SYNTHESIS OF DIPHTHERIA TOXIN IN *E. COLI* CELL-FREE LYSATE

by

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INTRODUCTION

Statement of the Problem

Strains of Corynebacterium diphtheriae infected with tox^+ corynebacteriophages synthesize a powerful exotoxin. Nontoxinogenic, nonlysogenic C. diphtheriae upon infection with certain phage mutants produce altered nontoxic proteins which cross react with antitoxin (CRM mutants). This phenomenon has provided evidence that diphtheria toxin is specified by the phage DNA. The role of toxin in the life cycle of corynebacteriophages remains to be elucidated. Although phage DNA carries the genetic code for the toxin, the amount of synthesis depends on the particular bacterial strain and on growth conditions, namely the concentration of iron in the medium. Maximum toxin synthesis occurs only under decreasing iron content.

In order to investigate factors controlling toxin synthesis at the molecular level, especially the regulatory role of iron, it was desirable to develop a cell-free protein synthesizing system in which de novo synthesis of toxin could be detected and its control studied.

The objectives of this research were to:

1. develop a cell-free protein synthesizing system derived from E. coli in which C. diphtheriae RNA was translated into proteins;

2. explore the parameters that governed translation of C. diphtheriae RNA in this system; and
3. focus on detecting the cell-free synthesis of a specific protein, namely diphtheria toxin by three methods:

a. Immunologically

by specific precipitation of toxin or toxin fragments with horse and rabbit antitoxins.

b. Enzymatically

by ADP-ribosylation of mammalian peptide elongation factor 2 by Fragment A of diphtheria toxin in the presence of NAD^+.

c. In vivo toxicity

by demonstrating localized skin necrosis following the intradermal injection of toxin into a susceptible animal.

The causative agent of the disease diphtheria, from the Greek word diphthera meaning "skin or membrane", was described by Klebs in 1883 in stained smears made from diphtheritic membranes. The organism was isolated in pure culture by Loeffler in 1884 from cases of diphtheria, and he produced experimental diphtheria infection closely simulating the human disease in guinea pigs. Loeffler found that the tissues of the guinea pigs fatally infected with Corynebacterium diphtheriae did not harbor the bacilli except at the site of inoculation. Careful study of other tissues showed damage in the heart, lungs, kidneys and adrenal glands. This fact forced Loeffler to conclude that the bacilli must have produced a soluble poison which was transported to the other tissues of the body by the blood. In 1888, Roux and Yersin showed that cell-free filtrate of the diphtheria cultures could be lethal to animals and that the symptomatic manifestation of diphtheria intoxication by infection with the causative organism C. diphtheriae could be produced by injection of the culture filtrate. The powerful exotoxin is a single antigenic type which is elaborated by certain strains of C. diphtheriae. The toxin specifically inhibits protein synthesis in mammalian cells. The mechanism of intoxication of mammalian cells has been explained at the molecular level.

Diphtheria toxin and its mode of action

The product of the gene tox^+ which is carried by toxinogenic corynebacteriophages has been shown to be a single polypeptide, MW

about 62,000 daltons (Collier and Kandel, 1971; Gill and Dinius, 1971). It is readily isolated from supernatants of toxinogenic C. diphtheriae cultures grown in complex, low-iron media (Kato, Nakamura, Uchida, and Katsura, 1960; Pope and Stevens, 1958; Iskierko, 1965; Relyveld and Raynaud, 1968). The amino acid composition and the end groups were recently reported by Michel, Zanen, Monier, Crispeeles and Kirkx (1972) who estimated total residues of 597 amino acids with glycine in the amino terminal and arginine in the carboxy terminal. Diphtheria toxin is lethal for man and susceptible animals in doses of 130 ng/kg body weight. When injected intradermally into sensitive unimmunized animals such as rabbits, 2×10^8 molecules of toxin are sufficient to cause skin necrosis (Barksdale, Garmise and Horibata, 1960). Diphtheria toxin is a powerful antigen but because of its extreme toxicity, it must be completely inactivated by formalin treatment before immunization of man or animals (Wilson, 1967).

Lennox and Kaplan (1957) reported that animal tissue cultures were sensitive to diphtheria toxin. They observed the destruction of the cell monolayer following cytopathic changes in the cells. The earliest effect of toxin, however, was found to be the inhibition of protein synthesis. A decrease in incorporation of radioactive amino acids in intoxicated HeLa cells was reported by Strauss and Hendee (1959). Aerobic respiration, oxidative phosphorylation and nucleic acid synthesis were not affected (Strauss, 1960). Collier and Pappenheimer (1964) working with cell-free protein synthesizing

system derived from HeLa cells, concluded that NAD^+ was required for inhibition of protein synthesis by diphtheria toxin. Collier (1967) demonstrated that one of the soluble factors required for protein synthesis was specifically inactivated by toxin. He subsequently identified this factor as the elongation factor 2 (EF2) enzyme, a soluble enzyme required for the GTP-dependent translocation of peptidyl-transfer RNA from the "acceptor" to the "donor" site of mammalian ribosomes (Collier and Traugh, 1969; Lucas-Lenard and Lipmann, 1971).

The requirement for NAD^+ in inactivation of EF2 enzyme was confirmed by others (Goor and Pappenheimer, 1967). The role of NAD^+ and toxin in the inactivation of EF2 enzyme was elegantly shown by using radioactive labeled NAD^+. In a series of experiments, various radioactive NAD^+ preparations labeled in adenine, adenosine, both phosphates, ribose and nicotanamide were incubated with EF2 enzyme and toxin. The acid insoluble fraction, i.e. EF2 enzyme, bound the radioactive labeled NAD^+ in every instance except when nicotinamide only was labeled. A stoichiometic quantity of nicotinamide was released during the reaction. The results indicated that the adenosine diphosphoribose moiety of NAD^+ was transferred to the acid insoluble material and the toxin acted as an enzyme in this reaction (Honjo, Nishizuka, Hayaishi and Kato, 1968; Honjo, Nishizuka and Hayaishi, 1969). This transfer reaction was found concommitant with the loss of activity of the EF2 enzyme (Raeburn, Goor, Schneider and Maxwell, 1968). However, it was

shown also that inactivation of EF2 enzyme could be prevented or even reversed in the presence of nicotinamide which suggested that NAD^+ functioned as a cofactor (Goor and Maxwell, 1969). In experiments with rat liver EF2 enzyme, Honjo, Nishizuka, Kato and Hayaishi (1971) demonstrated that the ADP-ribosylation of EF2 enzyme by the toxin is effective at pH 8.5 and that the reverse reaction takes place at pH 5.2.

$$EF2 + NAD^+ \rightleftharpoons \text{toxin} \rightleftharpoons \text{ADP-ribosyl-EF2} + \text{nicotinamide} + H^+$$

These workers found that no other protein except the EF2 enzyme could act as an acceptor in this reaction. The linkage of ADP-ribosyl EF2 was reported to be covalent and stable.

Studies on the relationship between the structure of diphtheria toxin and its mode of action, its enzymatic activity and its toxicity, were concommitantly reported by two groups (Collier and Kandel, 1971; Drazin, Kandel and Collier, 1971; Gill and Pappenheimer, 1971; Gill and Dinius, 1971). These workers reported that the toxin contained two disulfide bridges. Reduction of the disulfide bridges by a thiol reducing agent such as glutathione or dithiothreitol was necessary for the ADP-ribosylation activity. In absence of reducing agents, diphtheria toxin was found enzymatically inactive. Collier and Kandel (1971) showed that upon thiol treatment, some molecules of purified diphtheria toxin separated into two peptide fragments, the A fragment (MW 24,000 daltons) and the B fragment (MW 38,000 daltons) and some toxin molecules remained as intact toxin of MW

62,000 daltons. Furthermore, the intact diphtheria toxin remaining after this treatment, when treated with trypsin in presence of thiols, cleaved into two major polypeptides which were identical with the spontaneously cleaved Fragments A and B of the toxin. These results indicated that the toxin was preferentially cleaved at a specific point. Neither Fragments A nor B were toxic to HeLa cells or guinea pigs. Fragment A, which was found to be stable in acid, alkali and heat, also contained the thiol-dependent ADP-ribosylation activity. It has since been shown that the A fragment contains the amino terminal residue of the toxin (Michel et al., 1972). Fragment B, which was heat labile and aggregated at neutral pH was proposed to be necessary for attachment of the toxin to the cell membrane before the A fragment could enter the cell.

Supporting evidence for the role of B fragment was obtained by the study of altered toxins (CRM proteins) produced by cells lysogenized with mutant β phages. Uchida, Gill and Pappenheimer (1971) presented evidence to show that CRM45 protein which was produced by C_7 cells lysogenized with a mutant β phage, had the ADP-ribosylation activity of the A fragment of the toxin, was devoid of part of the B fragment and was nontoxic to mammalian cells. Furthermore, Uchida, Pappeheimer and Harper (1972) demonstrated that two nontoxic CRM proteins could form hybrid toxins i.e., Fragments A and B held together by a disulfide bond would intoxicate animals. Two CRM proteins, CRM45, which had a functional A fragment but was missing part of B fragment, and CRM197, which had a nonfunctional A fragment

but intact B fragment were mildly treated with trypsin and thiol. Then, the thiol was removed so that the nicked molecules could randomly interact and form disulfide bonds. The hybrid toxin thus formed was lethal to guinea pigs and HeLa cells, although none of the original proteins were toxic. Cukor, Solotorovsky and Kuchler (1973) have reported that isolated B fragment is able to delay the action of toxin on sensitive KB cell cultures, probably by competing with the toxin for the cell membrane. Because intoxicated animals were shown to have reduced protein synthesis in their tissues and high levels of ADP-ribosyl-EF2 enzyme, the lethal effect of diphtheria toxin has been suggested to be solely due to the inhibition of protein synthesis (Gill, Pappenheimer and Baseman, 1969; Baseman, Pappenheimer and Gill, 1970; Pappenheimer and Gill, 1973; Gill, Pappenheimer and Uchida, 1973).

Studies of the intoxicated cultured mammalian cells showed that susceptibility to toxin depended on the animal origin of the cell. Primary kidney cell cultures derived from man, rabbit, dog, pigeon, and monkey were found quite susceptible, those of mouse and rat were relatively resistant (Solotorovsky and Gabliks, 1965). The effect on established cell lines depended on the animal species and to some extent on the tissue from which the cells were derived. Thus, KB cells (human pharyngeal carcinoma) were quite susceptible. HeLa cells (human cervical carcinoma) were found 10 times less susceptible, HEp-2 cells (human laryngeal carcinoma) 100 times less susceptible. Mouse L-cells were found to be 100,000 times more

resistant than KB cells (Gabliks and Solotorovsky, 1962).

The cell membrane determines whether an animal or a given cell culture becomes intoxicated following treatment with a given concentration of diphtheria toxin. Even though the rat is a relatively resistant animal, Honjo et al. (1971) demonstrated that the cell-free protein synthesizing system from its liver is susceptible to Fragment A enzymatic activity. Also, the cell-free protein synthesizing system derived from mouse L-cells was found quite sensitive to inhibition by Fragment A of diphtheria toxin although mouse L-cells are not susceptible to toxin (Goor, Pappenheimer, and Ames, 1967). When resistant L-cells were treated with poly L-ornithine to facilitate the uptake of extracellular material by the cells, they were found sensitive to inhibition of protein synthesis by the toxin. It was concluded, therefore, that the barrier to the action of diphtheria toxin was the cell membrane, which did not allow toxin to enter the cell (Moehring and Moehring, 1968). Specific receptor sites on the membrane were postulated for diphtheria toxin which could be saturated with whole toxin, Fragment B or CRM protein having a functional Fragment B (Cukor, Solotorovsky and Kuchler, 1973; Uchida, Pappenheimer and Harper, 1973; Gill, Pappenheimer and Uchida, 1973). Whether only Fragment A enters the cell is not known but activation of the toxin at the cell membrane was reported to be necessary for subsequent inhibition of protein synthesis (Moehring and Moehring, 1972a and 1972b).

Virulence, toxinogeny and lysogeny in C. diphtheriae

Since toxin is not produced by all strains of C. diphtheriae, nontoxinogenic strains were ignored for a long time and only toxinogenic strains were studied. A classification of C. diphtheriae on the basis of colonial morphology and the severity of disease produced by toxinogenic strains was proposed by McLeod (1943). On the basis of growth on tellurite blood agar and the severity of the disease in the infected animal, three colonial "types", mitis, intermedius, and gravis were described. Toxinogenic strains with smooth colonies were generally from mild infection (mitis). The strains with semi-rough colonies were isolated from severe cases (gravis). The smooth-small colonies were from infections with intermediate severity (intermedius). However, establishment of infection by toxinogenic strains does not depend on the elaboration of toxin only, but also on the surface protein antigens of the bacterial cells. The K antigens are responsible for the particular colonial morphology and also the pseudomembrane formation in the throat and, therefore, the virulence of C. diphtheriae (Lautrop, 1950). Contribution of cell wall components to virulence became especially evident when the cause of toxinogeny in C. diphtheriae was discovered.

Freeman in 1951 discovered that all toxinogenic strains of C. diphtheriae were also lysogenic. Furthermore, he demonstrated that upon infection of nontoxinogenic cells with certain bacteriophages, which he called β corynebacteriophages, the nontoxinogenic

C. diphtheriae were converted to toxinogenic strains. Freeman's observation brought attention to the study of nontoxinogenic strains and their virulent properties. A virulent nontoxinogenic strain can produce pseudomembrane in a test animal such as a rabbit without the elaboration of toxin and the animal recovers. The same strain upon lysogenization with β corynebacteriophage not only produces the pseudomembrane in the rabbit, but also toxin and the animal dies. The separation of invasiveness from toxinogeny is consistent with the reported cases of diphtheria infection i.e., pseudomembrane formation by nontoxinogenic diphtheria bacillus, as well as those reports of diphtheria in individuals having circulating antitoxin (Barksdale, Garmise and Horibata, 1960; Barksdale, 1970).

Soon after Freeman's discovery and the isolation of the temperate bacteriophages carrying the gene tox^+, the study of pairs of toxinogenic and nontoxinogenic C. diphtheriae became a possibility. Barksdale and Pappenheimer (1954) showed that nontoxinogenic C. diphtheriae strains $C_4(-)$ and $C_7(-)$ could be converted to toxinogenic strains upon lysogenization with the β tox^+ corynebacteriophage. They also demonstrated that the lysogenic cells were sensitive to induction of the prophage by ultraviolet irradiation which resulted in release of the progeny phage and toxin. Groman (1955) also confirmed Freeman's results and established that the bacteriophage conversion of nontoxinogenic C. diphtheriae to toxinogeny by β phage was an inherent and irreputable property of every plaque-forming particle. Furthermore, he showed that not all corynebacteriophages

were able to endow the ability to produce diphtheria toxin on the host bacterium.

Some genetic studies on the toxinogenic and nontoxinogenic corynebacteriophages have been reported. Holmes and Barksdale (1969) demonstrated genetic recombination in β tox^+ bacteriophages. The $\underline{tox}^+$ genetic marker which caused the elaboration of diphtheria toxin by the bacteriophage-infected cell was inherited as a single unit among the β bacteriophage genetic markers. They also showed that the $\underline{tox}^+$ marker could be carried by corynebacteriophages which failed to genetically recombine (Holmes and Barksdale, 1970). This indicated that the $\underline{tox}^+$ gene could also be carried by genetically unrelated phages. Analysis of genetic material of β phage, which has been most extensively studied, has indicated that the β phage has a double-stranded DNA genome of about 2.2×10^7 daltons (Uchida, Pappenheimer and Greany, 1973). The phage, therefore, can code for 20-25 proteins of about 150 amino acids each.

Definite evidence that the genetic information for the elaboration of toxin by $\underline{C.\ diphtheriae}$ resides in the bacteriophage genome was presented by Uchida, Gill and Pappenheimer (1971). They succeeded in isolating a mutant of β bacteriophage which when it lysogenized $C_7(-)$ cells, the infected cells elaborated an altered toxin molecule. Briefly, their approach was to induce $C_7(\beta)$ cultures by ultraviolet irradiation. The induced cells were then treated with the chemical mutagen N-methyl-N-nitroso-N'-nitroguanidine. The phages released by the mutagen-treated cells were isolated and used to lysogenize

C_7(-) cells. The new lysogenic cells were then screened for their ability to produce diphtheria toxin. The extracellular proteins elaborated by the lysogenic cells were collected and tested by the intradermal injection of rabbit for presence of diphtheria toxin. One of the new lysogenes produced an extracellular protein which was nontoxic, had a smaller molecular weight than the toxin and immunologically cross-reacted with the antitoxin. Since the cross-reacting material (CRM45 with a MW 45,000 daltons as opposed to the toxin which is 62,000 (Gill and Dinius, 1971)) was produced by mutation in the bacteriophage genome, the results indicated that the genetic information for the toxin is carried by the bacteriophage DNA. Other phage mutants affecting toxin structural gene have been reported (Uchida, Pappenheimer and Greany, 1973; Matsuda, Kanei and Yoneda, 1972). The CRM proteins isolated from the culture supernatants had very little or no toxicity for animals and tissue culture cells but had the antigenic properties of the toxin.

Although the genetic information for the toxin is carried by the phage, control of synthesis of toxin seems to be different from that of other phage proteins. Synthesis of diphtheria toxin can occur without bacteriophage production; that is, prophage induction is not necessary for toxin synthesis. Miller, Pappenheimer and Doolittle (1966) reported that toxinogenic C_7 (β) cells in absence of phage synthesis elaborated toxin. The same conclusion had been drawn by Hatano (1956) who showed that very few bacteriophages were released by spontaneous lysis of the cells under conditions where

toxin synthesis by C_7 (β) cells was maximum. Toxin synthesis, however, was reported to increase following ultraviolet irradiation and induction of the prophage in C_7 (β) cells (Barksdale, Garmise and Horibata, 1960; Barksdale, Garmise and Rivera, 1961). Whether this increase in toxin synthesis is a consequence of replication of the bacteriophage genome which would result in an increase in the effective number of genes coding for the toxin is not known.

Integration of the prophage genome into the host DNA is not necessary for the production of toxin by the cells since toxin can also be produced by an obligatory lytic β phage. Matsuda and Barksdale (1966, 1967) studied synthesis of toxin and phage during the infection of C_7(-) cells with an obligatory lytic mutant of β phage (βhv_{64} tox^+). The mutant was obtained from a stock of clear plaque corynebacteriophage β. The virulent phage was used to observe toxin synthesis during a single cycle of viral growth. C_7(-) cells were synchronized in the presence of chloramphenicol for 20 min. Immediately after the removal of the inhibitor, intracellular toxin could be detected. Extracellular toxin began to increase 34 min after phage absorption had begun. Free phage was not detected until 85 min after the onset of phage absorption. The toxin synthesized during this lytic infection was antigenically and biologically identical with the toxin obtained from the lysogenic C. diphtheriae.

There is evidence that toxin can also be synthesized when the phage genome is carried as a non-replicating exogenote. Gill,

Uchida and Singer (1972) demonstrated that when lysogenic cells of C_7 (β 45) which produce CRM45 protein were superinfected with β *ch'* *tox*$^+$ phage (Holmes and Barksdale, 1969), both toxin and CRM45 were produced. The β *ch'* phage cannot maintain lysogeny and is thought to make no functional repressor, although it does respect the immunity conferred by *c*$^+$ marker carried by β 45 lysogens. The *h'* mutants can infect lysogenic cells such as C_7 (β) and C_7 (β 45). Following infection of C_7 (β 45) cells with β *ch'* phage, the cells were maintained in exponential growth for 5 generations. After each generation the extracellular proteins were tested for toxicity and analyzed on polyacrylamide gels. The results showed that while toxin and CRM45 were initially present in equal amounts in the supernatant, toxin production fell approximately by a factor of 2 for each doubling of the cell concentration suggesting that the toxin genes in the β *ch'* phage DNA were not replicated but were diluted out. Furthermore, analysis of the phage production during this experiment indicated that less than 5×10^6 cells/ml in a cell concentration of 3×10^9 cells/ml underwent spontaneous lysis. Therefore, release of toxin and CRM45 were not entirely due to lytic induction.

The cellular site of synthesis of diphtheria toxin in *C. diphtheriae* is probably the cell membrane. Uchida and Yoneda (1967) investigated antitoxin specific proteins associated with different cellular components of the PW8 strain. Following radioactive labeling of cellular proteins, the fraction of the cell components containing labeled polypeptides which specifically precipitated with antitoxin

was identified as the cell membrane. These authors concluded that the site of toxin synthesis must be closely associated with the cell membrane or the synthesis may actually take place on membrane-bound ribosomes.

From the evidence reviewed above, it seems that diphtheria toxin can be synthesized during the prophage stage, during the lytic cycle of phage replication or when the phage genome is present as a non-replicating exogenote. Expression of the tox gene is thus under separate control from that which regulates the genes concerned with phage replication. What role toxin plays in the life cycle of the phage is not well understood. Elwell and Iglewski (1972) presented evidence for presence of toxin B fragment in purified preparations of β phage. Whether this association is incidental or the B fragment is a structural protein of the phage has not been resolved.

Besides diphtheria toxin a number of other microbial toxins appear to be bacteriophage induced. Streptococcal erythrogenic toxin, staphylococcal α hemolysin, staphylococcal enterotoxin A and several types of botulinum toxins are induced by specific bacteriophages (Zabriskie, 1970).

Corynebacterium diphtheriae PW8

Park-Williams-8 first isolated by Park and Williams is a remarkable organism which deserves a special mention. The PW8 strain carries a tox^+ prophage P. It was isolated in 1896 from a diphtheric patient. This slow-growing strain is unusual in that it yields

10-20 times more toxin than the average diphtheria bacillus in low iron medium and is used throughout the world for the production of diphtheria toxin, toxoid and antitoxin. Because of its inability to form plaques on $C_7(-)$ indicator strain, the prophage P at one time was thought to be defective (Barksdale, Garmise and Horibata, 1960; Barksdale, Garmise and Rivera, 1961; Matsuda and Barksdale, 1966, 1967). Maximescu in 1968 found that PW8 strain would lyse spontaneously and liberate phage. The free phage could be detected if certain Corynebacterium diphtheriae var. ulcerans were used as indicator strains. The lysogenic conversion of C. ulcerans was also demonstrated. The resulting lysogenic C. ulcerans produced diphtheria toxin indistinguishable from the toxin produced by the PW8 strain. Lampidis and Barksdale (1971) showed that P phage was restricted in $C_7(-)$ cells but not in a strain of C. ulcerans. They studied a number of PW8 strains which had been maintained in the United States, England, Holland, Japan and Czechoslovakia. All the strains cultivated in widely separated laboratories were similar in colonial morphology and fermentation patterns which were characteristic of the mitis strain. All were lysogenic and produced large amounts of toxin indicating that in PW8 the prophage and its host have remained stable since first being isolated in 1896.

Influence of iron on growth and toxin production by C. diphtheriae

Locke and Main (1931) and Pope (1932) reported that presence of iron in large concentrations in the growth medium had an inhibitory

effect on toxin production by C. diphtheriae PW8 strain. Pappenheimer and Johnson (1936) confirmed the inhibitory effect of iron on toxin production using defined media. Mueller (1940), however, reported that iron was one of the necessary metals for maximum growth of C. diphtheriae. Barksdale, Garmise and Horibata (1960) reported that no toxin was synthesized by PW8 cells in the presence of 0.75 µg iron per ml. They also reported that inhibition of toxin production started at iron levels of 0.075 µg/ml. Edwards and Seamer (1960) showed that both ferrous and ferric iron were taken up by the PW8 cells, but ferrous iron appeared to inhibit toxin production much earlier in the growth period than the ferric iron. They suggested that perhaps conversion of ferric iron to ferrous was necessary before iron was inhibitory. Righelato and Van Hemert (1969) studying the growth and toxin production by PW8 strain in the steady state chemostat, reported that under iron starvation condition (6 µg atoms iron/liter) growth still occurred but that the level of iron-containing enzymes decreased. Under iron starvation condition, toxin production was maximum (0.3 g toxin per gram bacterial protein synthesized). They observed that when iron was added to the iron deficient culture at concentration of 90 µg atoms/liter, the synthesis of toxin stopped immediately.

Hatano (1956) studied the effect of iron on toxin synthesis and the spontaneous release of phage by C_7 (β) cells. He found that in the presence of 70 µg iron/ml toxin synthesis was one-tenth of the amount produced in 0.1 µg iron/ml, but complete shutdown of toxin

synthesis in high iron medium in these cells never occurred. He did not find any change in the number of phage particles spontaneously released in the high iron and low iron cultures. Similar results were reported by Matsuda and Barksdale (1966, 1967) who studied toxin synthesis during the one step growth cycle of βhv_{64} tox^+ phage in $C_7(-)$ cells. Ten times more toxin was produced in deferrated culture than in cultures containing 3 µg iron/ml. No significant difference in the number of phage particles produced in the two cultures was observed.

The mechanisms of inhibition of toxin synthesis in presence of iron is unknown. The decrease in the level of iron containing enzymes reported by Righelato and Van Hemert (1969) may also reflect a decrease in an iron containing regulatory protein which is normally elaborated by the cells. The regulatory protein may be present in high iron cells and cause partial or complete shutdown of toxin synthesis at the level of transcription or translation of RNA. In low iron cells, the level of synthesis of this regulatory protein may decrease enough for toxin synthesis to occur. An iron-containing protein having catalase activity was isolated from PW8 cells growing in high iron medium by Sato and Kato (1965, 1967). Using a cell-free protein synthesizing system from C. diphtheriae they claimed that the inhibitory protein specifically prevented toxin mRNA translation in vitro by interacting with the mRNA.

While the inhibitory effect of iron remains to be understood, still other factors such as host strain variation can influence rate

and duration of toxin synthesis. Matsuda, Kanei and Yoneda (1971) observed that C. diphtheriae var. ulcerans lysogenized with the phage isolated from PW8 cells does not produce the same amount of toxin as the PW8, indicating a host specificity in this instance. Furthermore, a strain of C_7 (β) treated with N-methyl-N'-nitro-N-nitrosoguanidine has been isolated which does not produce the same quantity of toxin as a normal C_7 (β) cell, indicating that mutation in the host bacteria can affect the amount of toxin synthesis (Uchida, Pappenheimer and Greany, 1973). Thus while toxin is produced only when the cells are infected with a tox^+ corynebacteriophage, there are factors in the host which affect the rate and duration of toxin synthesis by the Corynebacterium diphtheriae.

Cell-free protein synthesis with E. coli S-30

The cell-free protein synthesizing system from E. coli, first described by Nirenberg and Mathaei (1961), consists of a crude DNase treated extract of E. coli, freed of cells and cell debris. The S-30 refers to the supernatant which remains after the cells and the cell debris have been centrifuged at 30,000 x g for 30 min. It contains the components necessary for polypeptide synthesis (ribosomes; tRNA, amino acid activating enzymes; initiation, elongation and termination factors, etc) plus many other unidentified materials influencing amino acid incorporation. This extract, when supplemented with amino acids ATP, GTP, an ATP generating

system, plus adequate amounts of Mg^{++}, NH_4^+ and K^+ ions, incorporates amino acids into polypeptides directed by endogenous mRNA or exogenous natural or synthetic mRNA.

The S-30 system has the advantage of being more active and is more stable than most defined systems for protein synthesis (Goldman and Lodish, 1972). It uses the same initiation, elongation and termination mechanisms which work in vivo when natural mRNA is being translated. Recently Modolell (1971) has described the technical details of the growth of the E. coli cells and the preparation of an active S-30. The in vitro protein synthesizing system described by Modolell is similar to that originally described by Nirenberg and Mathaei (1961).

Fidelity of translation of RNA in the E. coli cell-free lysate can be tested by several methods, depending on the properties of protein being synthesized in vitro. The genome of RNA bacteriophages for instance were shown to be translated in the E. coli cell-free lysate into the phage coat and maturation proteins (Nathans, Notani, Schwartz and Zinder, 1962; Nathans, 1965; Lodish and Robertson, 1969). The products were identified by two-dimensional chromatography. Other viral RNAs have been translated with fidelity and the products identified. Van Ravenswaay, Claasen, Van Leeuwen, Duijts and Bosch (1961) translated RNA from alfalfa mosaic virus RNA and turnip yellow mosaic virus RNA; identifying the peptides by two-dimensional chromatography. Poliovirus RNA and avian myeloblastosis viral RNA have been translated in E. coli

cell-free systems (Rekosh, Lodish and Baltimore, 1970; Siegert, Konings, Bauer and Hofschneider, 1972). In addition, hemoglobin mRNA from rabbit reticulocytes was reported to direct the synthesis of rabbit globin in E. coli cell-free systems (Laycock and Hunt, 1969).

The ultimate test of translation fidelity is to show that a specific enzyme has been produced in vitro, since this requires not only near perfect translation of the mRNA into the protein, but also the subsequent correct folding of the polypeptide to give a three-dimensional structure. In vitro synthesis of a specific enzyme, namely lysozyme of T4 phage, was first reported by Salser, Gesteland and Bolle (1967). They showed that mRNA extracted from T4 phage-infected E. coli could direct the synthesis of biologically active T4 specific lysozyme (Salser, Gesteland and Bolle, 1967; Salser, Gesteland and Ricard, 1969). Subsequently, synthesis of another enzyme from the same phage-infected cells, glycosyl transferase, was reported (Young, 1970; Young and Van Houwe, 1970). The in vitro detection of these two enzymes has provided valuable information about production and control of T4 phage specific messenger RNA in the infected cells.

DNA-dependent in vitro protein synthesis was developed by Lederman and Zubay in 1967. In this system, the S-30 was not treated with DNase. Protein synthesis was primed with DNA in the presence of ATP, GTP, UTP and CTP for RNA synthesis. RNA was coupled to its translation.

The DNA-dependent synthesis of β galactosidase and the control of the synthesis of this enzyme was studied in vitro (Zubay and Lederman, 1969). The advantage of this coupled system was that it allowed the use of predetermined levels of β galactosidase repressor, DNA and β galactosidase inducer.

The DNA-dependent synthesis of diphtheria toxin and CRM45 protein was reported by Murphy and Pappenheimer (1972). The β phage DNA and β_{45} phage DNA were used to prime the E. coli in vitro system. In their experiment toxin synthesis directed by β phage DNA was measured by rabbit skin test only. Fragment A activity was reported to be present when CRM45 phage DNA primed the system. This activity was not found in the systems primed with β phage DNA.

The thesis objective was to use the RNA-dependent E. coli cell-free protein synthesizing system to translate RNA from C. diphtheriae into proteins. The RNA was obtained from toxinogenic, lysogenic PW8 strain, C_7(-) strain infected with a virulent β tox^+ corynebacteriophage and nontoxinogenic, nonlysogenic C_7(-) strain. Three methods were used to determine if synthesis of diphtheria toxin or toxin fragment by translation of toxin mRNA had occurred. They were precipitation of toxin and toxin fragment with specific antitoxin, detection of synthesized Fragment A of diphtheria toxin by its ADP-ribosylation activity of mammalian peptide elongation factor 2 enzyme and in vivo toxicity tests in rabbits to detect biologically active diphtheria toxin.

MATERIALS AND METHODS

Bacterial strains

1. *Corynebacterium diphtheriae*. Nontoxinogenic, nonlysogenic *C. diphtheriae* $C_7s(-)$ tox^- (Barksdale and Pappenheimer, 1954), hereafter referred to as $C_7(-)$, was obtained from Dr. L. Barksdale of New York University, School of Medicine.

2. *C. diphtheriae* Park-Williams No. 8. This strain, a high toxin-producing strain lysogenic with a phage P related to β corynebacteriophages (Barksdale, Garmise and Rivera, 1961; Lampidis and Barksdale, 1971) was obtained from Dr. N. B. Groman of the University of Washington School of Medicine.

3. *E. coli*. Strain Q_{13} deficient in RNase I and polynucleotide phosphorylase (Cammack and Wade, 1965) was provided by Dr. W. J. Iglewski of the University of Oregon Medical School.

Corynebacteriophages

Corynebacteriophage βhv_{64} tox^+ (Matsuda and Barksdale, 1966) hereafter referred to as βv tox^+ is an obligatory lytic bacteriophage, and directs synthesis of toxin upon infection of its host strain $C_7(-)$. βv tox^+ was a gift from Dr. L. Barksdale of New York University School of Medicine.

Media

1. Heart infusion broth (Groman and Lockart, 1953). The broth was prepared by dissolving 37 g dry powder (Difco) per liter of distilled water and sterilizing it in an autoclave for 15 min at 121

C and 15 lb pressure.

2. Heart infusion agar (Groman and Lockart, 1953). Forty g of the dry powder (Difco) was dissolved in 1 liter of distilled water and sterilized as above. The medium was dispensed in sterile petri dishes.

3. MS broth (Davis and Sinsheimer, 1963). Ten g tryptone (Difco), 1 g yeast extract (Difco) and 8 g NaCl were dissolved in 1 liter of distilled water and sterilized as before. Just prior to use, 2 ml sterile 50% v/w glucose and 2 ml sterile 1 M $CaCl_2$ were added to 1 liter of broth.

4. C-Y medium (Mueller and Miller, 1941; Pappenheimer, Uchida and Avery-Harper, 1972). Ten g casamino acids (Difco), 20 g yeast extract (Difco) and 5 g anhydrous KH_2PO_4 were dissolved in 300 ml distilled deionized water. After addition of 2 ml 50% v/w $CaCl_2 \cdot 2H_2O$, the pH was adjusted to 7.4 with 40% v/w NaOH and the solution was brought to boil and filtered. Two ml solution II and 1 ml solution III and 100 mg tryptophan (Matheson, Coleman and Bell) were added. The volume was adjusted to 1 liter with distilled deionized water and the pH was brought back to 7.4. The medium was sterilized as above. Twenty-five ml 50% v/w deferrated maltose (Difco) was added before use.

5. PGT medium (Mueller and Miller, 1941; Barksdale and Pappenheimer, 1954). Forty g casamino acids (Difco) was dissolved in 300 ml distilled deionized water. Five ml 10% v/w solution of $CaCl_2 \cdot 2H_2O$ was added and the pH was adjusted to 7.2 with 40% v/w

NaOH. The solution was brought to boil and filtered. The pH was adjusted to 7.2, 3 ml $CaCl_2 \cdot 2H_2O$ solution was added, and the solution was heated and filtered as before. This was repeated a third time. Then 5 g Na_2HPO_4 and 1.7 g KH_2PO_4 were added followed by 0.5 mg calcium pantothenate, 100 mg tryptophan and 0.5 glutamic acid (the last three supplements from Matheson, Coleman and Bell). Two ml solution II and 1 ml solution III were added. The volume was brought to 1 liter and the pH adjusted to 7.2. The medium was sterilized as above. Just before use, 25 ml 50% v/w deferrated maltose was added.

6. 50% v/w deferrated maltose (Matsuda and Barksdale, 1967). Crystalline maltose (Difco), 100 g, was dissolved in 200 ml distilled water. One g $CaCl_2 \cdot 2H_2O$ was added followed by 4 ml 10% v/w Na_2HPO_4 and the pH was adjusted to 7.4. The solution was heated in a boiling water bath for 3-4 min and filtered. The maltose solution was autoclaved for 10 min at 121 C and 15 lb pressure. It was added to the media just before culture.

7. Solution II (Mueller and Miller, 1941).

100 ml solution contained:

$MgSO_4 \cdot 7H_2O$		22.5 g
beta alanine		0.115 g (Matheson, Coleman & Bell)
nicotinic acid		0.115 g (Matheson, Coleman & Bell)
pimelic acid		0.0075 g(Matheson, Coleman & Bell)
$CuSO_4 \cdot 5H_2O$	1% solution	5.0 ml
$ZnSO_4 \cdot 7H_2O$	1% solution	4.0 ml
$MnCl_2 \cdot 4H_2O$	1% solution	1.5 ml

Conc HCl 3.0 ml

Distilled deionized water to volume.

8. Solution III (Mueller and Miller, 1941).

100 ml solution contained:

cystine 20.0 g (Matheson, Coleman & Bell)

conc HCl 20.0 ml (Matheson, Coleman & Bell)

distilled deionized water to volume.

Buffers

1. Standard buffer (Modolell, 1971).

10 mM Tris-HCl, pH 7.8 (Tris (hydroxymethyl) aminomethane-hydrochloride, Sigma)

60 mM NH_4Cl

10 mM Mg $(C_2H_3O_2)_2 \cdot 4H_2O$ (magnesium acetate)

6 mM 2-mercaptoethanol (Matheson, Coleman & Bell)

The standard buffer was made from a stock solution of 600 mM NH_4Cl and 100 mM Tris-HCl pH 7.8 which was diluted 10 times and supplemented with stock solution of 1 M magnesium acetate. Just prior to use, 2-mercaptoethanol was added.

2. Borate buffers (Campbell, Garvey, Cremer and Sussdorf, 1963). 0.08 M borate buffer pH 8.4 was prepared by dissolving 6.184 g boric acid, 9.636 g sodium tetraborate and 4.384 g NaCl in 1 liter of distilled deionized water. Borate-saline buffer was borate buffer diluted 1:20 with 0.85% v/w solution NaCl.

3. Sodium chloride, Tris, EDTA buffer (STE) (Franklin, 1966).

0.1 M NaCl

0.001 M EDTA (ethylenediaminetetraacetic acid, Matheson, Coleman & Bell)

0.05 M Tris-HCl pH 7.2

4. Sodium chloride, Tris buffer (ST) (Franklin, 1966).

0.1 M NaCl

0.05 M Tris-HCl pH 7.2

Growth of C. diphtheriae

1. C_7(-) cells. C_7(-) cells were grown as follows: A culture of C_7(-) kept at -20 C on heart infusion agar slant was transferred onto a fresh heart infusion agar slant and incubated overnight at 37 C. An overnight culture of C_7(-) cells was made from the slant culture in PGT broth containing 2% deferrated maltose and incubated at 37 C with aeration. One liter of PGT in a 6-liter Erlenmeyer flask containing 25 ml of 50% v/w deferrated maltose was inoculated with 10 ml of the overnight broth culture. The cells were incubated in a water bath at 37 C with vigorous shaking for maximum aeration and growth continued to cell concentration of 4×10^8 cells/ml. The culture was rapidly cooled in an ice bath, cells were separated by centrifugation at 10,000 x g in a GSA rotor using a Sorvall RC-2B centrifuge at 5 C and washed once with cold standard buffer. The packed cells were transferred into a small plastic bag and frozen on dry ice and stored at -70 C until RNA extraction. About 1 g cell was harvested from 1 liter of PGT.

2. $C_7\beta v$ tox$^+$ cells. Bacteriophage infected cells ($C_7\beta v$ tox$^+$) were prepared by growing C_7(-) cells as above to 2 x 10^8 cells/ml then βv tox$^+$ corynebacteriophage was added directly to the culture at a multiplicity of 4 plaque-forming units (PFU) per cell. After a 10 min adsorption period at 37 C, vigorous aeration was continued at 37 C for another 35 min at which time the culture was rapidly cooled, centrifuged, and the cells were washed and treated as C_7(-) cells. About 0.8 g $C_7\beta v$ tox$^+$ cells were harvested from 1 liter of PGT. When infection was allowed to proceed the cells started to lyse 60-70 min post-infection. Bacteriophage titer in such a culture was 1-2 x 10^{10} PFU/ml.

3. C. diphtheriae PW8. Stock cultures of C. diphtheriae PW8 were kept on heart infusion agar slants at -20 C. The culture was transferred onto a fresh heart infusion agar slant and incubated overnight at 37 C. An overnight broth culture was made from this slant culture in C-Y medium containing 1.25% deferrated maltose and incubated with maximum aeration at 37 C in a water bath. One liter of C-Y broth in a 6-liter Erlenmeyer flask containing 25 ml 50% v/w deferrated maltose was inoculated with 10 ml of the overnight broth culture. Growth continued at 37 C for 12 hr with maximum aeration. Cells were harvested, washed and stored as described for C_7(-) cells. About 2 g cells were harvested from 1 liter of culture. When growth was allowed to proceed, the cell mass continued to increase for another 6 hr.

Propagation of βv tox$^+$ corynebacteriophage (Matsuda and Barksdale, 1967)

Corynebacteriophage βv tox$^+$ was propagated in C_7(-) cells. PGT broth, 300 ml in a 2-liter Erlenmeyer flask, containing 2% deferrated maltose was inoculated with 3 ml of an overnight culture of C_7(-) in the same medium. The culture was incubated at 37 C with vigorous aeration. When the cell concentration had reached 2×10^8 cells per ml the culture was centrifuged and the medium was saved and kept warm. The cell pellet was suspended in 5 ml of the medium and well dispersed by repeated passage through a sterile needle and syringe. βv tox$^+$ bacteriophage was added to the cells at a multiplicity of 4 PFU/cell. After 10 min adsorption at 37 C, the cells were resuspended in the warm medium and incubated at 37 C for 50 min. Sterile sodium citrate solution, 12 ml of a stock solution of 51% v/w, was added to the culture and incubation continued for another 3 hr. The culture was then placed at 4 C overnight to complete cell lysis and phage release. Cells and cell debris were removed by centrifugation at 10,000 x g for 20 min. The phage suspension was titrated for plaque forming units (PFU) per ml.

Titration of corynebacteriophage βv tox$^+$ (Groman, 1955)

The indicator strain C_7(-) was grown overnight in heart infusion broth at 37 C with aeration. Heart infusion broth (20 ml) was inoculated with the overnight culture of C_7(-) and was incubated at 37 C with aeration. The culture was grown to a cell concentration of 5×10^8 cells/ml. Serial one to ten dilutions of the bacteriophage

suspension were made in heart infusion broth. The bacteriophage dilutions, 1 ml, were mixed with 0.15 ml of the indicator strain, 2.5 ml of the heart infusion agar overlay (1%) and layered on heart infusion agar in a petri dish. The agar overlay was allowed to harden at room temperature. The plates were incubated overnight at 34 C. βv tox^+ bacteriophage produced clear plaques which were counted using a Quebec colony counter. Usual titers of 2-4 x 10^{10} PFU/ml were obtained.

RNA extraction (Franklin, 1966)

C. diphtheriae cells (2-4 g) were ground in a pre-chilled mortar with an equal weight of washed alumina 503 (Sigma) for 5 min. STE buffer, 10 ml. containing 2% v/w sodium dodecylsulfate (SDS) (Sigma) was added followed by an equal volume of freshly distilled phenol washed with STE buffer. The mixture was transferred into a 60 ml extraction bottle fitted with a ground glass stopper. The bottle was attached to a Shaker-in-the-Round (Kraft) apparatus and shaken at full speed for 10 min at room temperature to extract the nucleic acids. The buffer phase was removed following centrifugation at 12,000 x g for 10 min in an SS-34 rotor in a Sorvall RC-2B centrifuge at 5 C. The interphase was also removed and put in about 4 ml STE buffer and extracted once more with an equal volume of washed phenol. The buffer phases were pooled and extracted two more times with phenol in the same manner. The residual phenol in the buffer phase was extracted with ether and the ether was evaporated

by bubbling nitrogen through the nucleic acid solution. NaCl to final concentration of 0.2 M was added and the nucleic acids were precipitated by addition of 2 vol absolute ethanol. After overnight at -20 C to complete the precipitation, the nucleic acids were concentrated by centrifugation and dissolved in 2 ml STE buffer. The nucleic acids were treated with 10 mg per ml DNase (electrophoretically purified, Worthington) at 37 C for 30 min in presence of 10 mM magnesium acetate. The RNA was precipitated in 0.2 M NaCl with 2 vol absolute ethanol. After overnight at -20 C the precipitated RNA was collected by centrifugation and dissolved in 2 ml distilled deionized water, aliquoted in 0.2 ml volumes and frozen at -70 C. Concentration of RNA was estimated by measuring the absorbency at 260 nm (A_{260}) of a suitable dilution in a Beckman spectrophotometer using 1 cm light path and the formula 25 A_{260} = 1 mg RNA (Young and Van Houwe, 1970). An aliquot of each RNA preparation was analyzed in a 5-20% v/w exponential sucrose gradient prepared in 5 ml STE. RNA (0.2 mg) was layered on the gradient and centrifuged at 204,000 x g for 3 hr in a SW50 rotor at 5 C using a Beckman L2-50 ultracentrifuge. The bottom of the tube was then punctured and 2-drop fractions were collected, diluted in 0.5 ml STE and the A_{260} of each fraction was determined.

Preparation of radioactive pulse-labeled RNA

Two 2-liter Erlenmeyer flasks, each containing 500 ml PGT broth plus 1.25% deferrated maltose were inoculated with 5 ml of an

overnight culture of C_7(-) cells in the same medium. The cultures were incubated in a water bath at 37 C with vigorous shaking for maximum aeration. Growth continued to cell concentration of 2×10^8 cells per ml. βv tox^+ corynebacteriophage was added at a multiplicity of 4 PFU/cell to one culture. After a 10 min adsorption period at 37 C vigorous aeration of both flasks was continued at 37 C for another 35 min. The cultures were pulse-labeled for 15 sec with tritiated uridine (Uridine 5-H^3, 25.6 C/mM, New England Nuclear) at a final concentration of 0.4 μ Ci per ml. The cultures were then poured over ice containing sodium azide and potassium cyanide to stop further incorporation of uridine. The final concentrations of the two inhibitors were 0.005 M and 0.01 M respectively. The cells were harvested and the RNA extracted as previously described. H^3-RNA was sedimented in 5-20% exponential sucrose gradient in STE under the same conditions as unlabeled RNA. Fractions were collected and diluted in 0.5 ml STE. The A_{260} of each fraction was determined. The RNA was precipitated by the addition of 0.5 ml 10% cold CCl_3COOH, to each fraction. Following 1 hr in an ice bath the precipitated RNA was collected on type HA filters (Millipore). The filters were washed three times with cold 5% CCl_3COOH, once with cold 95% ethanol and dried. They were then placed in glass scintillation vials and 10 ml Omnifluor (New England Nuclear)-tuoluene scintillation fluid was added (4 gm Omnifluor in 1 liter toluene). The vials were equilibrated in the dark overnight, and the radioactivity was determined using the H^3-window of a

Beckman LS-200B liquid scintillation counter. Background radiation was determined with a standard and subtracted automatically. No self-absorption was detected.

Preparation of S-30 extract from E. coli Q_{13} (Modolell, 1971)

E. coli Q_{13} was grown in 10 liter amounts in MS broth supplemented with 0.1% glucose and 2 mM $CaCl_2$ in 16-liter glass carboys. The medium was pre-warmed and then inoculated with 100 ml of an overnight culture of E. coli in MS broth. Growth at 37 C and under forced aeration (16 liter air per min) was continued until the cell concentration reached 4×10^8 cells per ml. The culture was then rapidly cooled in an ice bath. E. coli was harvested with the Szent-Gyorgyi and Blum continuous flow apparatus at 30,000 x g in the Sorvall RC2-B centrifuge at 5 C. The cells were washed once with cold standard buffer, placed in plastic bags and frozen on dry ice. The yield was about 1 g cell per liter of culture. All subsequent operations were carried out at 5 C. Four to 6 g cells were ground with washed alumina 503 in a pre-chilled mortar. The ground cells were extracted with 2 vol standard buffer and clarified by centrifugation at 20,000 x g for 15 min. The viscous supernatant was treated with 3 mg per ml DNase (electrophoretically purified, Worthington) for 5-10 min. The S-30 was obtained by centrifugation at 30,000 x g for 35 min, the supernatant was dialyzed against 500 vol standard buffer for 4 hr with 4 buffer changes. After dialysis the S-30 was clarified by centrifugation at 30,000 x g for 20 min.

It was immediately divided in 0.2 ml volumes, quick frozen in an acetone-dry ice bath and stored at -70 C. The amino acid incorporating activity of such preparations remained unchanged for several months. The A_{260} of a 1/500 dilution of each S-30 was measured and the approximate ribosomes plus ribosomal subunits were calculated by multiplying the A_{260} of the mixture by the factor 0.04 (Modolell, 1971).

In vitro protein synthesis (Modolell, 1971)

The composition of the reaction mixture for incorporation of amino acids with E. coli S-30 extract directed by C. diphtheriae RNA is shown in Table 1. Reaction mixtures of 50 to 500 µl volume in 2.0 ml glass conical tubes were routinely used. All the components except RNA were mixed at 0 C. The reaction was initiated by the addition of RNA and the incubation of the reaction mixture at 35 C. Samples (10 µl) were withdrawn from the reaction mixture, mixed with 1 ml 3% v/w casamino acids containing 0.075 mg/ml bovine serum albumin and 0.1 ml of 60% CCl_3COOH was added. The samples were then heated a 90 C for 15 min, cooled in an ice bath, filtered on type HA filters (Millipore), washed 3 times with 5 ml 5% CCl_3COOH, once with 5 ml 95% ethanol and dried. The filters were placed in glass scintillation vials, and 10 ml Omnifluor-toluene scintillation fluid was added. After overnight in the dark, the radioactivity of each sample was determined using the C^{14}-window of the Beckman LS-200B liquid scintillation counter. Background radiation was

Table 1

Components of the amino acid incorporating mixture directed by *C. diphtheriae* RNA

Tris-HCl pH 7.8 (Sigma)	50 mM
NH_4Cl	60 mM
$Mg(C_2H_3O_2)$	10 mM
ATP-Tris (Sigma)	1 mM
GTP (Sigma)	0.02 mM
Potassium phosphoenol pyruvate (Sigma)	5 mM
Pyruvate kinase (Sigma)	30 µg/ml
C^{14}-lysine (3.2 m Ci/mM, New England Nuclear)	4 µ Ci/ml
19 other C^{12}-amino acids (Mann)	0.05 mM
C. diphtheriae RNA	0.6-2 mg/ml
E. coli S-30 (10 mg ribosomes/ml)	0.2 vol

determined using a background standard and subtracted automatically. No self-absorption was detected. When unlabeled proteins were to be made in vitro, the amino acid composition was changed to 20 C^{12}-amino acids, and C^{14}-lysine was omitted from the reaction mixture.

Sucrose gradient analysis of the in vitro protein synthesizing mixture

Cell-free protein synthesizing mixtures were analyzed by centrifugation in 10-30% exponential sucrose gradients prepared in the standard buffer without 2-mercaptoethanol. Reaction mixtures of 100 µl volumes were incubated for 3 or 7 min at 35 C. The reaction mixtures were rapidly chilled and chloramphenical, 100 µl of a 0.1% w/v solution was added and gently mixed. Then 100 µl of the reaction mixture containing chloramphenicol was layered on the 5 ml sucrose gradient and centrifuged for 75 or 120 min at 165,000 x g at 5 C in the Beckman SW 50 rotor. The bottom of the tube was pierced and 2-drop fractions were collected. To a volume of 50 µl of each fraction 0.5 ml standard buffer without mercaptoethanol was added and the A_{260} was determined. Another 50 µl of each fraction was precipitated with CCl_3COOH and the radioactivity of the H^3-RNA or C^{14}-polypeptides in the fraction was determined.

Purification of horse antitoxin

Lyophilized horse diphtheria flocculating antitoxin (Lot No. DP 1252 from Wellcome Research Laboratories, Beckenham, England) was dissolved in water to a final concentration of 100 mg protein/ml. The antitoxin was tested for purity by double diffusion in agar gel

(Campbell, Garvey, Cremer and Sussdorf, 1963) against C_7(-) cell extract, crude diphtheria toxin (2.2 mg/ml, courtesy of Dr. R. Y. Gottshall, State of Michigan, Department of Public Health) and electrophoretically pure diphtheria toxin (1.2 mg/ml, a gift of Dr. R. J. Collier, University of California, Los Angeles). Gel diffusion showed that this horse antitoxin contained antibodies against C. diphtheriae C_7(-) cell extract. Furthermore, three lines of precipitation were observed against the crude toxin, one of which showed identity with the C_7(-) extract. The pure diphtheria toxin showed only one line of precipitation. The horse antitoxin was absorbed with C_7(-) cells and also E. coli Q_{13} to eliminate antibodies against these two cell extracts. E. coli Q_{13} and C_7(-) cells were grown in heart infusion broth overnight. Ten g of each cell preparation was ground with alumina. Following extraction with the borate-saline buffer and removal of alumina by centrifugation, the unbroken cells and cell extract were lyophilized. Horse antitoxin, 5 ml was absorbed with 100 mg lyophilized C. diphtheriae C_7(-) and 100 mg lyophilized E. coli Q_{13}. The mixture was incubated 1 hr at 37 C and overnight at 4 C. The precipitate was removed by centrifugation at 30,000 x g for 15 min. The absorbed antitoxin was re-tested by double diffusion in agar gel method against C_7(-) cells. The absorption was repeated 4 times until no line of precipitation against C_7(-) cell extract (15 mg/ml) could be detected with undiluted antitoxin in agar gel double diffusion. The titer of the adsorbed antitoxin was 500 units/ml.

Preparation of rabbit diphtheria antitoxin

Toxoid was prepared from electrophoretically pure diphtheria toxin by the method of Linggood, Stevens, Fulthorps, Woiwood and Pope (1963). Toxin, 1.5 mg, was dialyzed against 0.067 M phosphate buffer pH 7.8 containing 0.025 M lysine-HCl for 20 hr. Formaldehyde to final concentration of 0.5% v/v was added and the solution was stored at room temperature for 3 weeks. The toxoid solution in complete Freund's adjuvant was injected in the foot pads and the back of 3 adult male New Zealand rabbits (6 injection sites per rabbit). Each animal received 5 mg antigen distributed among the 6 sites. The animals were rested for 3 weeks and then bled through the ear vein. Serum was collected and tested for the presence of antitoxin by double gel diffusion in agar. Two rabbits produced antibodies against the toxin. The immune serums gave only one line of precipitation against both crude and electrophoretically pure diphtheria toxin, indicating that only monospecific antibodies were present. The serum from the rabbit with the higher antibody titer (220 units/ml) was used in the subsequent experiments.

Immune precipitation with horse and rabbit antitoxins

At the end of incubation time of the protein synthesizing mixtures, an equal volume of 0.2 M EDTA was added to release the ribosomes. After 15 min in an ice bath, the ribosomes were pelleted by centrifugation at 204,000 x g for 45 min in the Beckman SW 50 rotor. The supernatants were collected and dialyzed against borate-saline

buffer pH 8.4 for 48 hr with frequent changes of buffer to remove free C^{14}-lysine.

Following protein determination (Reiner and Cheung, 1961), a volume of 100 µl of each supernatant was precipitated with CCl_3COOH and total acid precipitable radioactivity was determined. A volume of 0.5 ml of each dialyzed supernatant containing approximately 750 µg protein was mixed with 100 µl absorbed horse antitoxin (500 units per ml) and 50 µl crude diphtheria toxin (2.2 mg per ml). The same volume of each supernatant was mixed with 100 µl rabbit antitoxin (220 units per ml) and 20 µl crude diphtheria toxin. An equal volume of each supernatant was mixed with 100 µl normal rabbit serum to determine the non-specifically precipitable proteins. The tubes, in triplicate, were incubated 1 hr at 37 C and 48 hr at 5 C. The precipitates were washed 3 times with 5 ml cold borate-saline buffer pH 8.4, collected on type HA filters (Millipore), dried and counted in a liquid scintillation counter as described previously for C^{14}-polypeptides. The counts were corrected for non-specific precipitation of the labeled proteins by subtracting the radioactivity precipitable with the normal rabbit serum from that precipitable with the antitoxins. The results were expressed as CPM/mg protein.

Preparation of mammalian polypeptide elongation factor 2 (EF2) (Allen and Schweet, 1962)

A partially purified EF2 enzyme from rabbit reticulocytes

was prepared as follows: Two New Zealand adult male rabbits weighing about 5 kg were injected subcutaneously for 6 days with 19 mg neutralized phenyl-hydrazine-HCl solution (Baker). Rabbits were bled by cardiac puncture on day 7 using a heparinized syringe. The collected blood was centrifuged for 15 min at 2500 x g at 5 C. All subsequent operations were carried out at 5 C. The packed cells were suspended in a solution containing 0.13 M NaCl, 5 mM KCl and 7.5 mM $MgCl_2$, filtered through gauze and centrifuged again. The cells were lysed by addition of 4 vol 2 mM $MgCl_2$ and stirred gently for 10 min. Solution of 1.5 M sucrose containing 0.15 M KCl was added (1/5 of total volume) to stop lysis. The mixture was stirred and clarified by centrifugation at 30,000 x g for 10 min. The clear supernatant was drawn off and centrifuged at 105,000 x g for 60 min to sediment the ribosomes. Tris-HCl (2.0 M, pH 7.5) was added to the supernatant to bring the Tris-HCl concentration to 0.10 M. Nucleic acids were precipitated with neutralized protamine sulfate at a final concentration of 0.17 mg/ml, by stirring for 30 min. The precipitated nucleic acids were separated by centrifugation at 12,000 x g for 20 min. The supernatant was fractionated with ammonium sulfate. The precipitate forming at 40% saturation was discarded. The concentration of the salt was increased to 60% saturation. After leaving the solution for 2 hr at this concentration, the precipitate formed was collected and dissolved in 0.1 M Tris-HCl pH 7.5. Ammonium sulfate to 70% saturation was added. The precipitate was collected and dissolved in 0.02 M Tris-HCl pH 7.5, 1 mM

glutathione (Sigma) and 0.1 mM EDTA and dialyzed against the same buffer. The solution was then concentrated to a small volume by pressure dialysis (Diaflo membrane 30,000: Amicon) and further purified by passage through a Sephadex G 100 column (Pharmacia) 2.5 cm^2 x 45 cm. The first protein peak which contained the EF-2 activity was collected and stored in small aliquots at -70 C.

Assay of ADP-ribosylation activity

At the end of 15 min incubation of the protein synthesizing mixtures at 35 C, an equal volume of 0.2 M EDTA was added to release the ribosomes. After 15 min in an ice bath the released ribosomes were pelleted by centrifugation at 204,000 x g for 45 min in the Beckman SW 50 rotor. The supernatants were collected and dialyzed against 50 mM Tris-HCl pH 8.2 containing 0.1 mM EDTA for 24 hr with frequent changes of buffer.

The dialyzed supernatants were collected and assayed for ADP-ribosylation of rabbit reticulocyte EF-2 enzyme in the presence of C^{14}-NAD by the method of Collier and Kandel (1971). The assay mixtures in total vol mes of 250 µl contained 50 mM Tris-HCl pH 8.2, 0.1 mM EDTA, 40 mM dithiothreitol (Sigma), 100 µl EF-2 enzyme, and 0.735 µM C^{14}-NAD^+ (adenine-C^{14}-(U), 136 mCi/mM; Amersham/Searle). The assay mixtures also contained one of the following: 150 µg trypsin digested toxin or 6 µg Fragment A of diphtheria toxin (a gift of Dr. R. J. Collier of University of California, Los Angeles) or 100 µl dialyzed supernatants of the in vitro protein synthesizing

mixture. Control EF-2 enzyme without the toxin, Fragment A or the supernatants was included. All the components except NAD^+ were mixed at 0 C, following 2 min incubation at 25 C the reaction was initiated by the addition of NAD^+, the mixtures were incubated at 25 C for various lengths of time. At the end of incubation, 250 µl cold 10% CCl_3COOH was added to each reaction mixture and the precipitate was collected on type HA filters (Millipore), washed 3 times with 5% CCl_3COOH, then once with 95% ethanol, dried and counted in the liquid scintillation counter in the same manner as the C^{14}-polypeptides.

Trypsin digestion of diphtheria toxin (Gill and Dinius, 1971)

To 1 ml pure diphtheria toxin (15 mg/ml) in 0.05 M Tris-HCl pH 8.0, 5% glycerol and 0.05 M dithiothreitol, freshly dissolved trypsin (Sigma, Chymotrypsin-free) was added to a concentration of 0.01 mg per ml. The tube was incubated 10 min at 37 C. The digestion was stopped by the addition of soy bean trypsin inhibitor (Sigma) in a final concentration twice that of trypsin. The digested toxin was immediately used or was frozen at -20 C.

Rabbit skin test (Matsuda and Barksdale, 1967)

The dialyzed supernatants of the in vtiro reaction mixtures (prepared as for the ADP-ribosylation assay) were concentrated tenfold in Microsolute Concentrators (Amicon) and 3 mg protein of each supernatant in 0.2 ml was injected intradermally into the depilated back of a rabbit. The same volume was neutralized with 20 µl rabbit

antitoxin and injected at the opposite site 6 hr later. Diphtheria toxin (5 MRD) and the same amount of toxin neutralized with rabbit antitoxin was also injected as controls. Results were read 48 to 96 hr after the first inoculation.

RESULTS

A. Isolation of biologically active messenger RNA from C. diphtheriae and its translation in E. coli cell-free lysate

1. RNA extraction from C. diphtheriae. C. diphtheriae cells: $C_7\beta v$ tox^+, PW8 and $C_7(-)$ were ground with alumina and the RNA was extracted by phenol-SDS method. Sedimentation pattern of the RNA was then examined in 5-20% exponential sucrose gradients to determine molecular size and distribution of the RNA. The results for $C_7\beta v$ tox^+, PW8 and $C_7(-)$ RNA are shown in Figs. 1, 2 and 3. A standard RNA preparation containing 23S, 16S, and 4S E. coli RNA was used as a sedimentation marker to identify the position of each peak in the sucrose gradient. C. diphtheriae RNA sedimented into three discernible peaks of 23S, 16S and 4S RNA. Furthermore in all RNA preparations from C. diphtheriae examined in sucrose gradients the 4S peak was larger than the one observed in total RNA extracted from E. coli (Gesteland, Salser and Ricard, 1969). There may be more 4S RNA in C. diphtheriae cells than in E. coli cells. Another possibility is that some C. diphtheriae cells were not broken open by grinding with alumina and remained intact; subsequently, only 4S RNA was obtainable from these cells. The latter possibility is substantiated by preliminary experiments attempting to extract RNA with phenol-SDS from C. diphtheriae without prior grinding with alumina which yielded only 4S RNA.

Since the sedimentation profiles of the RNA were not skewed towards the top of the gradients, it appeared that degradation

Figure 1

An amount of 0.2 mg RNA from $C_7\beta v$ tox^+ cells (45 min post-infection) was layered on a 5 ml 5-20% exponential sucrose gradient in STE buffer. The gradient was centrifuged at 204,000 x g for 3 hr at 5 C. Two-drop fractions were collected and diluted in 0.5 ml STE buffer. The A_{260} was determined for each fraction. Arrows indicate the position of sedimentation marker RNA from E. coli (courtesy Dr. W. J. Iglewski).

Sucrose gradient sedimentation of RNA
from C. diptheriae $C_7 \beta_v$ tox+

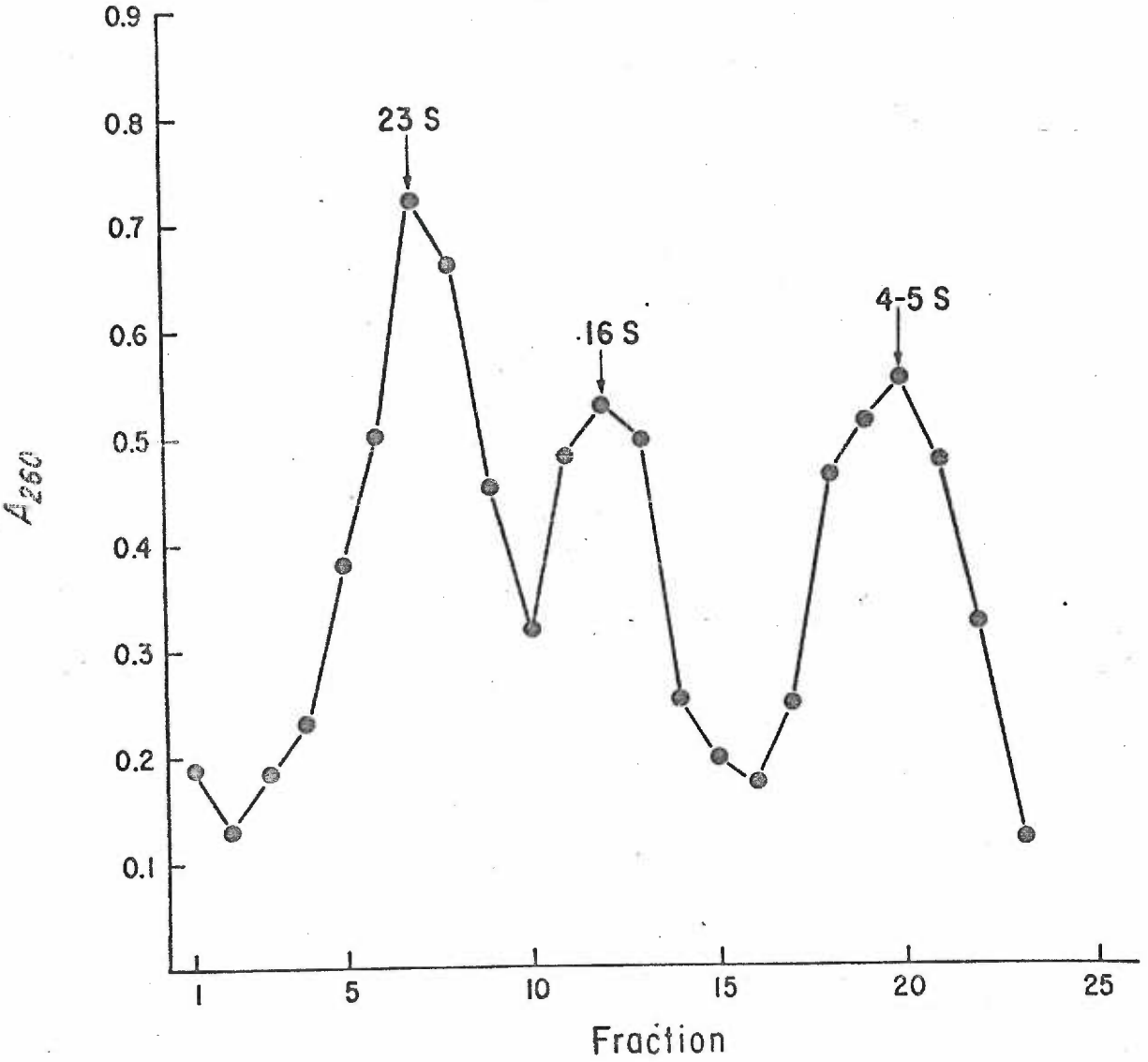


Figure 2

An amount of 0.2 mg RNA from PW8 cells was analyzed in the sucrose gradient as described in Fig. 1.

Sucrose gradient sedimentation of RNA from C. diphtheriae PW8

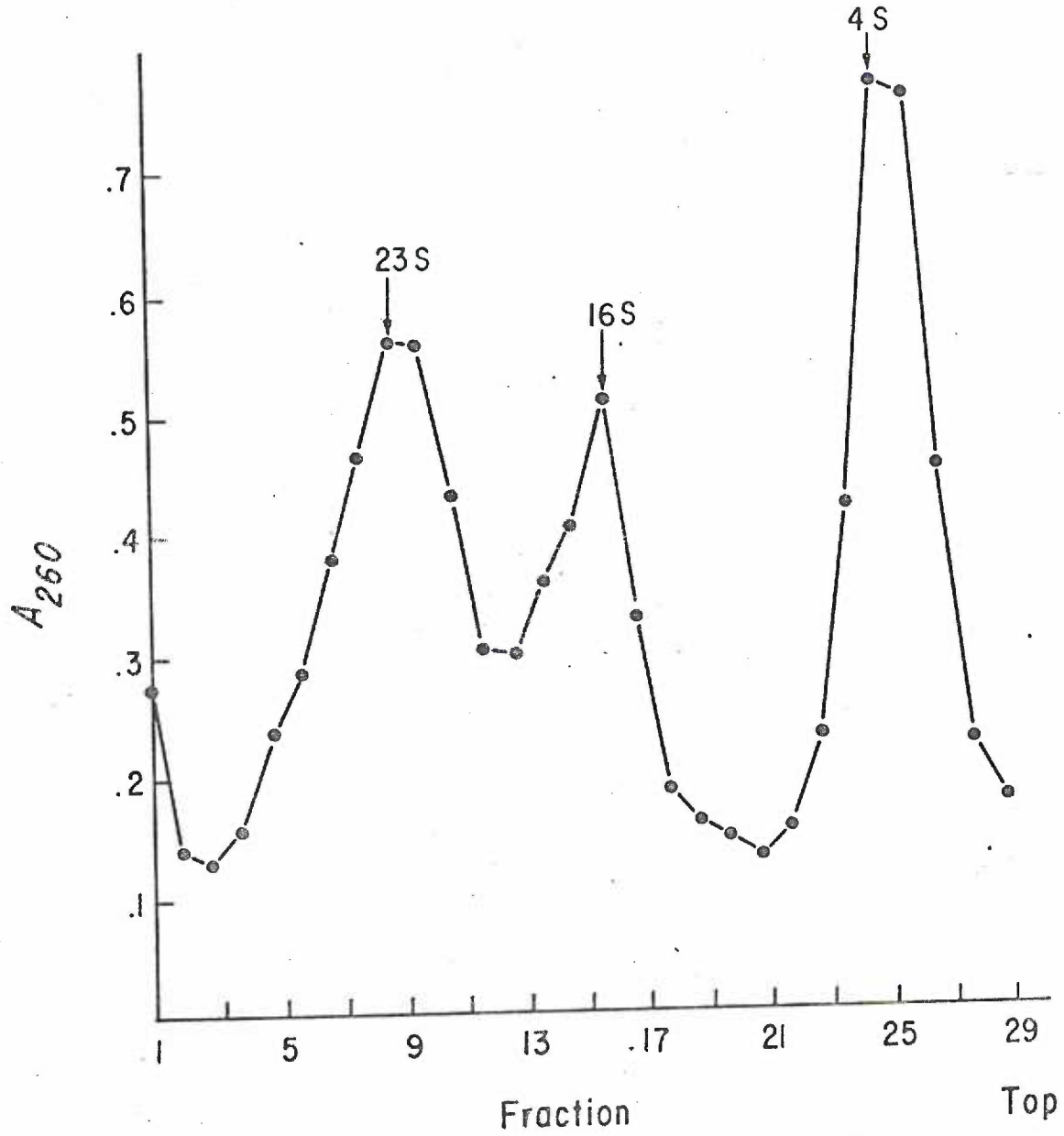
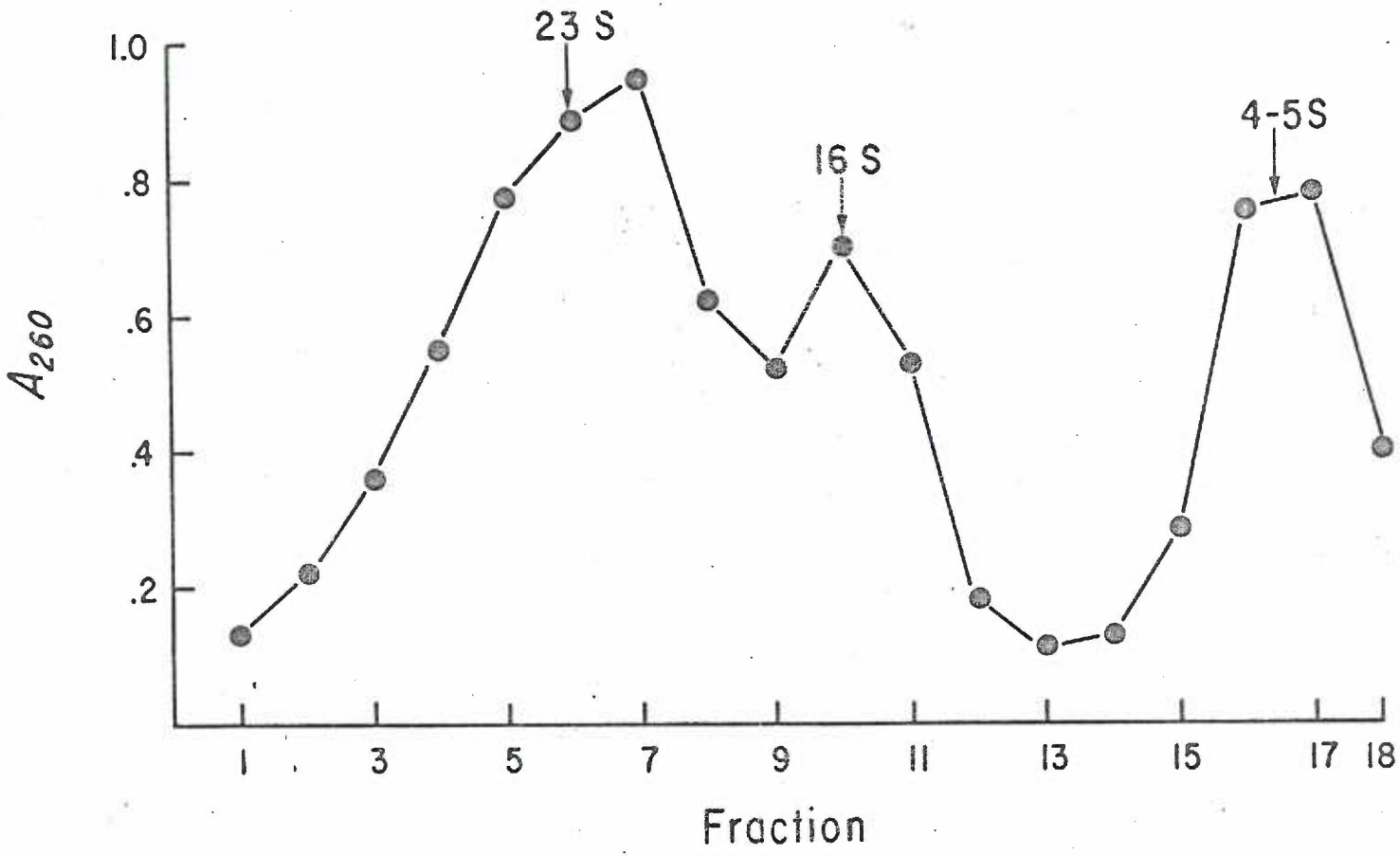


Figure 3

An amount of 0.2 mg RNA from $C_7(-)$ cells was analyzed in the sucrose gradient as described in Fig. 2. Three drop fractions were collected.

Sucrose gradient sedimentation of RNA
from C. diptheriae $C_7(-)$ tox^-



of the RNA due to nuclease activity, or physical shearing was minimal during the extraction.

2. Incorporation of short pulse of H^3-uridine into RNA in $C_7\beta v$ tox^+ (45 min post-infection) and $C_7(-)$ cells. The purpose of this experiment was to investigate RNA synthesis in the bacteriophage-infected $C_7\beta v$ tox^+ and non-infected $C_7(-)$ cells at the time of cell harvest.

C. diphtheriae $C_7\beta v$ tox^+ and $C_7(-)$ cells were exposed to a 15 sec pulse of H^3-uridine prior to harvest. Sodium azide, potassium cyanide and rapid chilling were used to arrest further uptake and incorporation of H^3-uridine. The RNA was isolated by the method previously described. The specific activity of each preparation of RNA was determined. The specific activity of the RNA was found to be the same for both infected and non-infected cells (about 5.6×10^5 cpm/mg). Therefore, the bacteriophage infected cells, as well as the non-infected C. diphtheriae were actively synthesizing RNA at the time of their harvest. The sedimentation patterns of the H^3-RNA in a 5-20% exponential sucrose gradient was examined under similar conditions as previously described for non-labeled RNA. The A_{260} and the distribution of H^3-RNA from the bacteriophage infected and non-infected C. diphtheriae cells are shown in Figs. 4 and 5. The A_{260} profiles were similar to that of the unlabeled RNA shown in Figs. 1, 2, and 3. The sedimentation profiles of H^3-RNA indicated that larger molecular species of pulse-labeled RNA were present in

Figure 4

An amount of 0.2 mg H^3-RNA from $C_7\beta v$ tox^+ cells (15 sec H^3-uridine, 45 min post-infection) was layered on a 5 ml 5-20% sucrose gradient and sedimented under the same conditions as Fig. 1. Two drop fractions were collected and diluted in 0.5 ml STE. Following measurement of the A_{260} of each fraction, the RNA was precipitated with cold CCl_3COOH and the radioactivity in the precipitate was determined.

Sucrose gradient sedimentation of
H^3-uridine labeled RNA from
C. diphtheriae C_7 βv tox^+

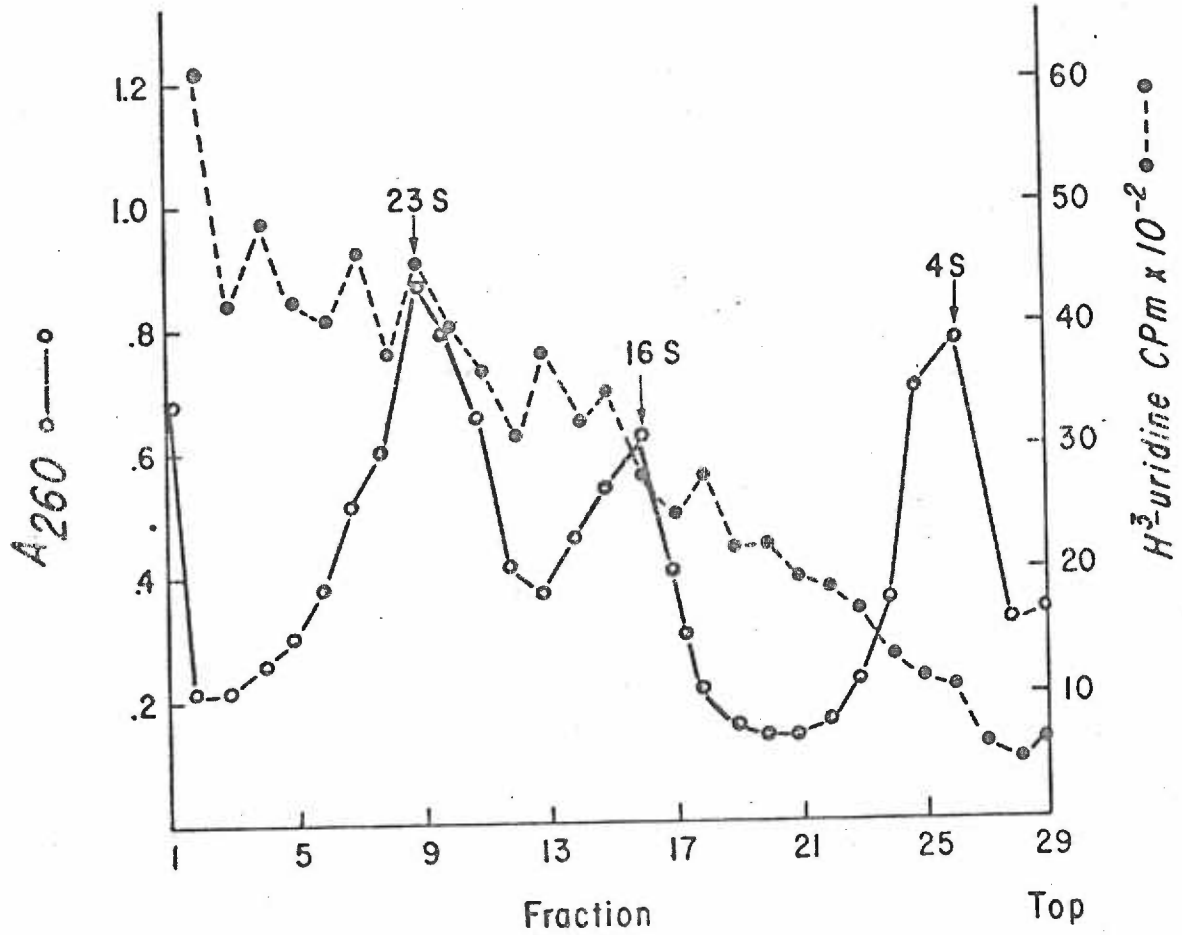


Figure 5

An amount of 0.2 mg H^3-RNA from C_7(-) cells (15 sec pulse of H^3-uridine) was examined in 5-20% sucrose gradient as described in Fig. 4.

Sucrose gradient sedimentation of H^3 - uridine labeled RNA from C. diphtheriae C_7 (-) tox^-

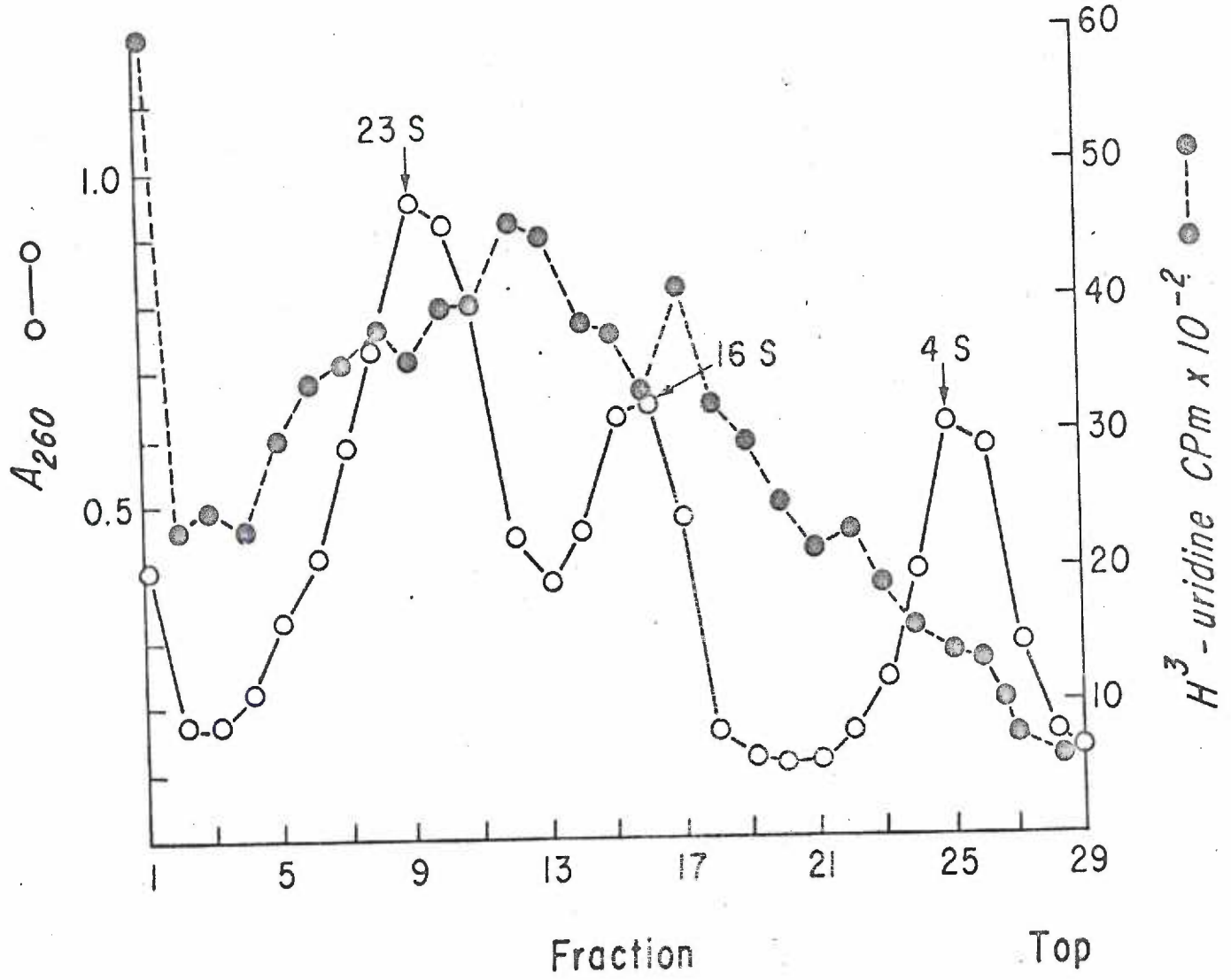
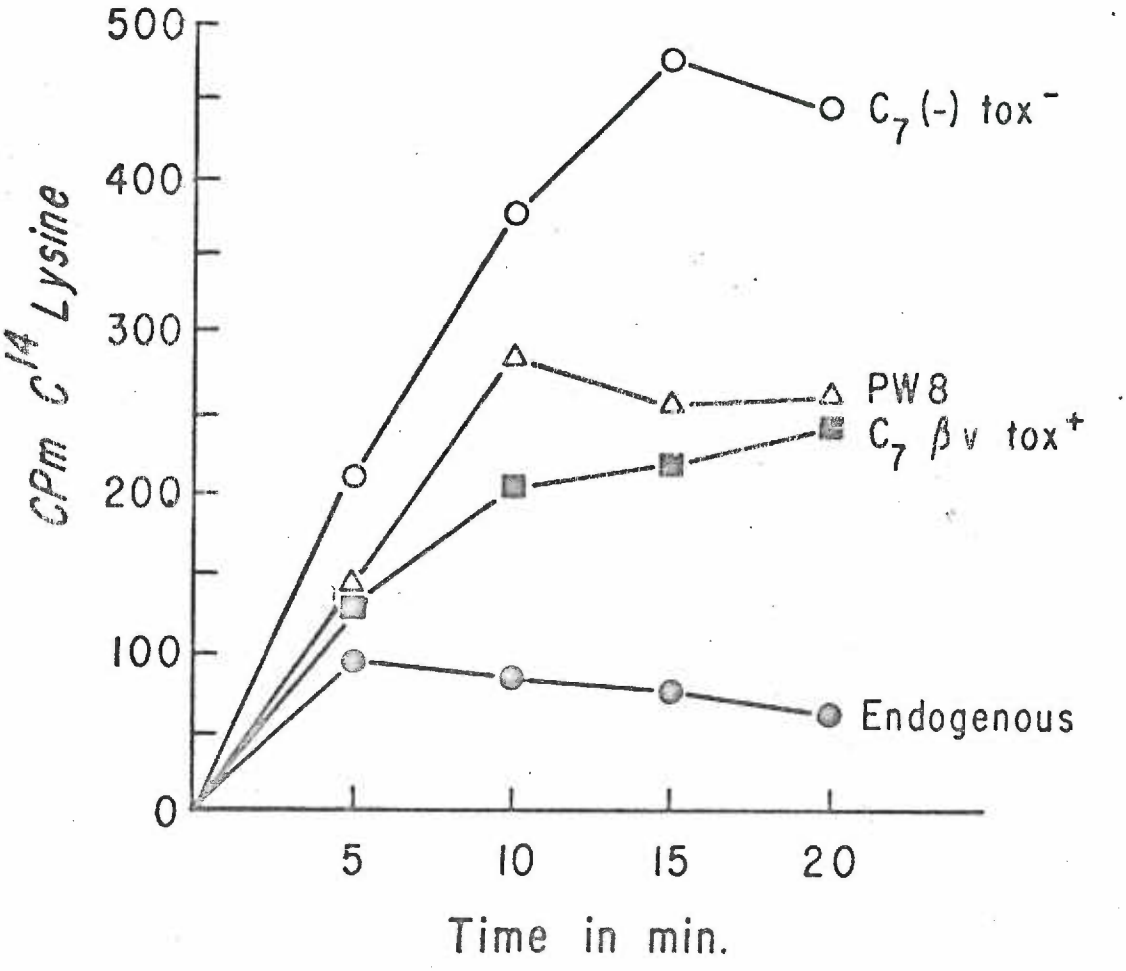


Figure 6

The 100 µl reaction mixtures were primed with 0.6 mg/ml C_7(-) RNA, 2 mg/ml PW8 RNA and 2 mg/ml C_7βv tox^+ RNA and incubated at 35 C. Samples of 5 µl were removed and hot CCl_3COOH precipitable radioactivity in each sample was determined. The reading is the average of two samples taken at the indicated times.

Incorporation of C^{14}-Lysine in E. coli in vitro system stimulated by C. diphtheriae RNA



the bacteriophage infected cells than in the non-infected cells. Since the specific activities of the RNA isolated from the infected cells was the same as that from the uninfected cells, there was no apparent inhibition of RNA synthesis in the infected cells. Therefore, the change in molecular size of the RNA being synthesized probably reflected a shift in the RNA synthesis from that of host-specific RNA to that of the bacteriophage RNA.

3. Kinetics of amino acid incorporation of E. coli protein synthesizing system directed by RNA from C. diphtheriae. The E. coli cell-free protein synthesizing system was initially tested for its ability to translate exogenous mRNA by using R17 bacteriophage RNA (a gift of Dr. B. H. Iglewski, Univesity of Oregon Medical School). Following 15 min incubation at 35 C with 1 mg R17 RNA/ml reaction mixture, the incorporation of C^{14}-lysine into material insoluble in hot CCl_3COOH was stimulated 15 times over that of the endogenous reaction mixture. The cell-free protein synthesizing system was therefore able to translate exogenous mRNA.

RNA from C. diphtheriae $C_7\beta v$ tox^+, PW8 or $C_7(-)$ when used as messenger RNA in the cell-free protein synthesizing system stimulated C^{14}-lysine incorporation at least 3 times over that of the endogenous. Furthermore, the C^{14}-lysine incorporation was linear during the first 10 to 15 min of incubation (Fig. 6).

Optimum concentration of RNA for maximum incorporation of C^{14}-lysine was determined for three preparations of RNA from $C_7\beta v$ tox^+ (45 min post-infection), PW8 and $C_7(-)$ cells. Reaction

mixtures containing increasing amounts of RNA were incubated at 35 C for 15 min. The amount of C^{14}-lysine incorporation in each reaction mixture was determined. An endogenous reaction mixture incubated under the same conditions was used to determine the background incorporation due to the endogenous mRNA in the cell-free system. The stimulation of incorporation of C^{14}-lysine by the added RNA was determined by subtracting the background incorporation from the total incorporation of the reaction mixtures containing the various RNA concentrations. The results are presented in Figs. 7, 8 and 9 which show that maximum incorporation of C^{14}-lysine for the three RNA preparations shown here occurred at RNA concentrations of 2 mg/ml reaction mixture for $C_7\beta v$ tox^+, 1.2 mg/ml for PW8 and 0.8 mg/ml for $C_7(-)$ RNA. RNA concentrations higher than optimum reduced amino acid incorporation. Therefore, the optimum concentration for each new RNA preparation was determined prior to its use for toxin synthesis. The mRNA activity depended on the particular preparation, rather than its cell source. While some RNA preparations were quite active in C^{14}-lysine incorporation (6-15 times over endogenous) sometimes preparations from the same cell source were less active (3-5 times over endogenous).

4. Dependence of the in vitro protein synthesis directed by C. diphtheriae RNA on the concentration of magnesium ion. The purpose of this experiment was to determine the magnesium ion concentration which produced maximum incorporation of C^{14}-lysine in the E. coli cell-free lysate directed by C. diphtheriae RNA. The optimum

Figure 7

The dependence of C^{14}-lysine incorporation by E. coli cell-free protein synthesizing system on concentration of $C_7\beta v$ tox^+ RNA.

The 50 µl reaction mixtures were primed with increasing amounts of RNA and incubated for 15 min at 35 C. Each point represents the average hot CCl_3COOH precipitable radioactivity in duplicate 5 µl samples withdrawn from the reaction mixtures and corrected for the endogenous amino acid incorporation.

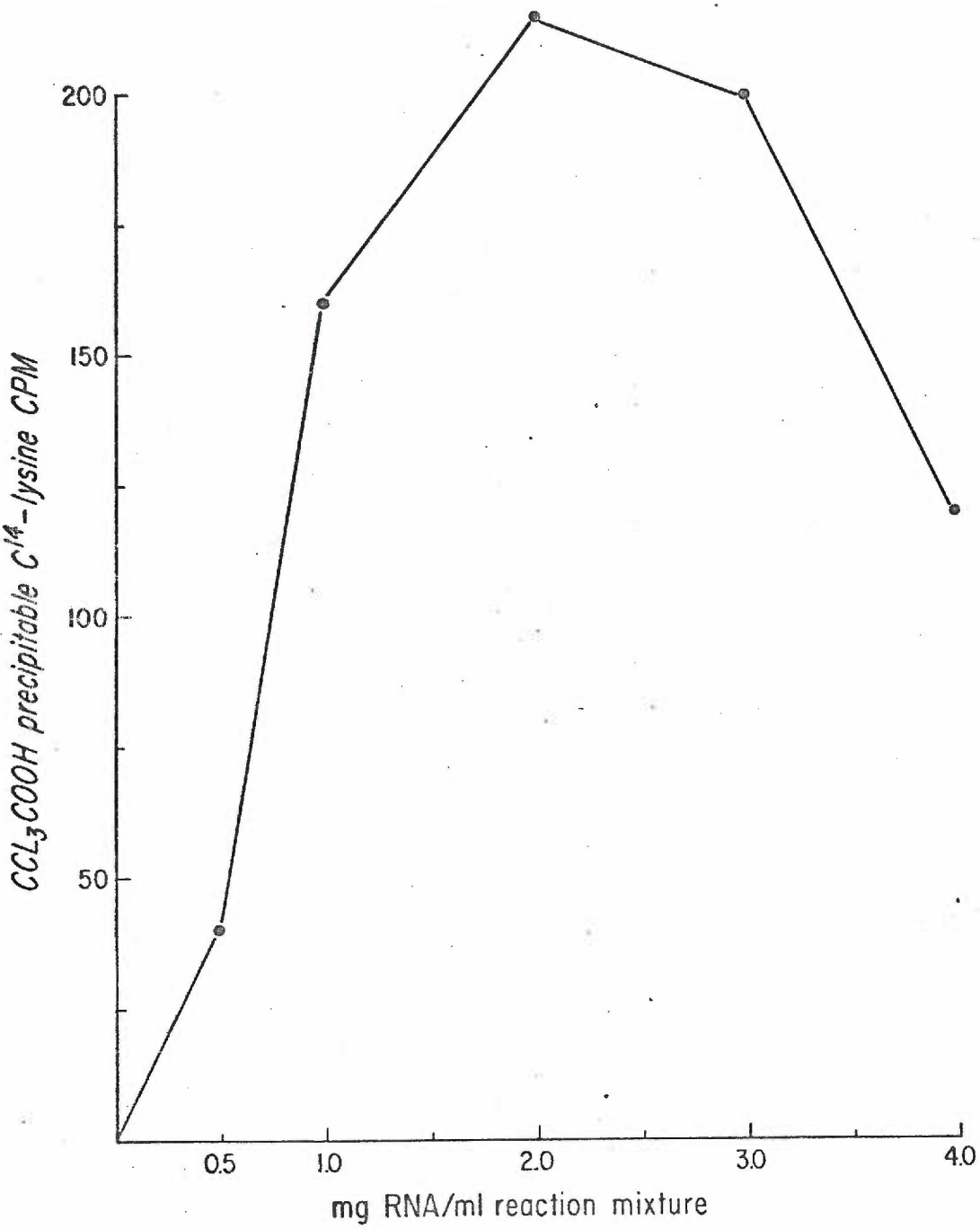

200
150
100
50
CCL_3COOH precipitable C^{14}-lysine CPM
0.5
1.0
2.0
3.0
4.0
mg RNA/ml reaction mixture

Figure 8

Dependence of C^{14}-lysine incorporation by E. coli cell-free protein synthesizing systems on concentration of PW8 RNA.

The 50 µl reaction mixtures were primed with increasing concentrations of PW8 RNA and incubated for 15 min at 35 C. Samples were withdrawn and treated as in Fig. 7.

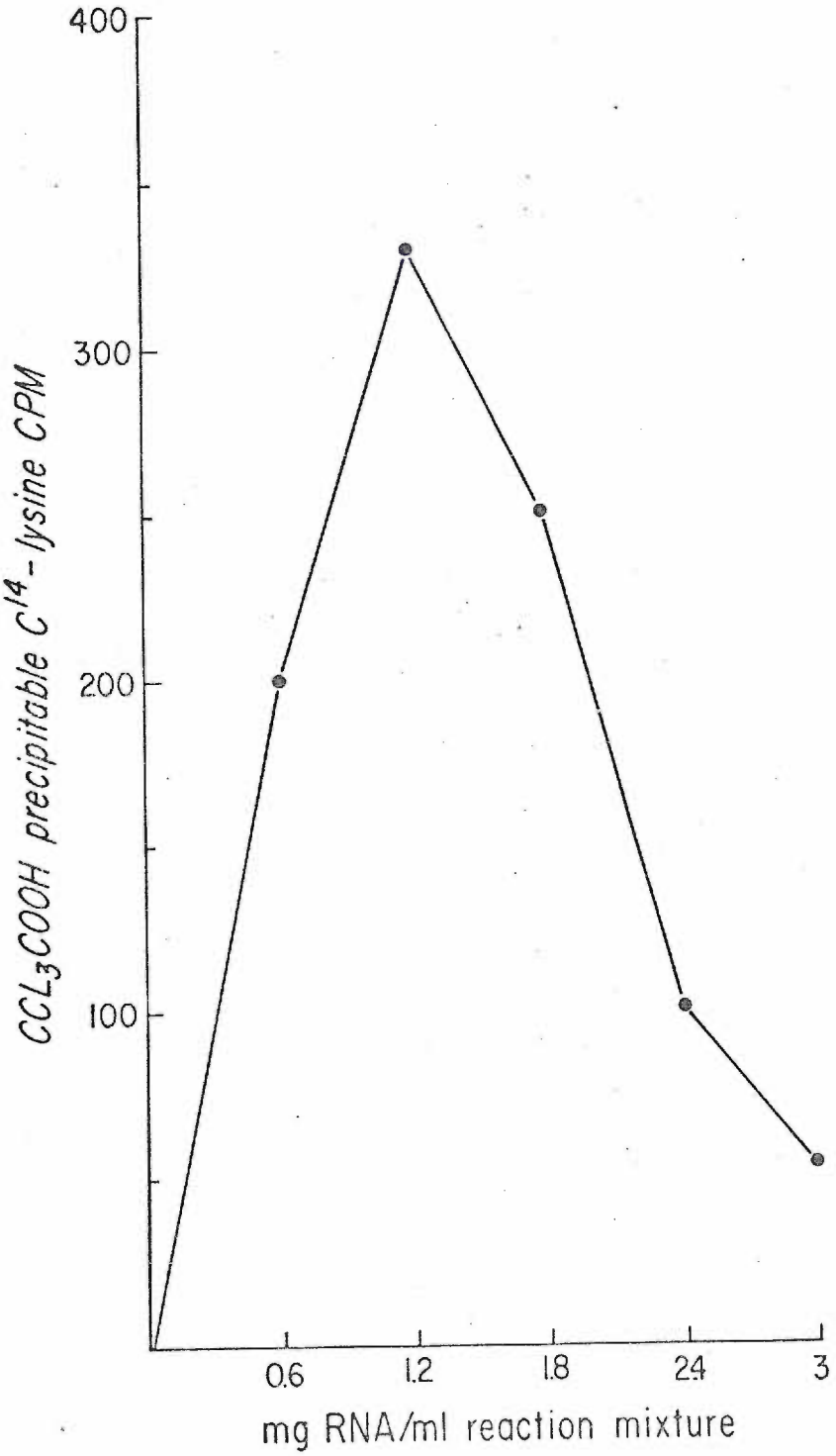
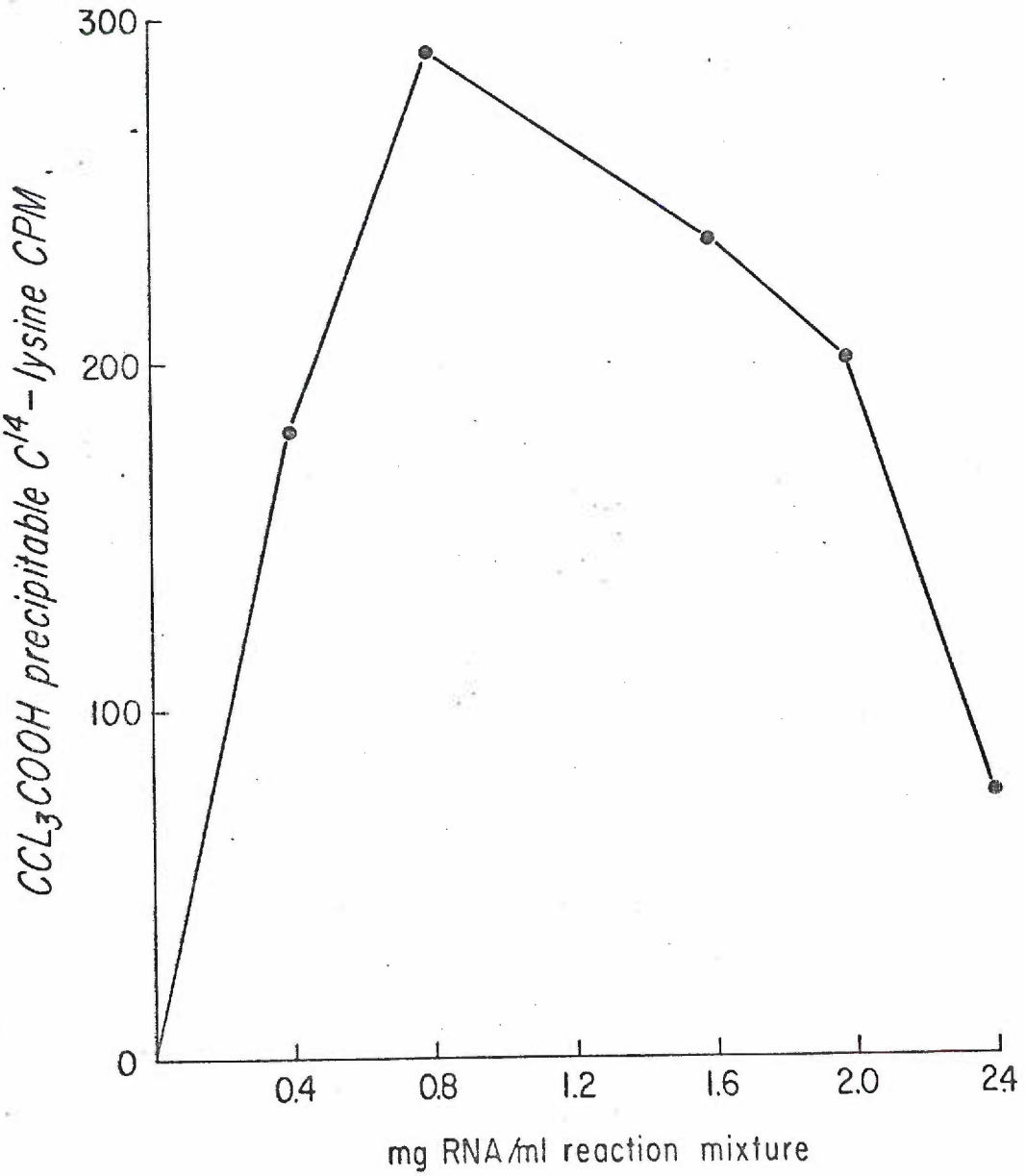

400
300
200
100
CCL_3COOH precipitable C^{14}-lysine CPM
0.6
1.2
1.8
2.4
3
mg RNA/ml reaction mixture

Figure 9

The dependence of C^{14}-lysine incorporation by E. coli cell-free protein synthesizing systems on concentration of C_7(-) RNA.

The 50 µl reaction mixtures were primed with increasing concentrations of C_7(-) RNA and incubated for 15 min at 35 C. Samples were withdrawn and treated as in Fig. 7.


300
200
100
0
CCL_3COOH precipitable C^{14}-lysine CPM
0.4
0.8
1.2
1.6
2.0
2.4
mg RNA/ml reaction mixture

magnesium ion concentration for cell-free protein synthesis with natural mRNA is generally lower than that with synthetic RNA such as polyuridylic acid. Those natural mRNA reported on, require 7-12 mM magnesium ion, (Modolell, 1971) for the initiation of protein synthesis with N-formylmethionyl tRNA and the initiation factors (Lucas-Lenard and Lipmann, 1971). Ribosomal interaction with synthetic mRNA requires higher magnesium ion concentrations (14-18 mM) and does not require N-formylmethionyl tRNA or the initiation factors. The magnesium ion concentration promoting maximum incorporation of C^{14}-lysine was therefore measured and used as an indication that normal initiation processes were involved in the initiation of protein synthesis with C. diphtheriae RNA.

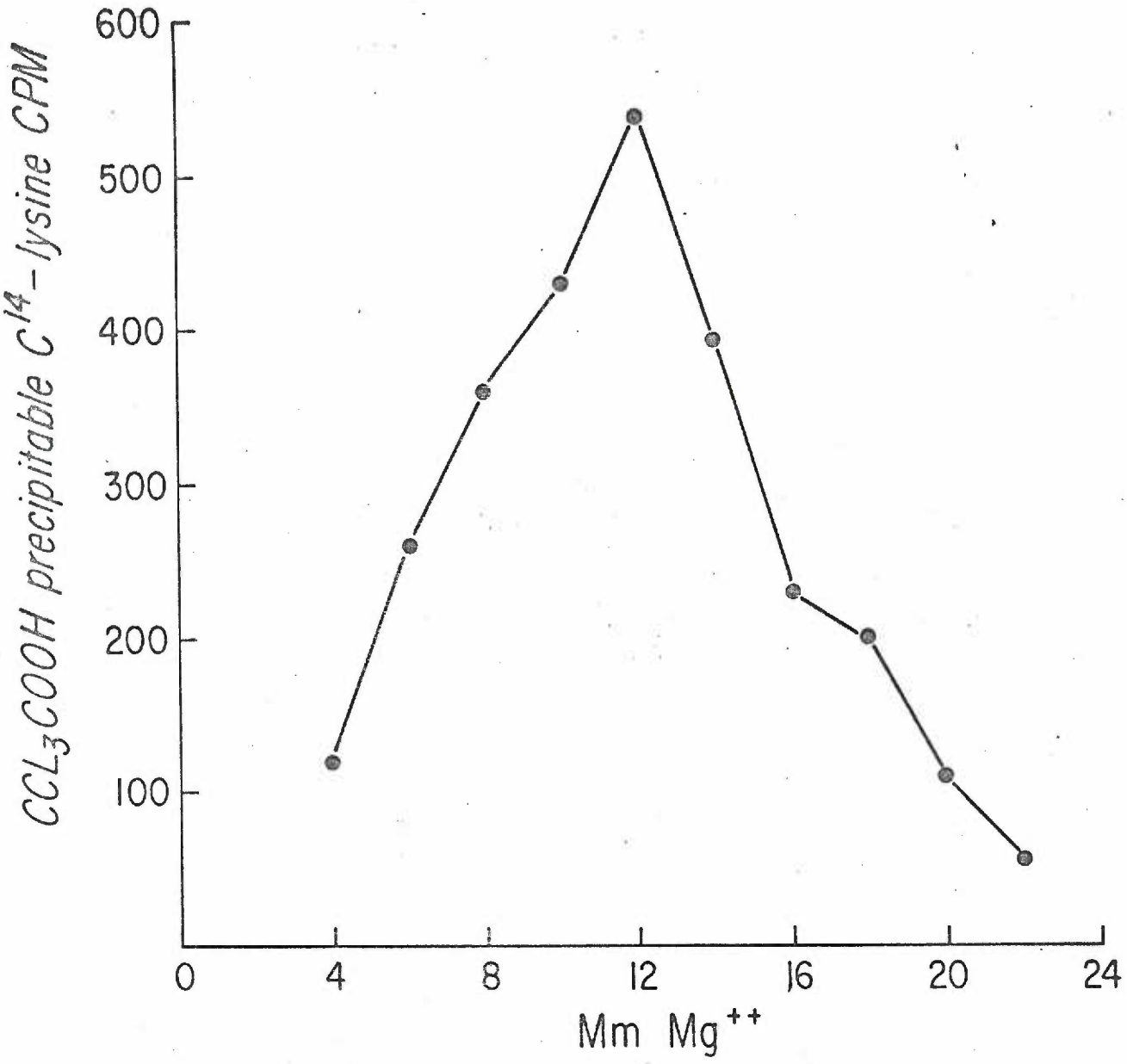
C. diphtheriae C_7(-) RNA was used to stimulate the incorporation of C^{14}-lysine into proteins in the presence of various concentrations of magnesium ion. The reactions were stopped after 20 min incubation at 35 C. Fig. 10 shows that the concentration of magnesium ion which maximally stimulated amino acid incorporation was 12 mM and that higher concentrations of magnesium ion were inhibitory. This indicated that the requirement for magnesium ion in the cell-free protein synthesizing system directed by C. diphtheriae RNA was similar to that reported for other natural mRNA and therefore probably involved the formation of normal initiation complex.

Fidelity of translation of natural mRNA is improved at a slightly lower than optimum magnesium ion concentration (Salser, Gesteland and Bolle, 1967; Capecchi, 1967). Therefore, 10 mM

Figure 10

The dependence of the C^{14}-lysine incorporation by E. coli cell-free protein synthesizing system primed with C_7(-) RNA on the concentration of magnesium ion.

The 100 µl reaction mixtures were primed with 0.8 mg C_7(-) RNA/ml reaction mixture and incubated at 35 C for 20 min. Duplicate 10 µl samples were withdrawn and hot CCl_3COOH precipitable radioactivity was determined for each sample. Each point represents the average radioactivity in the duplicate samples.


CCL_3COOH precipitable C^{14}-lysine CPM
600
500
400
300
200
100
0
4
8
12
16
20
24
Mm Mg^{++}

magnesium ion was routinely used to reduce misreading of the C. diphtheriae RNA in the E. coli cell-free lysate.

5. Association of H^3-RNA from C. diphtheriae with E. coli ribosomes. The aim of these experiments was to determine if H^3-labeled RNA from C. diphtheriae would bind with E. coli ribosomes under the conditions used for the in vitro protein synthesis. Fifteen-sec pulse-labeled-H^3-RNA from $C_7\beta v$ tox^+ and $C_7(-)$ cells was used to prime the E. coli cell-free protein synthesizing system. C^{14}-lysine was omitted and all the amino acids in the reaction mixtures were unlabeled. The reaction mixtures were incubated for 7 min at 35 C. They were then rapidly chilled and chloramphenicol was added to prevent ribosomal run-off. The reaction mixtures were then analyzed in 10-30% exponential sucrose gradients in the standard buffer minus 2-mercaptoethanol. The gradients were centrifuged at 5 C for 75 min at 165,000 x g. Fractions were collected and the radioactivity and the A_{260} of each fraction were determined as described in Materials and Methods. The A_{260} and H^3-RNA profiles indicated that the labeled RNA from C. diphtheriae cells sedimented with and just ahead of the 70S single ribosomes of E. coli (Fig. 11 and 12). Although some H^3-RNA also sedimented in the polysomes region, more RNA was found associated with the single ribosomes. In order to obtain a better resolution of the single ribosomal region in subsequent experiments the gradients were centrifuged for 120 min. The results are shown in Figs. 13 and 14. The single ribosomal peak was well resolved under these conditions and association of H^3-RNA from $C_7\beta v$ tox^+ and $C_7(-)$

Figure 11

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with H^3-RNA from C. diphtheriae $C_7\beta v$ tox^+.

The 100 µl reaction mixture was primed with H^3-RNA (0.64 mg/ml) and incubated at 35 C for 7 min. Chloramphenicol was added and the reaction mixture rapidly chilled. 100 µl was layered on a 5 ml 10 - 30% exponential sucrose gradient in standard buffer minue 2-mercaptoethanol. The gradient was centrifuged for 75 min at 165,000 x g at 5 C. Fractions were collected. The A_{260} and radioactivity in 50 µl samples of each gradient fraction were determined.

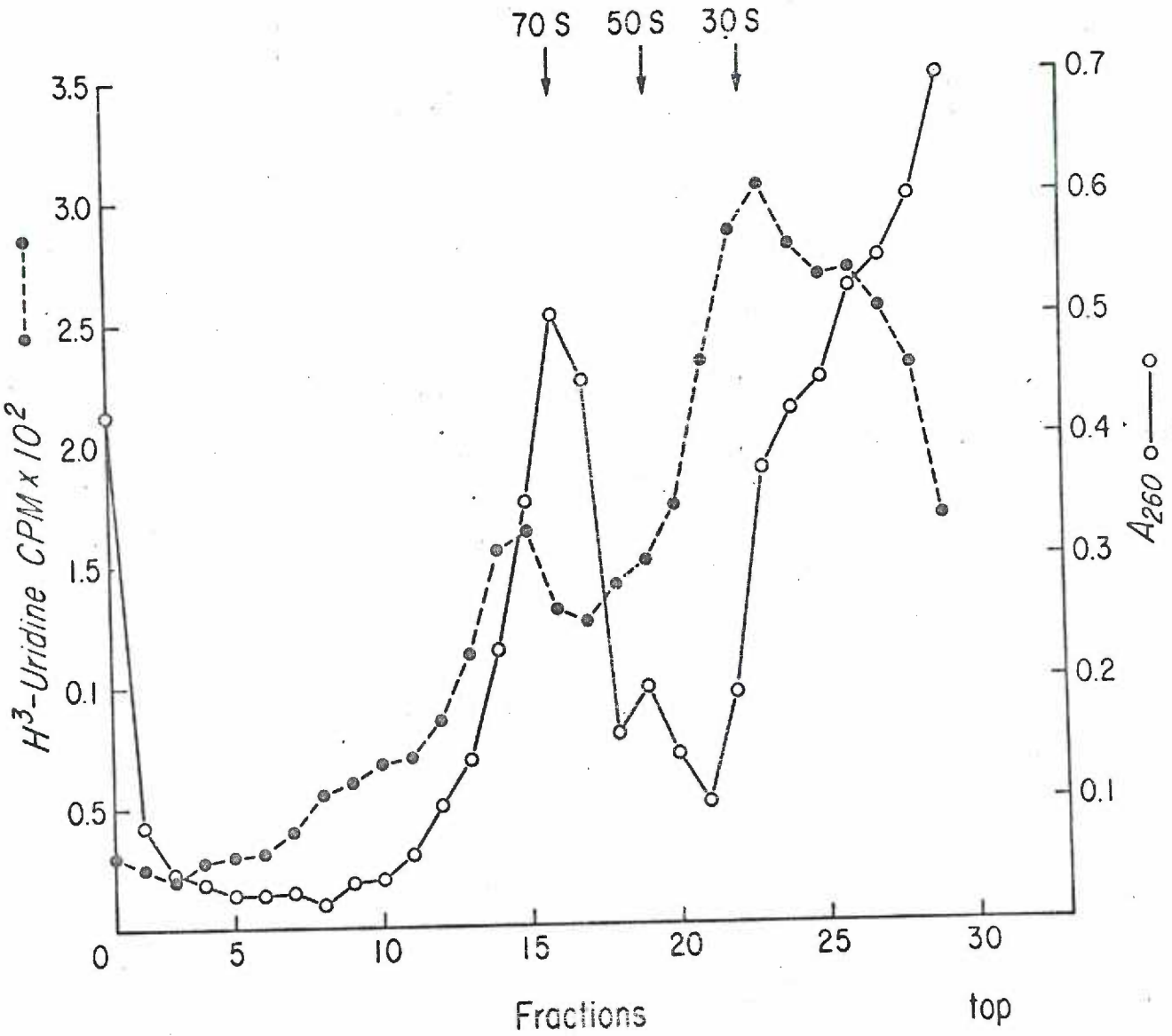

70 S
50 S
30 S
3.5
3.0
2.5
2.0
1.5
0.1
0.5
0
5
10
15
20
25
30
Fractions
top
H^3-Uridine CPM x 10^2
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A_{260}

Figure 12

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with H^3-RNA from C. diphtheriae $C_7(-)$.

The 100 µl reaction mixture was primed with H^3-RNA (0.7 mg/ml) and incubated at 35 C for 7 min. The reaction mixture was analyzed as in Fig. 11.

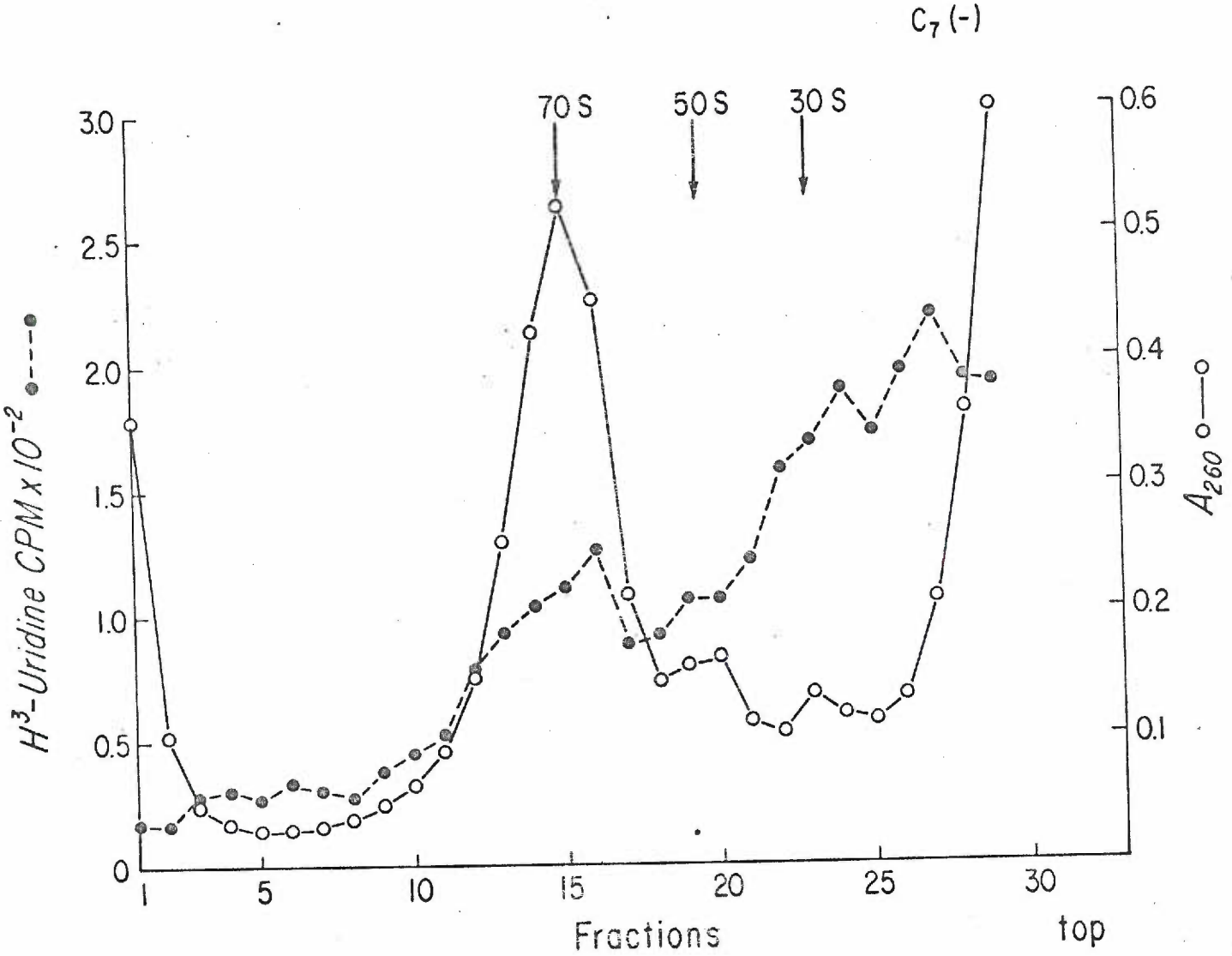

C7 (-)
70 S
50 S
30 S
3.0
2.5
2.0
1.5
1.0
0.5
0
0.6
0.5
0.4
0.3
0.2
0.1
H3-Uridine CPM x 10-2
A260
1
5
10
15
20
25
30
Fractions
top

Figure 13

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with H^3-RNA from C. diphtheriae $C_7\beta v$ tox^+.

The 100 µl reaction mixtures were primed with the RNA and treated as in Fig. 11 with one exception. The centrifugation time was increased to 120 min.

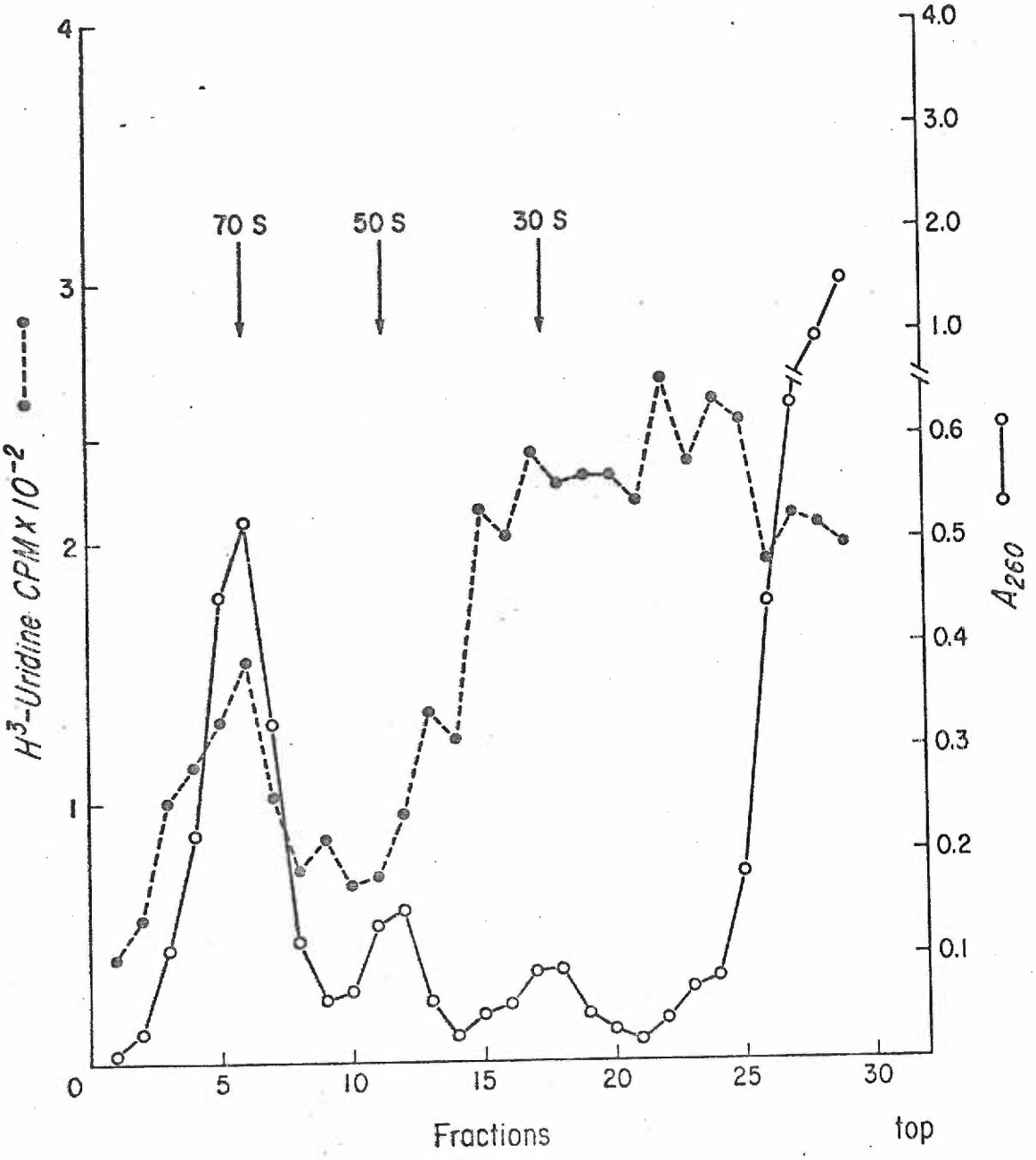
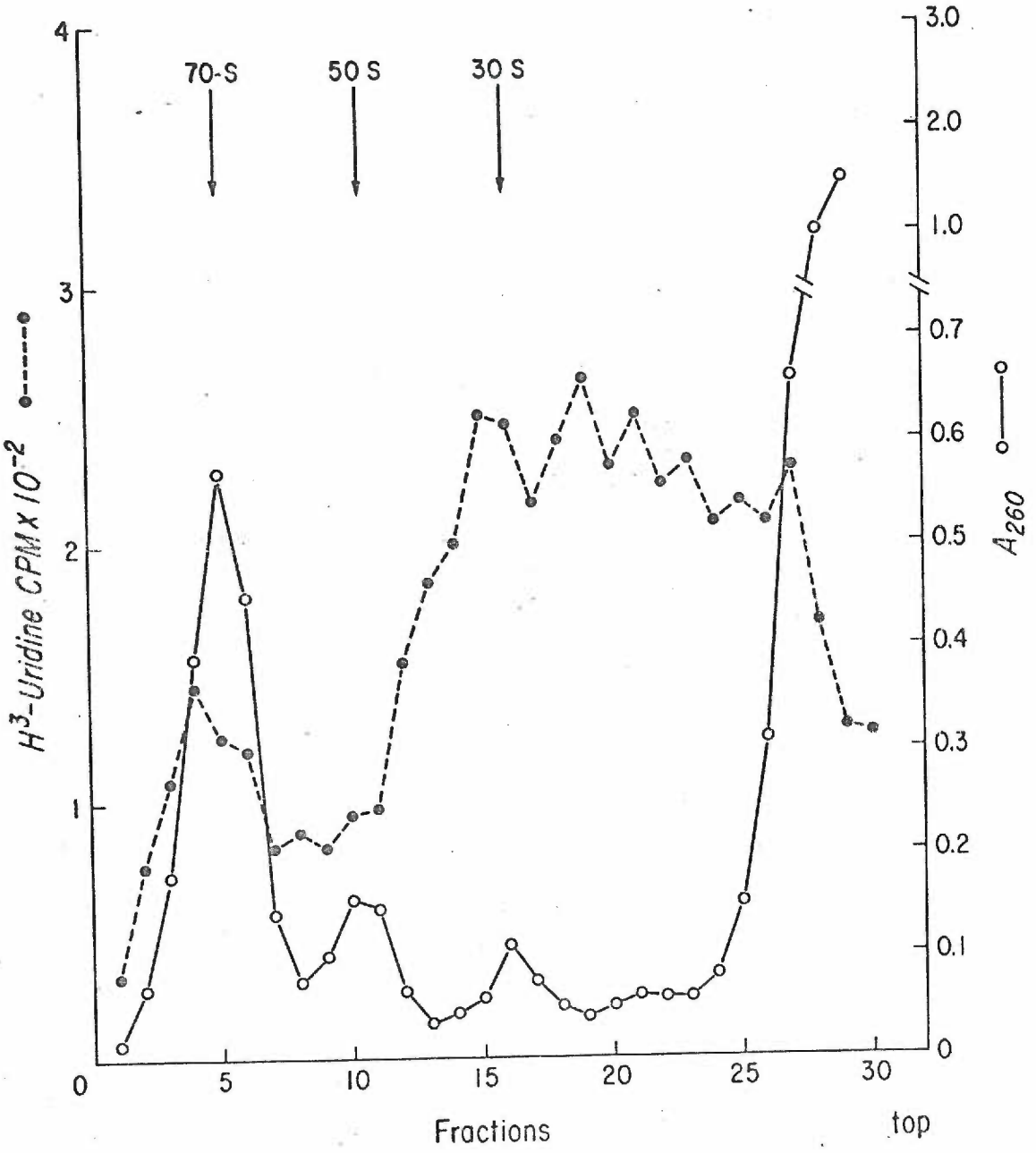

70 S
50 S
30 S
H^3-Uridine CPM x 10^{-2}
A_{260}
Fractions
top
0
1
2
3
4
5
10
15
20
25
30
0.1
0.2
0.3
0.4
0.5
0.6
1.0
2.0
3.0
4.0

Figure 14

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with H^3-RNA from C. diphtheriae $C_7(-)$.

The 100 µl reaction mixtures were primed with the RNA and treated as in Fig. 11 with one exception. The centrifugation time was increased to 120 min.


70-S
50 S
30 S
H^3-Uridine CPM x 10^{-2}
A_{260}
Fractions
top
0
1
2
3
4
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1.0
2.0
3.0
5
10
15
20
25
30

cells with the E. coli ribosomes was evident. Labeled RNA from both sources in the absence of ribosomes sedimented in a different fashion under identical conditions and did not show definite peaks in the 70S region of the gradients (Figs. 15 and 16). These results indicated that RNA from both bacteriophage-infected cells ($C_7\beta v$ tox^+) and non-infected $C_7(-)$ C. diphtheriae were able to bind and form complexes with the E. coli ribosomes.

6. Association of C^{14}-lysine-labeled polypeptides with the E. coli ribosomes directed by mRNA from C. diphtheriae. The results of the previous experiments indicated that in presence of 10 mM magnesium ion C. diphtheriae RNA formed complexes with the E. coli ribosomes. The objective of these experiments was to demonstrate the newly synthesized polypeptides on the E. coli ribosomes directed by RNA from C. diphtheriae. The RNA from C. diphtheriae PW8 and $C_7(-)$ were incubated with the E. coli complete protein synthesizing system. The polypeptides were labeled with C^{14}-lysine for 3 min at 35 C. The reaction mixtures were rapidly chilled and chloramphenicol was added to arrest the protein synthesis and to preserve the polypeptides bound to the ribosomes. The reaction mixtures were then analyzed in 10-30% exponential sucrose gradients in the standard buffer minus 2-mercaptoethanol. The gradients were centrifuged at 5 C for 75 min at 165,000 x g. Fractions were collected and hot acid precipitable radioactivity and the A_{260} of each fraction was determined as described in Materials and Methods. Figs. 17, 18 and 19 show the sedimentation pattern of the ribosomes and the C^{14}-polypeptides in the

Figure 15

Sucrose gradient sedimentation profile of the H^3-RNA from C. diphtheriae $C_7\beta v$ tox^+ in presence and absence of E. coli S-30.

The 100 µl reaction mixtures contained 0.64 mg/ml H^3-RNA and were incubated in presence or absence of E. coli S-30 for 7 min at 35 C. Chloramphenicol was added and the reaction mixtures were analyzed in the 10 - 30% exponential sucrose gradients in the standard buffer minus 2-mercaptoethanol. Centrifugation was at 165,000 x g for 120 min at 5 C. Fractions were collected and radioactivity in 50 µl of each gradient fraction was determined.

O———O, complete reaction mixture with E. coli S-30 (from Fig. 13);
●------●, without the S-30.

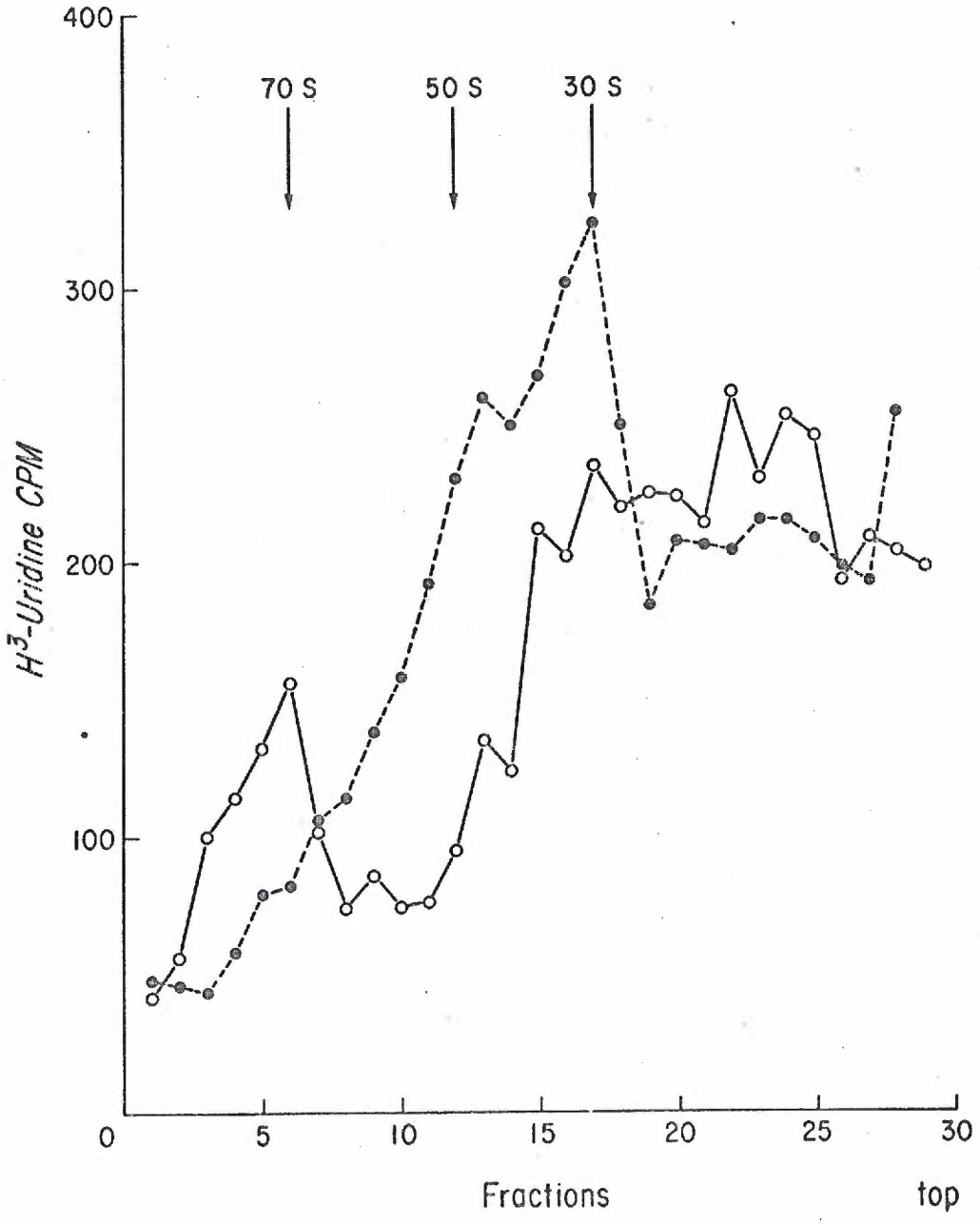

400
300
200
100
0
70 S
50 S
30 S
H³-Uridine CPM
5
10
15
20
25
30
Fractions
top

Figure 16

Sucrose gradient sedimentation profile of the H^3-RNA from C. diphtheriae C_7(-) in presence and absence of E. coli S-30.

The reaction mixturs, RNA concentration and other conditions of the experiment were as in Fig. 15.

O———O, complete reaction mixture with E. coli S-30 (from Fig. 13);

●-----●, without the S-30.

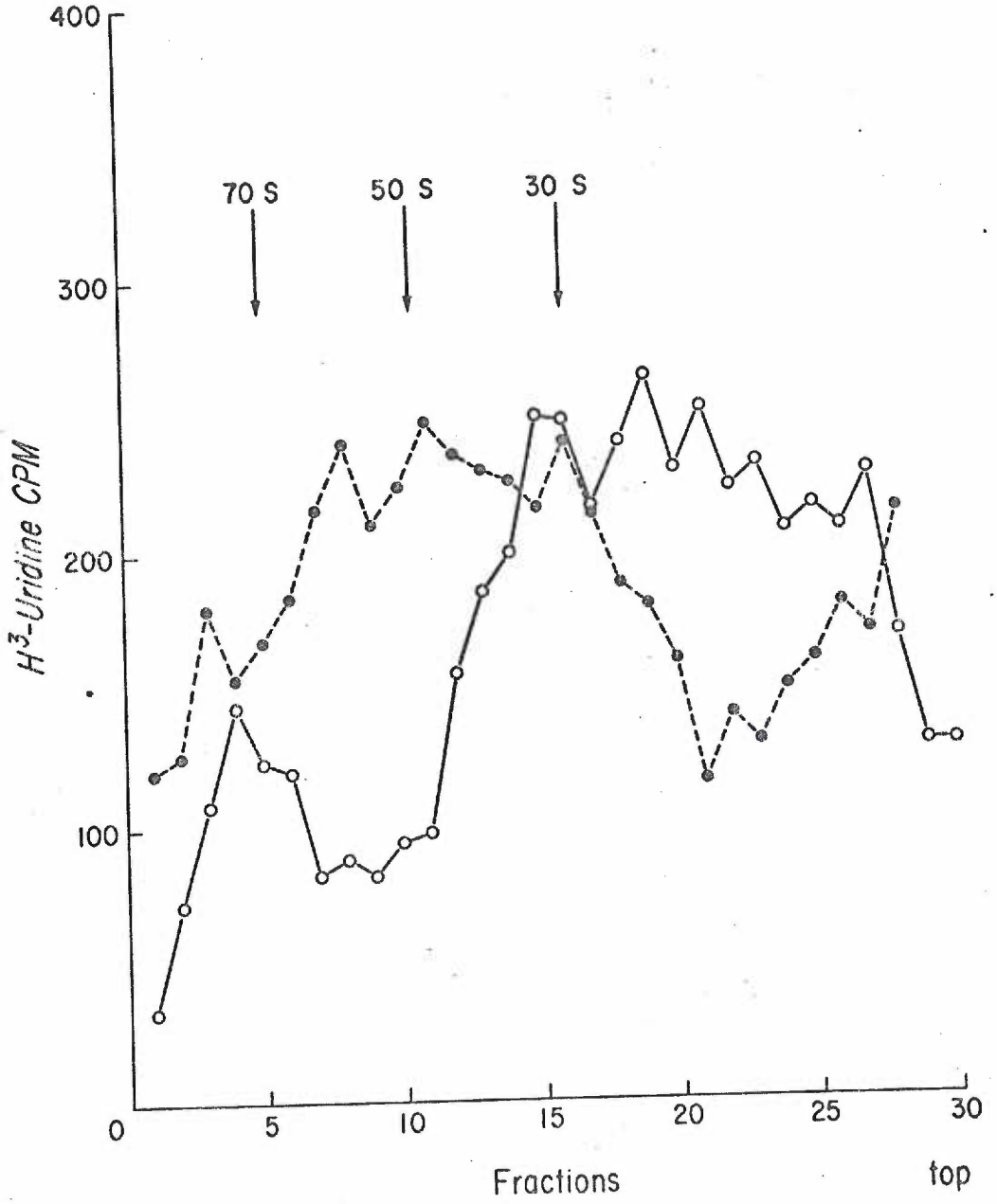

400
300
200
100
0
70 S
50 S
30 S
H³-Uridine CPM
5
10
15
20
25
30
Fractions
top

Figure 17

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with C. diphtheria PW8 RNA.

The 100 µl reaction mixture was primed with 1.2 mg/ml PW8 RNA. Polypeptides were labeled with C^{14}-lysine for 3 min at 35 C. Chloramphenicol was added and the reaction mixture rapidly chilled. 100 µl was layered on a 5 ml 10 - 30% exponential sucrose gradient in standard buffer minus 2-mercaptoethanol. The gradient was centrifuged for 75 min at 165,000 x g at 5 C. Fractions were collected. The A_{260} and the hot acid insoluble material in 50 µl samples of each gradient fraction were determined.

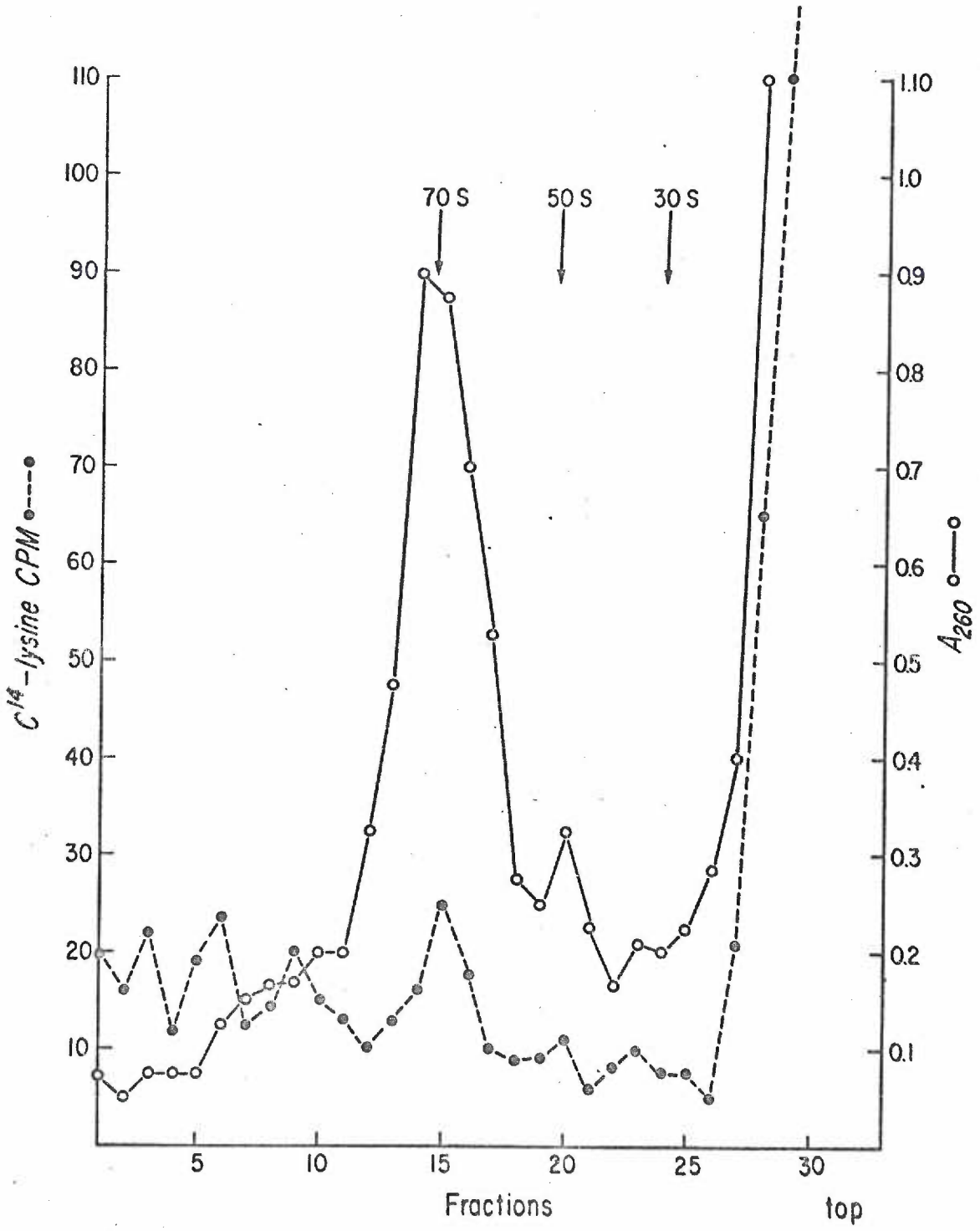

110
100
90
80
70
60
50
40
30
20
10
C14-lysine CPM
70 S
50 S
30 S
1.10
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A260
5
10
15
20
25
30
Fractions
top

Figure 18

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with C. diphtheriae $C_7(-)$ RNA.

The 100 µl reaction mixture was primed with 0.8 mg/ml $C_7(-)$ RNA. All other operations were as described in Fig. 17.

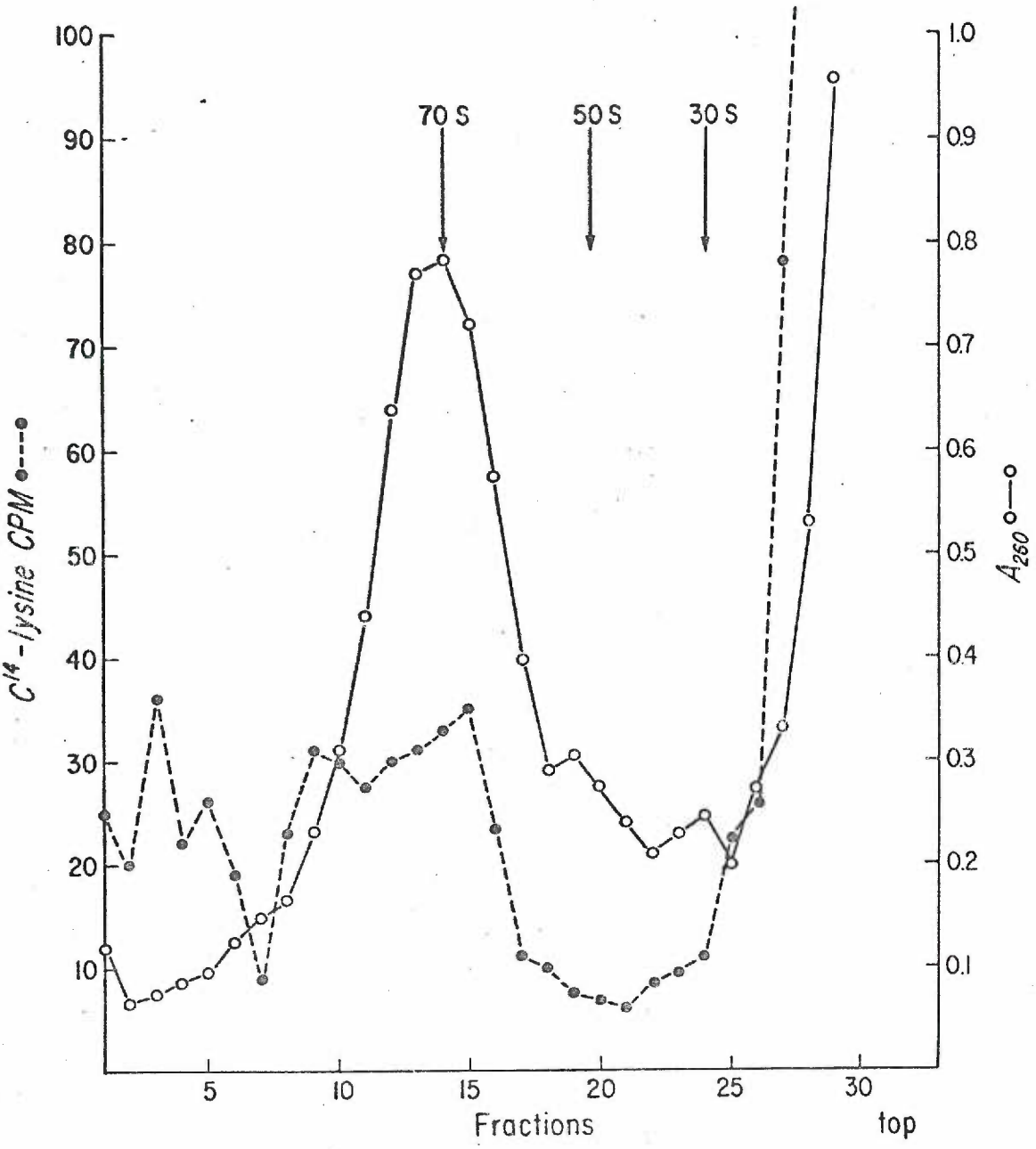
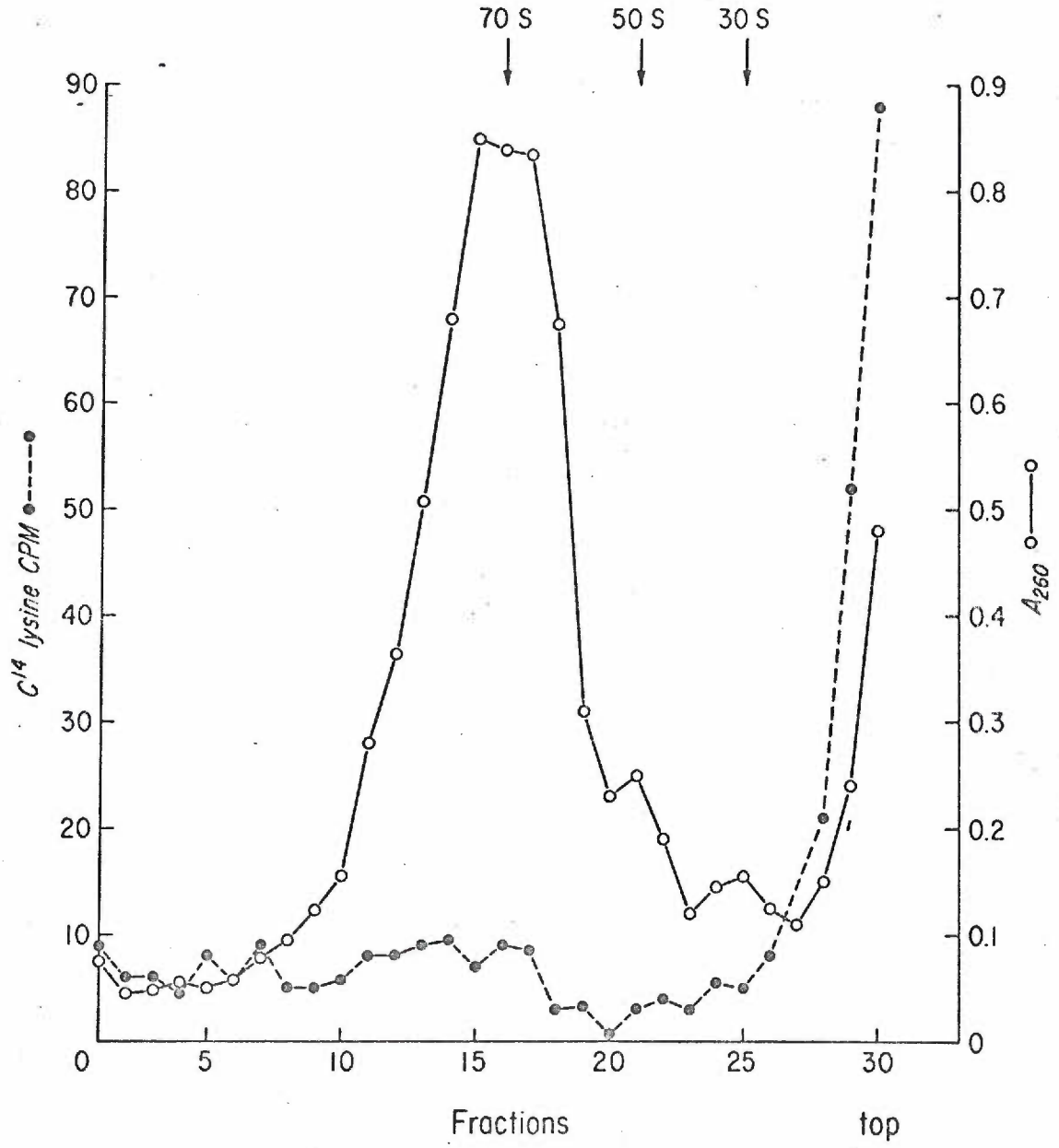

70 S
50 S
30 S
C14-lysine CPM
A260
Fractions
top
100
90
80
70
60
50
40
30
20
10
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30

Figure 19

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with endogenous RNA.

The 100 µl reaction mixture was incubated at 35 C for 3 min in absence of added RNA. All other operations were as described in Fig. 17.


70 S
50 S
30 S
90
80
70
60
50
40
30
20
10
0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C14 lysine CPM
A260
0
5
10
15
20
25
30
Fractions
top

reaction mixtures primed with PW8 RNA, C_7(-) RNA and endogenous RNA. The results indicated that more labeled polypeptides were associated with the ribosomes in the reaction mixtures primed with the exogenous C. diphtheriae RNA than with the endogenous RNA. Labeled polypeptides were present in the single ribosome region and also in the polyribosomes region, although definite peaks of dimers and trimers were not discernible.

An endogenous reaction mixture analyzed after incubation for 3 min in an ice bath (Fig. 20) showed that the polypeptide synthesis was temperature-dependent since there were less C^{14}-polypeptides present in the ribosomes region than when the same reaction mixture had been incubated at 35 C for 3 min (Fig. 19). These experiments indicated active protein synthesis on the E. coli ribosomes directed by C. diphtheriae RNA and the temperature dependence of the polypeptide synthesis by E. coli cell-free lysate.

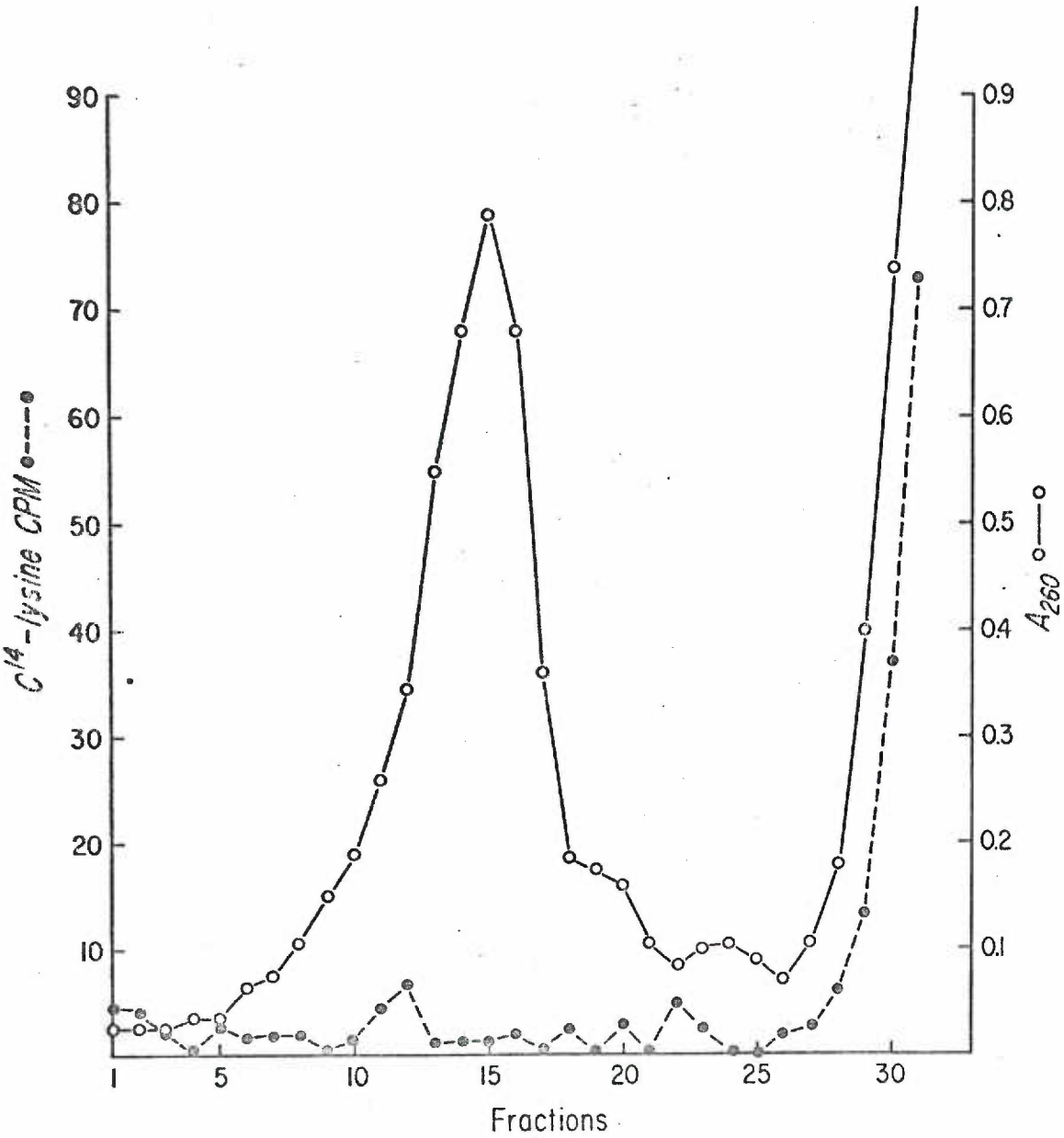
B. Analysis of the in vitro synthesized polypeptides directed by RNA from C. diphtheriae for presence of diphtheria toxin or toxin fragments

1. Immune-specific precipitation of the in vitro synthesized polypeptides with diphtheria antitoxin. The purpose of this experiment was to detect which, if any, of the RNA preparations were capable of directing the synthesis of diphtheria toxin in vitro. RNA from $C_7\beta v$ tox^+, PW8 and C_7(-) cells was used to prime the in vitro system.

Figure 20

Sucrose gradient sedimentation profile of the cell-free protein synthesizing mixture primed with endogenous RNA and incubated at 0 C.

The 100 µl reaction mixture was incubated for 3 min at 0 C. All other operations were as described in Fig. 17.


90
80
70
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0.9
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0.4
0.3
0.2
0.1
1
5
10
15
20
25
30
Fractions
C14-lysine CPM •---•
A260 o—o

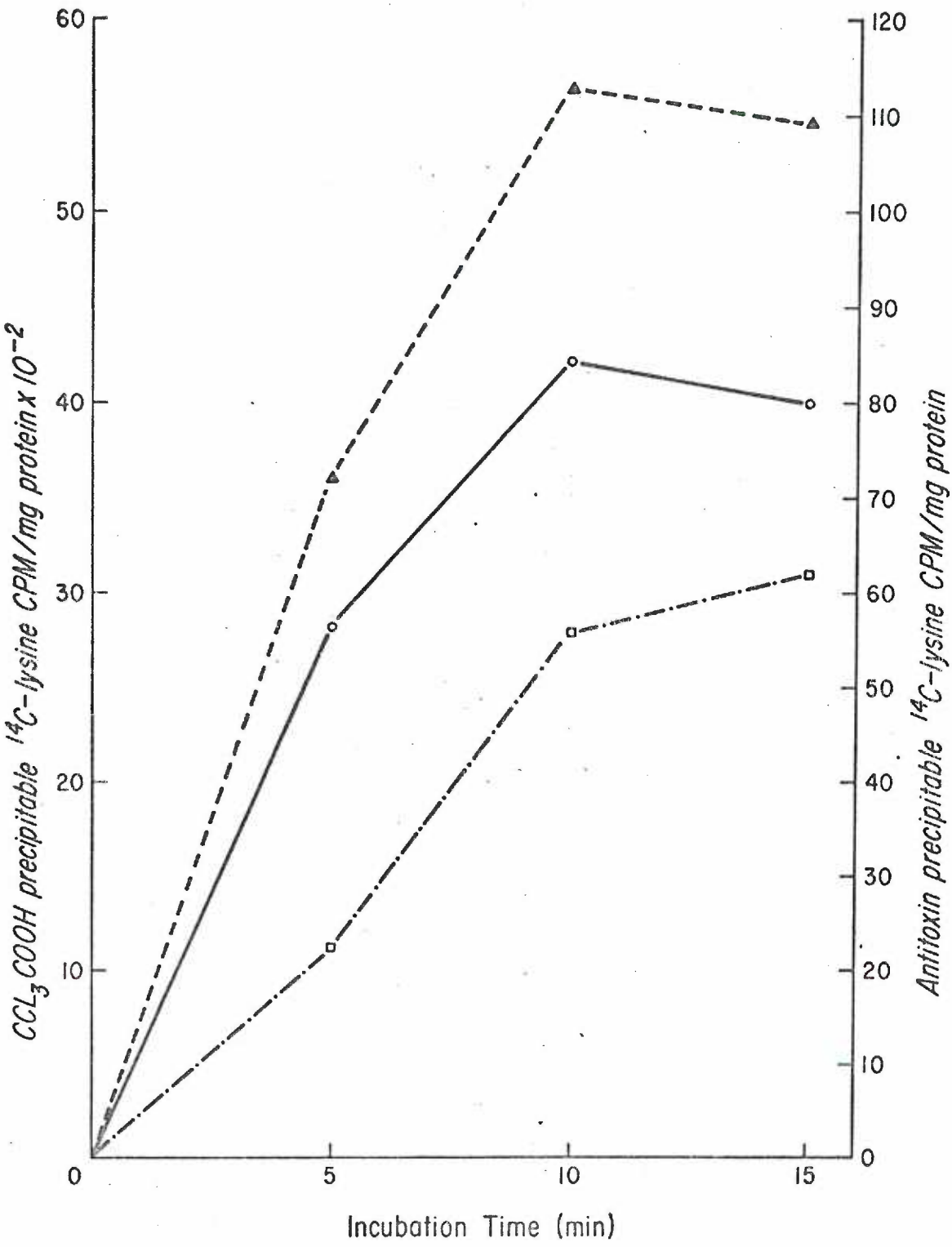
The reaction mixtures were incubated for 5, 10, or 15 min at 35 C with the test RNA. Polypeptides were released with EDTA and tested for the presence of proteins specifically precipitable with the purified horse or rabbit antitoxins as described in Methods and Materials. The total hot CCl_3COOH precipitable radioactivity was also -etermined for each supernatant. After correction for non-specifically precipitable radioactivity, the results were expressed as counts per min (cpm) per mg protein in each supernatant. Fig. 21 and 22 indicate that immune-specific polypeptides were precipitable from the cell-free reaction mixtures primed with RNA from toxinogenic C. diphtheriae $C_7\beta v$ tox^+ and PW8.

The purified horse antitoxin precipitated a higher proportion of the antitoxin-specific polypeptides than the rabbit antitoxin. The reason for this may have been that the horse antitoxin, being a flocculating antibody, was able to react more avidly with the synthesized toxin and/or the horse antitoxin contained antibodies to the other bacteriophage proteins which were also synthesized in vitro. The horse antitoxin precipitated about 2.7% of the total acid insoluble polypeptides from the reaction mixtures primed with $C_7\beta v$ tox^+ RNA and about 1.6% of the polypeptides from the reaction mixtures primed with PW8 RNA. This ratio was 1.6% and 1.0% respectively with rabbit antitoxin. Although $C_7(-)$ RNA was capable of stimulating amino acid incorporation in the cell-free lysate (Fig. 23), less than 0.4% of the polypeptides synthesized in the reaction mixtures

Figure 21

Immune precipitation of C^{14}-polypeptides from supernatants of the cell-free protein synthesizing mixture primed with $C_7\beta v$ tox^+ RNA.

The reaction mixture primed with $C_7\beta v$ tox^+ RNA were incubated at 35 C for 5, 10, and 15 min. The supernatants containing the labeled polypeptides were collected and precipitated with horse and rabbit antitoxins and normal rabbit serum. Total hot CCl_3COOH precipitable material in 100 µl of each supernatant was determined. 500 µl of each supernatant containing about 750 µg protein was mixed with 100 µl absorbed horse antitoxin (500 units/ml) and 50 µl crude diphtheria toxin (2.2 mg/ml). The same volume of each supernatant was mixed with 100 µl rabbit antitoxin (220 units/ml) and 20 µl diphtheria toxin. All toxin antitoxin precipitations were at equivalence zone. 500 µl of each supernatant was mixed with 100 µl normal rabbit serum to determine non-specifically precipitable radioactivity. All tubes (in triplicate) were incubated 1 hr at 37 C and 48 hr at 4 C. The precipitates were washed 3 times with 5 ml chilled borate saline buffer collected on type HA filters and the radioactivity on each filter was determined. Non-specifically precipitable radioactivity with normal rabbit serum was subtracted from that precipitable with the antitoxins. Results are presented CPM/mg protein in the supernatants. O——O hot CCl_3COOH precipitable material; Δ----Δ horse antitoxin precipitable polypeptides; □-·-·-□ rabbit antitoxin precipitable material.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20
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120
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10
15
Incubation Time (min)
CCl_3COOH precipitable ^{14}C-lysine CPM/mg protein x 10^{-2}
Antitoxin precipitable ^{14}C-lysine CPM/mg protein

Figure 22

Immune precipitation of C^{14}-polypeptides from the supernatants of cell-free protein synthesizing mixtures primed with PW8 RNA.

The experimental procedure and presentation of results is as in Fig. 21. 0——0 hot CCl_3COOH precipitable material; Δ----Δ horse antitoxin precipitable material; □-.-.-□ rabbit antitoxin precipitable material.

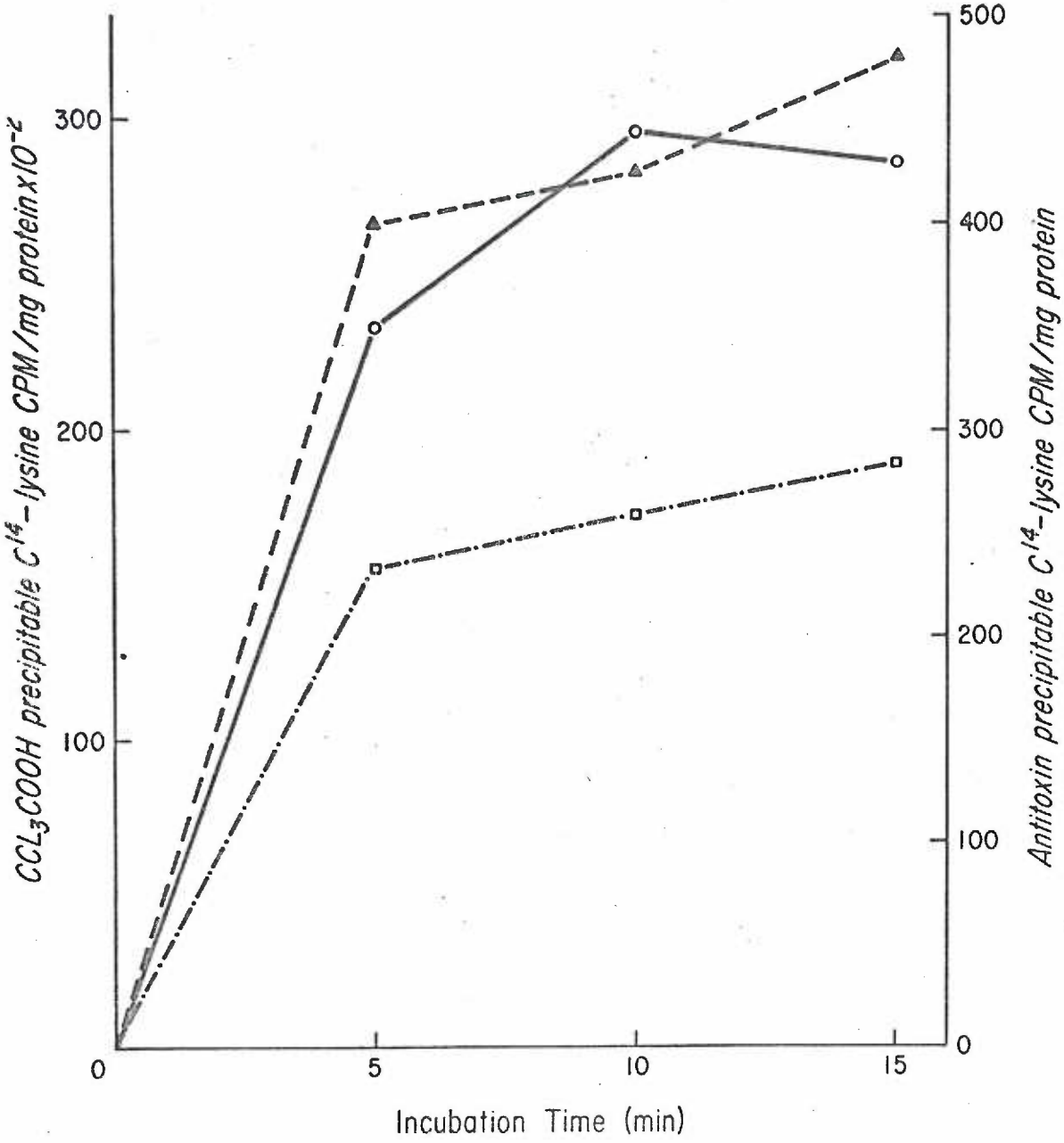
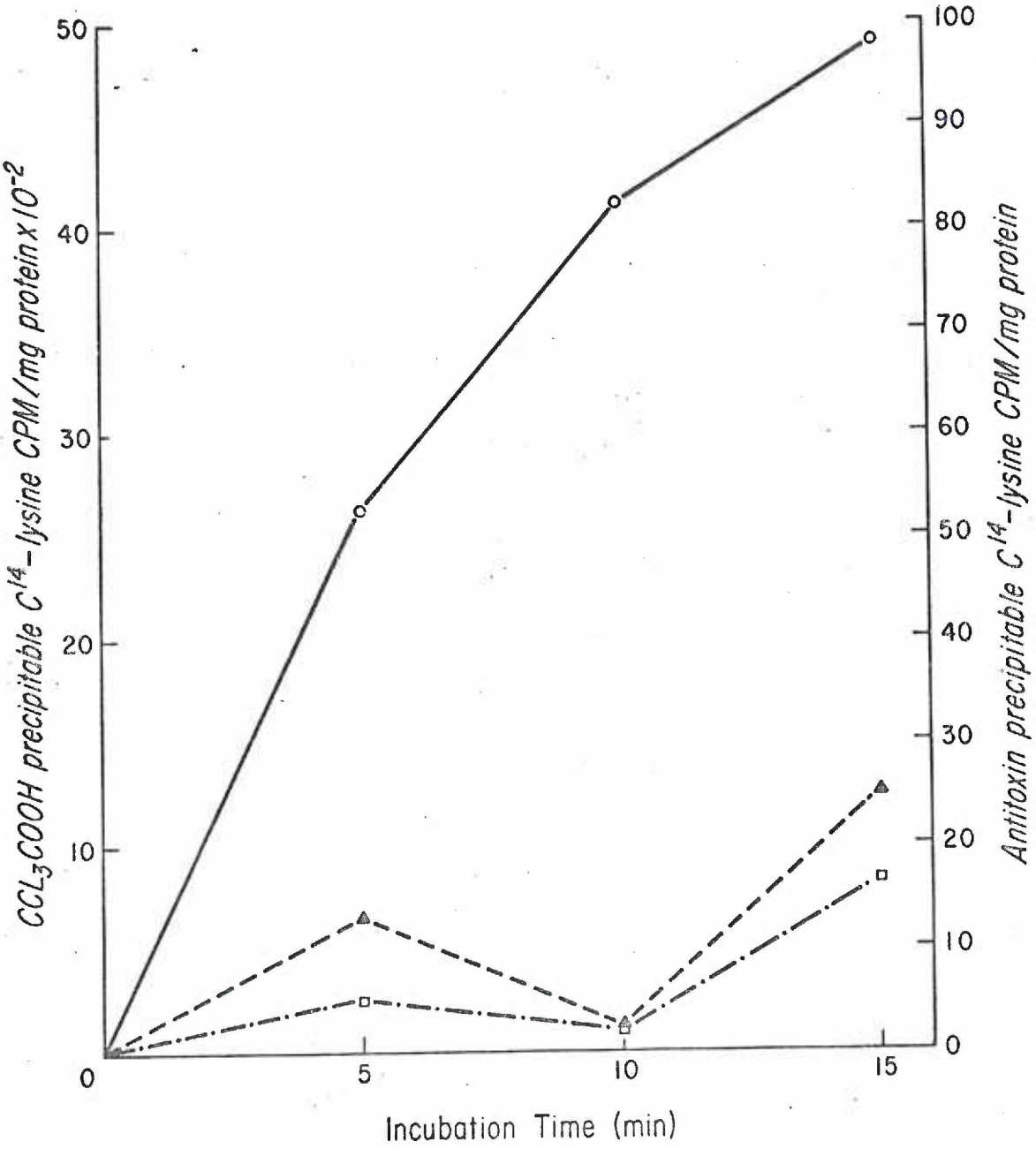

CCL_3COOH precipitable C^{14}-lysine CPM/mg protein x 10^{-2}
Antitoxin precipitable C^{14}-lysine CPM/mg protein
Incubation Time (min)
0
5
10
15
100
200
300
400
500

Figure 23

Immune precipitation of C^{14}-polypeptides from the supernatants of cell-free protein synthesizing mixtures primed with C_7(-) RNA.

The experimental procedure and presentation of results were as in Fig. 21. O——O hot CCl_3COOH precipitable material; Δ-----Δ horse antitoxin precipitable material; □-·-·-□ rabbit antitoxin precipitable material.


50
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20
10
0
5
10
15
Incubation Time (min)
CCL_3COOH precipitable C^{14}-lysine CPM/mg protein x 10^{-2}
Antitoxin precipitable C^{14}-lysine CPM/mg protein

primed with C_7(-) RNA precipitated with the antitoxins.

The results indicated that while total acid insoluble protein synthesized in each in vitro reaction mixture depended upon the particular RNA preparation, the synthesis of antitoxin precipitable proteins depended on the source of RNA. RNA from toxinogenic cells directed the synthesis of polypeptides which were specifically precipitable with the antitoxins, RNA from nontoxinogenic cells did not. Furthermore, the kinetics of incorporation of C^{14}-lysine into hot acid precipitable proteins and immune precipitable proteins were the same when RNA from toxinogenic cells directed the protein synthesis indicating that immune-specific proteins were synthesized at the same rate as the other proteins.

2. Investigation of ADP-ribosylation of mammalian elongation factor 2 by Fragment A of diphtheria toxin in the presence of E. coli cell-free protein synthesizing mixture. Fragment A of diphtheria toxin has a unique enzymatic activity. It covalently binds the adenosine-diphosphoribose moiety of NAD^+ to the mammalian elongation factor 2. This enzymatic reaction was to be used to determine if Fragment A of diphtheria toxin was synthesized in the in vitro system. Therefore, the purpose of this experiment was to investigate the possible interference of the E. coli cell-free protein synthesizing mixture with the enzymatic activity of Fragment A of diphtheria toxin.

Complete E. coli protein synthesizing system was mixed with either trypsin-digested diphtheria toxin or Fragment A. After

15 min incubation at 35 C, a volume of 110 µl of each mixture was assayed for ADP-ribosylation of rabbit reticulocyte elongation factor 2 enzyme in presence of C^{14}-NAD^+. The E. coli cell-free protein synthesizing system without the added Fragment A or digested toxin was also tested for its ADP-ribosylation activity to rule out the possibility that the E. coli S-30 could non-specifically bind NAD^+ to the EF2 enzyme or any other protein. The ADP-ribosylation could not be detected in any of the assay mixtures. Collier and Kandel (1971) have shown that ADP-ribosylation by Fragment A is inhibited by NH_4^+, Mg^{++}, Cl^- and RNA. All these molecules are present in the E. coli cell-free system. Therefore, the ADP-ribosylation could have been inhibited by the presence of these molecules. Furthermore, the concentration of unlabeled NAD^+ in the S-30 was not known, but it could have been enough to effectively dilute the C^{14}-NAD to a level at which the detection of ADP-ribosylation by the radioactive labeling technique became impossible. Therefore, to remove the small inhibitory ions and the unlabeled NAD^+, the E. coli cell-free lysate containing the digested toxin or Fragment A, along with the control E. coli cell-free lysate were dialyzed against 50 mM Tris-Hcl pH 8.2 and 0.1 mM EDTA for 48 hr with frequent changes of buffer. ADP-ribosylation of elongation factor 2 was then assayed using the dialyzed mixtures. The results of both assays before and after dialysis are presented in Table 2. Following dialysis ADP-ribosylation, activity was detected in the reaction mixtures which contained digested

Table 2

ADP-ribosylation of mammalian elongation 2 (EF2) enzyme by Fragment A and trypsin-digested diphtheria toxin in presence and absence of *E. coli* cell-free lysate and the effect of dialysis prior to ADP-ribosylation.

Additions to the ADP-ribosylation assay[a]	µg Toxin or Fragment A	CPM ADPR-EF2*
Digested toxin	150	1700
Fragment A	6	1650
None	-	40
Before dialysis:[b]		
Digested toxin + *E. coli* lysate	150	33
Fragment A + *E. coli* lysate	6	37
E. coli lysate	-	31
After dialysis:[c]		
Digested toxin + *E. coli* lysate	150	2100
Fragment A + *E. coli* lysate	6	1110
E. coli lysate	-	35

* adenosine-diphosphoribose elongation factor 2 precipitated by CCl_3COOH

[a] ADP-ribosylation assay in total volume of 250 µl contained in final concentrations 50 mM Tris-HCl pH 8.2, 0.1 mM EDTA, 40 mM dithriothreitol, 100 µl EF2 enzyme and 50 nM (C^{14}-adenine)NAD^+. Incubation time 15 min at 25 C.

Table 2, continued

[b] Complete E. coli cell-free lysate (500 µl) was mixed with 50 µl (750 µg) trypsin-digested diphtheria toxin or 50 µl (30 µg) Fragment A and incubated 15 min at 35 C. A volume of 110 µl of each mixture was assayed. E. coli lysate control (500 µl) received 50 µl H_2O.

[c] Mixtures in [b] were dialysed against 50 mM Tris-HCl pH 8.2, 0.1 mM EDTA and were reassayed.

toxin or Fragment A. Compared to the controls the enzymatic activity of the digested toxin increased and that of Fragment A decreased. The increase in activity of digested toxin may have been due to further digestion of some remaining intact toxin molecules by the proteolytic enzymes of the E. coli S-30 during the dialysis. The same proteolytic enzymes may have hydrolyzed some Fragment A and effectively reduced its concentration. The E. coli cell-free protein synthesizing system did not bind C^{14}-NAD^{+} in the absence of digested diphtheria toxin or Fragment A.

The results of this experiment indicated that extensive dialysis of the E. coli cell-free protein synthesizing system to remove the inhibitory molecules was necessary before the ADP-ribosylation could be detected. Therefore, in experiments where this assay system was to be used for the detection of in vitro synthesized Fragment A of diphtheria toxin, the ribosomes were pelleted to reduce the concentration of particulate matter and the supernatants were extensively dialyzed before the ADP-ribosylation assay.

3. ADP-ribosylation activity of the cell-free protein synthesizing mixtures primed with C. diphtheriae. While immune precipitation detected de novo synthesis of toxin or toxin fragment in vitro, it could not distinguish between biologically active and inactive products. The aim of this experiment was to detect de novo synthesis of Fragment A of diphterhia toxin in the E. coli cell-free lysate primed with C. diphtheriae RNA by the ADP-ribosylation activity

of Fragment A.

The E. coli cell-free protein synthesizing mixtures were primed with RNA from $C_7\beta v$ tox^+, PW8 and $C_7(-)$ C. diphtheriae. After 15 min incubation at 35 C to allow protein synthesis, the polypeptides were released in 0.1 mM EDTA and the ribosomes were pelleted. The supernatants were dialyzed against 50 mM Tris-HCl pH 8.2 and 0.1 mM EDTA for 48 hr. A volume of 100 µl of each supernatant was assayed for the presence of ADP-ribosylation activity using elongation factor 2 enzyme from rabbit reticulocyte and C^{14}-NAD^+. Table 3 shows that when the supernatants of the E. coli cell-free lysate primed with RNA from C. diphtheriae were tested for the ADP-ribosylation activity adenosine diphosphoribose-EF2 complex was only formed in the presence of the supernatants of the cell-free lysate primed with $C_7\beta v$ tox^+ RNA or PW8 RNA. This ADP-ribosylation activity was absent in assay mixtures containing the supernatant of the cell-free lysate primed with $C_7(-)$ RNA. Finally in order to rule out the possibility that the RNA used to prime the cell-free lysate was contaminated with Fragment A of toxin, PW8 RNA was tested for its ADP-ribosylation activity and was found to be free from Fragment A activity under these conditions.

4. Rabbit skin test. Intact molecules of diphtheria toxin injected intracutaneously into rabbit skin leads to localized erythema and necrosis evident 48 to 96 hr later. An amount of 10^5 MRD (minimum reactive dose) corresponds to 2 µg (1 Lf) toxin protein (Matsuda and Barksdale, 1967). Therefore, taking the molecular

Table 3

ADP-ribosylation of mammalian elongation factor 2 by the supernatant of the cell-free protein synthesizing system primed with C. diphtheriae RNA.

Additions to the ADP-ribosylation assay[a]	CPM[b] ADPR-EF2	
Supernatant of cell-free protein synthesizing system primed with RNA from:		
C. diphtheriae PW8	100 ± 15[c]	$P < 0.05$
C. diphtheriae $C_7\beta v$ tox^+	129 ± 12	$P < 0.05$
C. diphtheriae $C_7(-)$	43 ± 17	$P > 0.05$
None	42 ± 11	
Phenol extracted PW8 RNA (2 mg)	44 ± 10	$P > 0.05$

[a] The assay mixture was similar to that in Table 2 with exception that 0.735 μM C^{14}-NAD was used with 2 hr incubation at 25 C.

[b] Each value in the table is the result of 10 ADP-ribosylation assays carried out with each supernatant. ADPR-EF2:adenosine diphosphoribose-EF2 enzyme precipitable by CCl_3COOH.

[c] 95% confidence interval.

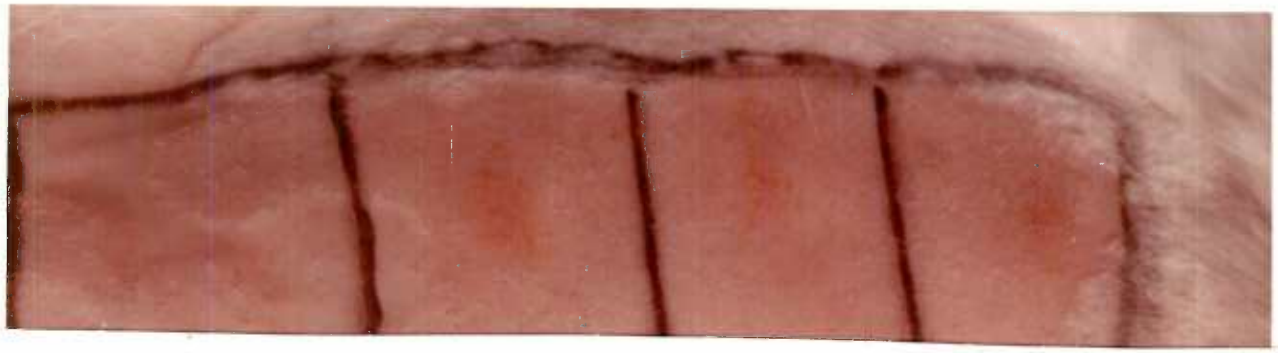
weight of the toxin as 62,000 (Gill and Dinius, 1971) approximately 2×10^8 molecules of toxin corresponding to 1 MRD are sufficient for the skin reaction. The erythema and necrosis is specifically prevented if the toxin is first neutralized with antitoxin or the animal is protected by administration of antitoxin intravenously or peritoneally (Matsuda and Barksdale, 1967). Only intact molecules of diphtheria toxin i.e., the Fragment A and Fragment B attached together by a peptide bond and/or a disulfite bridge can cause necrosis. Fragment A or B alone do not give positive skin reaction (Gill, Pappenheimer and Uchida, 1973). Since this reaction is very specific and sensitive it was used to detect de novo synthesis of biologically active intact diphtheria toxin in E. coli cell-free lysate directed by RNA from C. diphtheriae.

E. coli cell-free protein synthesizing system was primed with $C_7\beta v$ tox^+ RNA, PW8 RNA or $C_7(-)$ RNA and incubated at 35 C for 15 min. The reaction mixtures were treated with EDTA and the ribosomes were pelleted. Following dialysis against 50 mM Tris-HCl pH 8.2 containing 0.1 mM EDTA the supernatants were concentrated tenfold and 3 mg protein of each supernatant was injected intradermally into the back of a rabbit as described in Materials and Methods. Fig. 24 shows that erythema and necrosis were evident at the injection sites of the toxin and the supernatants of the cell-free protein synthesizing mixtures primed with $C_7\beta v$ tox^+ RNA and PW8 RNA. There was no necrosis where toxin or the above supernatants neutralized with rabbit antitoxin had been injected. Furthermore, supernatant of the

Figure 24

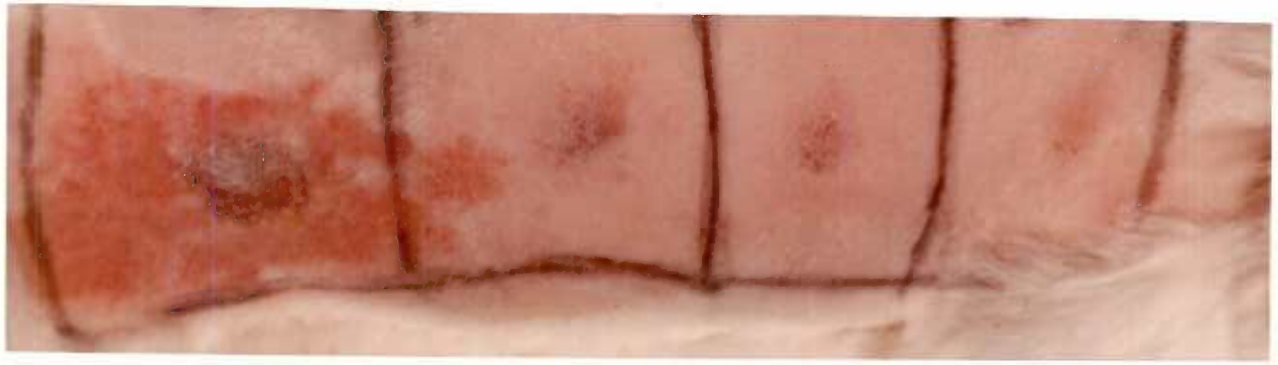
Rabbit skin test for toxicity. 3 mg concentrated supernatant in 0.2 ml volume, derived from the in vitro reaction mixtures primed with C. diphtheriae RNA was injected intradermally. Another 3 mg of each concentrated supernatant was mixed with 20 µl rabbit antitoxin and was injected in the opposite site 6 hr later. Results were read 72 hr after the first inoculation

1 A. Diphtheria toxin (5 MRD)	1 B. Diphtheria toxin (5 MRD) + antitoxin
2 A. PW8 supernatant	2 B. PW8 supernatant + antitoxin
3 A. $C_7\beta v$ tox^+ supernatant	3 B. $C_7\beta v$ tox^+ supernatant + antitoxin
4 A. $C_7(-)$ supernatant	4 B. $C_7(-)$ supernatant + antitoxin



1 B 2 B 3 B 4 B

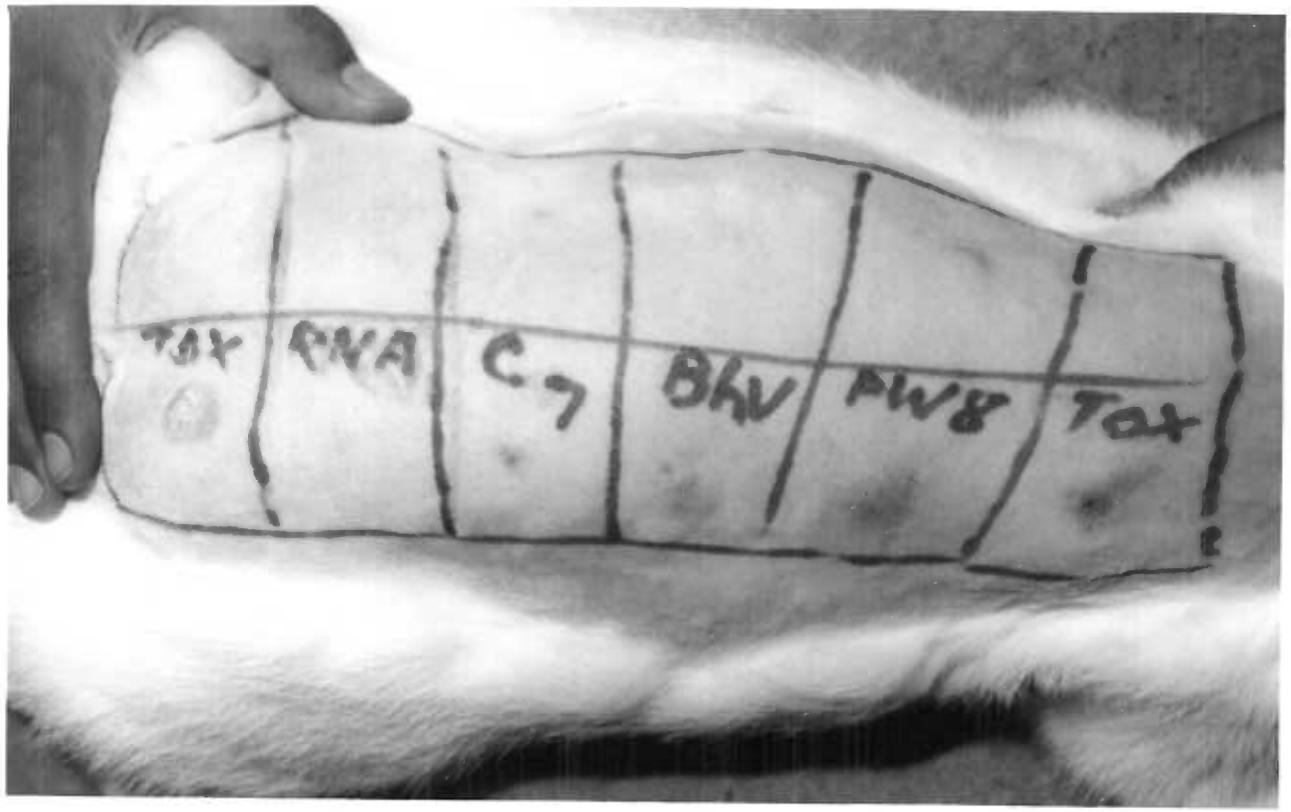
1 A 2 A 3 A 4 A



cell-free protein synthesizing mixture primed with $C_7(-)$ RNA did not cause necrosis. In a second experiment essentially the same results were obtained (Fig. 25). To insure that the rabbit skin reaction was due to the diphtheria toxin synthesized in vitro and not due to contaminating toxin in the RNA from toxinogenic C. diphtheriae, 2 mg PW8 RNA was also injected intradermally into the rabbit. The RNA did not show any skin reaction indicating absence of toxin in the RNA preparation from toxinogenic C. diphtheriae. The results indicated that biologically active diphtheria toxin was synthesized by translation of RNA from toxinogenic C. diphtheriae only. RNA from non-toxinogenic cells did not direct the synthesis of toxin.

Figure 25

Rabbit skin test for toxicity. Concentrated supernatants were prepared and treated as in Fig. 24. From left to right, bottom row, 2 MRD toxin, 2 mg PW8 RNA, 3 mg $C_7(-)$ supernatant, 3 mg $C_7\beta v$ tox^+ supernatant, 3 mg PW8 supernatant, 1 MRD toxin. Top row, the above materials neutralized with rabbit antitoxin and injected 6 hr later. Test read 72 hr later.


TAX
RNA
C7
BKV
HW8
TAX

DISCUSSION

Two aspects of the in vitro translation of C. diphtheriae RNA in the E. coli cell-free lysate were explored. One was to isolate RNA which had messenger activity from C. diphtheriae and to determine parameters influencing the translation of this RNA in the E. coli lysate. The second aspect was to determine if diphtheria toxin or toxin fragments were synthesized as the result of translation of mRNA from the toxinogenic C. diphtheriae. Three separate methods were used to detect the de novo synthesis of diphtheria toxin and toxin fragments. These methods were immune precipitation of toxin with antitoxin, NAD^+-dependent ADP-ribosylation of mammalian peptide elongation factor 2 enzyme (EF2) catalyzed by the A fragment of the diphtheria toxin, and necrosis of rabbit skin produced by intradermal injection of diphtheria toxin.

Three sources of C. diphtheriae were used for extraction of RNA: Park-Williams No. 8, which is a lysogenic, toxinogenic C. diphtheriae, $C_7(-)$ cells infected with a hypervirulent tox^+ β corynebacteriophage (C_7 βv tox^+) and the nontoxinogenic, nonlysogenic $C_7(-)$ cells. The latter source of RNA acted as a suitable control since $C_7(-)$ cells do not synthesize toxin. The RNA was extracted from the C. diphtheriae cells cultured in low iron medium under optimum conditions for toxin synthesis. The PW8 cells and the $C_7(-)$ cells were harvested in mid-log phase.

The C_7 βv tox^+ cells were harvested at 45 min post-infection, a time when the cells were actively engaged in phage and toxin synthesis (Matsuda and Barksdale, 1966). Attempts to isolate C. diphtheriae RNA without prior grinding of the cells to disintegrate the cell wall was not successful. Only 4S RNA was obtained from these cells and this RNA did not have messenger activity. Therefore, the cells were ground with alumina to break up the cell wall and the RNA was then extracted. The sucrose gradient sedimentation of the RNA extracted by this method indicated that three species of 23S, 16S and 4S were present. The 4S peak was large which may indicate that some cells remained intact after grinding with alumina and subsequently yielded 4S RNA. On the other hand, C. diphtheriae may contain more 4S RNA than has been observed in RNA extracted from E. coli (Salser, Gesteland and Ricard, 1969). The sedimentation profile of C. diphtheriae RNA in the sucrose gradients was not skewed towards the top of the gradients indicating that nuclease activity or shearing of the RNA was minimal during the extraction.

To investigate the synthesis of RNA in the βv tox^+ infected cells at the time of harvest, these cells were pulsed with H^3-uridine for 15 sec before harvest. The specific activity of the H^3-RNA isolated from phage-infected cells was the same as the control cells. This indicated that there was not an overt decrease or increase in RNA synthesis in the phage-infected cells. However,

Elwell and Iglewski[1] noticed a decrease in ribosomal RNA synthesis in the phage-infected cells at 45 min post-infection. Therefore it appears that in the infected cells there is a shift from the bacterial to viral RNA synthesis without a change in the overall level of uridine incorporation. The pulse-labeled H^3-RNA from infected and noninfected cells sedimented differently in sucrose gradients. The H^3-RNA from the infected cells contained larger molecular species than the H^3-RNA from the noninfected cells. This observation reflected that the shift in the RNA synthesis from that of the host to that of the phage was accompanied by a change in the size of RNA molecules being synthesized during the 15 sec pulse of H^3-uridine.

The phenol extracted RNA from C. diphtheriae PW8, C_7 βv tox^+ and C_7(-) stimulated amino acid incorporation in the E. coli cell-free lysate at least three times over that of the endogenous. The level of incorporation depended on the RNA preparation rather than the cell source. When increasing amounts of RNA were present, the incorporation of C^{14}-lysine increased, but it eventually reached a maximum after which increasing the concentration of RNA actually inhibited amino acid incorporation. This may be the same observation which was reported by Salser, Gesteland and Bolle (1967) with RNA from E. coli and E. coli infected with T4 phage. The presence of ribosomal RNA may actually interfere with the translation of mRNA at high RNA concentrations. Therefore, the optimum concentration of RNA for maximum incorporation of amino acids was determined for

[1]Elwell, L. P. and Iglewski, B. H., Personal communication, 1971.

each new batch of RNA before the RNA was used in subsequent experiments. This concentration depended on the particular preparation of RNA rather than its cell source.

A comparison between the messenger activity of R17 RNA which is all messenger RNA and C. diphtheriae RNA indicated that the latter was probably as active as R17 RNA in directing protein synthesis in the E. coli cell-free system. The concentration of mRNA in the C. diphtheriae cells is not known but results reported for E. coli infected with T4 bacteriophage, E. coli and Bacillus subtilis indicated 3%, 5%, and 9% of the total RNA was mRNA (Nygaard and Hall, 1964; Salser, Janin and Levinthal, 1968). If one assumes that the mRNA concentration in C. diphtheriae is 10% or less of the total RNA in C. diphtheriae the RNA preparations used in these experiments were more active in directing protein synthesis than an equivalent amount of R17 RNA.

The in vitro translation of natural mRNA occurs at a lower magnesium concentration than the translation of synthetic mRNA (Modolell, 1971). Binding of natural mRNA with the ribosomes through the AUG initiation codon, the f-met tRNA and the initiation factors requires 7-12 mM Mg^{++}. C. diphtheriae RNA directed incorporation of amino acid maximally at Mg^{++} concentration of 12 mM. This observation suggested that C. diphtheriae RNA was using the normal E. coli initiation process involving f-met tRNA and the E. coli initiation factors (Rekosh, Lodish, Baltimore, 1969). Higher

concentrations of Mg^{++} were inhibitory to translation of C. diphtheriae RNA. Increased misreading of the RNA in vitro was reported to occur at optimal Mg^{++} concentrations (Salser, Gesteland, and Bolle, 1967; Capecchi, 1967). To reduce misreading of the message and to obtain better fidelity of translation 10 mM Mg^{++} concentration was used in the present studies. With 10 mM Mg^{++} concentration the H^3-RNA from C_7(-) cells and C_7 βv tox^+ cells attached to the E.coli ribosomes. The complex of H^3-RNA and the ribosomes sedimented in the sucrose gradients just ahead of the single ribosomal peak. Furthermore nascent polypeptides were found attached to this complex of ribosomes and C. diphtheriae RNA.

The polypeptides synthesized in vitro by translation of C. diphtheriae RNA were tested for presence of diphtheria toxin or toxin fragments by immune precipitation with specific antitoxins. The horse antitoxin from Wellcome Research Laboratories contained antibodies to C_7(-) cells and E. coli cell extract. Therefore, it was absorbed 4 times with the two cell extracts to remove these antibodies. Furthermore rabbit antitoxin was prepared by immunization of rabbit with toxoid made from ultra-purified toxin and used in the present studies.

Only polypeptides synthesized by translation of RNA from C_7 βv tox^+ and PW8 cells precipitated with horse and rabbit antitoxins. The horse antitoxin precipitated a larger percentage of the

polypeptides from both C_7 βv tox^+ and PW8 RNA-primed in vitro systems than did the rabbit antitoxin. There are several possible explanations for this observation. The horse antitoxin may have contained antibodies to more antigenic determinants on the toxin molecule than the rabbit antitoxin and therefore the horse antitoxin precipitated more of the synthesized toxin polypeptides than the rabbit antitoxin. Although the horse antitoxin was absorbed with C_7(-) and E. coli extracts and as judged by immunodiffusion in agar gel it no longer reacted against these two antigens it still might have contained antibodies to nontoxin proteins of corynebacteriophages or C. diphtheriae which were synthesized in the cell-free system. Another possibility for the horse antitoxin precipitating more toxin specific polypeptide than the rabbit antitoxin is that the latter may have contained antibodies with greater affinity for the toxin. Horse antitoxin is mainly 19S immunoglobulin, the precipitate formed is more complete and the antigen-antibody precipitates do not dissociate on washing with buffered saline as much as the rabbit antibody-antigen precipitates (Kabat and Mayer, 1961). Any one or all of the above factors may have contributed to the observation of greater avidity of the horse antitoxin. Although the rabbit antitoxin was not as avid as the horse antitoxin, the former was made against purified toxin, thus it was more specific in precipitating toxin and toxin fragment than the horse antitoxin.

Although both C_7 βv tox^+ RNA and PW8 RNA directed the synthesis of antitoxin-specific polypeptides, a higher percentage of polypeptides from the reaction mixtures primed with C_7 βv tox^+ RNA precipitated with both antitoxins. In the cell-free lysates primed with C_7 βv tox^+ RNA and incubated for 15 min, 2.7% of the total protein precipitated with the horse antitoxin and 1.6% with rabbit antitoxin. The ratios in the cell-free systems primed with PW8 RNA, were 1.7% and 1% respectively.

The immune-specific polypeptides increased in the cell-free reaction mixtures at the same rate as the other proteins indicating that preferential translation of toxin mRNA did not occur. Michel et al. (1972) determined that there are 42 lysine residues in the diphtheria toxin molecule equally distributed between Fragment A and Fragment B of the toxin. According to the specific activity of the C^{14}-lysine used in these experiments and the efficiency of the liquid scintillation counter employed for C^{14} radio-isotope counting, 1.5 p mole lysine was equivalent to 1000 CPM. The rabbit antitoxin precipitable CPM was equivalent to 0.09 p mole lysine incorporated into rabbit antitoxin-specific polypeptides when the cell-free systems primed with C_7 βv tox^+ RNA were incubated for 15 min. In the cell-free systems primed with PW8 RNA 0.42 p mole lysine was incorporated into rabbit antitoxin specific polypeptides. Calculating the amount of toxin synthesized (assuming equal distribution of lysine in the toxin

molecule and 42 lysine residues per molecule) approximately 0.002 p mole toxin was synthesized in cell-free protein synthesizing systems primed with C_7 βv tox^+ RNA and 0.010 p mole in the systems primed with PW8 RNA. However, PW8 RNA preparation used for these experiments had a higher overall messenger RNA activity than the βv tox^+ RNA. The PW8 RNA stimulated C^{14}-lysine incorporation seven times more than the βv tox^+ RNA. The ratio of rabbit antitoxin specific protein to total protein synthesized was higher in the system primed with C_7 βv tox^+ RNA than with PW8 RNA (1.6% to 1%). This observation may reflect that RNA from C_7 βv tox^+ cells, which were actively synthesizing toxin and phage (Matsuda and Barksdale, 1966), contained more copies of toxin messenger RNA than the RNA from PW8 cells which were lysogenic but were not induced (Barksdale, Garmise and Horibata, 1961). The cell-free systems primed with C_7(-) RNA incorporated amino acids efficiently but only about 0.3% of the total proteins were precipitable with rabbit antitoxin. The radioactivity in polypeptides precipitable with rabbit antitoxin from the in vitro reaction mixtures primed with C_7(-) RNA was low and probably within the limits of the experimental error for detection of C^{14}-lysine.

While immune precipitation detected de novo synthesis of toxin by translation of RNA from C_7 βv tox^+ cells and PW8 cells, it could not distinguish between biologically active and inactive products. Fragment A of diphtheria toxin has a unique enzymatic activity. It covalently links the adenosine diphosphoribose moiety of NAD^+ to the

mammalian elongation factor 2 enzyme thereby inhibiting protein synthesis in the cell (Honjo et al., 1968). This specific reaction takes place with EF2 enzyme from all eucaryotic sources (Pappenheimer and Gill, 1973) and no other protein can replace Fragment A or EF2 enzyme in this reaction. (Gill and Pappenheimer, 1973). The G factor of procaryote (equivalanet to EF2 enzyme) is not a substrate for the reaction (Gill and Pappenheimer, 1973). The results in this thesis showed that when the ADP-ribosylation assay was carried out with trypsin-digested toxin or isolated A fragment in the presence of E. coli S-30, the ADP-ribosylation was detectable only after the dialysable molecules had been removed. There are at least two explanations for this observation. NH_4^+, Mg^{++}, Cl^- and/or RNA present in the S-30 may have inhibited the ADP-ribosylation reaction. The inhibitory effect of these molecules has been reported (Collier and Kandel, 1971). On the other hand, the short dialysis of the S-30 during its preparation (4 hr only) may have left enough unlabeled NAD^+ in the S-30 so that the C^{14}-NAD^+ was diluted to a level which made detection of adenine C^{14}-diphosphoribose EF2 enzyme impossible. After extensive dialysis, the NAD^+-dependent ADP-ribosylation of EF2 enzyme was detected only in the assay systems containing Fragment A or trypsin digested toxin. There was probably some proteolysis taking place during the dialysis since the enzymatic activity of Fragment A decreased and that of digested toxin increased. The proteolytic enzymes might have hydrolysed some Fragment A and therefore decreased its concentration whereas some

intact toxin remaining after mild trypsin digestion were cleaved by the proteolytic enzymes of the S-30 and therefore the effective concentration of the A fragment in the reaction mixture containing digested toxin increased. None of the proteins present in the E. coli S-30 mediated the ADP-ribosylation of EF2 enzyme in absence of the A fragment.

The A fragment of the diphtheria toxin is the smaller polypeptide derived from the cleavage of toxin by trypsin or other proteolytic enzymes (Gill and Pappenheimer, 1971), and it is the amino terminal fragment of the toxin (Michel et al., 1972). Thus, correct translation of toxin mRNA in the E. coli cell-free lysate even if premature termination of polypeptides synthesis did take place should have resulted in synthesis of some Fragment A. When the in vitro synthesized polypeptides were assayed for the ADP-ribosylation of EF2 enzyme in the presence of C^{14}-NAD^{+}, this specific enzymatic activity was detected in the presence of polypeptides derived from translation of mRNA from toxinogenic cells. The polypeptides derived from translation of C_7(-) RNA did not have the ADP-ribosylation activity. Neither was this activity present in phenol-SDS extracted PW8 RNA preparations.

These results indicated that some enzymatically active Fragment A of diphtheria toxin had been synthesized in the E. coli cell-free lysate by correct translation of toxin mRNA. Although the C^{14}-adenine bound to the EF2 by the in vitro synthesized Fragment

A was low, it was significantly higher than the background radioactivity precipitable in the control assay mixtures. The low ADP-ribosylation activity may have been due to inactivation of some Fragment A by the proteolytic enzymes of the E. coli S-30 during the synthesis of the proteins or the subsequent dialysis step.

In vivo toxicity with diphtheria toxin can occur only when the A and the B fragments of the diphtheria toxin are attached by a peptide bond and/or by a disulfide bridge (Drazin, Kandel and Collier, 1971). When toxin is injected intradermally into unimmunized animals such as rabbits, 0.0003 p mole or 2×10^8 molecules of toxin (1 MRD) are sufficient to cause erythema and skin necrosis at the site of injection. The intoxication of tissues can be specifically prevented if the toxin is first neutralized with antitoxin or the animal is protected by passive or active immunization. Since relatively few molecules of toxin are necessary to elicit the skin reaction this assay system is probably the most sensitive technique for detection of diphtheria toxin. But its disadvantage is that only intact, biologically active, diphtheria toxin can be detected since toxin fragments do not cause intoxication of the cells (Drazin, Kandel and Collier, 1971). The rabbit skin test for toxicity was performed to detect the presence of biologically active diphtheria toxin in the polypeptides synthesized by translation of mRNA from C. diphtheriae. The erythema and necrosis which was specifically prevented by antitoxin appeared at

the injection site of the control toxin and the polypeptides derived from the translation of C_7 βv tox^+ RNA and PW8 RNA. The polypeptides derived from the translation of C_7(-) RNA did not elicit the skin reaction and neither did the RNA preparation from the toxinogenic cells. The size of erythema and necrosis indicated that probably 1-2 MRD toxin was present in the polypeptides synthesized by translation of toxinogenic mRNA.

Theoretically and on the basis of rabbit antitoxin precipitable proteins, the polypeptides derived from the in vitro translation of C_7 βv tox^+ RNA injected into the rabbit skin contained 0.006 p mole or 20 MRD toxin. However, the size of the lesion produced in rabbits indicated that only 1-2 MRD was present as biologically active diphtheria toxin. Therefore 5-10% of the polypeptides which were precipitable with rabbit antitoxin were biologically active toxin. Similarly the translation of PW8 RNA also lead to the synthesis of antitoxin specific proteins but only 1-2% of the proteins were biologically active toxin. Based on the observed proteolytic activity present in the S-30 against diphtheria toxin (Table 2) it appears likely that during the incubation period of the cell-free protein synthesis and the subsequent dialysis period, the proteolytic enzymes cleaved some toxin molecules which had been synthesized. This is supported by the detection of free Fragment A in the polypeptides synthesized by the translation of toxinogenic RNA. While the rabbit skin test required intact biologically active

toxin molecules, the immune precipitation could also detect toxin and toxin fragment. Therefore the difference observed in these studies between the two assays is not surprising.

The results of the current experiments indicated that RNA from C. diphtheriae could be translated in the E. coli cell-free protein synthesizing system probably by the normal process of initiation of protein synthesis. The detection of synthesized diphtheria toxin and its fragments following translation of RNA from C_7 βv tox^+ and PW8 cells by immune precipitation was more specific when it was carried out with rabbit antitoxin made against pure diphtheria toxoid than with horse antitoxin. There was sufficient amount of Fragment A of diphtheria toxin synthesized in the reaction mixtures by translation of C_7 βv tox^+ RNA and PW8 RNA to make this assay method applicable for detection of in vitro synthesized and biologically active Fragment A. The assay, however, required prior removal of the dialysable molecules. Diphtheria toxin was synthesized in vitro and sufficient amounts of it remained biologically active, to be detected by its toxicity for rabbit skin.

Detection of diphtheria toxin, synthesized in vitro indicated that RNA from lysogenic but non-induced C. diphtheriae and RNA from C. diphtheriae infected with a lytic phage both contained toxin mRNA which could direct the synthesis of toxin in the E. coli cell-free system. The inclusion of RNA from nontoxinogenic, nonlysogenic $C_7(-)$ was probably the most appropriate control which could be incorporated in these experiments since this RNA in every aspect

was similar to C_7 βv tox^+ RNA except for the absence of phage-specific mRNA.

Diphtheria toxin, synthesized by translation of toxin mRNA in the E. coli cell-free lysate, could be detected because of its unique immunological and biological properties. The availability of these different assay systems for detection of diphtheria toxin made in vitro makes the study of the control of toxin synthesis a possibility. This system provides an interesting model for understanding the control and synthesis of bacteriophage mediated toxins.

SUMMARY AND CONCLUSION

The RNA derived from C. diphtheriae was translated into proteins by an E. coli cell-free protein synthesizing system and synthesis of diphtheria toxin was detected. C. diphtheriae RNA sources were the nontoxinogenic, nonlysogenic C. diphtheriae $C_7(-)$, $C_7(-)$ infected with a virulent β tox^+ corynebacteriophage (C_7 β tox^+, 45 min post-infection) and C. diphtheriae PW8 lysogenic with P tox^+ corynebacteriophages. The cells were cultured in deferrated media and ground with alumina. RNA was extracted with phenol and sodium dodecyl sulfate. C. diphtheriae RNA stimulated amino acid incorporation at least 3 times over that of the endogenous incorporation at Mg^{++} concentration of 10 mM. Optimum Mg^{++} concentration was 12 mM and higher concentrations were inhibitory to amino acid incorporation. The magnesium ion requirement indicated that initiation of protein synthesis with C. diphtheriae RNA was probably with f Met-tRNA and the normal initiation factors of the E. coli. Sucrose gradient analysis of the cell-free protein synthesizing mixtures revealed that C. diphtheriae RNA was bound to the E. coli ribosomes and that nascent polypeptides were associated with this complex.

Antitoxin-specific polypeptides were synthesized only in the cell-free protein synthesizing systems primed with RNA from toxinogenic C. diphtheriae, i.e. C_7 β tox^+ and PW8 cells.

Furthermore, synthesis of antitoxin-specific polypeptides was at the same rate as the synthesis of other proteins. Polypeptides from the cell-free protein synthesizing systems primed with RNA from C. diphtheriae $C_7(-)$ cells did not precipitate with antitoxin, indicating specificity of the immune precipitation reaction for detection of the in vitro synthesized toxin or toxin fragments.

Polypeptides derived from the in vitro reaction mixtures, primed with RNA from toxinogenic cells, had the ADP-ribosylation activity specific for the A fragment of diphtheria toxin. The results showed that enzymatically active Fragment A had been synthesized in vitro by translation of the tox^+ mRNA from the toxinogenic cells.

Finally, to detect the synthesis of biologically active toxin in the cell-free protein synthesizing system, the intradermal toxicity test was performed in rabbits. Necrosis of the skin at the site of injection of the polypeptides derived from the cell-free protein synthesizing mixture primed with RNA from toxinogenic cells indicated the presence of biologically active toxin.

In conclusion, RNA with messenger activity was isolated from C. diphtheriae. Translation of toxin mRNA from the toxinogenic cells in the E. coli cell-free lysate led to synthesis of biologically active toxin and toxin fragments. This indicated that toxin mRNA could be isolated from cells infected with a

virulent tox^+ corynebacteriophage and also from the cells infected with a lysogenic tox^+ corynebacteriophage. C. diphtheriae $C_7(-)$ is nontoxinogenic and nonlysogenic and therefore the RNA from this strain acted as a suitable control in the assay systems used to detect de novo synthesis of toxin.

The methods developed to detect de novo synthesis of toxin in vitro should prove useful in studies of the control of diphtheria toxin synthesis at the molecular level, and the role of regulatory substances, especially iron-containing proteins in the synthesis of toxin.

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