the solution was clarified by centrifugation at 2°C for 30 minutes at 20,000 RPM (30,000 xg) in the Ti-50 rotor. The supernatant from this spin was

finally centrifuged at 2°C for 4 hours at 50,000 RPM (93,000 xg), again in the Ti-50 rotor. The supernatant from this spin contained the crude initiation factors, along with other molecules washed off the ribosomes by the high salt wash.

This supernatant was made 35% saturated (at 2°C) with enzyme grade ammonium sulfate by the appropriate amount of buffer $A_S f_2$. After allowing flocculation to occur for 40 minutes, the precipitate was collected by centrifugation in the SS-34 rotor of the Servall centrifuge for 15 minutes at 15,000 RPM (22,000 xg). The precipitate was discarded.

To the above 35% saturated supernatant was added sufficient buffer $A_S f_2$ to bring the ammonium sulfate saturation to 75%. The solution was again allowed to flocculate for 40 minutes and centrifuged as was the 35% precipitate. The supernatant was discarded and the precipitate retained as the source of crude initiation factors.

The pellet of crude initiation factors was rinsed with 75% ammonium sulfate saturated buffer $A_S f_2$ and stored at 2°C with a volume of 75% saturated buffer $A_S f_2$ sufficient to cover the pellet.

Such preparations retained their stimulatory activity for purified in vitro systems for up to three weeks, after which time their stimulatory activity decreased to less than 10%.

For in vitro incorporations, crude initiation factors from 8 g. of cells were dialyzed on the forced flow dialyzer against two 1-liter changes of buffer LMSN-16. The ultraviolet spectra of the clear

solubilized product was then taken, and the solution was used immediately. The final concentration of crude initiation factors was usually between 5 and 10 A_{280} optical density units per ml.

(n) In vitro incorporation procedures

Incorporation with S-30 extracts

Incubations were carried out in 12-ml. ribonuclease free conical centrifuge tubes. All glassware, pipettes and materials were also ribonuclease free. One or two milliliter incubation volumes were used, depending on the intended use. The contents of a standard one-ml. incubation were as follows, all reagents being made up in buffer LMSN:

0.05 ml. of 0.08 M ATP

0.05 ml. of 5×10^{-4} M GTP and CTP

0.05 ml. of 0.005 M PEP

0.05 ml. of 30.0 IU/ml. pyruvate kinase

0.10 ml. of 4 mg./ml. *E. coli* tRNA

0.10 ml. of 10 μCi/ml. ^{14}C-lysine

0.10 ml. of 10 μCi/ml. ^{14}C-arginine

0.05 ml. of a solution 10^{-3} M in each L-amino acid except histidine, arginine and lysine which were deleted

0.20 ml. of pre-incubated and dialyzed S-30

0.10 ml. of 1 mg./ml. MS2 RNA

0.15 ml. of buffer LMSN

Each one-milliliter incorporation therefore contained the following ingredients:

4.0 μM ATP

0.025 μM GTP and CTP

0.025 μM PEP

5×10^{-2} μM each L-amino acid (except histidine, arginine and lysine)

1.5 IU pyruvate kinase

1.0 μCi ^{14}C-lysine

1.0 μCi ^{14}C-arginine

400 μg. E. coli tRNA

100 μg. MS2 RNA

0.2 ml. S-30 fraction

10 μM Tris

80 μM NH_4Cl

8.5 μM $Mg(OAc)_2$

Each 12-ml. incubation tube was supplied with all ingredients except the MS2 mRNA. When all the tubes were ready, MS2 mRNA was added using either a 100-μl. micropipette or a 100-μl. Eppendorf micropipetter. The mixture was then gently agitated utilizing a Vortex Jr mixer and the tube placed in a 37°C water bath for the appropriate interval of time.

Upon completion of the incubation, either: a) the radioactive polypeptide products were precipitated with trichloroacetic acid, or b) the ^{14}C-peptides were digested by trypsin and fingerprinted.

Counting of total acid precipitable counts. When counting of total acid precipitable counts was desired, one milliliter incubation volumes were employed. At the termination of the 37°C incubation, 0.3 mg. of bovine serum albunin (1 mg./ml. distilled water) was added as a protein carrier and the solution was mixed. Six milliliters of 5% trichloroacetic acid was then added and the solution was mixed well. Heating the mixture to 90°C for 15 minutes was next performed to hydrolyze the acyl bonds of amino acids and polypeptides esterified to tRNA molecules. The mixture was cooled to 2°C and filtered through Millipore HAWP 2.5-cm. nitrocellulose filters. Each filtered precipitate was subsequently washed with at least 100 ml. of 5% trichloroacetic acid, a vital step in reducing background radioactivity because of the ^{14}C-amino acids retention on the filter. The filters were then removed from the Millipore filtration apparatus, mounted on appropriate counting planchets, and dried at 70°C for 15 minutes. The samples were counted in a Nuclear-Chicago model 4312 3-cm. windowed gas flow counter.

Typical incubations gave well over 12,000 cpm/ml. incubation, with a background of less than 150 cpm.

Fingerprinting of the ^{14}C *in vitro* peptides.

Part 1: Preparation of the ^{14}C-products for tryptic digestion. When fingerprinting of ^{14}C *in vitro* peptides was desired, 2-ml. incorporation volumes were employed. In addition, the amount of MS2 $_m$RNA added was carefully adjusted to give at least 50,000 acid precipitable counts per 2-ml. incubation, see Fig. 10.

Upon termination of the 37°C incubation, the entire 2-ml. sample was loaded into 2-ml. Beckman cellulose nitrate centrifuge tubes (Stock #303369). These small centrifuge tubes were maintained at salt/ice bath temperatures throughout the loading procedure.

The caps were tightened, the tubes placed in the Ti-50 rotor adaptors, and the sample centrifuged for 2 hours at 40,000 RPM (105,000 xg) in the Beckman model L type Ti-50 rotor.

The supernatant from this centrifugation was carefully removed with a Pasteur pipette and transferred to a clean 12-ml. conical centrifuge tube. The pellet was then gently rinsed with buffer LMSN and the wash saved. The pellet was finally dissolved in 1.0 ml. of glass distilled water and set aside at 2°C for later use.

Three milligrams of lysozyme were added to the 105,000 xg supernatant (1 mg./ml. distilled water) as a protein carrier. A ten-fold excess of ^{12}C-arginine and lysine was added (0.1 ml. of a solution of 7.31 mg. lysine and 8.25 mg. arginine to each 1.0 ml. of supernatant). The mixture was stirred and subsequently precipitated by adding 1.49 ml. of

100% saturated ammonium sulfate to each ml. of solution (60% final saturation). The cloudy white suspension was allowed to flocculate at 2°C for 30 minutes.

The precipitate was collected by centrifugation at top speed in a clinical centrifuge at 2°C for 20 minutes. The supernatant was discarded and the precipitate washed twice with 60% saturated ammonium sulfate by resuspension and recentrifugation. The precipitate was finally suspended in 1.0 ml. of glass distilled water.

Six milliliters of 5% trichloroacetic acid was next added and the solution heated at 90°C for 15 minutes to hydrolyze the esterified bonds of the t-RNA moieties. After cooling at 2°C for 20 minutes, the precipitate was again collected by centrifugation. Discarding this supernatant, the precipitate was washed twice at room temperature with 5% trichloroacetic acid by resuspension and recentrifugation.

The resulting precipitate, still moist from the 5% trichloroacetic acid wash, was washed twice with 6.0 ml. of an equal parts solution of reagent grade ethanol/ether, followed by a final wash of 6.0 ml. of reagent grade ether. This final washed precipitate was collected by centrifugation and the supernatant was discarded.

The washed product was dried under a jet of nitrogen at 2°C and stored at -90°C in the Revco cold chest until used.

The precipitate which resulted from the initial 105,000 xg centrifugation (dissolved in the 1.0 ml. of distilled water), was treated in an

identical manner as the supernatant. This washed and dried product was also stored at -90°C until used.

Part 2: Oxidation and tryptic digestion of MS2 coat protein — native and *in vitro*. The following procedure involved the use of either purified native MS2 coat protein or the washed and dried MS2 $_{m}$RNA *in vitro* peptides. In the case of native MS2 coat protein, the pure product alone was used, whereas with an *in vitro* product additional native MS2 coat protein (or lysozyme) had been added as a carrier.

Two milliliters of reagent grade formic acid and 0.1 ml. of 30% reagent grade hydrogen peroxide were added to a 12-ml. conical centrifuge tube. The mixture was allowed to sit in a watery ice bath for two hours at which time the titer of peroxyformic acid was at a maximum.

Three-tenths milliliter of this liquid was added to 3 mg. of the dried protein to be oxidized and allowed to sit in the ice bath for one hour. The reaction was stopped by the addition of 1.0 ml. of chilled distilled water and the mixture immediately lyophylized to dryness.

One milliliter of 0.05 M ammonium bicarbonate and 0.12 mg. of TPCK treated trypsin were added to the dried oxidized product. The mixture was incubated at room temperature for three hours, followed by lyophylization to dryness in a 250-ml. teardrop lyophylization flask.

Care was taken at this juncture to prevent the extremely light lyophylized powder from being drawn into the lyophylization apparatus. This was accomplished by blocking the neck of the teardrop flask with

a fine Teflon screen and in all cases avoiding the use of conical centrifuge tubes for lyophylization. Repeated thawing and freezing of the lyophylization sample during this process to allow the residue to become more concentrated also appeared to help in this regard. The product was stored at -90°C in the Revco cold chest until fingerprinting was performed.

Part III: Fingerprinting procedure.

1. Electrophoresis. Twenty to 40 µl. of glass distilled water was added to the dried, oxidized and digested product and the sample dissolved so that the resulting solution was without any precipitate. In some cases the product would not dissolve completely and approximately 1 µl. of 1% NH_4OH was added to facilitate solubility. In some instances the dried product was dissolved directly in buffer EBN-1. The results, however, were identical no matter which method was employed.

Using a 5-µl. micropipette, the sample was carefully applied to the corner of a 30 cm. x 40 cm. piece of Whatman 3-mm. filter paper, taking care that the following criteria were met:

1. The sample applied was always without any visible precipitate.
2. The final spot was always between 0.4 cm. and 0.6 cm. in diameter.
3. Five microliters was applied followed by air drying for 15 minutes before applying the next 5 µl.

4. The filter paper was not torn during the process of spotting.
5. The filter paper was always placed on a sheet of clean plastic during spotting.
6. The sample was applied to the origin as a 5-µl. drop and not as a continuous flow.

After the applied sample had been allowed to air dry for at least 1 hour, the filter paper was laid onto a large piece of clean clear plastic. The paper was now completely moistened with buffer EBN-1 using a 10-ml. pipette. The sample spot itself was moistened by carefully allowing buffer to diffuse along four directions towards the center of the spot. The filter paper was then immediately blotted by placing it on a larger sheet of filter paper (Whatman No. 3mm) and covering it with a second large sheet of the same filter paper. A 6" wide roller (Mayfield Co., Inc.) was then rolled over the filter paper, and the sandwiched filter paper thoroughly blotted. A second top and bottom sheet of Whatman No. 3mm filter paper was exchanged for the now wet sheets and blotting resumed for about one minute.

The blotted filter paper was next positioned on the bottom of a water cooled electrophoresis plate and the cellulose separators and Whatman #1 wicks mounted (see Fig. 7). Buffer EBN-1 was added to the electrophoresis buffer tanks and allowed to seek the same level by virtue of a tube joining both tanks. A sheet of plastic was then placed over the moistened and blotted filter paper and the top plate was

lowered and clamped into place. The system was then allowed to equilibrate for one-half hour. The tube joining the two buffer tanks was then pinched off with a screw clamp.

Electrophoresis continued for 2.5 hours at a voltage flux of 30 v/cm. (1100 volts total), 25 milliamperes current. The unit was disarmed of the high voltage and the electrophoretogram removed. The paper was first dried at room temperature for 15 minutes followed by drying in an oven at 70°C for 30 minutes. Chromatography was then performed.

2. Chromatography. A cylinder was formed out of the dried electrophoretogram by connecting points A-A and B-B with plastic paper clips (see Fig. 7). The tube was then placed (edge A-A down) into a round glass chromatography tnak, 30 cm. in diameter and 60 cm. in height. In the bottom of the tank was placed a 10-cm. glass Petri dish containing about 50 ml. of buffer CBN-1. A glass cover was placed over the tank and vapor equilibration was allowed to proceed for 12 hours.

At the termination of equilibration, 200 ml. of buffer CBN-1 was introduced into the bottom of the tank with a 40-cm. long stemmed funnel. Ascending chromatography was allowed to progress for 8 to 10 hours. The chromatogram was then removed and dried as was the electrophoretogram.

The dried fingerprint was quickly dipped into a 0.25% solution of ninhydrin in acetone, and then dried at 70°C for 10 minutes. The resulting ninhydrin spots (lysozyme or MS2 coat protein) gave a quick check

as to the separation of the tryptic spots. Visualization of the ^{14}C *in vitro* peptides was next achieved by autoradiography.

3. Autoradiography. A sheet of 8" x10" Kodak X-ray film was placed next to the surface of the dry fingerprint, the sheets placed into an appropriate X-ray film holder and the film allowed to expose for 10 to 15 days. The film was developed using Kodak Rapid X-ray developer and the size and distribution of the exposed spots was noted both on the X-ray film and the fingerprint itself.

4. Scintillation counting of ^{14}C-tryptic peptides. The individual ^{14}C radioactive spots were cut out of the filter paper fingerprint and the radioactive level determined by one of two counting procedures. Either method gave proportional results when compared to the other method.

Method A. The ^{14}C radioactive filter paper discs were placed into new 25-ml. Beckman scintillation vials and 20 ml. of scintillation fluid added (42 ml. Spectrafluor PPO-POPOP to 958 ml. of tolulene). Radioactive levels were then registered on the Nuclear-Chicago model 6801 liquid scintillation counter.

Method B. The radioactive material was eluted from each disc with 20 ml. of glass distilled water and the eluent placed into Beckman 25-ml. scintillation vials. The aqueous solution was then dried at 70°C and the residue subsequently solubilized by the addition of 1 ml. of Hydroxide of Hyamine. The scintillation fluid was added (20 ml.) and radioactive counting recorded as in Method A.

Incorporation with purified ribosomes and initiation factors

In vitro incorporations using purified ribosomes, initiation factors and MS2 messenger RNA were carried out in 0.5-ml. or 1.0-ml. volumes. The concentrations of the various reagents added were as follows. All reagents were made up in buffer LMSN-16.

0.025 ml. of 0.08 M ATP

0.025 ml. of 0.005 M PEP

0.025 ml. of 5×10^{-4} M GTP/CTP

0.025 ml. of 30.00 IU/ml. pyruvate kinase

0.050 ml. of 10 μCi/ml. ^{14}C-lysine

0.050 ml. 10 μCi/ml. ^{14}C-arginine

0.025 ml. of a solution 10^{-3} M in each L-amino acid except histidine, arginine and lysine which were deleted

0.025 ml. of 30 mg./ml. washed ribosomes

0.050 ml. of 10 A_{280} units/ml. crude initiation factors

0.050 ml. of buffer LMSN-16

0.050 ml. of 1 mg./ml. MS2 RNA

0.050 ml. of 4 mg./ml. E. coli tRNA

0.050 ml. of pH 5 fraction

Each one-milliliter incorporation therefore contained the following ingredients.

4.0 μM ATP

0.025 μM GTP/CTP

0.25 μM PEP

5×10^{-3} μM each L-amino acid (except histidine, arginine and lysine)

1.5 IU pyruvate kinase

1.0 μCi ^{14}C-lysine

1.0 μCi ^{14}C-arginine

400 μg. *E. coli* t-RNA

100 μg. MS2 RNA

0.5 A_{280} units crude initiation factors

750 μg. washed ribosomes

1 A_{280} units pH 5 fraction

10 μM Tris

80 μM NH_4Cl

16.0 μM $Mg(OAc)_2$

Each incubation tube was supplied with all ingredients except the MS2 $_m$RNA. Sufficient buffer was then added to bring the total volume of each incubation mixture to 1.0 ml. When all the tubes were ready, MS2 $_m$RNA was added using a ribonuclease free 100-μl. Eppendorf micropipette. The mixture was then gently agitated utilizing a Vortex Jr. mixer and the tube was placed in a 37°C water bath for the appropriate length of time.

One hundred fifty micrograms of bovine serum albumin was added as a protein carrier (optional) prior to precipitation of the radioactive

products with the addition of 6.0 ml. of 5% trichloroacetic acid (for a 1.0-ml. incorporation). The mixture was agitated with a Vortex Jr. mixer and heated to 90°C for 15 minutes. After cooling at 2°C for 30 minutes, the precipitate was filtered on Millipore HAWP 2.5 cm. nitrocellulose filters. The filters were next dried at 70°C for 15 minutes. Radioactivity was determined by counting on a Nuclear-Chicago model 4312 3-cm. gas flow counter.

Typical incubations gave over 2500 cpm/0.5 ml. using 100 μg. of MS2 $_{m}$RNA. Background radioactivity averaged 50 to 100 cpm. A greater incorporation was sometimes desired and obtained by the addition of larger amounts of MS2 $_{m}$RNA.

4. EXPERIMENTAL RESULTS

(a) In vitro fingerprints

Development of the system

Effect of Mg^{++} concentration on S-30 incorporations. The rate of formation of acid precipitable radioactivity varies when the Mg^{++} concentration is varied in an in vitro protein synthesizing system. Nathans, Notani, Schwarz & Zinder (1962) have found that 11 mM Mg^{++} concentration resulted in maximal incorporation of ^{14}C-amino acids in an S-30 assisted MS2 $_m$RNA directed system. Clark (1965) found, however, that 8 mM Mg^{++} was optimum under the same conditions. The effects shown in Fig. 8 were produced when the magnesium ion concentration of a typical S-30 incubation was varied over the range of 7 mM to 16 mM. The value of 8 mM to 9 mM magnesium was thus considered optimum for an MS2 directed S-30 incorporation, and all reagents were made up in a buffer containing 8.5 mM magnesium (buffer LMSN).

Kinetics of ^{14}C-amino acid incorporation. It can be estimated that the time required for the completion of an MS2 coat protein molecule is about 3 to 5 seconds at 37°C (Goldstein, 1964). In vitro studies indeed indicate that there is an increase in the acid precipitable radioactivity as a function of time, but the relationship is not linear.

In an effort to determine the kinetics of an MS2 $_m$RNA directed S-30 assisted system, samples were simultaneously started by the

addition of MS2 $_m$RNA and stopped at specified times by the addition of 5% trichloroacetic acid. The results are shown in Fig. 9. The most efficient incubation period was 30 to 40 minutes, as the ^{14}C-amino acid incorporation plateaued after this time. Accordingly, all ensuing incorporations were incubated for 40 minutes.

Stimulatory effect of MS2 messenger RNA. Varying amounts of the RNA were added to an S-30 system in an attempt to measure the stimulatory effect of phage MS2 $_m$RNA directed incoporation of ^{14}C-amino acids. As can be seen from Fig. 10, a clear stimulation was produced at 20 μg./ml. Furthermore, the message-dependent MS2 $_m$RNA directed ^{14}C-amino acid incorporation was increased more than tenfold over a 20 μg./ml. control by the addition of 200 μg./ml. of $_m$RNA. From this experiment, 100 μg./ml. was considered a concentration of message high enough to produce statistically accurate counts, yet was economical in the use of MS2 $_m$RNA.

Dependence of incorporation on added ^{14}C-amino acids. In order to best gauge the most efficient concentration of ^{14}C-arginine and ^{14}C-lysine present in an incubation, the concentration of these ^{14}C-amino acids was varied from 0.1 μCi/ml. to 1.0 μCi/ml. As can be seen in Fig. 11, the amount of acid precipitable material increased as the concentration of added radioactive amino acids increased. It was concluded that

to minimize the limiting nature of the added ^{14}C-amino acids, 1.0 μCi of lysine and arginine would be added to each 1.0-ml. incubation.

Effect of chloramphenicol on total acid precipitable counts. The percent inhibition of total acid precipitable counts when chloramphenicol (CAP) is added to a protein synthesizing system varies with the polynucleotide being used as a message. Julian (1965) showed that 67 μg./ml. of CAP was sufficient to produce 60% inhibition of protein synthesis in a polyadenylic acid directed system, whereas Hahn & Wolfe (1965) demonstrated a requirement of 300 μg./ml. of CAP to give 80% inhibition in a polyuridylic acid directed system.

To determine CAP's effect on the S-30 assisted system, varying amounts of CAP were added to standard incubation tubes. From the graph shown in Fig. 12 it is evident that a concentration of only 2.5 μg./ml. of chloramphenicol produced a 50% inhibition of total acid precipitable counts.

Effect of chloramphenicol on peptides released in vitro. MS2 specific polypeptide chains that have been completed and released from the ribosomal complex are termed "released" peptides. Those peptides remaining attached to the ribosomal complex are termed "bound" peptides.

An experiment was designed to examine the effect of CAP on released peptides. A 2.0-ml. sample was incubated, with aliquots removed at varying times, centrifuged at 105,000 xg for 2 hours and the

supernatant examined for acid precipitable counts. Fig. 13 depicts the results of such an experiment. From this one may conclude that the appearance of released peptides was inhibited by chloramphenicol.

Peptide map of native MS2 coat protein. A tryptic fingerprint was made of 3.0 mg. of native MS2 coat protein to determine the number and distribution of tryptic peptides. The details of oxidation, digestion and fingerprinting have been previously described in "Materials and Methods". The methodology followed that of Nathans (1965), although the ^{14}C-labeled amino acids were different. The resulting fingerprint is depicted in Fig. 14, as a tracing of the original fingerprint. Eleven ninhydrin positive spots were produced. Position 5 was attributed to free lysine, as confirmed by an independent fingerprint of lysine alone.

Peptide map of in vitro MS2 directed released peptides. Fingerprinting was performed on the 105,000 xg supernatant of a 2.0-ml. incubation to compare the number and distribution of tryptic peptides in the released in vitro coat protein. Three mg. of lysozyme were added as a protein carrier and a standard peptide map was made. After exposure to X-ray film for one week, a tracing was made of the radio-opaque areas. Fig. 15 depicts this peptide map. Again the number and distribution of spots closely approximates th e of the native coat protein. The numbering sequence of the spots is analogous to the native coat protein peptide map.

Effect of chloramphenicol on the peptide map of peptides released in vitro. The released in vitro peptides are inhibited by CAP at a concentration of 2.5 μg./ml., as shown in Fig. 13. Figs. 17 and 19 show that this inhibition is also applied to the ribosomal bound material and that all ribosomal bound tryptic peptides are equally inhibited.

A fingerprint was made of the 105,000 xg supernatant of a 2.0-ml. incubation to determine the effect of CAP on the released in vitro peptides. The results, depicted in Fig. 16, again show the same number and distribution of spots. A quantitative analysis of each spot by scintillation counting yielded results qualitatively proportional with those of Fig. 20. As with the ribosomal bound peptides, released tryptic peptides were inhibited proportionally. These results confirm those of Nathans (1965).

Experimental results

Effect of chloramphenicol on acid precipitable counts of the ribosomal bound in vitro peptides. This experiment was designed to examine the effect of chloramphenciol on the formation of MS2 $_m$RNA specific ribosomal bound polypeptide chains. The precedure was identical to that of Fig. 13.

Fig. 17 depicts the results of this experiment, indicating a lowered quantity of radioactivity associated with the ribosomal bound peptides in the presence of CAP. This suggested that the level of

^{14}C-nascent peptides associated with the ribosome was reduced.

Peptide map of the in vitro MS2 directed ribosomal bound peptides. A tryptic fingerprint was made of the 105,000 xg in vitro ^{14}C-labeled product to determine the nature of the in vitro products produced under the direction of MS2 $_{m}$RNA. The incubation was a standard 2.0-ml. sample incubated and prepared as described in "Materials and Methods". The supernatant from the 105,000 xg centrifugation was saved for later use.

Fig. 18 shows a tracing of a fingerprint autoradiograph of the in vitro incorporation. The number and distribution of these spots closely approach the fingerprint of the native MS2 coat protein. One additional spot can be identified as belonging to free arginine, a remnant of the ^{14}C-amino acids added during the incubation. It can be concluded from this fingerprint that the major product produced in vitro was MS2 coat protein.

Effect of chloramphenicol on the peptide map of the ribosomal bound in vitro peptides. At a concentration of 2.5 µg./ml., CAP inhibits 50% of the total acid precipitable ^{14}C-amino acid labeled material (Fig. 12). A sample was incubated with 2.5 µg./ml. of chloramphenicol to determine CAP's effect on the in vitro peptides. A peptide map was made

of the 105,000 xg precipitate and an autoradiograph produced by exposing Kodak X-ray film to the fingerprint.

Fig. 19 is a tracing of the radio-opaque areas of the autoradiogram. It is quite apparent from this that the number and distribution of the tryptic peptides remain the same as the fingerprint of native coat protein. Each radioactive spot was counted to compare the differences in radioactivity between the control in vitro tryptic peptides and those in vitro tryptic peptides produced in the presence of 2.5 μg./ml. CAP. As explained in detail in "Materials and Methods", the areas on the fingerprint corresponding to radio-opaque areas on the X-ray film were cut out and the radioactivity determined by liquid scintillation counting. The results are summarized in Fig. 20. The radioactivity in each spot was reduced by a proportionally equal amount. All tryptic peptides were inhibited equally, producing no change in the C to N terminal peptide gradient of labeling. These results suggest CAP's effect was on peptide chain initiation and not peptide chain extension.

(b) The role of ribosomal wash factors

Mg^{++} titration curve of the purified in vitro system. Several groups have reported that the Mg^{++} concentration optimum shifts to a higher value when purified ribosomes are used in conjunction with initiation factors in an in vitro natural $_{m}$RNA stimulated system as compared to a crude S-30 assisted system (Revel, Lelong, Brawerman &

Gros, 1968; Revel, Herzberg, Becarevic & Gros, 1968; Wahba, Chae, Iwasaki, Mazumder & Miller et al., 1969; Kolakofsky, 1966). In light of this possibility, incubations of purified ribosomes, high speed supernatant factors in the form of a pH 5' fraction (Julian, 1965), and MS2 $_m$RNA were carried out at varying concentrations of Mg^{++} to determine the optimum concentration of Mg^{++}. Fig. 21 depicts the results of this experiment, and shows an optimal radioactive incorporation at 16 mM Mg^{++}. Accordingly, all further incubations were carried out at 16 mM Mg^{++}. At this concentration of Mg^{++}, the system is entirely dependent upon added initiation factors (Fig. 22).

Henceforth, any reference to a purified system refers to an in vitro system containing DEAE washed ribosomes, added crude initiation factors, an energy source, purified MS2 $_m$RNA and a pH 5' fraction.

Titration curve of crude initiation factors against the purified system. The addition of increasing amounts of MS2 $_m$RNA to an in vitro system produces a proportional increase in the incorporation of ^{14}C-amino acids. Such a result probably indicates the limiting nature of the $_m$RNA. Since a similar relationship might exist with respect to crude initiation factors, a titration curve of crude initiation factos was performed to determine the optimum concentration of these factors. The crude factors exhibited an A_{260}/A_{280} ratio of 1:2 when prepared according to the details given in "Materials and Methods".

Fig. 22 depicts this titration curve. Throughout the range of concentrations used, the addition of crude initiation factors caused a proportional increase in total acid precpitable counts. Accordingly, in all further incorporations, 0.5 A_{280} units of initiation factors were used per ml. of incorporation sample.

Kinetics of ^{14}C-amino acid incorporation of the purified system. In Fig. 9, the kinetics of ^{14}C-amino acid incorporation of the S-30 system was investigated. Over 80% of the ^{14}C-amino acid incorporating ability remained in such a system after the first ten minutes of incubation. Such residual incorporating ability represents both elongation and re-initiation collectively. It was decided to determine the residual ^{14}C-amino acid incorporating potential of the purified system.

Fig. 23 depicts the kinetics of ^{14}C-amino acid incorporation. All samples were treated identically and the incubations terminated at the indicated times by the addition of 5% trichloroacetic acid. It is clear from this graph that only 20% of the radioactivity was incorporated in the first five minutes.

Effect of chloramphenicol on total acid precipitable counts of the purified system. An S-30 assisted MS2 $_m$RNA directed in vitro system is inhibited 50% at a concentration of chloramphenicol equal to 2.5 μg./ml. (Fig. 12). Varying concentrations of CAP were therefore tested with the purified system to make a comparison with the S-30 system.

Fig. 24 depicts this experiment. Chloramphenicol was more than twice as effective in the purified system as in the S-30 system, requiring only 0.9 μg./ml. CAP concentration to yield a 50% inhibition.

Effect of crude initiation factors on a purified system re-incubated after a two-hour centrifugation. The presence of crude initiation factors in a purified system is a necessary adjunct for the successful incorporation of ^{14}C-amino acids. When such factors are withheld, negligible amounts of amino acids are incorporated. It was decided to investigate the role of crude initiation factors in a purified system.

One milliliter of a standard incubation mixture was incubated for 5 minutes at 37°C to initiate the translation of $_{m}$RNA. Subsequently, each mixture was quickly chilled to 0°C in a salt/ice bath and centrifuged at 105,000 xg for 2 hours in a 2.0-ml. Beckman centrifuge tube containing a 0.2-ml. pad of 10% sucrose. Thus the soluble initiation factors were removed from the ribosomal aggregate. Upon completion of the centrifugation, the 10% sucrose pad plus 0.1 ml. of supernatant directly above the pad was transferred to a mixture containing all incubation reagents except ribosomes and message. The system readily incorporated additional ^{14}C-amino acids when transferred to a mixture containing crude initiation factors (Fig. 25). There was a 65% loss of incorporating ability if the re-incubation was lacking initiation factors. This substantial loss in incorporating ability was therefore a result of the absence of crude initiation factors.

It may be concluded that this incorporating system can be re-initiated from polysomal complexes that were washed free of initiation factors. Such re-initiation is dependent upon addition of crude initiation factors not found in the pH 5' precipitate. The lack of crude initiation factors in the 105,000 xg pellet clearly results in the loss of ^{14}C-amino acid incorporation. This system allows the differentiation of of elongation and initiation. The 65% gain of radioactivity in Fig. 25 is thus entirely due to initiation catalyzed by the presence of crude initiation factors.

Dependence of the purified system on the pH 5' fraction. Although the crude initiation factor preparation probably differs from those enzymes found in the pH 5' fraction, the possibility of complementary enzymes cannot be ruled out. The re-initiated ribosomal pellet would not be dependent solely on initiation factors if this were true.

In Fig. 26, 1.0-ml. samples were incubated for 5 minutes to begin initiation, centrifuged at 105,000 xg to pellet the ribosomal bound peptides and the ribosomal material re-incubated in a standard mixture minus ribosomes and messenger. The system was complete and further incorporation occurred when both initiation factors and pH 5' precipitate were present. When, however, the pH 5' precipitate was omitted, the system would not continue amino acid incorporation. Thus the two enzyme preparations are complementary. The initiation factor preparation alone will not support protein synthesis in a purified system.

Effect of chloramphenicol on initiation factor dependent and independent ^{14}C-amino acid incorporation. It is now feasible to test CAP's action on our system which is dependent upon added ribosomal wash factors. Fig. 27 represents this experiment.

In this experiment, purified incubation mixtures containing all ingredients except ^{14}C-arginine and ^{14}C-lysine (but including ribosomal wash factors) were incubated for five minutes at 37°C. The 1.0-ml. mixtures were then loaded into 2.0-ml. Beckman cellulose nitrate centrifuge tubes containing a 0.2-ml. pad of 10% sucrose (special sucrose density grade) in buffer LMSN. The material was centrifuged for two hours at 105,000 xg in the Model L-2 ultracentrifuge. The procedure thus far was analogous to Fig. 25. Upon completion of this centrifugation, 0.3 ml. of the sucrose pad was transferred to a purified incubation system with ^{14}C-arginine and ^{14}C-lysine present and re-incubated for 25 minutes at 37°C.

In one case, the re-incubated mixture contained a complete complement of reagents including ribosomal wash factors. As expected, it incorporated ^{14}C-amino acids and produced 1710 CPM (Fig. 27). When 1.0 µg./ml. of CAP was added to a re-incubation mixture containing ribosomal wash factors, the incorporation was reduced to 763 CPM (Fig. 27), representing a 45% inhibition.

Such results would be expected if either initiation or elongation were inhibited. The re-incubated system allowed both initiation and

elongation to proceed. CAP might, however, have acted at either step. Initiation and elongation can be differentiated by investigating CAP's effect on the residual ^{14}C-amino acid incorporating potential when re-incubation occurs in the absence of ribosomal wash factors. Such residual incorporating potential may be attributed to peptide chain extension.

Fig. 27 also represents an experiment in which ribosomal complexes were re-incubated without ribosomal wash factors present, producing 398 CPM. Since ^{14}C-amino acids were added only during the 25-minute reincubation, these counts represent peptide chain extension alone, as the system was without crude initiation factors. When 1.0 μg./ml. of CAP was added to the re-incubation no inhibition was noted. Ten μg./ml. of CAP again produced a neglible inhibition dropping the incorporation to 379 CPM (Fig. 27). Such a concentration of CAP would be expected to produce a 100% inhibition of the system. These results suggest that CAP has a significantly stronger effect on initiation as compared to elongation.

5. DISCUSSION

The preceding experiments indicate that at low concentrations of chloramphenicol (CAP), inhibition of protein synthesis is at the level of initiation in a natural $_{m}$RNA directed system. Furthermore, such inhibition is directed at initiation factor dependent incorporation of ^{14}C-amino acids.

Julian (1965) showed that formation of the di- and tri-lysine peptides was not affected by CAP, whereas formation of the larger peptides was inhibited. He suggested that CAP acted at a peptide bond forming step. Traut & Munro (1964) found that CAP inhibited the effect of puromycin in releasing phenylalanine peptides from polyphenylalanyl-sRNA attached to ribosomes. On the basis of this finding they suggested that CAP acted as an inhibitor of the peptide bond forming enzyme, at the same site as puromycin. Weisberger (1967) has suggested that CAP acted on a ribosomal site at a stage after binding of $_{m}$RNA and during peptide synthesis to prevent the final condensation of amino acids and the growth of polypeptide chains. Thus the general consensus was that CAP acted on the elongation process in protein synthesis.

Armentrout & Weisberger (1967) had previously concluded that protein synthesis directed by polyuridylic acid was more resistant to CAP inhibition than that induced by natural $_{m}$RNA, and suggested that the inhibitory effect of CAP may be peculiar to the RNA preparation

used. Since the work previously described utilized synthetic $_m$RNAs, it was now desirable to test this model of elongation inhibition on an *in vitro* system directed by a natural $_m$RNA.

The translation of the coat protein cistron of the MS2 virus natural $_m$RNA may be envisioned as involving a population of ribosome/messenger complexes in all stages of translation. Consequently, every complex will have incorporated an N-terminal amino acid, but only those complexes in the final stages of polypeptide synthesis will have incorporated a C-terminal amino acid. If CAP were to block a step in elongation, we would expect a population of ribosome/messenger complexes frozen in the various stages of elongation. Hence, we might not expect any decrease in the ribosomal ^{14}C-nascent peptides as compared to a normal protein synthesizing system. Furthermore, as elongation was inhibited on a $_m$RNA strand, an increase in the quantity of the N-terminal peptides might be expected as newly initiated ribosomes were allowed to synthesize peptides before being inhibited in some stage of elongation.

Nathans (1965) examined the effect of CAP on the released ^{14}C-labeled MS2 *in vitro* peptides. His methodology utilized a fingerprint of a tryptic digest of the non-ribosomal bound ^{14}C-labeled MS2 *in vitro* peptides. He found no increase in the tryptic N-terminal peptides over the C-terminal peptides when such peptides were synthesized in the presence of 3.5 μg./ml. of CAP. This was in contrast to the released

peptides formed in the presence of puromycin, which showed an increase in the number of N-terminal peptides. These results were in accord with an elongation inhibition model for CAP, as one would expect the released peptides to be whole and possess an equal number of N and C-terminal peptides. His work also tended to rule out CAP induced premature release, for in this case we should expect results similar to those seen with the released peptides synthesized in the presence of puromycin. However, he did not examine CAP's effect on the ribosomal bound ^{14}C-nascent peptides. The true test for an elongation inhibition model exists here, since the peptides resulting from inhibition would likely be trapped on the messenger/ribosome complex. It was decided to compare the ribosomal bound to released products using Nathan's procedures.

A normal distribution of tryptic peptides resulted when a peptide map was made of tryptic hydrolysates of released peptides synthesized in the presence of 2.5 µg./ml. of CAP, with no preponderance of any tryptic peptide over any other tryptic peptide (Fig. 16). These results confirm those presented by Nathans (1965) on the released MS2 in vitro peptides. Thus we may rule out CAP induced premature release, but offer no conclusions regarding an elongation inhibition model for the mechanism of action in natural $_m$RNA directed systems.

A peptide map was made of the 105,000 xg ^{14}C-lysine and ^{14}C-arginine labeled products associated with the precipitate of an in vitro

MS2 directed system. Such products were allowed to be synthesized in the presence of 2.5 μg./ml. of CAP. A distribution of tryptic peptides identical to that of native MS2 coat protein was produced, Fig. 19. As this material primarily represents nascent peptides (Nathans, Notani, Schwarz & Zinder, 1962; Nathans, 1965), the results are in accord with a model of normal growing peptide chains. Again, the normal distribution and number of tryptic peptides rules out premature release of nascent peptides.

A quantitative analysis of the radioactivity of each tryptic peptide revealed that there was a proportional decrease in the radioactivity of all the tryptic peptides and there was no change in the quantity of N-terminal peptides in the presence of CAP, Fig. 20. Total acid precipitable counts are inhibited by 50% at a concentration of CAP of 2.5 μg./ml. Seventy-five percent of this inhibition occurs in the ribosomal bound material, whereas the released material is inhibited by only 25%. Therefore the primary effect of CAP on the MS2 $_{m}$RNA directed system is the reduction of ribosomal bound ^{14}C-labeled material. This is clearly not in agreement with a model of elongation inhibition.

A conflict thus exists between the results of this study and that model generally ascribed to CAP's action. To examine these conflicts, it is helpful to consider other mechanisms that may account for the inhibition of protein synthesis by CAP in the MS2 $_{m}$RNA directed system. We may rule out inhibition of termination, as the release of ^{14}C-labeled

material is facilitated rather than inhibited. We have previously discounted premature release. Since the data presented in this study do not support an elongation inhibition model, we are forced to finally consider initiation.

If CAP were to block a step in initiation, previously initiated ribosome/messenger complexes would be allowed to complete translation of the $_{m}$RNA. Any ^{14}C-labeled nascent peptides would be released and leave the $_{m}$RNA in either an inhibited state of initiation or prevent any initiation complexes from forming at all. The net effect would be to decrease the ribosomal bound ^{14}C-labeled nascent peptides. We would not expect any increase in the number of N-terminal nascent peptides over a control, as any nascent material remaining would be in a normal process of peptide chain elongation. Therefore, in partially inhibitory concentrations of CAP, a percentage of the ribosome/messenger complexes would be stripped of ^{14}C-labeled material, and the remaining uninhibited fraction would appear as normally growing nascent material. The data presented in this study strongly support this model of inhibition of protein synthesis by CAP.

Approximately one year after this effect on initiation of the MS2 system was discovered, Irvin & Julian (1970) published their findings on the effect of CAP on the polycytidylic acid directed synthesis of ^{14}C-polyproline. They noted that not only was this system significantly more sensitive to CAP than other synthetic $_{m}$RNAs, but that CAP

inhibited the synthesis of all peptides including the di- and tri-proline residues. This was in contrast to the polyadenylic acid and polyuridylic acid directed systems where CAP inhibited only peptides larger than the tri-peptide (Julian, 1965; Pestka, 1969). Irvin and Julian concluded that this effect was attributable to inhibition of an initiation step and not peptide chain extension as it appears to act in the polyadenylic acid and polyuridylic acid directed systems.

The CAP sensitivity of the MS2 $_{m}$RNA and polycytidylic acid directed systems suggest that the mode of action of the drug differs from the inhibitory mechanism observed in the less sensitive synthetic $_{m}$RNA directed systems. It is generally accepted that inhibition is at the level of elongation in the polyadenylic acid and polyuridylic acid directed systems, when high levels of CAP are used. In the MS2 $_{m}$RNA and polycytidylic acid directed systems, the evidence presented in this discussion suggests inhibition of an initiation step.

It is illuminating to consider the levels of CAP required to inhibit the polyadenylic acid and polyuridylic acid directed systems versus the natural $_{m}$RNA and polycytidylic acid directed systems. This evidence points to two possible mechanisms of inhibition (elongation and initiation). The levels of CAP required for inhibition fall into two groups and further suggest that one mechanism of inhibition is a low concentration phenomenon while the second effect is found only at much higher concentrations of the drug. In addition, systems which require

ribosome wash factors appear to be most sensitive to the drug. At a concentration of CAP of 2.5 μg./ml. total MS2 ^{14}C-peptide synthesis is inhibited 50% when assisted by an S-30 extract. Similarly, only 2.5 μg./ml. of CAP are required to bring about 60% inhibition of the polycytidylic acid directed system (Irvin & Julian, 1970). On the other hand, 67 μg./ml. of CAP are required to inhibit by 60% the polyadenylic acid directed system (Julian, 1965). At a concentration of CAP of 180 μg./ml., total phenylalanine peptide synthesis is uninhibited (Pestka, 1969). The distinct effects at the two levels suggest that at low concentrations of CAP initiation is inhibited, as in the MS2 $_m$RNA and polycytidylic acid directed systems, and at high concentrations elongation is effected as with the other two synthetic $_m$RNAs. This suggests that there are similar mechanisms of initiation in the MS2 and polycytidylic acid directed systems which are different from the other two synthetic $_m$RNAs.

In 1971, Cameron, Rogers, & Julian noted that the addition of crude ribosomal wash factors stimulated the incorporation of ^{14}C-proline in a polycytidylic acid directed system. In addition, a crude high salt ribosomal wash fraction was required as a supplement when a pH 5' fraction was used as a source of supernatant factors in the polycytidylic acid directed system. In contrast, an ammonium sulfate fraction of the S-100 supernatant alone stimulated the polycytidylic acid

directed system. Since a high salt ribosomal wash fraction was known to contain the three bacterial protein initiation factors FI, FII and FIII (Dubnoff & Maitra, 1969; Ochoa, 1969; Revel, Lelong, Brawerman & Gros, 1968; Revel, Herzberg, Becaravic & Gros, 1968), it was possible that CAP's apparent inhibition of initiation in the MS2 $_{m}$RNA and polycytidylic acid directed systems was related to the function of these factors. The stimulatory effect of the ribosomal wash factors, normally only seen with the natural $_{m}$RNA system, would suggest that initiation in the polycytidylic acid directed system was similar to initiation in the MS2 system.

A series of experiments was required to bridge the gap between the general hypothesis of inhibition of initiation in the MS2 system by CAP, and the detailed roles, if any, of the recognized bacterial initiation factors.

An MS2 $_{m}$RNA directed in vitro protein synthesizing system was developed which was highly dependent upon added ribosomal wash factors. The CAP sensitivity of this system was found to be over twice as sensitive to CAP as the S-30 assisted system, Fig. 24. This increased sensitivity to CAP was highly suggestive of an interaction between the drug and some factor(s) present in the high salt ribosomal wash.

A purified system was needed to differentiate protein synthesis which had an absolute requirement for ribosomal wash factors from protein synthesis which occurred in the absence of these factors. By

subjecting a pre-incubated in vitro MS2 directed system to centrifugation at 105,000 xg for two hours, the ribosomal complex could be pelleted onto a 10% sucrose pad, collected and placed into new incubation mixture, complete with crude initiation factors. A significant amount of ^{14}C-amino acids was incorporated.

The pH 5' fraction alone would not stimulate the ribosomal complex recovered by centrifugation. It was concluded that the stimulation by ribosomal wash factors resulted from their requirement for re-initiation of $_{m}RNA$. When ribosomal wash factors were not added to the centrifuged complexes, the system would not incorporate ^{14}C-amino acids due to removal of initiation factors by the centrifugation. By this means it was possible that a ^{14}C-amino acid incorporation involving initiation alone could be distinguished from one involving both initiation and elongation. It was now possible to test CAP's effect on crude initiation factor dependent and crude initiation factor independent incorporation of ^{14}C-amino acids.

It was found that CAP had a significantly greater effect on ^{14}C-amino acid incorporation specifically catalyzed by the ribosomal wash factors, producing a 45% inhibition. Residual ^{14}C-amino acid incorporation with crude initiation factors absent was not affected (Fig. 27). These results suggest that CAP exerts its effect during initiation. After initiation, it exerts a significantly lower effect. The methodology utilized to differentiate initiation and elongation dependent ^{14}C-amino

acid incorporation involved the selective presence or absence of crude initiation factors. This suggests that initiation catalyzed by the initiation factors themselves is inhibited by CAP.

The work presented here therefore rules out models of premature release, random initiation, inhibition of termination and inhibition of elongation as the mechanism of action of CAP at low concentrations in the MS2 $_{m}$RNA directed system. In addition, the data strongly argue in favor of a model whereby CAP inhibits the initiation of polypeptide synthesis specifically catalyzed by the recognized bacterial initiation factors.

Table 1

Properties of the E. coli initiation factors

Designation	Molecular Weight (Wahba, 1969)
A...f_1...FI	10,000
B...f_3...FII	20,000
C...f_2...FIII	80,000

A..B..C.. notation (Revel, 1966)

f_1..f_2..f_3.. notation (Ochoa, 1969)

FI..FII..FIII.. notation (Dubnoff, 1971)

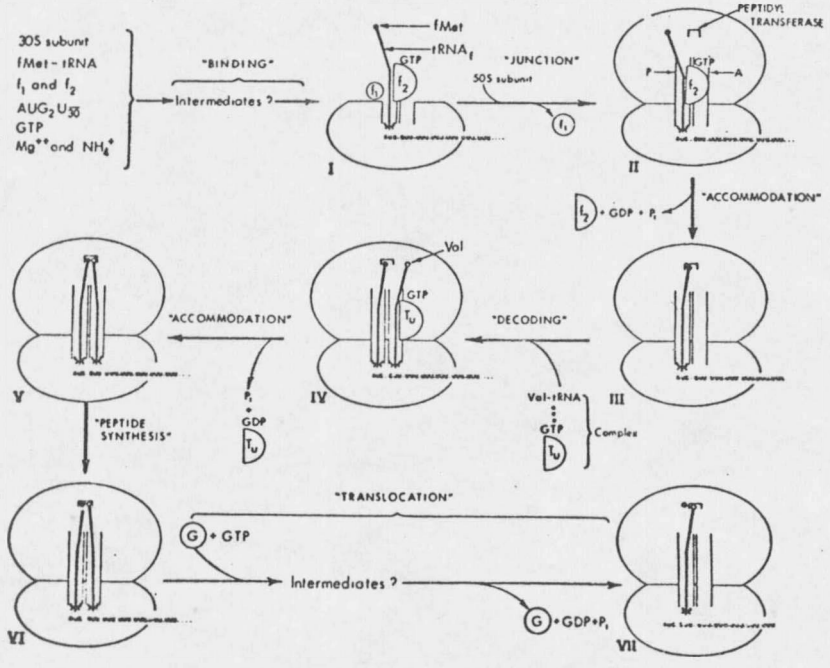
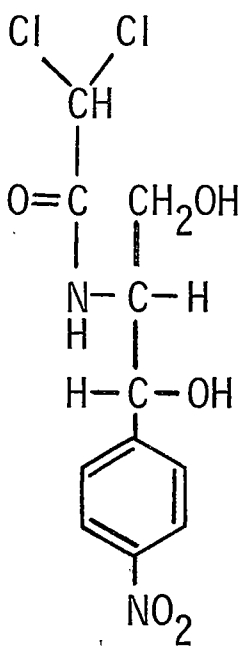


Fig. 1. Schematic representation of the events during protein synthesis (after Thach & Thach, 1971).

Fig. 2.



STRUCTURE OF CHLORAMPHENICOL*

*D(-)threo-2-DICHLOROACETAMIDO-1-p-NITROPHENYL-1,3-PROPANEDIOL

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	ALA	SER	ASN	PHE	THR	GLN	PHE	VAL	LEU	VAL	ASN	ASP	GLY	GLY	THR
16	GLY	ASN	VAL	THR	VAL	ALA	PRO	SER	ASN	PHE	ALA	ASN	GLY	VAL	ALA
31	GLU	TRP	ILU	SER	SER	ASN	SER	ARG	SER	GLN	ALA	TYR	LYS	VAL	THR
46	CYS	SER	VAL	ARG	GLN	SER	SER	ALA	GLN	ASN	ARG	LYS	TYR	THR	ILU
61	LYS	VAL	GLU	VAL	PRO	LYS	VAL	ALA	THR	GLN	THR	VAL	GLY	GLY	VAL
76	GLU	LEU	PRO	VAL	ALA	ALA	TRP	ARG	SER	TYR	LEU	ASN	MET	GLU	LEU
91	THR	ILU	PRO	ILU	PHE	ALA	THR	ASN	SER	ASP	CYS	GLU	LEU	ILU	VAL
106	LYS	ALA	MET	GLN	GLY	LEU	LEU	LYS	ASP	GLY	ASN	PRO	ILU	PRO	SER
121	ALA	ILU	ALA	ALA	ASN	SER	GLY	ILU	TYR						

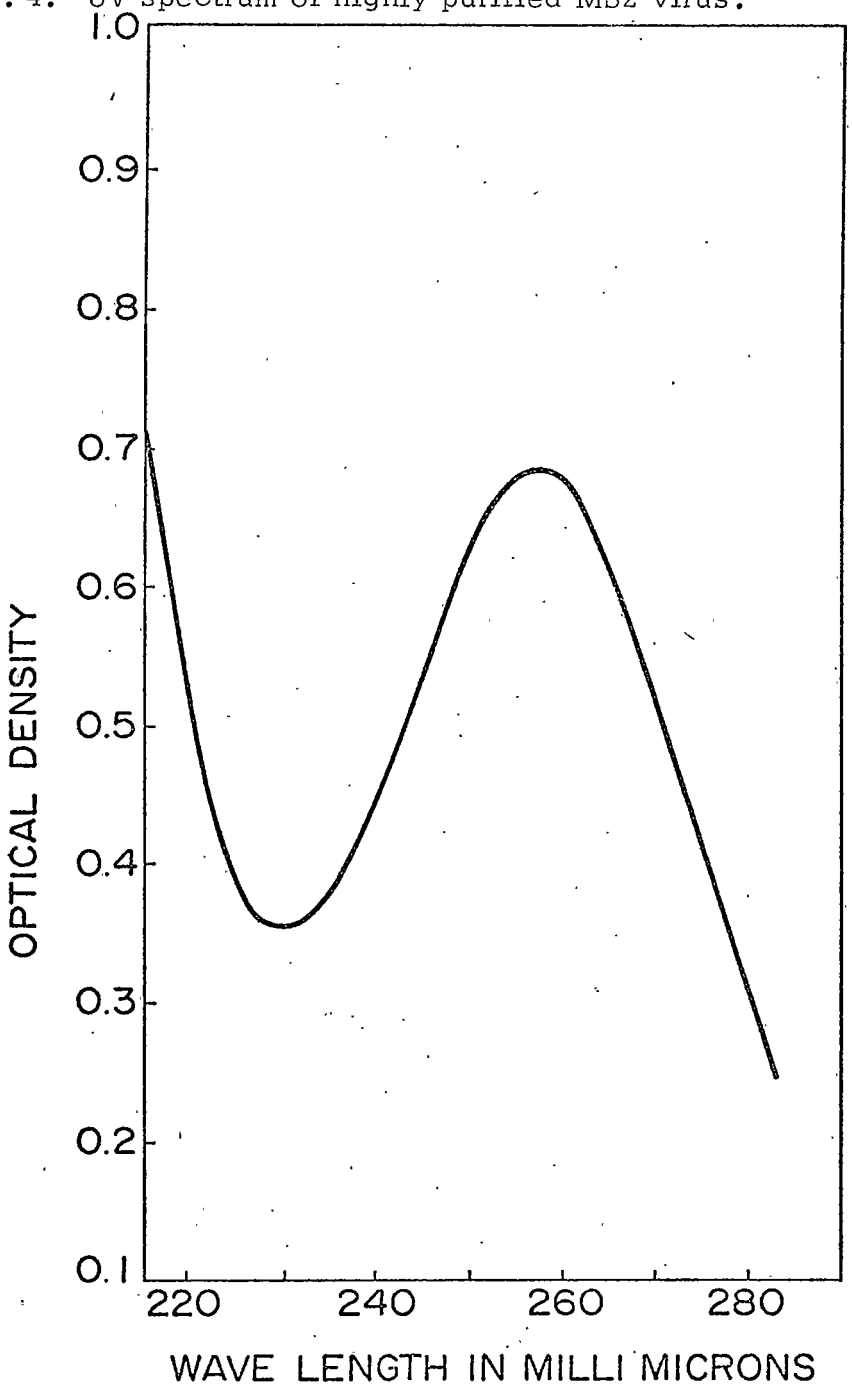
COMPOSITION

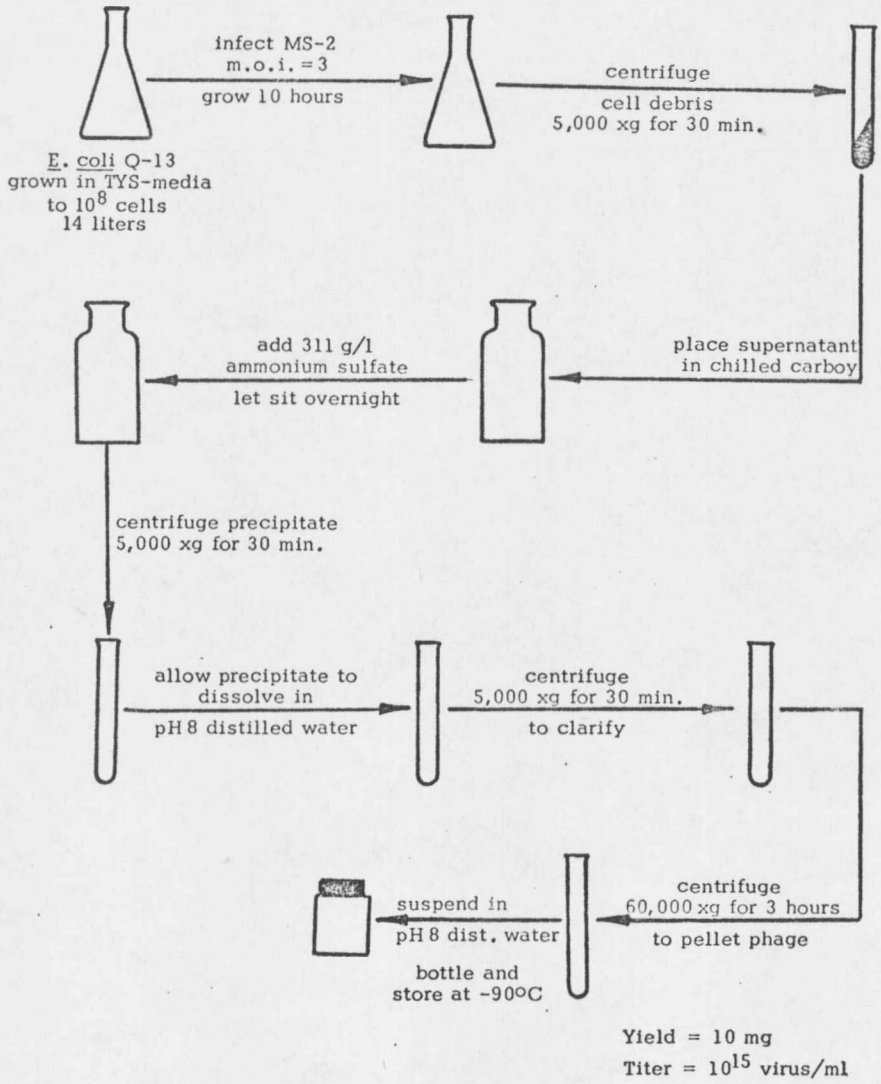
14	ALA	2	CYS	3	ASP	5	GLU
4	PHE	9	GLY	0	HIS	8	ILU
6	LYS	7	LEU	2	MET	11	ASN
4	TYR	6	PRO	6	GLN	4	ARG
13	SER	9	THR	14	VAL	2	TRP

TOTAL NO. OF ACIDS = 129

Fig. 3. Amino acid sequence of MS2 virus coat protein (Weber & Konigsberg, 1967).

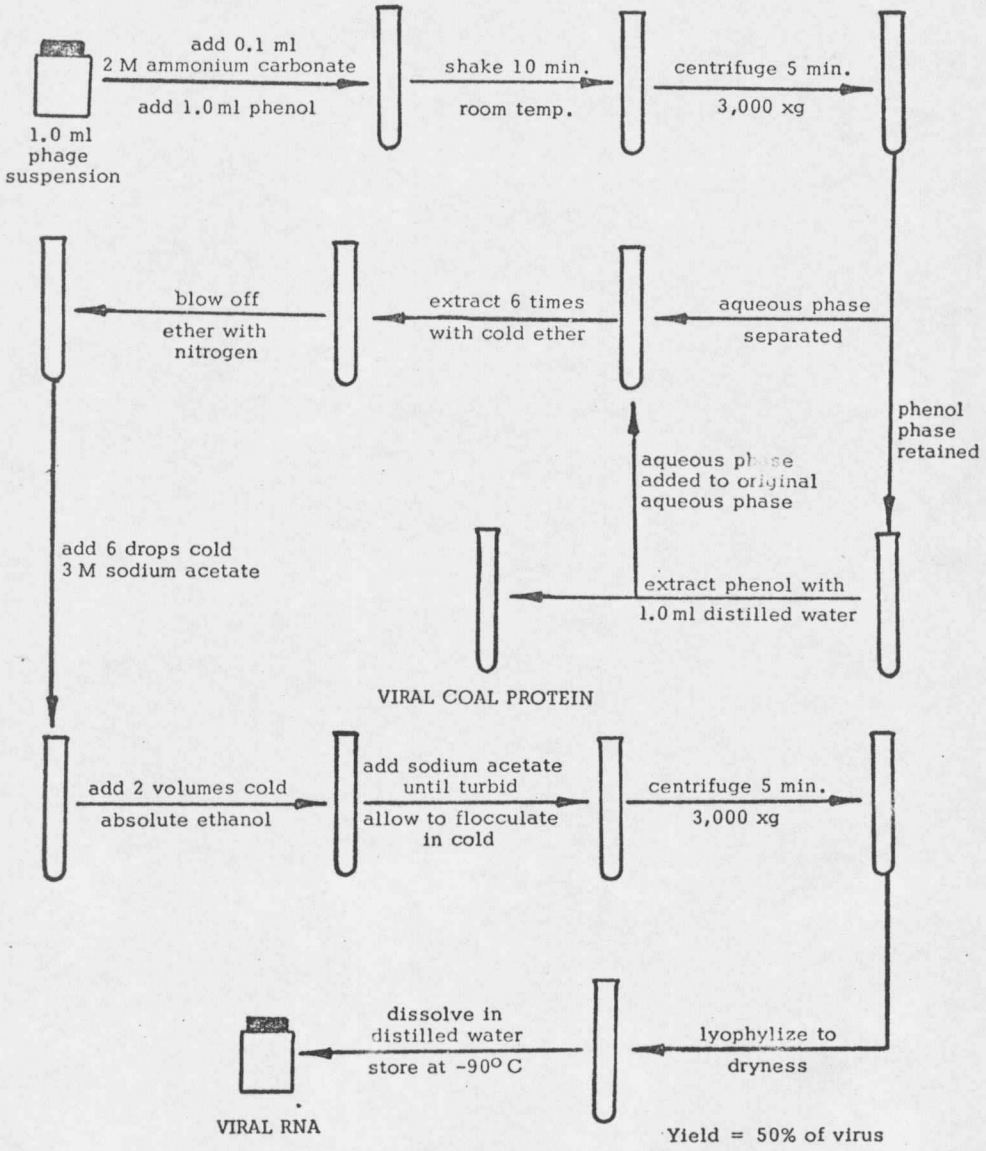
Fig. 4. UV spectrum of highly purified MS2 virus.





GROWTH AND PURIFICATION OF MS-2 VIRUS

Fig. 5. Flow diagram illustrating the growth and purification of MS2 virus. Details are described in "Materials and Methods".



EXTRACTION OF MS-2 m-RNA AND COAT PROTEIN

Fig. 6. Flow diagram illustrating the extraction of MS2 RNA and coat protein from the bacteriophage MS2. Details are given in "Materials and Methods".

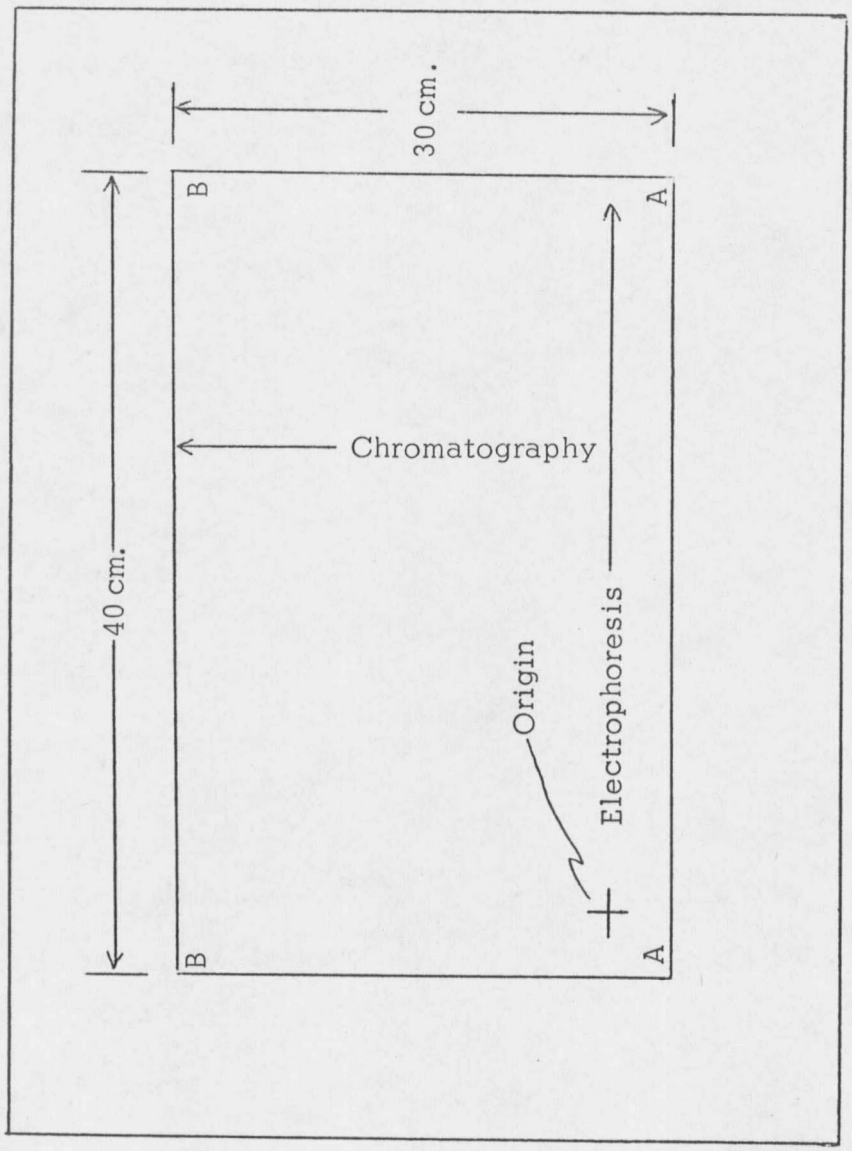


Fig. 7. Diagram of the dimensions of an MS2 fingerprint. Electrophoresis was performed in the indicated direction at a voltage flux of 30 v/cm. for 2.5 hours. After air drying overnight, points A-A and B-B were connected by plastic paper clips, forming a cylinder. Chromatography was then allowed to ensue for 8 hours after 12 hours of equilibration with the chosen buffer.

Fig. 8. Effect of Mg^{++} concentration on S-30 incorporations. Only the Mg^{++} concentration was varied by using an LMSN base buffer. The incubation time was 40 minutes. One hundred micrograms of MS2 RNA was added to each sample. The radioactive counting techniques and the concentration of reagents are described in "Materials and Methods".

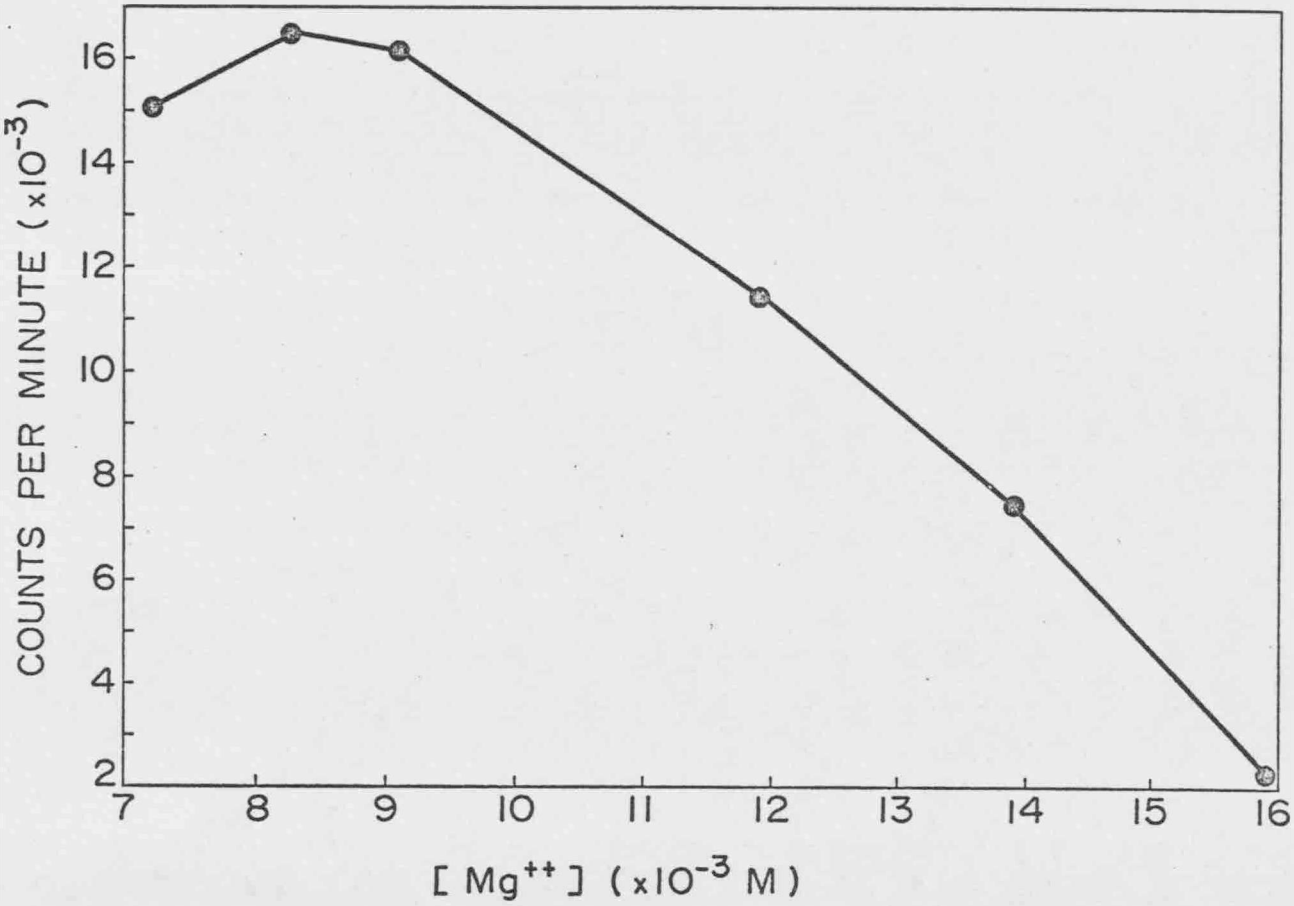

COUNTS PER MINUTE (x10^{-3})
16
14
12
10
8
6
4
2
7
8
9
10
11
12
13
14
15
16
[Mg^{++}] (x10^{-3} M)

Fig. 9. Kinetics of ^{14}C-amino acid incorporation. One hundred micrograms of MS2 $_m$RNA were added to each sample containing the reagents as described in "Materials and Methods". Incorporation was terminated at the appropriate time by the addition of 6 ml. of 5% trichloroacetic acid. The ensuing sample preparation and radioactive counting technique are described in "Materials and Methods".

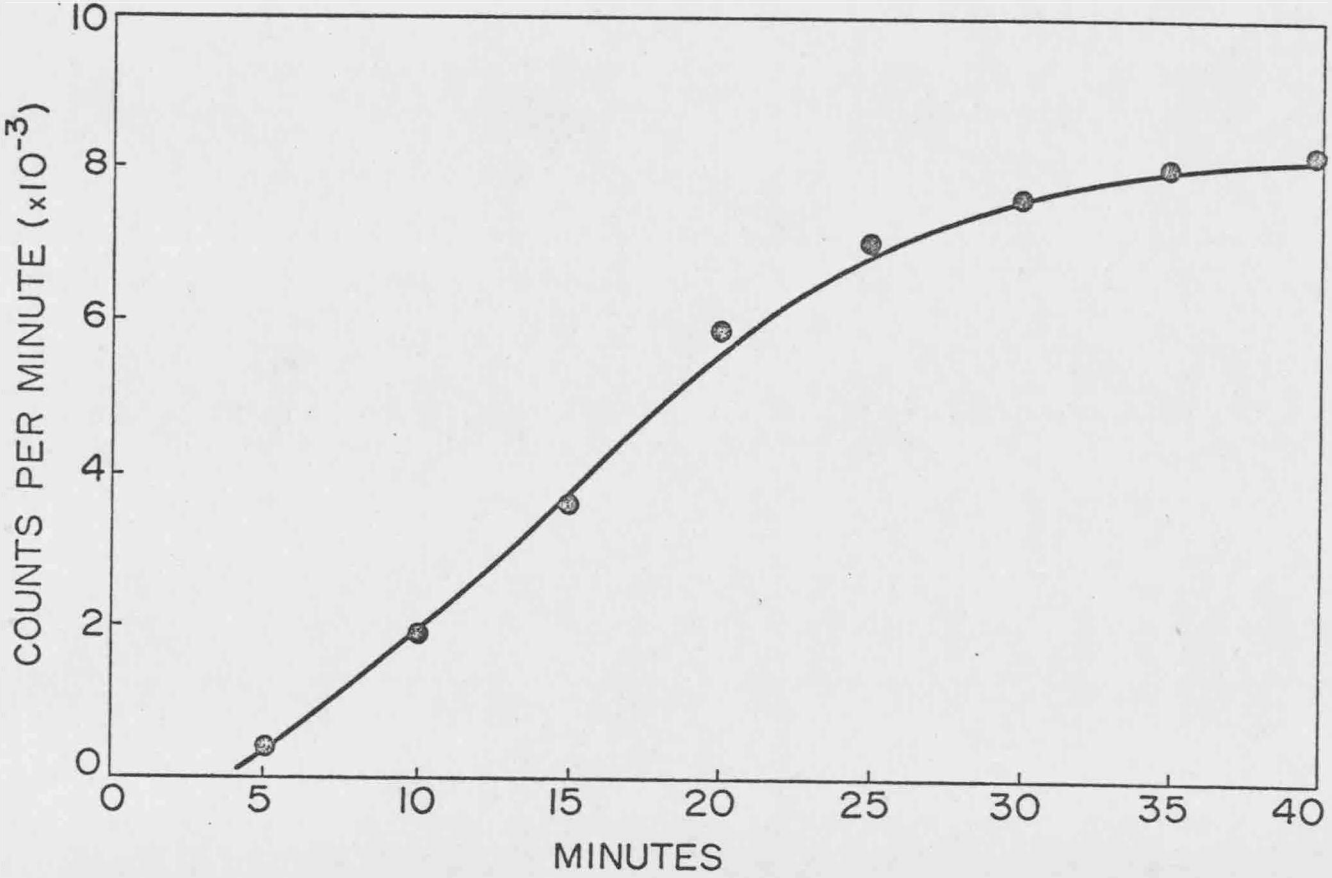

COUNTS PER MINUTE (x10^{-3})
0
2
4
6
8
10
0
5
10
15
20
25
30
35
40
MINUTES

Fig. 10. Stimulatory effect of MS2 messenger RNA. Varying amounts of MS2 RNA were added to an S-30 assisted protein synthesizing system. A Mg^{++} concentration of 8.5 mM, an incubation time of 40 minutes, and a temperature of 37°C were used. Further details of the incubation are described in "Materials and Methods".

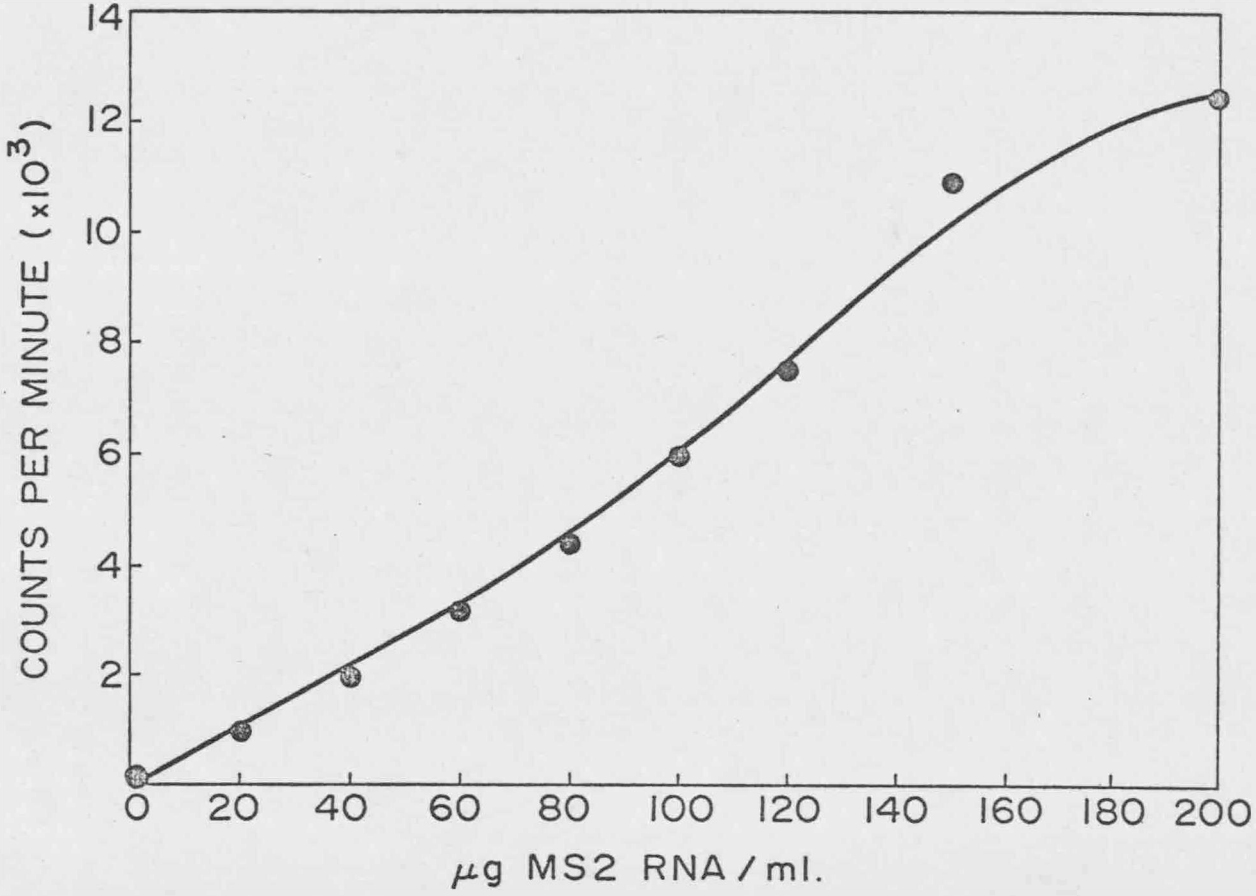

COUNTS PER MINUTE (x10³)
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
μg MS2 RNA / ml.

Fig. 11. Dependence of incorporation on added ^{14}C-amino acids. The final incubation volume was 1.0 ml., and the Mg^{++} concentration was 8.5 mM in all cases. Varying amounts of ^{14}C-lysine and ^{14}C-arginine were added to the incubation tubes, with the volumes adjusted by the addition of buffer LMSN. All other details are described in "Materials and Methods".

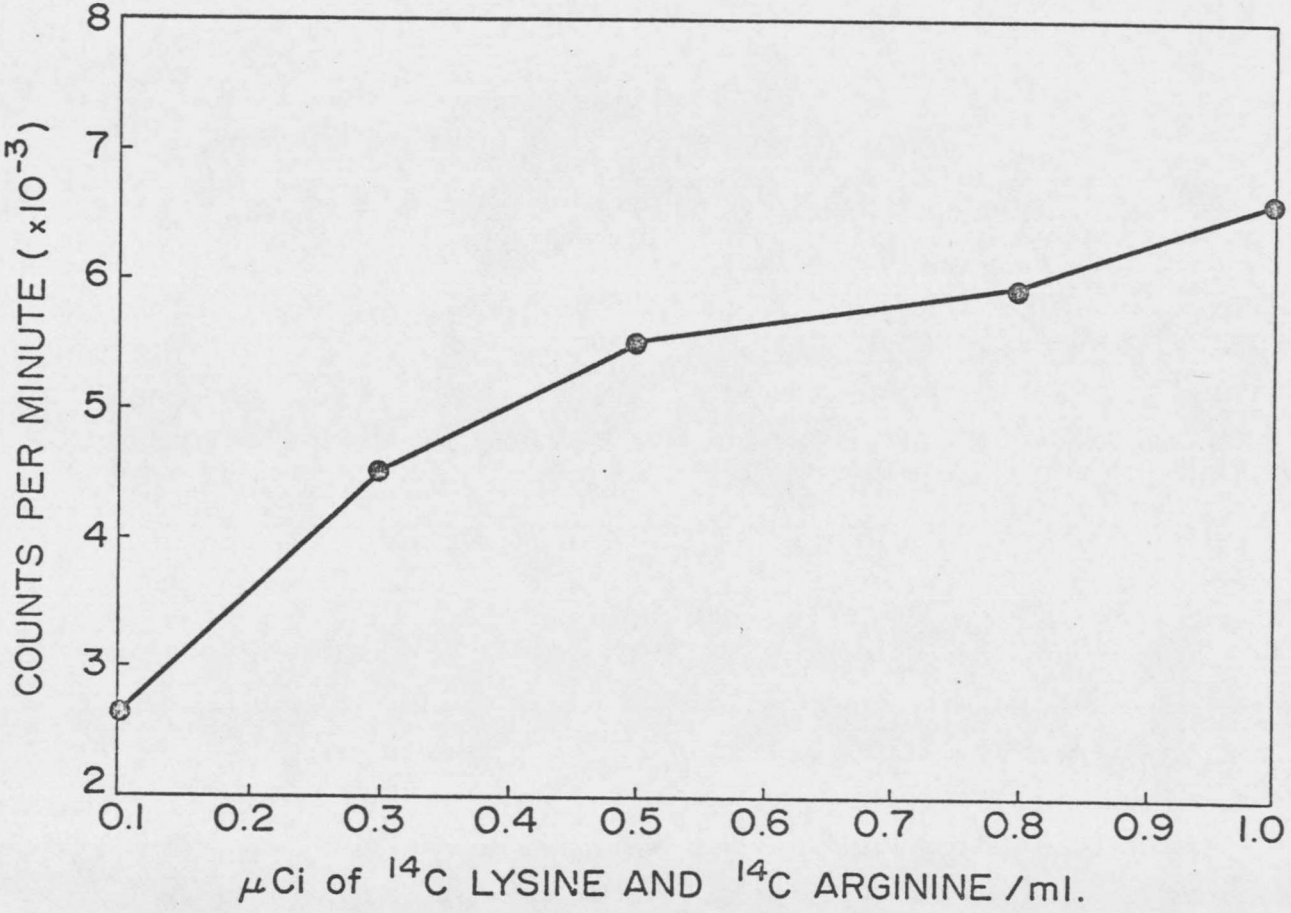

COUNTS PER MINUTE (x10^{-3})
8
7
6
5
4
3
2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
μCi of ^{14}C LYSINE AND ^{14}C ARGININE /ml.

Fig. 12. Effect of chloramphenicol on total acid precipitable counts. The incubation volumes were in all cases 1.0 ml., with an incubation time of 40 minutes and a temperature of 37°C. Varying amounts of chloramphenicol were added from a stock solution of 1.0 mg. chloramphenicol per 10 ml. buffer LMSN. All other details are described in "Materials and Methods".

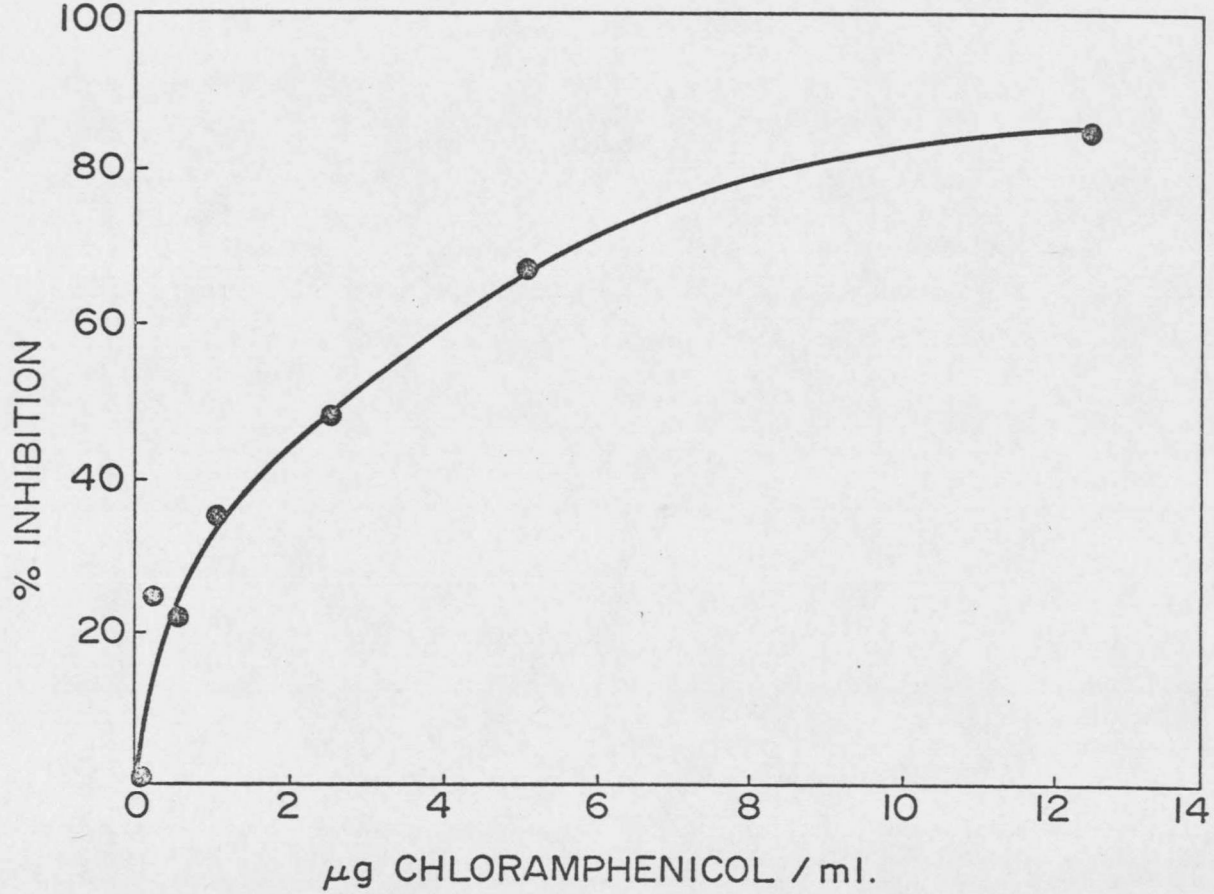
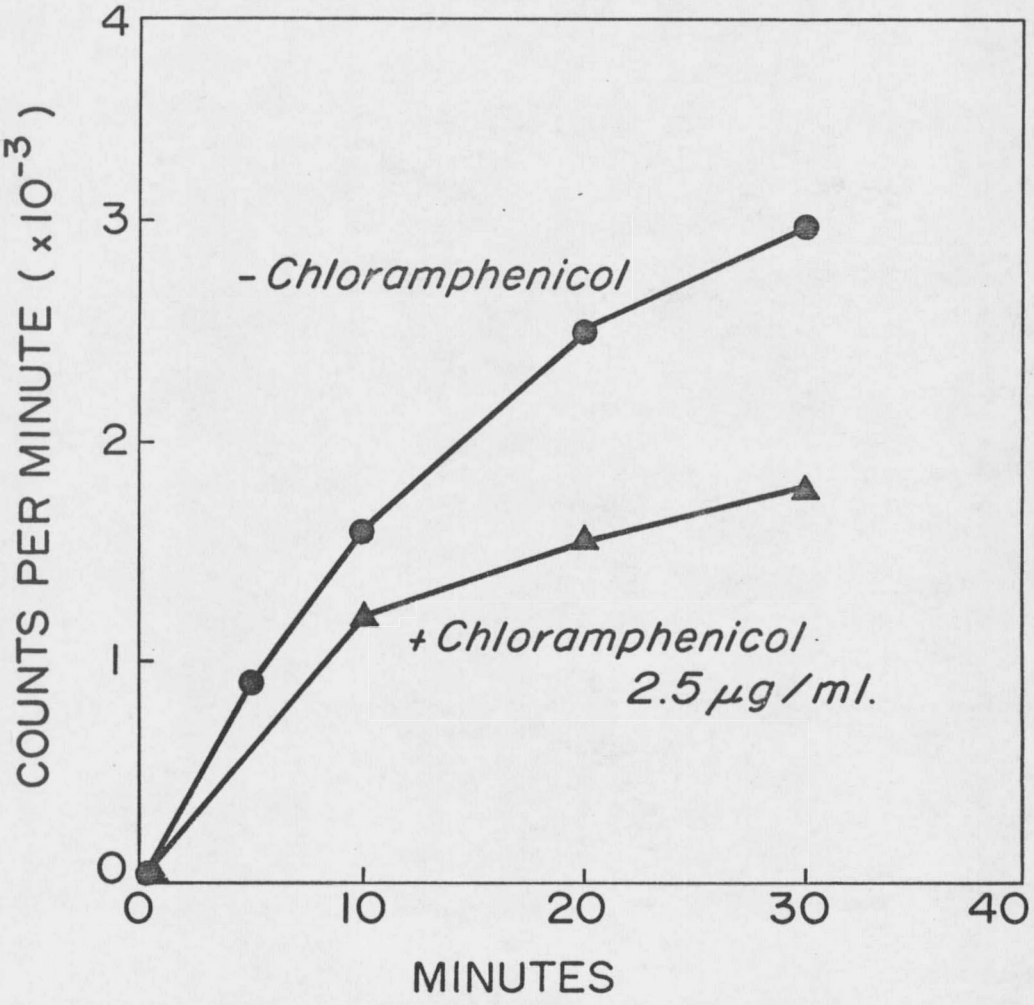
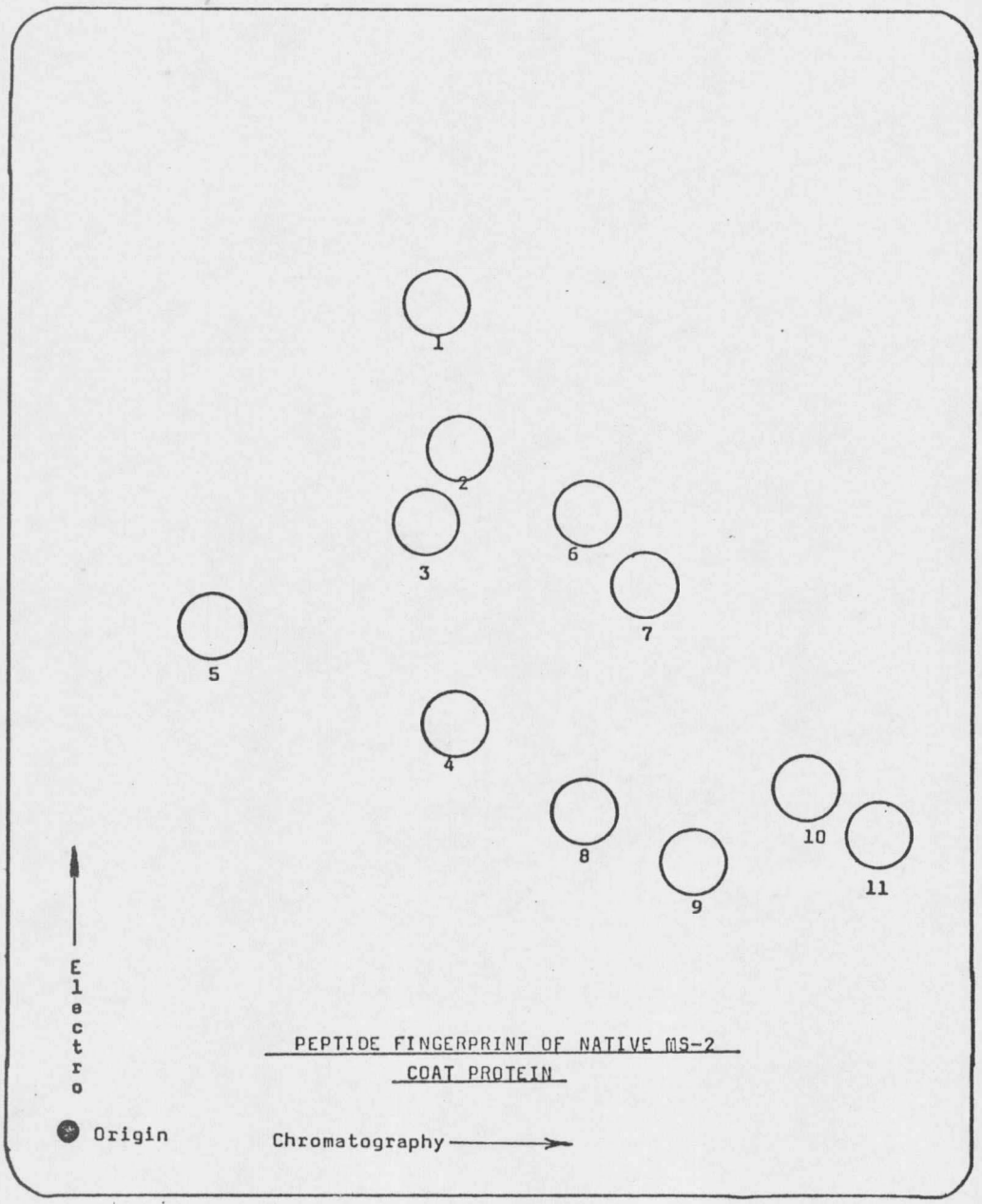

100
80
60
40
20
% INHIBITION
0
2
4
6
8
10
12
14
μg CHLORAMPHENICOL / ml.

Fig. 13. Effect of chloramphenicol on peptides released in vitro. The final incubation volume was in all cases 2.0 ml. The samples were incubated for the indicated time at 37°C and chilled to 0°C in an ice/salt bath at the end of the incubation. Samples were centrifuged at 105,000 xg for 2 hours as described in "Materials and Methods". At the conclusion of this centrifugation, the supernatant was removed and 3.0 mg. lysozyme added as a protein carrier. The supernatant ^{14}C products were then precipitated with 5% trichloroacetic acid and radioactivity determined as described in "Materials and Methods".



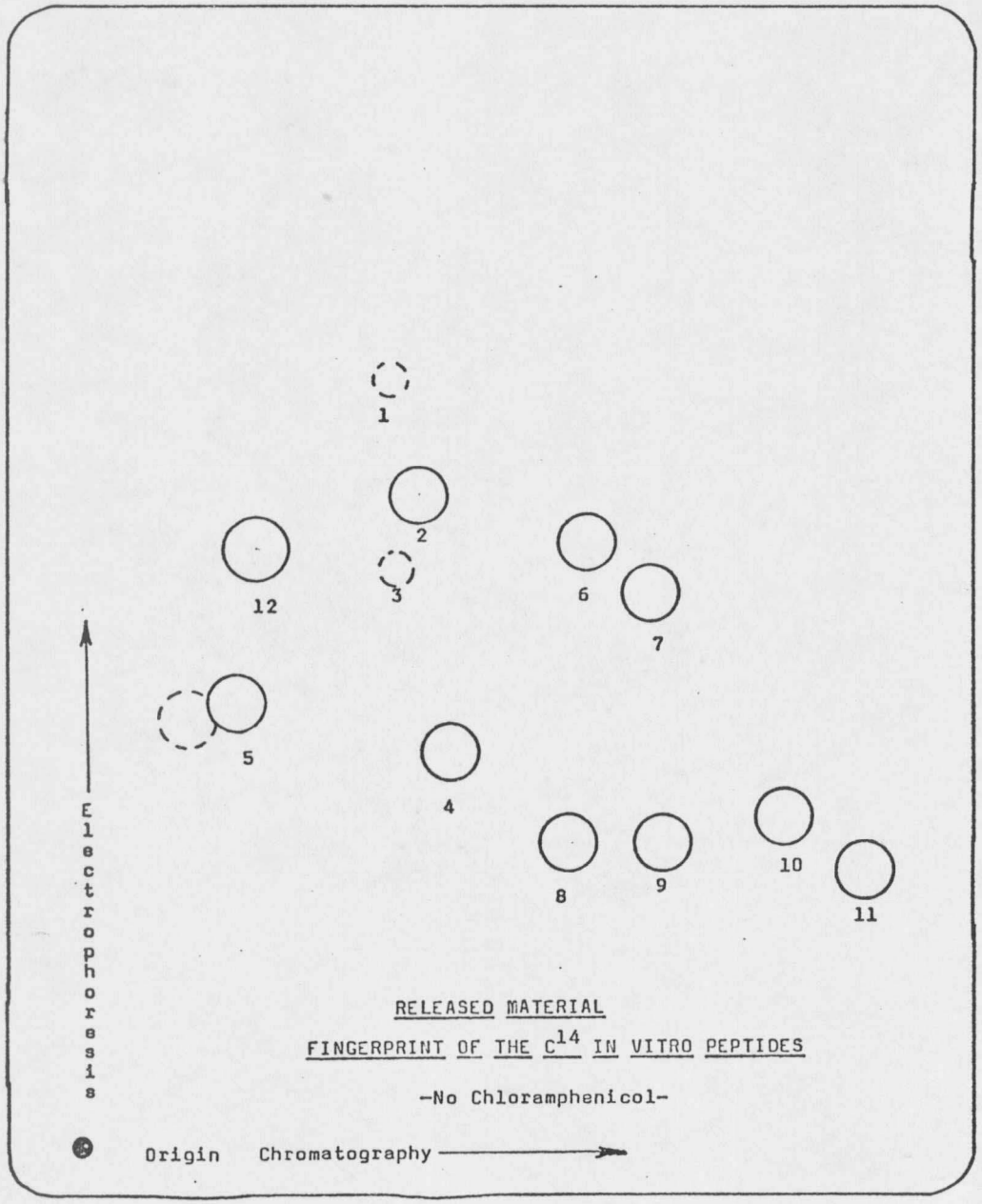
Released Peptides

Fig. 14. Peptide map of native MS2 coat protein. Three mg. of purified native MS2 coat protein were oxidized and digested with TPCK-treated trypsin. The resulting dried peptides were subjected to electrophoresis in buffer EBN-1 on Whatman No. 3mm filter paper. After air drying the electrophoretogram, chromatography was performed for 8 to 10 hours in buffer CBN-1. Refer to "Materials and Methods" for complete details.



PEPTIDE FINGERPRINT OF NATIVE MS-2 COAT PROTEIN

Fig. 15. Peptide map of the in vitro MS2 directed released peptides. A standard 2.0 ml. incubation mixture was incubated for 40 minutes at 37°C. The sample was centrifuged for two hours at 105,000 xg and the supernatant was fingerprinted. Three mg. of lysozyme were added as a protein carrier and oxidation, digestion, and fingerprinting carried out as described in "Materials and Methods". The fingerprint was exposed to Kodak 8" x10" X-ray film for 10 days and developed. A tracing was then made of the radio-opaque spots as shown here.

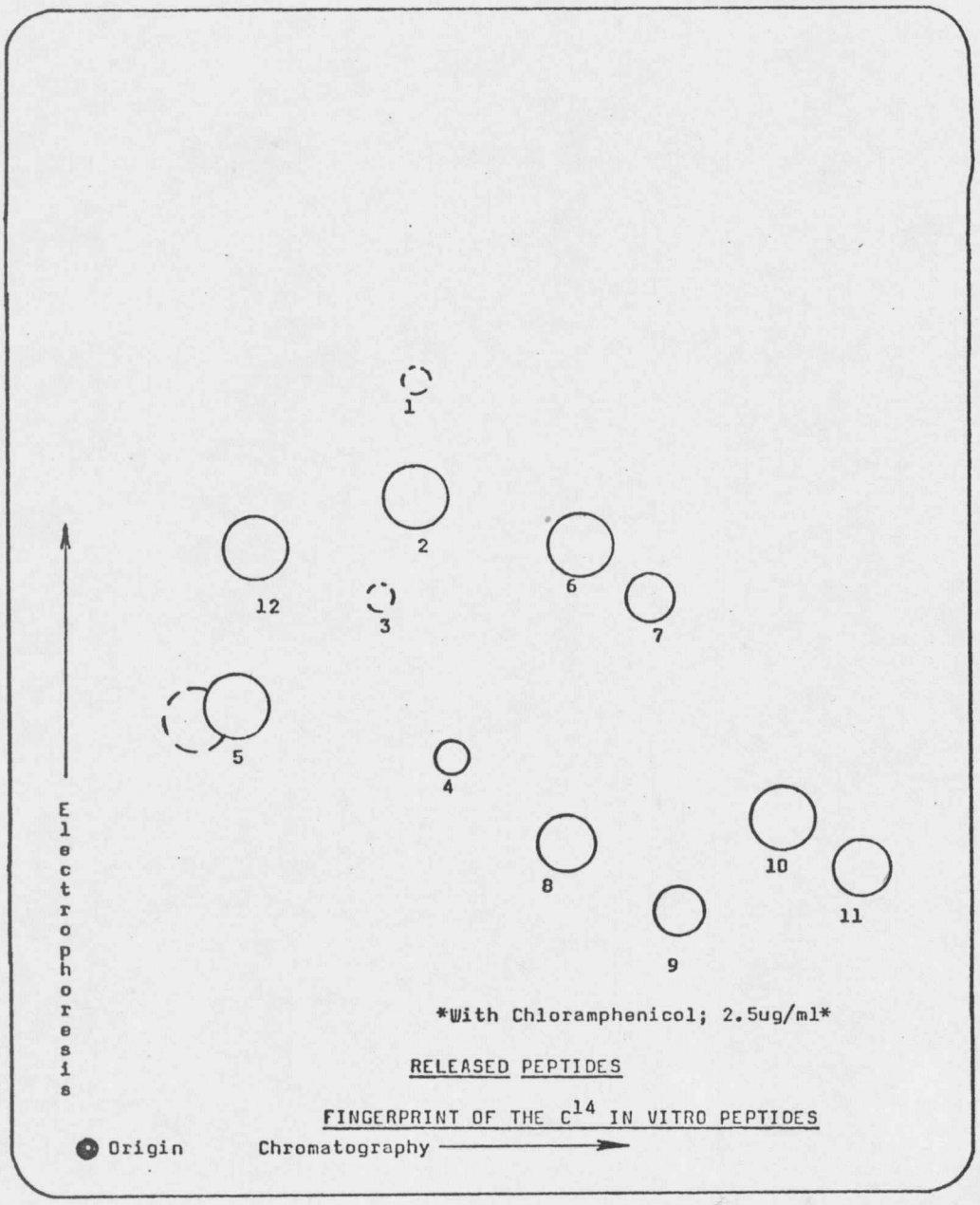


RELEASED MATERIAL

FINGERPRINT OF THE C^{14} IN VITRO PEPTIDES

-No Chloramphenicol-

Fig. 16. Effect of chloramphenicol on the peptide map of peptides released *in vitro*. A standard 2.0 ml. incubation mixture was incubated for 40 minutes at 37°C in the presence of 2.5 µg./ml. of chloramphenicol. The procedure after incubation was identical to that of Fig. 15, and a standard fingerprint was made of the 105,000 xg supernatant. Details of the three peptide maps are described in "Materials and Methods".

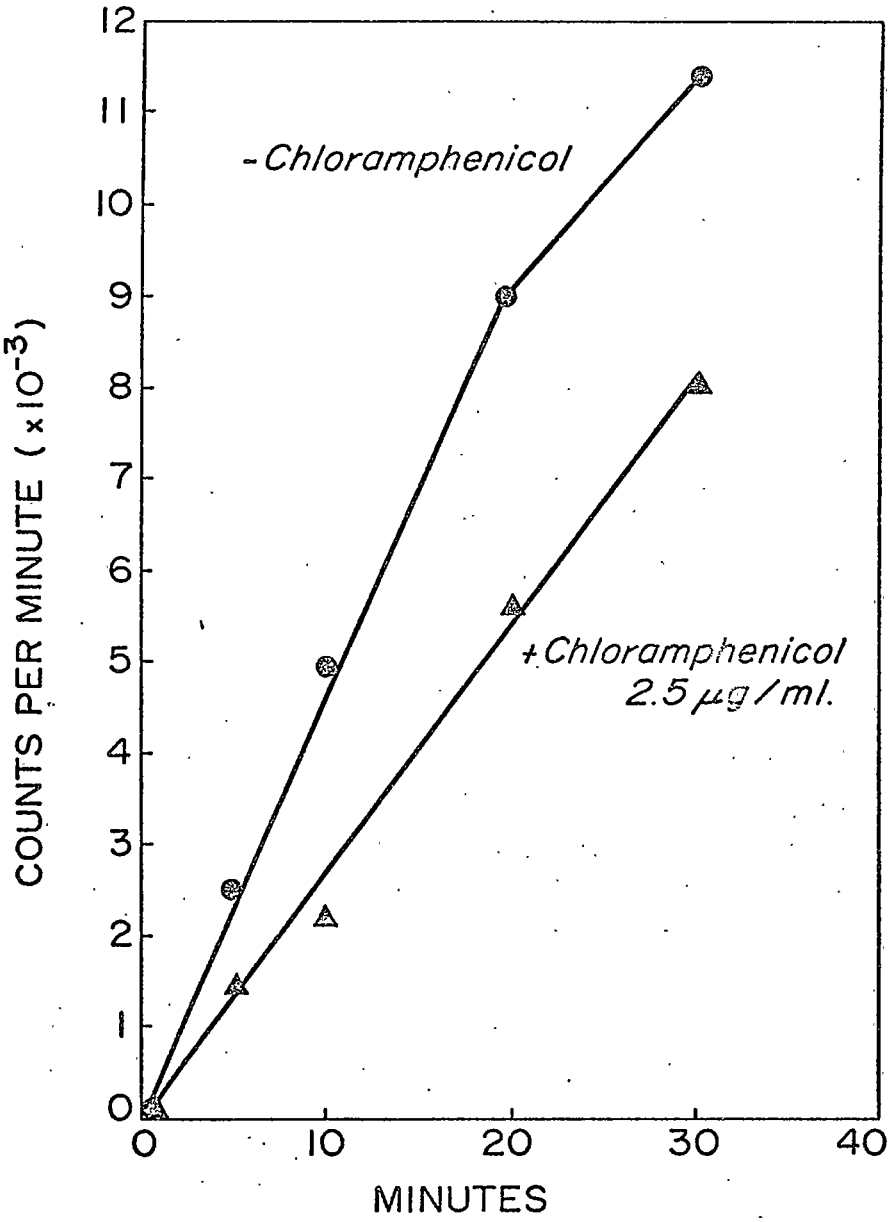


With Chloramphenicol; 2.5ug/ml

RELEASED PEPTIDES

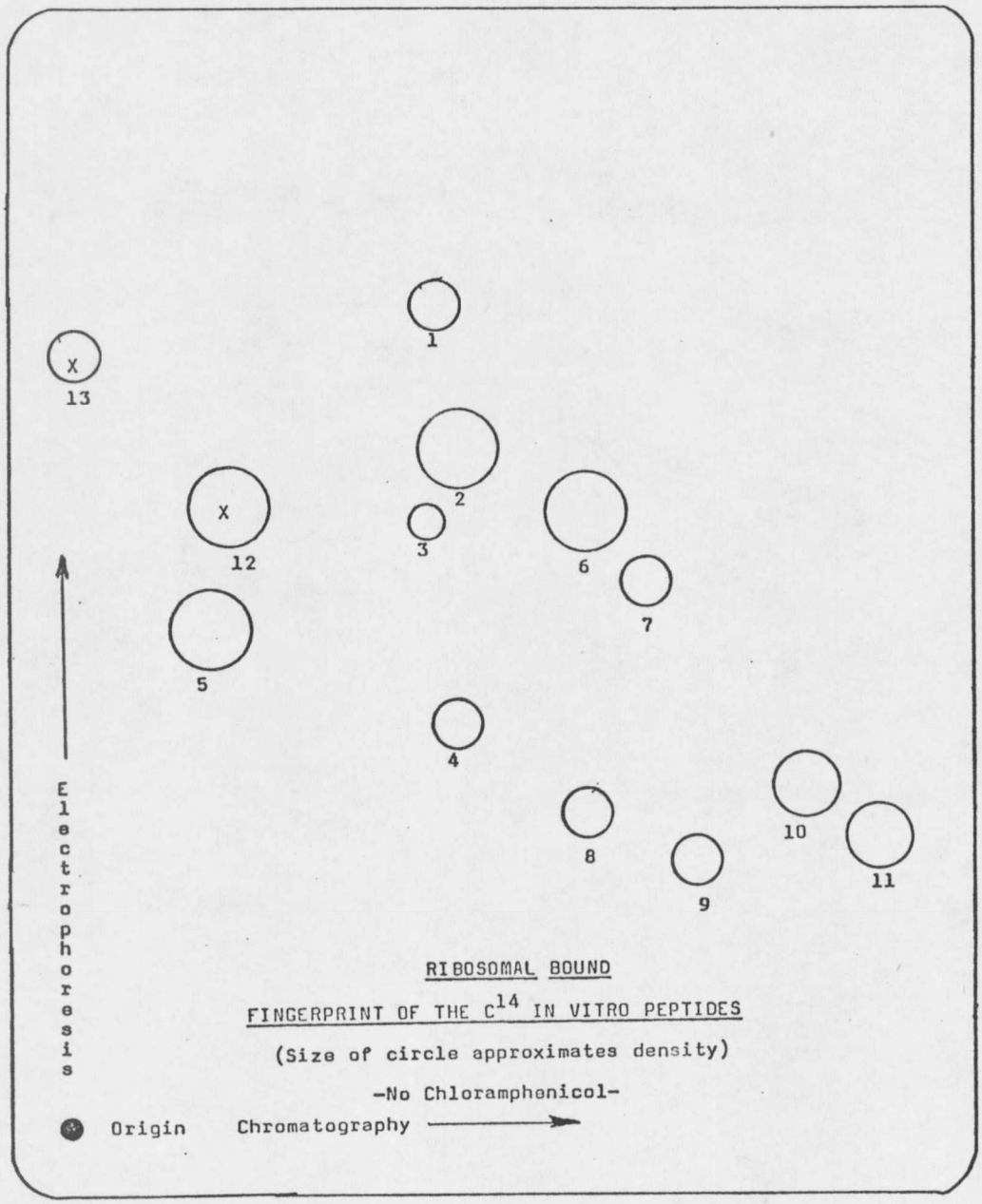
FINGERPRINT OF THE C^{14} IN VITRO PEPTIDES

Fig. 17. Effect of chloramphenicol on acid precipitable counts of the ribosomal bound in vitro peptides. The incubation procedure was identical to those for Fig. 16, except the supernatant was removed from the 105,000 xg centrifugation, and the precipitate was solubilized in 2.0 ml. of buffer LMSN. Three mg. of lysozyme was then added and the acid precipitable radioactivity determined as described in "Materials and Methods".



Ribosomal Bound Material

Fig. 18. Peptide map of the in vitro MS2 directed ribosomal bound peptides. A standard 2.0 ml. incubation was centrifuged at 105,000 xg for 2 hours and the supernatant removed. The precipitate was solubilized in 2.0 ml. of buffer LMSN and 3.0 mg. of lysozyme added as a protein carrier. The solution was then treated as described in "Materials and Methods", and a standard peptide map was made.



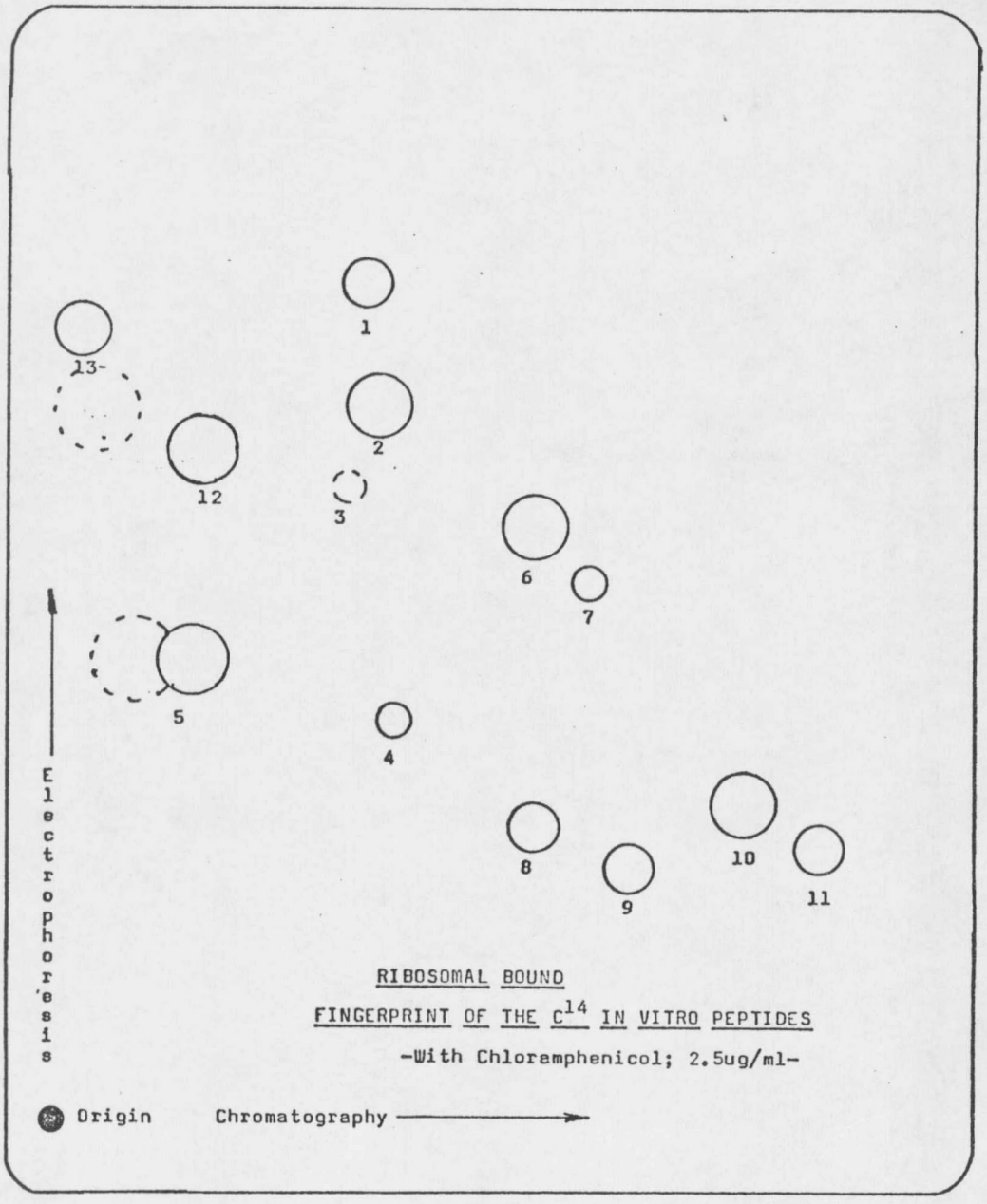
RIBOSOMAL BOUND

FINGERPRINT OF THE C^{14} IN VITRO PEPTIDES

(Size of circle approximates density)

-No Chloramphenicol-

Fig. 19. Effect of chloramphenicol on the peptide map of the ribosomal bound in vitro peptides. A standard 2.0 ml. incubation mixture was incubated for 40 minutes at 37°C in the presence of 2.5 μg./ml. of chloramphenicol. The sample was centrifuged for two hours at 105,000 xg and a standard fingerprint was made of the material precipitated at 105,000 xg. The details of the procedure are summarized in "Materials and Methods". The completed fingerprint was exposed for 10 days to Kodak X-ray film, and upon development of the autoradiogram, a tracing was made of the radio-opaque spots.



RIBOSOMAL BOUND

FINGERPRINT OF THE C^{14} IN VITRO PEPTIDES

-With Chloremphenicol; 2.5ug/ml-

Fig. 20. Quantitative effect of chloramphenicol on the ribosomal bound in vitro tryptic peptides. Each of the fingerprints from Figs. 18 and 19 were matched to their respective autoradiograph. Using a soft lead pencil, the areas on the fingerprints were marked corresponding to the radio-opaque areas on the autoradiograph. These areas on the fingerprint were cut out and the radioactivity determined by liquid scintillation counting. This methodology is described in detail in "Materials and Methods". The points plotted represent the radioactive level of the cut out fingerprint spot minus a control spot from the edge of the same fingerprint.

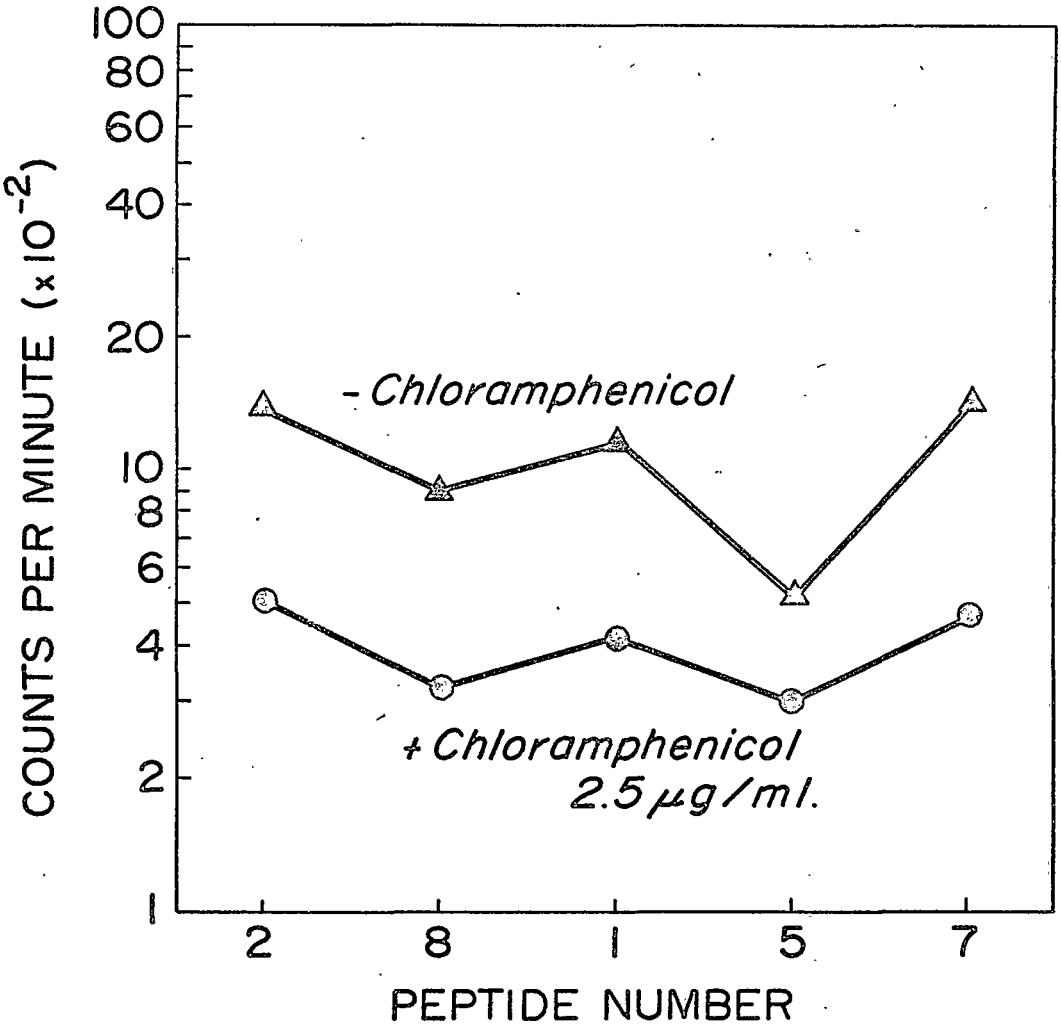

100
80
60
40
20
10
8
6
4
2
1
COUNTS PER MINUTE (x10^{-2})
- Chloramphenicol
+ Chloramphenicol
2.5 µg/ml.
2
8
1
5
7
PEPTIDE NUMBER

Fig. 21. Mg^{++} titration curve of the purified *in vitro* system. The concentration of Mg^{++} was varied over the range indicated and the 0.5-ml. sample was incubated at 37°C for 40 minutes. Standard concentrations of all reagents was used. Total acid precipitable radioactivity was determined as described in "Materials and Methods".

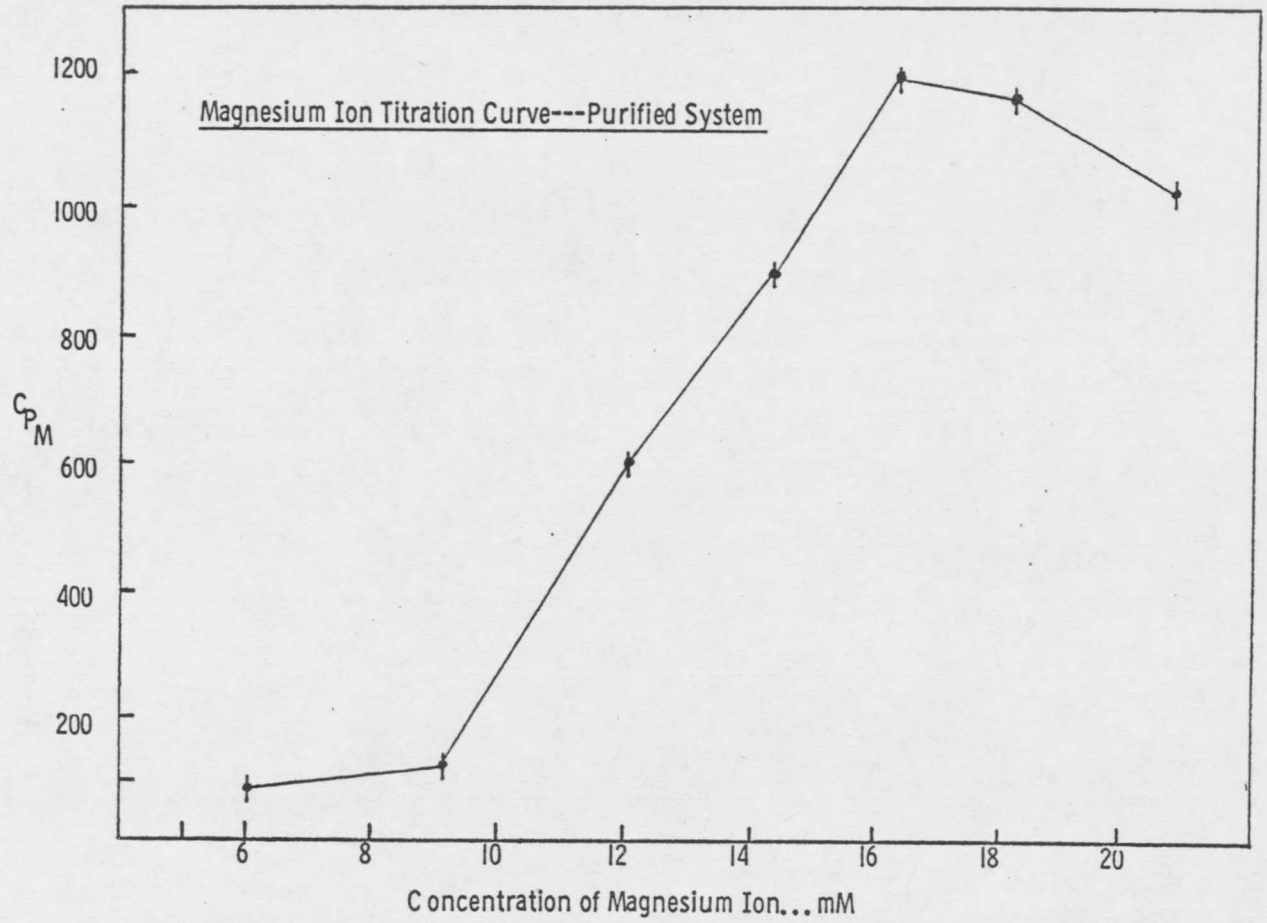

Magnesium Ion Titration Curve---Purified System
1200
1000
800
600
400
200
CPM
6
8
10
12
14
16
18
20
Concentration of Magnesium Ion... mM

Fig. 22. Titration curve of crude initiation factors against the purified system. The amounts of crude initiation factors varied as described. A standard incubation volume of 0.5 ml. was used. Incubation was for a duration of 30 minutes at 37°C. Concentrations of all other incubation reagents were held constant. Radioactive counting is described in "Materials and Methods".

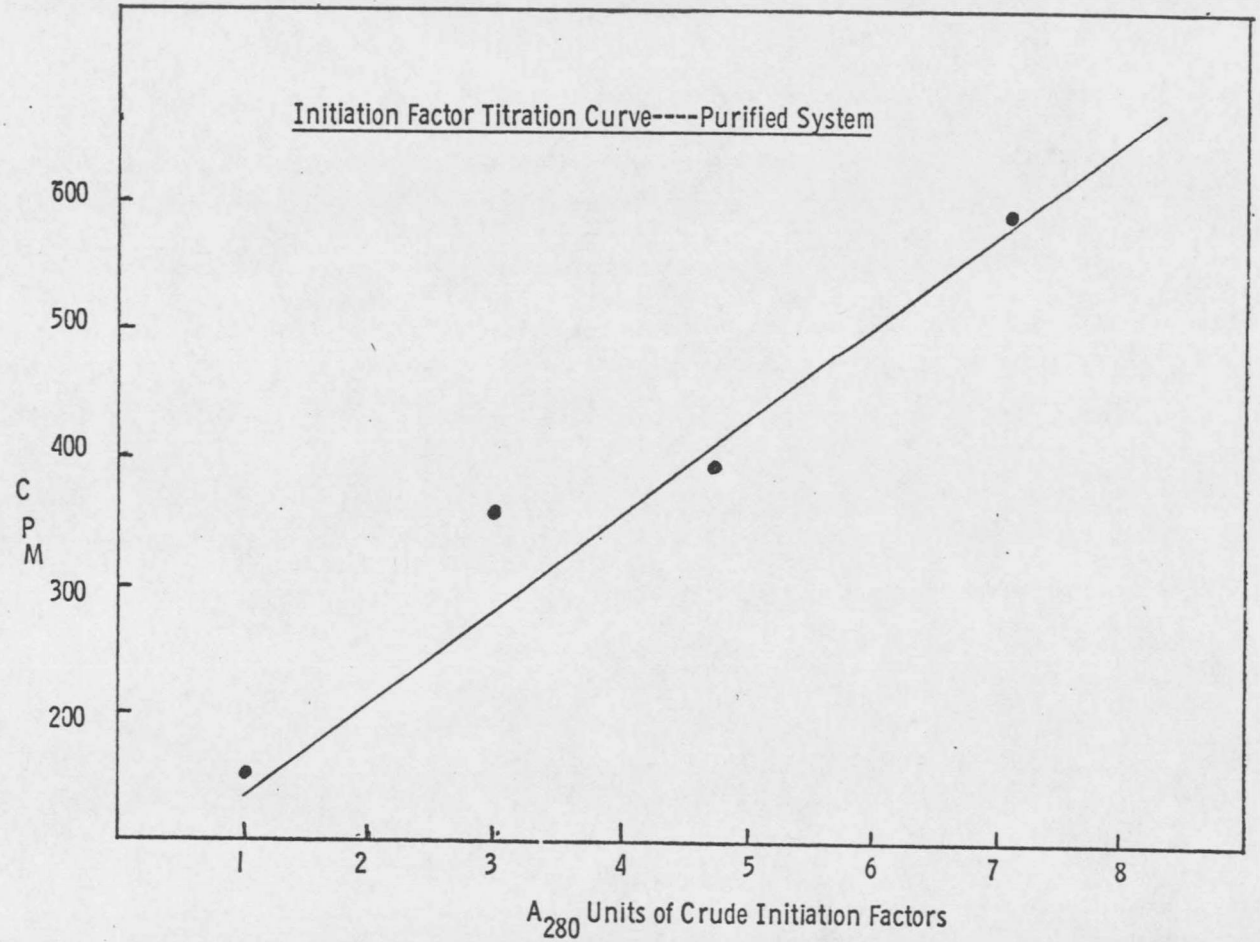

Initiation Factor Titration Curve----Purified System
600
500
400
C
P
M
300
200
1
2
3
4
5
6
7
8
A280 Units of Crude Initiation Factors

Fig. 23. Kinetics of ^{14}C-amino acid incorporation of the purified system. Standard 0.5-ml. incubation mixtures were incubated for the indicated times, and 5% trichloroacetic acid added to terminate incorporation. The samples were then counted as described in "Materials and Methods".

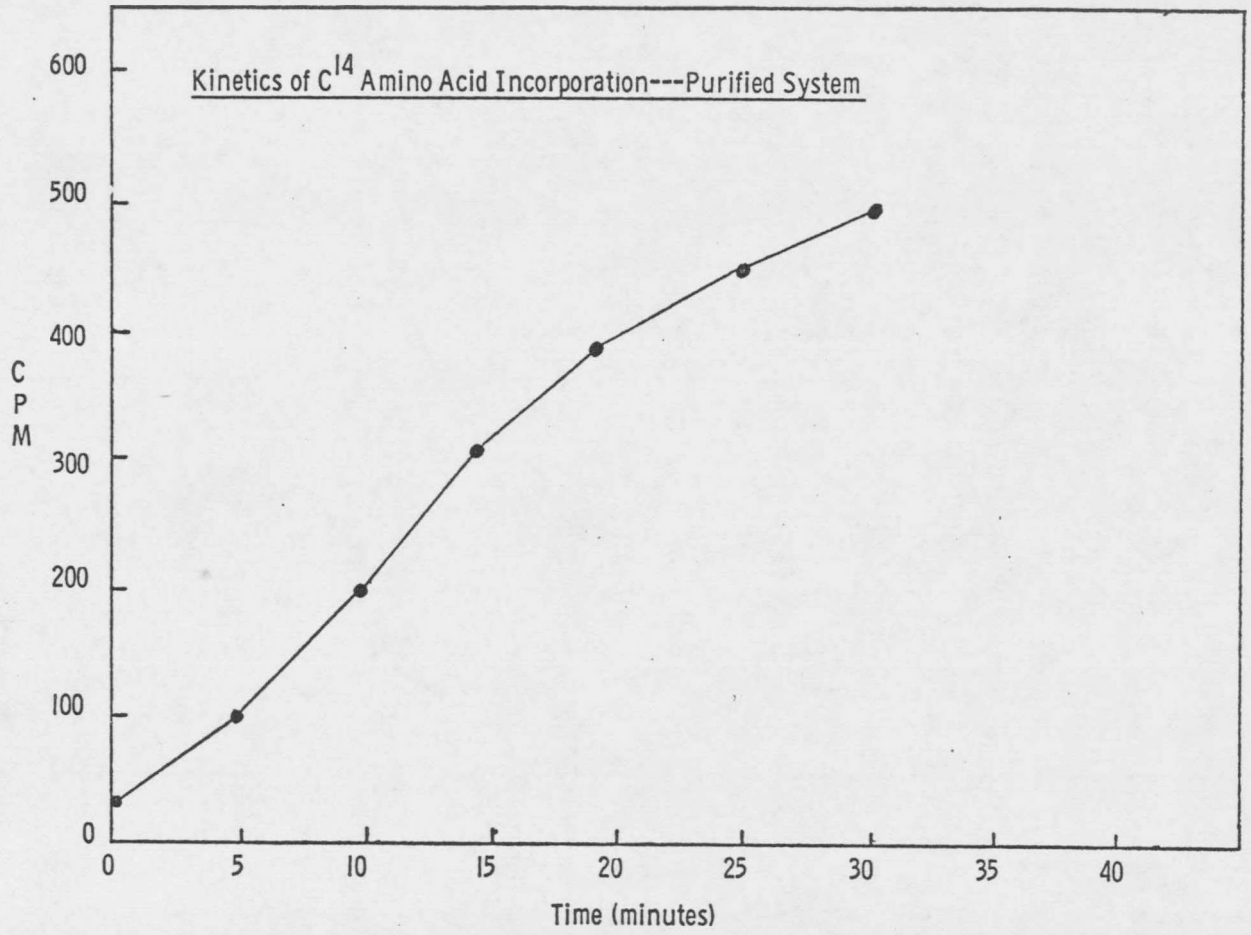

Kinetics of C14 Amino Acid Incorporation---Purified System
600
500
400
300
200
100
0
C
P
M
0
5
10
15
20
25
30
35
40
Time (minutes)

Fig. 24. Effect of chloramphenicol on total acid precipitable counts of the purified system. Standard incubation samples of 0.5 ml. were incubated for 40 minutes at 37°C with the indicated concentration of chloramphenicol. Radioactive counting is described in "Materials and Methods".

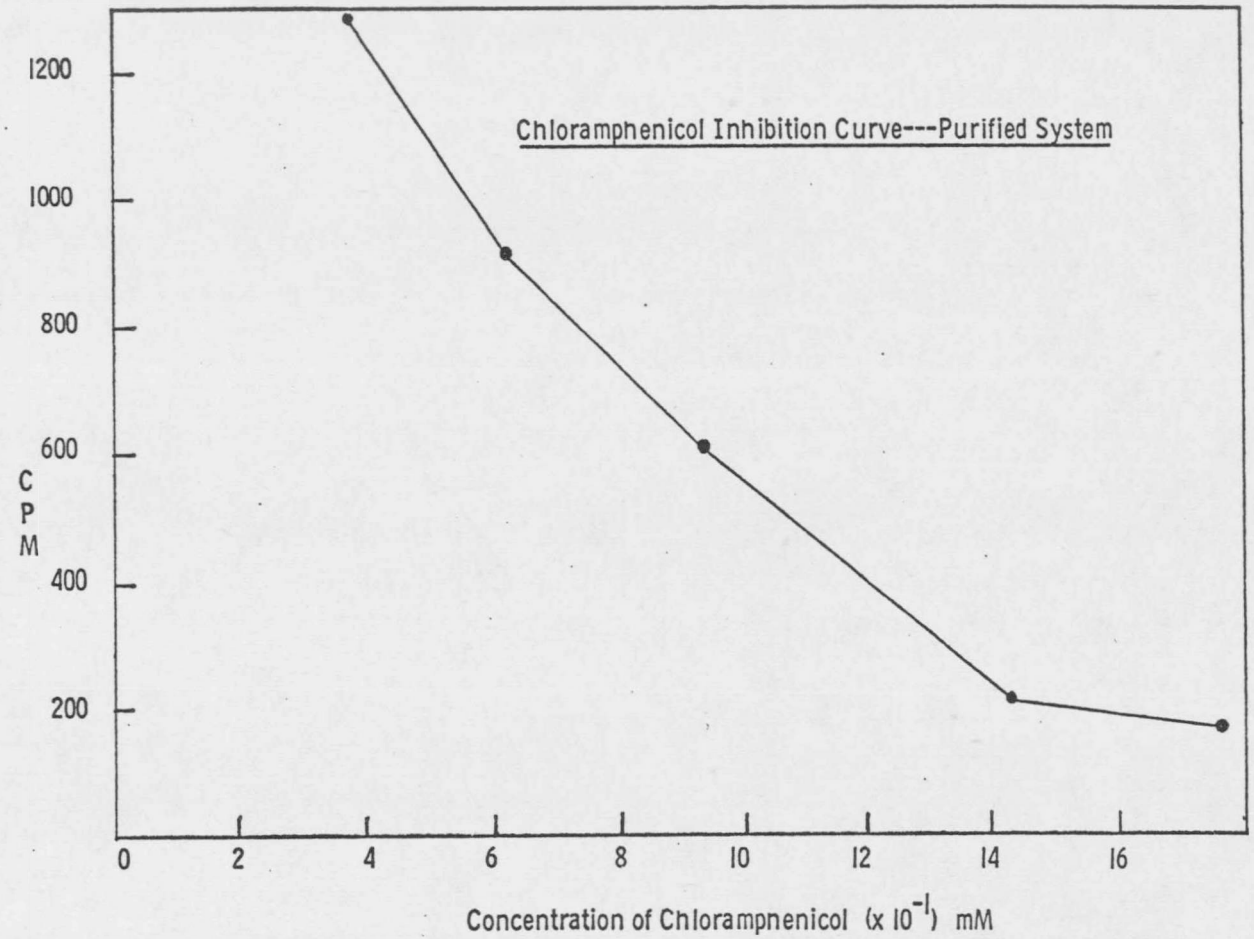

Chloramphenicol Inhibition Curve---Purified System
1200
1000
800
600
400
200
C
P
M
0
2
4
6
8
10
12
14
16
Concentration of Chloramphenicol (x 10⁻¹) mM

Fig. 25. Effect of crude initiation factors on a purified system re-incubated after a 2-hour centrifugation. Standard complete 1.0-ml. mixtures were incubated for 4 minutes at 37°C, and chilled to 0°C in an ice/salt bath. The samples were carefully loaded into Beckman 2.0-ml. nitrocellulose centrifuge tubes, containing a 0.2-ml. pad of 10% sucrose in buffer LMSN-16, bringing the final volume to 2.0 ml. with buffer LMSN-16. After centrifugation at 105,000 xg for 2 hours in the Beckman Model L, the supernatant was carefully removed leaving a total volume in the centrifuge tube of 0.3 ml. This 0.3-ml. sucrose pad was added to a chilled 1.0-ml. incubation mixture minus ribosomes and $_{m}$RNA. In addition, crude initiation factors were added to one sample. After mixing, the samples were incubated at 37°C for 25 minutes and the radioactivity determined as described in "Materials and Methods".

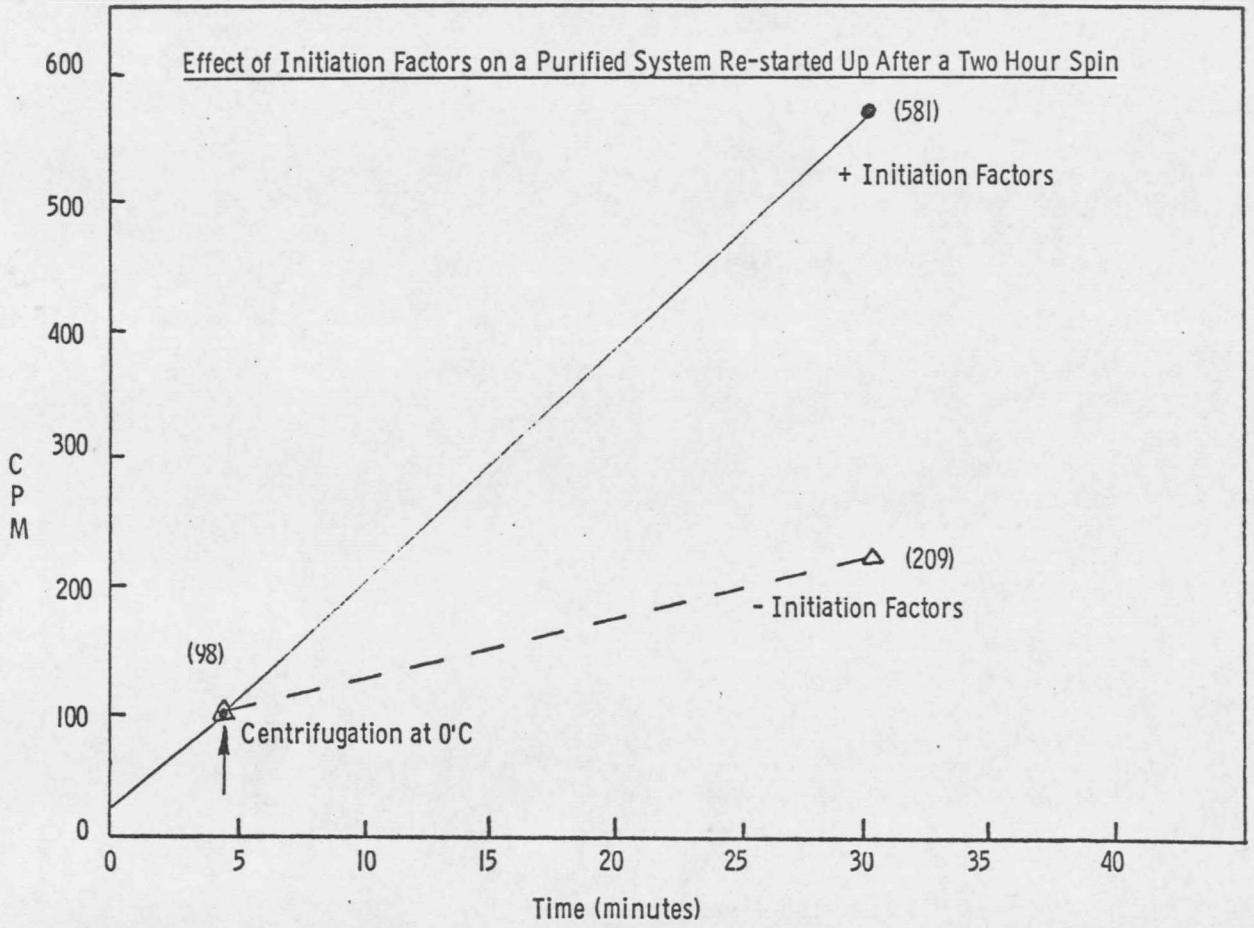
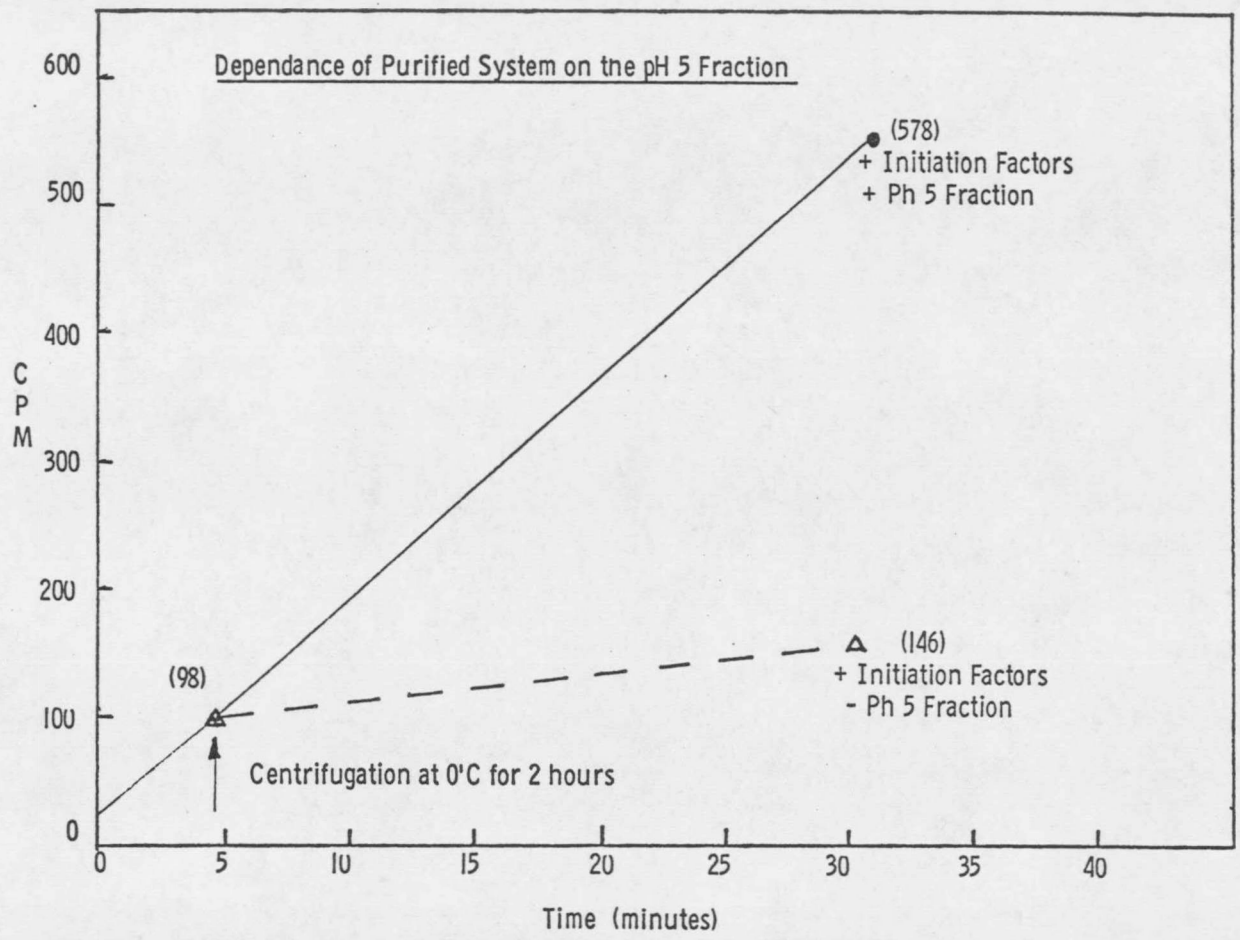
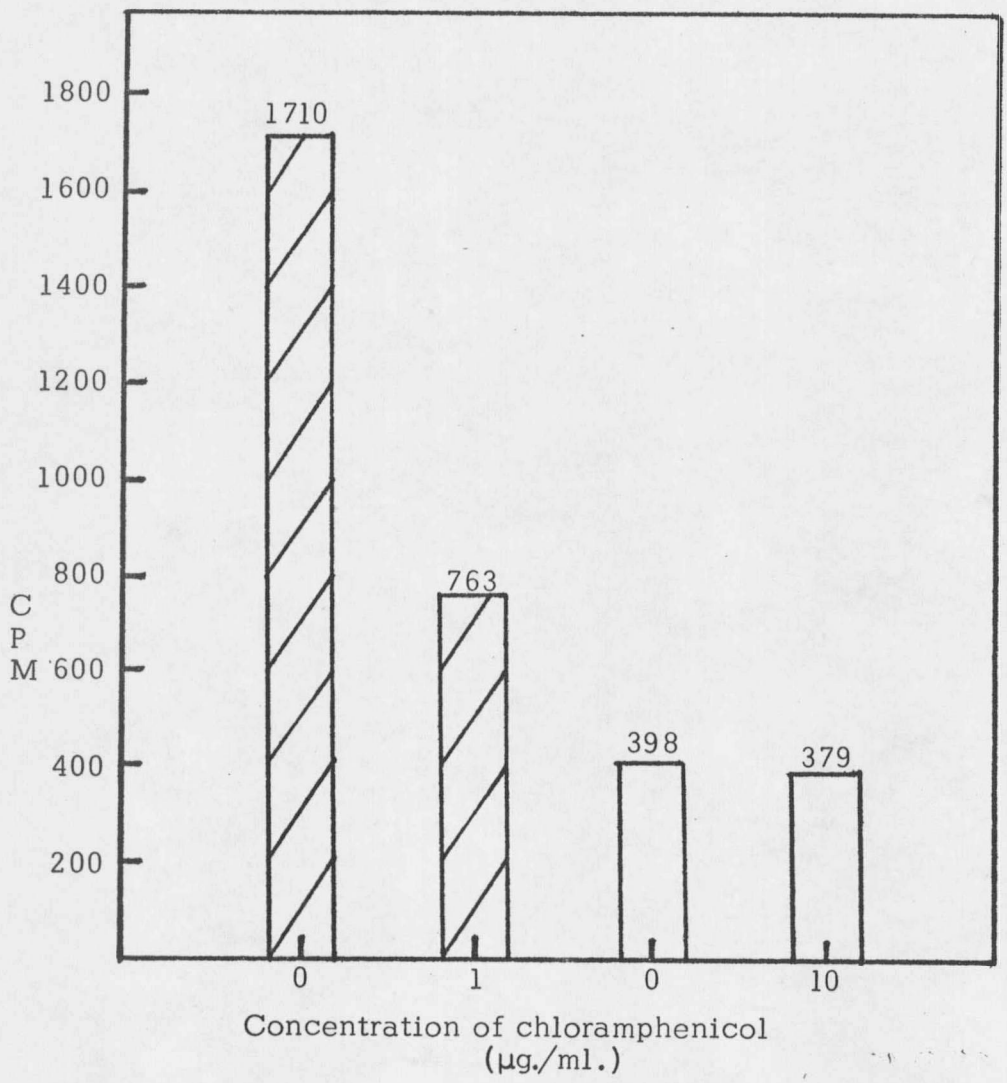

Effect of Initiation Factors on a Purified System Re-started Up After a Two Hour Spin
600
500
400
300
200
100
0
C
P
M
(581)
+ Initiation Factors
(209)
- Initiation Factors
(98)
Centrifugation at 0°C
0
5
10
15
20
25
30
35
40
Time (minutes)

Fig. 26. Dependence of the purified system on the pH 5' fraction. The re-incubation mixtures contained all ingredients as a standard sample, except that in one case the pH 5' fraction was deleted. The samples were removed at the end of the 25-minute re-incubation and terminated with 5% trichloroacetic acid. Radioactive counting was as described in "Materials and Methods".


Dependance of Purified System on the pH 5 Fraction
600
500
400
300
200
100
0
C
P
M
(578)
+ Initiation Factors
+ Ph 5 Fraction
(146)
+ Initiation Factors
- Ph 5 Fraction
(98)
Centrifugation at 0°C for 2 hours
0
5
10
15
20
25
30
35
40
Time (minutes)



= crude initiation factors present (during re-incubation)

= crude initiation factors absent (during re-incubation)

Fig. 27. Effect of chloramphenicol on initiation factor dependent and independent incorporation of ^{14}C-amino acids.

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