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
Montana State University


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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EDWARD J. MORGAN, III

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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in

Chemistry

Approved:

Charles N. Caughlan
Head, Major Department

Gordon C. Linderman
Chairman, Examining Committee

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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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

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 $_m$RNA directed in vitro protein synthesizing system, inhibition of protein synthesis by chloramphenicol is at the level of initiation.

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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.