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**Studies on Regulation of Genetic Transcription and Messenger RNA  
Decay in Escherichia coli.**

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**77SC01938**

## Preface

Recent works on genetic transcription have been shown the main outline of the mechanism by which primary step of gene expression is regulated, though some facets of it such as transcriptional termination are relatively not so clear. In contrast, the mechanisms which regulate template activity of the messenger RNA are much less understood. Clarification of the mechanism of post-transcriptional control in the pathway by which genetic information is translated into the amino acids sequence of proteins is obviously one of the most important problems in understanding the genetic regulation.

I will report here some aspects of regulation of the transcriptional termination and messenger RNA decay in Escherichia coli with particular regards to the tryptophan operon.

Part 1.

Diversity of Regulation of Genetic Transcription ; Differential  
Chain Termination in Transcripts Originating at the trp Promoter  
and P<sub>L</sub> Promoter of  $\lambda$ trp Phage.

### Summary

The trp operon translocated in the early region of phage  $\lambda$  can be transcribed under the control of two promoters, the authentic  $P_{trp}$  promoter and the  $P_L$  promoter of the N gene (Imamoto & Tani, 1972; Ihara & Imamoto, 1976a). To determine trp mRNA from  $\lambda trp$ , appropriate  $\phi 80 trp$  DNAs were used as a DNA complement in DNA-RNA hybridization assays.

In the absence of N function, transcription of the trp operon from the  $P_L$  promoter in  $\lambda trp$  terminates at a specific site in trp leader gene. The effect can be observed in the experiment employing  $\lambda trp$  phage retaining an amber mutation in the N gene or a host strain groN785 or nusA-B27-1 infected with normal  $\lambda trp$  phages. Result obtained through base sequence analysis by two dimensional thin layer chromatography lead me to a conclusion that the termination signal is the DNA sequence which is rich in AT base pairs. In  $\lambda trp$  phage employed, the  $t_L$  terminator has been deleted (Fiandt *et al.*, 1974). The protein product of the tof gene has no connection with this effect.

In contrast to this observation, trp mRNA synthesis originated at the  $P_{trp}$  promoter is not sensitive to this termination signal even in absence of functional N gene product. Possible mechanisms for these findings are discussed.

## 1. Introduction

When the bacterial trp or gal operons are translocated into  $\lambda$  phage, transcription can occur starting either at the bacterial operon promoter or at a proximal  $P_L$  promoter of the N gene and continuing through the operon sequences (Franklin, 1971; Imamoto & Tani, 1972; De Crombrugghe *et al.*, 1973; Tani & Imamoto, 1975; Ihara & Imamoto, 1976a). Transcription from the  $P_L$  promoter shows at least four major differences from transcription originated at the authentic promoter of the operon; 1) the transcription is not deleteriously affected by blockage of translation by antibiotics (Imamoto & Tani, 1972; Ihara & Imamoto, 1976b) or by inactivation of temperature-sensitive ribosomal elongation factors G, Ts or Tu (Kuwano & Imamoto, unpublished results); 2) the translational termination at nonsense mutation sites of  $P_L$ -promoted transcript fails to produce polarity (Segawa & Imamoto, 1974; Franklin, 1974; Adhya *et al.*, 1974); 3) the signals for terminating transcription at the end of trp operon (Segawa & Imamoto, 1976; Franklin, personal communication), at  $t_X$  (Franklin, 1974), and at the end of the bioA gene (Adhya *et al.*, 1974) are ignored in read-through transcription from the  $P_L$  promoter; 4) the trp mRNA (Yamamoto & Imamoto, 1975) and gal mRNA (Court *et al.*, 1975) formed as a part of  $P_L$ -promoted transcript are chemically stable.

Many of these phenomena are concerned with general insensitivity of the  $P_L$ -promoted transcription to events of translational arrest. Here I report, in contrast to these changes, a fifth difference, in the opposite direction: transcription of the trp operon in  $\lambda$ trp phage from the  $P_L$  promoter terminates at a specific site in trp leader gene.

effected most possibly by rho factor in the absence of N function;  
while the transcription initiated at the authentic trp promoter is  
insensitive to this termination signal.

## 2. Materials and Methods

### (a) Bacterial and phage strains

The following strains of E. coli K12 were used for infection experiments; W3110 trpR<sup>+</sup> trpAE1, a large deletion mutant covering the whole trp operon W3110 trpR<sup>+</sup> trpAE1 suI, groN785 (Georgopoulos, 1971) and W31C2 nusA-B27-1 and the parental W3102 K37 (Friedman & Baron, 1975; Friedman, personal communication). The mutations of groN785 and nusA-B27-1 block  $\lambda$  development at the stage of N gene function.

The following phages were used for infection experiments; non-defective transducing phage,  $\lambda$ trp(h <sup>$\lambda$</sup>  att<sup>80</sup> trp60-3imm <sup>$\lambda$</sup>  QSR <sup>$\lambda$</sup> ), which replaces the  $\lambda$  genes to the left of N with the whole trp operon of E. coli, and its derivatives  $\lambda$ trpN<sub>53</sub><sup>-</sup> and  $\lambda$ trpN<sub>7</sub><sup>-</sup> tof<sub>12</sub><sup>-</sup> which were obtained by crosses with  $\lambda$ N<sub>7</sub><sup>-</sup> N<sub>53</sub><sup>-</sup> or  $\lambda$ tof<sub>12</sub><sup>-</sup> (Takeda et al., 1975). The genetic map of the  $\lambda$ trp phage is shown in Figure 1.

The following phages were used as sources of DNA for DNA-RNA hybridization assays;  $\phi$ 80, the non-transducing parental phage, non-defective transducing phages  $\phi$ 80trpED and  $\phi$ 80trpC-A. The trp operon segments carried by the  $\phi$ 80trp phages are shown in Figure 1.

### (b) Preparation of $\lambda$ trp phages

$\lambda$ trp phages were prepared by lytic infection of sensitive bacteria E. coli K12 W1485suIII on agar plates (10g bacto-tryptone, 2.5g NaCl, 12g agar and one liter H<sub>2</sub>O). After extraction with dilution fluid [ $1 \times 10^{-2}$  M Tris-HCl buffer (pH 7.3) and  $1 \times 10^{-2}$  M MgCl<sub>2</sub>], phages were concentrated with one cycle of low-and high-speed centrifugation and suspended in  $\lambda$  dilution fluid containing 20  $\mu$ g/ml bovine serum albumin.



(c) Preparation of phage DNA

Lysates of  $\phi 80$  and  $\phi 80_{trp}$ 's were prepared by lytic growth on sensitive bacteria E.coli K12 W1485. After low- and high-speed centrifugation, phage particles were suspended in T1 dilution buffer [ $6 \times 10^{-4}$  M  $MgSO_4$ ,  $5 \times 10^{-4}$  M  $CaCl_2$ ,  $1 \times 10^{-3}$  % gelatin and  $6 \times 10^{-3}$  M Tris-HCl buffer (pH 7.3)] and treated with DNase ( $3 \mu g/ml$ ). After DNase treatment, phages were purified by a combination of one cycle of low- and high-speed centrifugation and CsCl density centrifugation. Phages were suspended at a concentration of  $2 \times 10^{12}/ml$  in 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1 M NaCl after dialysis against the buffer. Phage DNA's were extracted by phenol treatment (Kaiser and Hogness, 1960) and dissolved in a saline-citrate solution (1 X SSC: 0.15 M NaCl-0.015 M sodium citrate) after dialysis against the solution.

(d) Preparation of pulse-labeled RNA

Bacteria were grown with aeration to  $6 \times 10^8$  cells/ml in an enriched medium (L-broth) (Lennox, 1955) supplemented with L-tryptophan ( $50 \mu g/ml$ ). The cells were collected by centrifugation, washed <sup>twice</sup> with cold T1-dilution buffer and resuspended in the same buffer to give a final density of about  $6 \times 10^{10}$  cells/ml. About  $6 \times 10^9$  cells of bacteria were infected with each of the  $\lambda_{trp}$  phages at a multiplicity of about 5 in 1.5 ml T1-dilution buffer containing  $1 \times 10^{-3}$  M KCN by incubating for 10 minutes at  $30^\circ C$  or in 1.5 ml of 0.02M  $MgSO_4$  by incubating for 10 minutes at  $30^\circ C$  in the experiments of Figs. 2, a and 6. Employment of the

latter condition lessened inequality of the rates of mRNA synthesis at opposite ends of the trp operon (Imamoto, 1969).

The cells were collected by centrifugation and suspended in 0.1 ml of cold minimal medium (Vogel & Bonner, 1956) and stored at 0°C less than an hour until labeling. The cell suspension was transferred to 2.8 ml of prewarmed (30°C) minimal medium supplemented with 19 amino acids (each 0.5 mM) excluding tryptophan, and the cell suspension was shaken vigorously in a water bath at 30°C. L-tryptophan (50 µg/ml) was added in experiments to demonstrate uniquely  $P_L$ -promoted synthesis of trp mRNA. At a suitable time during incubation the cell suspension was pulse-labeled as indicated with 100 to 300 µCi of tritiated uridine (19.0 Ci/mmol).

Labeling was stopped by rapidly pouring the suspension onto 35 ml of crushed frozen medium containing  $1 \times 10^{-2}$  M Tris-HCl buffer at pH 7.3,  $5 \times 10^{-3}$  M  $MgCl_2$ ,  $1 \times 10^{-2}$  M  $NaN_3$  and 250 µg of chloramphenicol/ml. RNA was prepared by the procedure reported previously (Imamoto et al., 1965). The RNA obtained was filtered through a Millipore filter, precipitated by ethanol and resolved in  $1 \times 10^{-2}$  M Tris-HCl buffer containing 0.5M KCl and  $1 \times 10^{-3}$  M  $Na_2EDTA$  or in  $H_2O$ . Pulse-labeling with  $^{32}P$  was performed in the same way as labeling with [ $^3H$ ]uridine except that cells were grown in trypton-λ medium, washed with cold buffer free from phosphate (see Materials and Methods of part III) and labeled in the same buffer containing 20 amino acids with about 10 mCi of  $^{32}P$ -phosphate.

#### (e) DNA-RNA hybridization

The hybridization procedure was as follows; DNA of  $\Phi 80$  or  $\Phi 80trp$  was diluted to a concentration of 100 µg/ml in  $1 \times SSC$  and heated in boiling water for 10 minutes followed by rapid cooling in ice water. The DNA was further diluted to a concentration of 8 µg/ml in  $3 \times SSC$ . Five milliliters of the DNA

solution was filtered through a Millipore filter (type HA, 0.45  $\mu$ m pore size) of 25 mm diameter. The filter was washed with 40 ml of 3 x SSC, cut into 8 pieces and dried at 80°C for 2 hours. Assay of trp mRNA was carried out by immersing one of these filter bits in 100  $\mu$ l of [ $^3$ H]mRNA solution in  $1 \times 10^{-2}$  M Tris-HCl buffer, pH 7.3, containing 0.5 M KCl and  $1 \times 10^{-3}$  M Na<sub>2</sub>-EDTA, and incubating at 66°C for 18 hours. Afterwards, the filters were treated with RNase (5  $\mu$ g/ml) in  $1 \times$  SSC at 37°C for 30 minutes, washed with  $1 \times$  SSC, dried and counted in toluene-based scintillation fluid. Total radioactivity of [ $^3$ H]uridine incorporated into RNA (total [ $^3$ H]RNA) was measured as the material precipitable by cold trichloroacetic acid.

(f) Sucrose density-gradient analysis

RNA preparations were sedimented in 5 to 30 % linear sucrose gradients containing  $2 \times 10^{-2}$  M Tris-HCl buffer (pH 7.3), 0.1 M NaCl, 0.5 % sodium dodecyl sulfate and  $5 \times 10^{-3}$  M EDTA for 120 minutes at 63,000 rev/min in a SW65 rotor or 240 minutes at 45,000 rev/min in a SW65 rotor at 15°C. After centrifugation the bottom of the tube was punctured and appropriate fractions were collected.

(g) Chromatography on PEI-cellulose

The isolated RNA (containing 20  $\mu$ g of cold carrier RNA) was digested with T<sub>1</sub> RNAase and applied to the thin-layer plates. Chromatography was carried out on PEI-cellulose by the techniques as described by Mirzabekov and Griffin (1972). The condition of T<sub>1</sub> RNAase hydrolysis is also the same as described by them.

(h) Reagents

Tritiated uridine was purchased from the Daiichi Chemical Company, Osaka, and was used without the addition of carrier. Millipore filters were purchased from the Millipore Filter Company, Bedford, Mass. Chloramphenicol was from Park Davis. DNase and RNase were obtained from the Worthington Biochemical Company. RNase was used after heating

at 80°C for 20 min in 0.15 M NaCl to inactivate any contaminating DNase.

### 3. Results

The trp mRNA synthesized specifically from the translocated trp operon of  $\lambda$ trp phages was assayed after infecting a deletion host trpAEl, which lacks the whole trp operon. It has been directly demonstrated that trp mRNA synthesis of  $\lambda$ trp which possesses an intact trp operon is controlled by two promoters, the authentic trp (P<sub>trp</sub>) and the P<sub>L</sub> promoter, and by two operators, the trp and the O<sub>L</sub> operator (Imamoto & Tani, 1972; Segawa & Imamoto, 1974; Tani & Imamoto, 1975; Ihara & Imamoto, 1976a). In order to demonstrate uniquely P<sub>L</sub>-promoted synthesis of trp mRNA, strain trpAEl which retains a tryptophan regulator (trpR) gene located near thr on the chromosome of E. coli, far removed from the clustered tryptophan structural genes (Cohen & Jacob, 1959), was infected with  $\lambda$ trp phage in the presence of L-tryptophan. In all the experiments, DNA of phage  $\phi$ 80trps carrying various trp gene segments was used as a DNA complement in DNA-RNA hybridization assays to demonstrate trp mRNA for the translocated trp operon. Evidence has been presented establishing that the hybridization reaction effectively distinguishes between different genetic and physiological states of regulation of the translocated trp operon in  $\lambda$ trp phage (Segawa & Imamoto, 1974; Yamamoto & Imamoto, 1975).

#### (a) Decrease in trp mRNA production upon N<sup>-</sup> mutation

After infection of trpAEl with  $\lambda$ trp phage in the presence of L-tryptophan at 30°C, the synthesis of trp mRNA reaches a sharp maximum

during the first three to seven minutes after infection, and then declines until it reaches a steady state (Yamamoto & Imamoto, 1975). The rate of synthesis remains nearly constant during the period from 12 to at least 20 minutes after infection. This is shown in Figure 2(a). The decline in the rate of synthesis seen after several minutes of infection is believed to be caused by the function of the tof gene, whose product acts at the operator of the N gene and substantially reduces transcription with the l-strand of the early region of  $\lambda$  soon after the initiation of the phage development (Szybalski *et al.*, 1970). The appearance of trp mRNA after infection is sequential in the order of trpE to trpA, thus supporting a notion that the trp mRNA is asymmetrically synthesized from the l-strand of  $\lambda$ trp DNA as is predicted by the genetic evidence (see Fig. 1).

In Figure 2(b), I show changes in the rate of  $P_L$ -promoted synthesis of trp mRNA in  $\lambda$ trp phage which retains an amber (53) mutation of the N gene. Seemingly, the rates are much lower than those of normal  $\lambda$ trp phage during incubation for about 23 minutes after infection. Yet significant production of trp mRNA from only the operator-proximal portion of the trp operon (mRNA hybridized to  $\phi 80$ trpED DNA) was observed.

Besides the above observation, further evidence has been available supporting the notion that, in  $\lambda$ trp phage employed here, the  $t_L$  terminator has been deleted: (1) location of the right endpoint of the bacterial substitution in the phage falls well within the imm21 region, most probably to the right of the  $t_L$  site (Fiandt *et al.*, 1974); (2) the  $P_L$ -promoted transcription is permitted to extend from the  $\lambda$  genome into the trp operon, even when synthesis of the N product is blocked by antibiotics (Imamoto & Tani, 1972); and (3) under conditions in which

the product of the N gene loses its activity in the E. coli K95 nus host at non-permissive temperature (Friedman et al., 1973), the steady-state level of the synthesis of mRNA for the Q, S and R genes from the P<sub>R</sub> promoter in  $\lambda$ trp phage is severely suppressed, while trp mRNA synthesis from the P<sub>L</sub> promoter is not so affected (data not shown).

Unlike changes characteristic of the initial trp mRNA synthesis originating at the P<sub>L</sub> promoter by  $\lambda$ trp N<sub>53</sub> phage, the synthesis of the trp mRNA by  $\lambda$ trp N<sub>7</sub> tof<sub>12</sub> phage exhibited an alleviation of the turn-off phenomenon and continued at nearly a constant rate for at least 25 minutes after infection (Fig. 2,c). Yet the level of trp mRNA synthesis from the operator-distal trpC, B and A genes was considerably lower throughout the incubation than that of the synthesis from the operator-proximal portion of the operon. Reduction in the overall rate of trp mRNA production from both the operator-proximal and distal region of the operon was partially relieved when an amber mutation of the N gene in  $\lambda$ trp N<sub>7</sub> tof<sub>12</sub> phage was suppressed by introduction of su1 into host bacteria (Fig. 2,d).

(b) Premature arrest of P<sub>L</sub>-promoted trp mRNA synthesis in the absence of N function

From the foregoing experiments it would be expected that trp mRNA molecules produced from the P<sub>L</sub> promoter by  $\lambda$ trp phages retaining an amber mutation of the N gene should be smaller in size than those of trp mRNA molecules from the normal  $\lambda$ trp phage. This possibility was examined in sedimentation studies with trp mRNA from cultures pulse-labeled with [<sup>3</sup>H]uridine at a steady-state of transcription of the trp operon.

In Fig. 3(a) sedimentation profiles are presented for  $P_L$ -promoted trp mRNA from trpAE1 infected with  $\lambda$ trp  $N_{53}^-$  phage. When compared with the profile of  $P_L$ -promoted trp mRNA by normal  $\lambda$ trp phage (Segawa & Imamoto, 1974), the trp mRNA molecules produced when the activity of the N gene product is low are found to be extraordinarily small in size, sedimenting at a position of about 12S. A sedimentation profile essentially similar to this was observed when the experiment was carried out with the trp mRNA synthesized by a host strain groN785 (Georgopoulos, 1971) infected with normal  $\lambda$ trp phage [in which the N antiterminating function is not expressed (Fig. 3,b)]. Though loss of function of the N gene product might be somehow leaky in the strain nusAB27-1 (Friedman et al., 1975) infected with normal  $\lambda$ trp phage, production of the truncated trp mRNA molecules due to transcriptional blockage at a site around the trp leader gene was also obvious in this host, when compared the profile of trp mRNA from the mutant (Fig. 4,a) with that of the mRNA from the parental strain infected with the phage (Fig. 4,b). The reason why mRNA hybridizable with  $\phi 80$ trpCBA DNA sedimented at 12s. is obscure, however it may reflect some function of these mutations for mRNA metabolism.

Although suppressed level of trp mRNA synthesis by  $\lambda$ trp  $N_{53}^-$  phage is lessened in  $\lambda$ trp  $N_7^-$  tof<sub>12</sub> phage (Fig. 2,c), the effect is not attributed to alleviation of premature arrest of the mRNA synthesis but to increase in frequency of transcription at initiation step: the majority of the trp mRNA molecules produced by  $\lambda$ trp  $N_7^-$  tof<sub>12</sub> phage is still as small in size as those from  $\lambda$ trp  $N_{53}^-$  phage, thus indicating that arrest of transcription is effectively occurring at the beginning of trp operon (Fig. 5,a). Upon suppression of the amber mutation of the N gene by introduction of su1 into host bacteria, the  $P_L$ -promoted synthesis of the trp mRNA is greatly enhanced (Fig. 2,d).

This seeming enhancement of the trp mRNA synthesis results from production of the larger mRNA molecules (Fig.5,b); i.e., a block of transcription at the beginning of trp operon is at least in part alleviated by function of the suppressed N gene.

The fact that the effect couples with loss of function of the N gene product lead me to consider the possibility that in the absence of N gene product, rho factor functions at a site located near the beginning of the trpE gene and blocks the transcription.

(c) Sequence analysis of truncated  $P_L$  trp mRNA produced under N-deficient condition.

Figure 6 shows fingerprints of the  $^{32}$ P-trp mRNA segment isolated by hybridization with  $\phi 80$ trpED DNA from a strain trpAEI infected with  $\lambda$ trpN7tofl2 phage. The mRNA was digested completely with  $T_1$  RNAase. The oligonucleotides obtained range from one to about 15 nucleotides in length and are adequately separated from each other. Results show that spots for large nucleotides 2, 3, 4, 5, 8, 9, 11, 13 and 15 can correspond to the nucleotides sequence of the trp leader region which has been determined by Bertrand et al (1975). Further analysis of the oligonucleotides eluted from a spot 15 by two-dimensional thinlayer chromatography following to pancreatic RNAase A revealed that the spot corresponded to 5'pUpUpUpUpUpUpUpUOH3' (data not shown). Thus it can be concluded that trp mRNA synthesis initiated at the  $P_L$  promoter in trpAEI infected with  $\lambda$ trpN7tofl2 frequently terminates at the site where AT nucleotides pairs cluster in the trp leader region.

(d) Escape of  $P_{trp}$ -promoted trp mRNA synthesis from the terminator

An experiment was carried out to investigate if trp mRNA synthesis originating at the P<sub>trp</sub> promoter is also blocked by the signal for



terminating transcription created upon a loss of N function. In a non-lysogenic host trpAE1 infected with  $\lambda$ trp N<sub>7</sub><sup>-</sup> phage in the absence of tryptophan, both P<sub>trp</sub> promoter and P<sub>L</sub> promoter function<sup>(Ihara & Imamoto, 1976a)</sup>

The sedimentation profile of Fig. 7 shows that the majority of the trp mRNA molecules synthesized originating at the P<sub>trp</sub> promoter are much larger than those from P<sub>L</sub> promoter. A small peak in the profile of trp mRNA hybridizable with  $\phi$ 80trpED DNA may suggest that small trp mRNA molecules are still produced from the P<sub>L</sub> promoter in  $\lambda$ trp N<sub>7</sub><sup>-</sup> phage in the absence of tryptophan.

#### 4. Discussion

My major inference is that when functional N gene product is deficient, the P<sub>L</sub>-promoted trp mRNA synthesis is frequently blocked at a site of trp<sub>A</sub><sup>leader</sup> gene, whereas trp mRNA synthesis originated at the P<sub>trp</sub> promoter in the same condition is not sensitive to this termination signal.

In P<sub>L</sub>-mRNA molecules synthesized by  $\lambda$ trp N<sub>53</sub><sup>-</sup> (about 12S or 800 nucleotides length), trp mRNA is covalently linked distal to the N mRNA sequence (Tani & Imamoto, 1975). In the  $\lambda$ trp (60-3) phage employed here, the right terminus of the trp60-3 substitution is within the imm21 region just to the left of gene N at 71.8 %  $\lambda$  units; i.e., the  $\lambda$ trp60-3 endpoint is preceded in  $\lambda$  duplex DNA by 1.8 %  $\lambda$  units or about 770 nucleotide pairs to the left of the P<sub>L</sub> promoter at 73.6 %  $\lambda$  units (Fiandt et al., 1974). Since at least the first about 170 nucleotides of the N mRNA synthesized from P<sub>L</sub> promoter are probably cut off in vivo by processing (Lozeron et al., 1975), the bacterial mRNA sequence in the distal portion of the molecule is estimated to be about 200 nucleotides length. The initiation codon for the polypeptide specified by the trpE gene is preceded in P<sub>L</sub> mRNA by a "leader" sequence of about 160 nucleotides in length (Bertrand et al., 1975) and probably some part of bacterial RNA sequences ahead of the leader mRNA portion. Thus, the signal for

transcription seems to be located within the trp leader region, which is supported by the more direct evidence from the two dimensional thin layer chromatographic analysis (Fig. 6). In Fig. 6a radioactivity in spot 10 which represent  $(U_8)^{3'}OH$  is smaller than the other spots. It would be attributable to some nuclease activity in vivo, which degrades mRNA from 3' to 5'.

Recently, new regulatory elements for the trp operon have been suggested. One might wonder whether, when N gene product is deficient, transcription is somehow arrested in the leader region at the "attenuator," where the level of extended transcription is believed to be limited (Bertrand et al., 1975). Such events might occur, but it can be pointed out that more than one signal for transcription termination is created in vivo in the trpD gene translocated into  $\lambda$ trp46 phage (Franklin, 1974) when the N function is lost (Nakamura & Imamoto, unpublished results). It has also been demonstrated that there are several sites in the trp (Shimizu & Hayashi, 1974) and gal (De Crombrughe et al., 1973) operons where rho factor can function in vitro.

N gene products has been shown to function by inhibiting rho-mediated terminations in  $\lambda$  phage, thus allowing transcription of the distal genes downstream from the rho-binding sites (Roberts, 1970). This would suggest that a deficiency of activity of the N gene product would not decrease the rate of mRNA synthesis for the N gene. An alternate possibility has been inferred from the observation that a loss of activity of N protein results in a co-ordinate decrease in the amount of mRNA made complementary to the N gene and of mRNA complementary to the distal genes of the operon, this could inhibit initiation of RNA synthesis at the promoter (Wood & Konrad, 1974). Present observations that the action of the N protein is as an anti-terminator favor the former interpretation. In fact, when N mRNA synthesis was measured by employing l-strand DNA from  $\lambda$ h<sub>i</sub><sup>80 434</sup> phage

(Fig. 1) as a DNA complement in DNA-RNA hybridization assay of mRNA specific for N gene of  $\lambda$ trp phage, the rate of the synthesis was almost comparable (86 % in  $\lambda$ trpN<sup>-</sup> phage of the normal) to that in the control  $\lambda$ trp phage (data not shown).

Considering the termination of transcription, P<sub>trp</sub>-promoted transcription is markedly different from P<sub>L</sub>-promoted transcription: only the former is sensitive to the polarity effected by general translational blockage, and to the natural terminators for transcription; but only the latter is sensitive to the terminations, probably rho-mediated, reported here. Recently, it has been reported that rho factor is the product of the suA gene (Richardson et al., 1975; Ratner, 1975), which mutation can partially relieve the polarity effect of nonsense mutations of the operon of E. coli without suppressing the mutant phenotype itself (Beckwith, 1963). Relief of polarity by suA is associated with partial detectability of mRNA for genes distal to the mutated gene (Morse & Primakoff, 1970). It has therefore been suggested that the suA strain suppresses polarity because it lacks normal activity of rho factor, the product of the wild-type suA allele.

However, this statement is in apparent conflict with the present finding of the insusceptibility of P<sub>trp</sub>-promoted transcription to rho-mediated termination. This paradox is difficult to resolve by the simplest prevailing models for the termination of transcription. One might argue that rho factor can have two different modes of action - one on sites in DNA (Roberts, 1969; Beckman et al., 1971) or on RNA polymerase (Schäfer & Zillig, 1973), and the other on sites in mRNA exposed in the absence of translating ribosomes (Richardson et al., 1975; Galluppi et al., 1975). The first mechanism would then apply to P<sub>L</sub>-

directed trp transcription, and the second to  $P_{\text{trp}}$ -transcription. However, since the expression of trp genes is normal and seemingly very similar in both  $P_L\text{-trp}$  and  $P_{\text{trp}}\text{-trp}$  transcripts (Yamamoto & Imamoto, 1975), the second mechanism should hold as much for one species of transcript as the other!

Therefore, one must consider other alternatives. As one speculative possibility, one might assume that free-running RNA polymerase can be programmed to at least two classes of termination signals, one independent of rho factor, and the other rho-mediated. Rho-independent transcription stops could then occur with  $P_{\text{trp}}$ -transcripts - at attenuator sites, or at the end of the operon, for example. Instead, under certain conditions (e.g., blocked ribosomes), the RNA polymerase would be reprogrammed to respond to rho-specific stops. The reprogramming would be transient, because it would be reversed by a resumption of translation (as in the case of gradients of polarity). N gene or other "positive factors" could differentially change the susceptibility of RNA polymerase to one or another type of attenuation or termination signal. Alternative like this have the advantage of consistency with all the data; but they assume that rho factor, contrary to many current discussions, is not involved at all in normal RNA chain termination by RNA polymerase.

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## Legends to Figure

Figure 1.

Simplified molecular maps of coliphages.

The relative size of each trp gene carried by  $\phi 80$ trp and  $\lambda$ trp phages is estimated from the molecular weight of the corresponding polypeptides (Imamoto & Yanofsky, 1967). The genetic map of the transducing phage  $\lambda$ trp,  $h^{\lambda} att^{80} trp60-3imm^{\lambda}$ , is based on the data of Nishimune (1973) and Fian dt et al. (1974). Location of the right endpoint of the trp substitution in the phage is represented aligning on a relative scale for  $\lambda$  chromosome length (Davidson & Szybalski, 1971). For the markers describing the maps see Szybalski (1970). Dotted, solid, zigzag and double lines indicate the region of  $\phi 80$  genome,  $\lambda$  genome, 434 genome and bacterial chromosome including trp genes, respectively.

Figure 2.

Time course of transcription of the translocated trp operon in  $\lambda$ trp phages.

Strain trpAE1 was infected with  $\lambda$ trp(a),  $\lambda$ trpN<sub>53</sub><sup>-</sup>(b) or  $\lambda$ trpN<sub>7</sub>tof<sub>12</sub><sup>-</sup> phages(c) or strain trpAE1 suI was infected with  $\lambda$ trpN<sub>7</sub>tof<sub>12</sub><sup>-</sup> phages(d). The infected cells were resuspended in cold minimal medium (Vogel & Bonner, 1956) to give a final concentration of  $1.2 \times 10^{11}$  cells/ml. A portion (0.2 ml) of the cell suspension was transferred to prewarmed (30°C) minimal medium (3 ml) supplemented with 19 amino acids (each 0.5 mM) with L-tryptophan (50  $\mu$ g/ml), and the cell suspension was shaken vigorously in water bath at 30°C. The cultures were pulse-



labeled with 100  $\mu$ Ci to 200  $\mu$ Ci of [ $^3$ H]uridine for 1 min at the times indicated. 7.5 to 19.0  $\mu$ g of [ $^3$ H]RNA (spec. act.  $5.0 \times 10^4$  to  $3.4 \times 10^4$  cts/min per  $\mu$ g) were used for each hybrid assay. Trp mRNA corresponding to the trpE and trpD genes or trpC, trpB and trpA genes was assayed by hybridization of [ $^3$ H]RNA with DNA from  $\phi 80$ trpED or  $\phi 80$ trpC-A, respectively. The values with  $\phi 80$ DNA background were subtracted from each hybrid value. The values represented are the percentage of hybridized RNA relative to the total labeled RNA and the averages of duplicate determinations. Data are plotted in the middle of each period of pulse-labeling. The other conditions are as described in Materials and Methods. (●) trp mRNA hybridized specifically with  $\phi 80$ trpED DNA; (o) trp mRNA hybridized specifically with  $\phi 80$ trpC-A DNA.

Figure 3.

Sedimentation profiles of trp mRNA synthesized originating at the P<sub>L</sub> promoter in trpAEI infected with  $\lambda$ trp N<sub>53</sub> phages (a), groN<sub>785</sub> infected with  $\lambda$ trp phages (b) or trpAEI infected with  $\lambda$ trp phages.

Tritiated RNAs sedimented were pulse-labeled with 150  $\mu$ Ci of [ $^3$ H]-uridine for 1 min at the 10th min (a), 20th min (b) or 15th min (c) of incubation. About 100  $\mu$ g of [ $^3$ H]RNA [spec. act.,  $7.4 \times 10^3$  to  $2.1 \times 10^4$  cts/min per  $\mu$ g] were cosedimented with 10  $\mu$ g [ $^{14}$ C]RNA ( $2 \times 10^4$  cts/min per  $\mu$ g) prepared from the rifampicin-sensitive strain, as described previously (Imamoto, 1973). Centrifugation was carried out for 240 min at 45,000 rpm (a) or 120 min at 63,000 rpm (b) and (c). After centrifugation, about 30 fractions were collected, and 10  $\mu$ l portions from each fraction were used for the determination of [ $^{14}$ C]RNA. The neighboring 2 fractions

were combined and diluted to a volume of about 0.6 ml (a) or 0.4 ml (b) at concentration of  $3 \times \text{SSC}$  containing  $1 \times 10^{-3} \text{M Na}_2\text{EDTA}$ . 100  $\mu\text{l}$  of each combined fraction was hybridized with DNA from  $\phi 80$ ,  $\phi 80\text{trpED}$  or  $\phi 80\text{trpC-A}$  phages. The other conditions are as in Fig. 2 and Materials and Methods. (●) trp mRNA hybridized specifically with  $\phi 80\text{trpED}$  DNA; (○) trp mRNA hybridized specifically with  $\phi 80\text{trpC-A}$  DNA; (-----) total [ $^3\text{H}$ ]RNA. Arrows in the Figure indicate positions of 23S rRNA, 16S rRNA and 4S rRNA.

Figure 4.

Sedimentation profiles of trp mRNA synthesized originating at the  $P_L$  promoter in nusA-B27-1 (a) or the parental strain K37 (b) infected with  $\lambda\text{trp}$  phages.

Tritiated RNAs sedimented were pulse-labeled with 150  $\mu\text{Ci}$  of [ $^3\text{H}$ ]uridine for 2 min at the 20th min (a) or for 1 min at 14th min (b) of incubation. The conditions and representations were the same as described in the legend to Fig. 3, except that the centrifugation was carried out for 120 min at 63,000rpm. The amount of [ $^3\text{H}$ ]RNA sedimented was 86  $\mu\text{g}$  of  $3.6 \times 10^4$  cts/min per  $\mu\text{g}$  (a) or 200  $\mu\text{g}$  of  $6.0 \times 10^4$  cts/min per  $\mu\text{g}$  (b).

Figure 5.

Sedimentation profiles of trp mRNA synthesized originating at the  $P_L$  promoter in trpAE1 (a) or trpAE1 suI (b) infected with trpN<sub>7</sub><sup>-</sup>tof<sub>12</sub><sup>-</sup> phages.

Tritiated RNAs sedimented were pulse-labeled with 100  $\mu\text{Ci}$  of

[<sup>3</sup>H]uridine for 1 min at the 23th min of incubation. The conditions and representations were the same as described in the legend to Fig. 3, except that the centrifugation was carried out for 110 min at 63,000 rpm. The amount of [<sup>3</sup>H]RNA sedimented was 23 µg of  $2.0 \times 10^4$  cts/min per µg (a) or 46 µg of  $7.3 \times 10^3$  cts/min per µg (b).

#### Figure 6

Autoradiogram of <sup>32</sup>P-oligonucleotide chromatographed on  
PEI-cellulose

Cells infected with λtrpN7tofl2 were pulse-labeled with <sup>32</sup>P in presence of L-trptophan. P<sub>L</sub>trp mRNA was prepared by hybridization with φ80trpED DNA. NmRNA which is covalently associated with P<sub>L</sub>trp mRNA but not hybridize with φ80trpED DNA was broken down by RNAase treatment. From the hybrid trp mRNA was eluted by heating. After digesting <sup>the mRNA</sup> with T<sub>1</sub> RNAase oligonucleotides are applied on two-dimentional thin layer chromatography. PEI-cellulose was kept contact with X-ray film to visualize the radioactivity.

a, Autoradiogram. b, Schematic drawing of spots in a.

c, Diagrams illustrating the relationship between nucleotide composition and position of spots. For instance spot 8 means (AC<sub>2</sub>U<sub>3</sub>)G, although it might be confused with (A<sub>3</sub>CU<sub>3</sub>)G or (A<sub>5</sub>U<sub>3</sub>)G.

#### Figure 7.

Sedimentation profile of trp mRNA synthesized originating at the P<sub>trp</sub> and P<sub>L</sub> promoters in trpAE1 infected with λtrp phages in the absence of L-tryptophan.

Tritiated RNA sedimented was prepared and assayed as described in the legend to Figure 3, a, except that the incubation was carried out in the absence of tryptophan. Representations were the same as in Fig. 3. The amount of [<sup>3</sup>H]RNA sedimented was about 100 µg of  $3.0 \times 10^3$  cts/min per µg.

Fig. 1

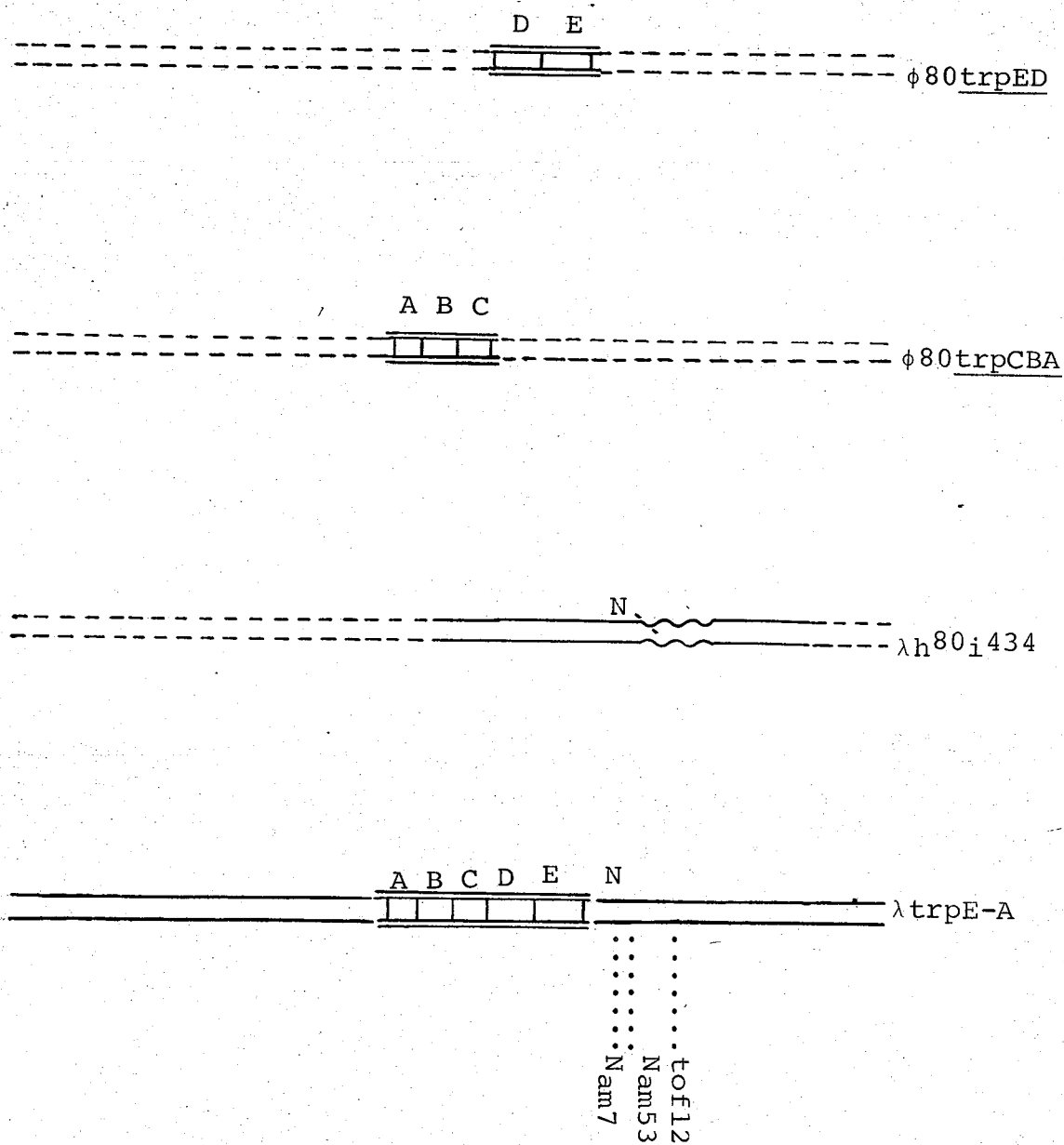


Fig. 2

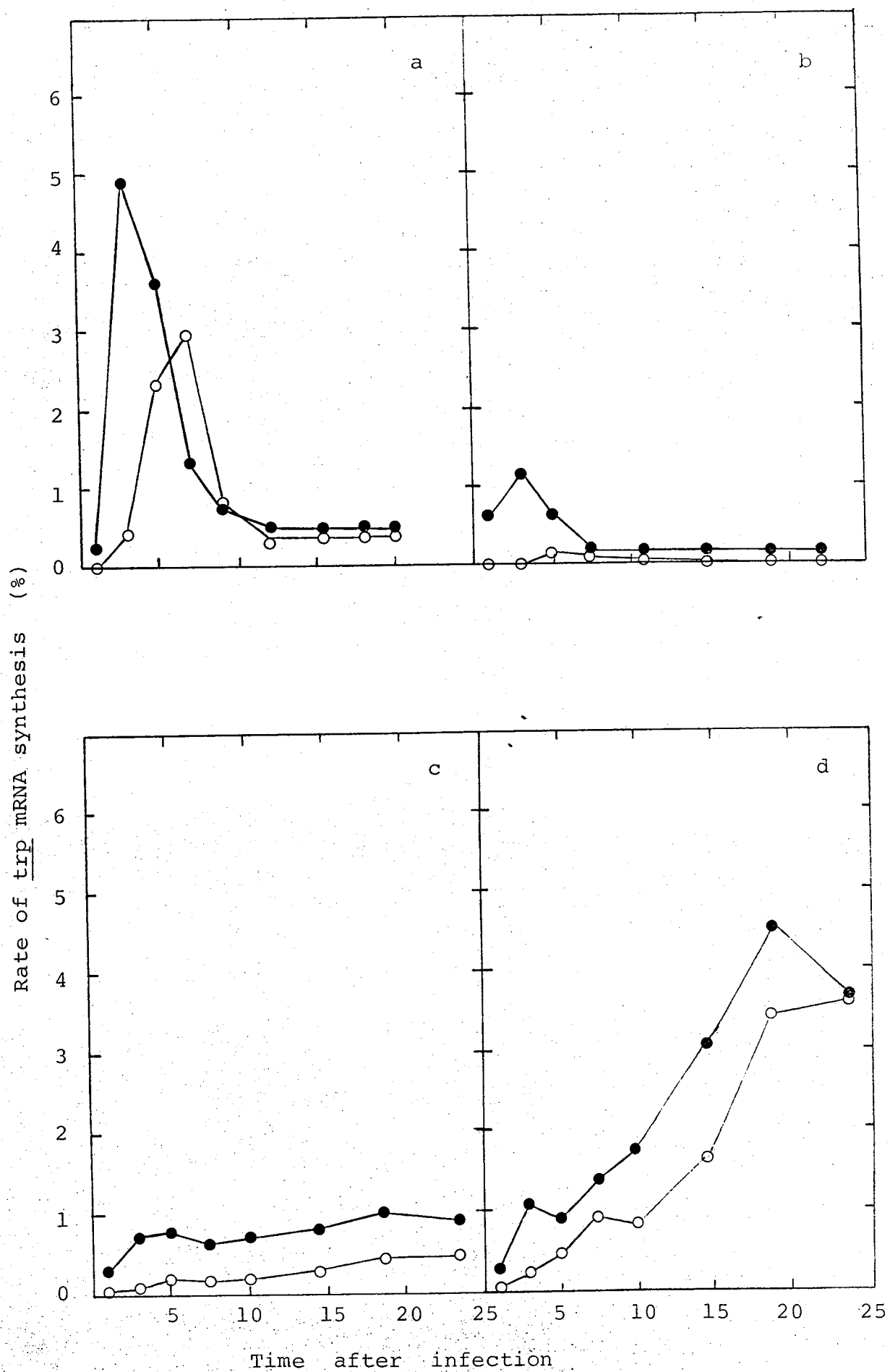


Fig. 3

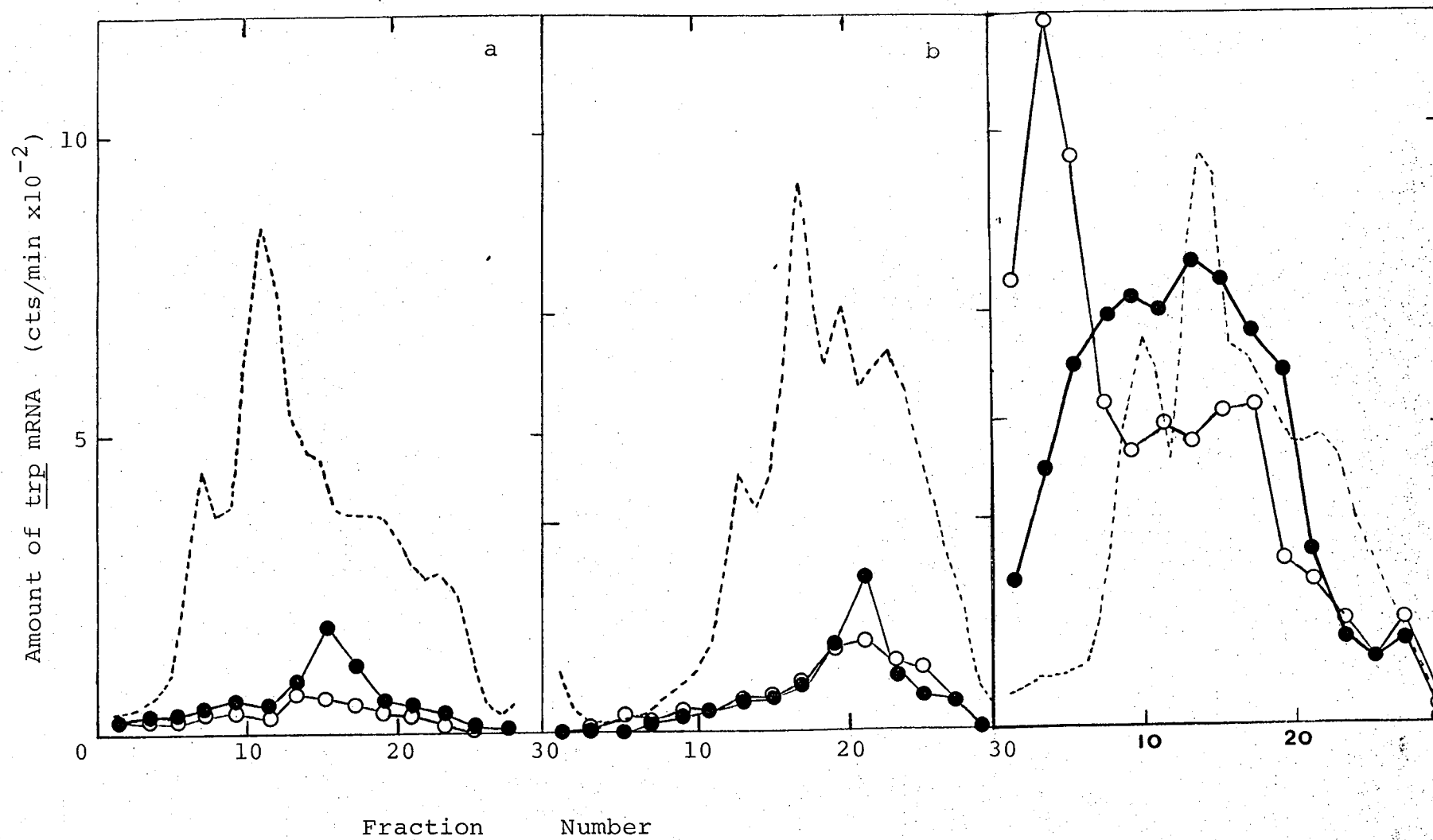


Fig. 4

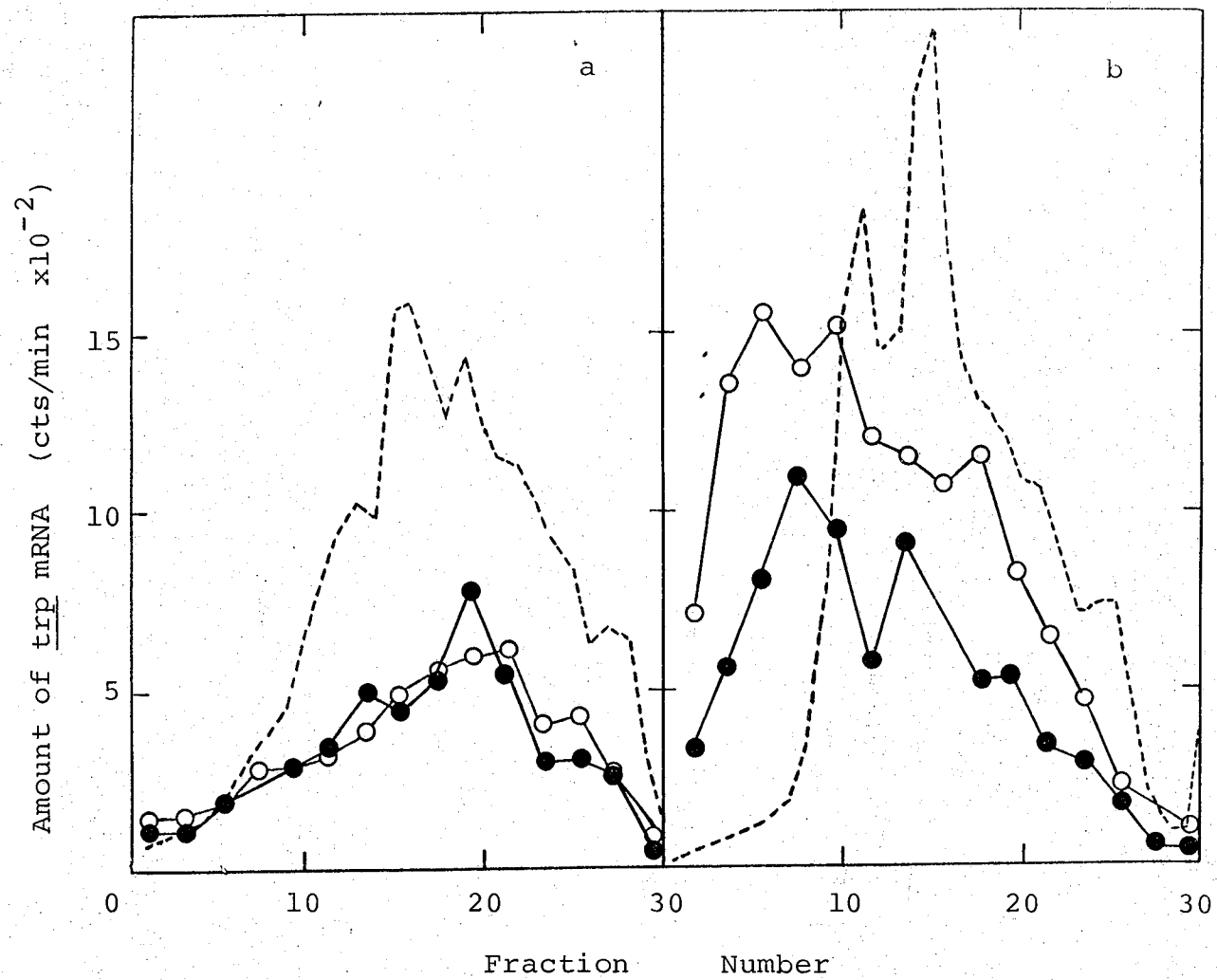


Fig. 5

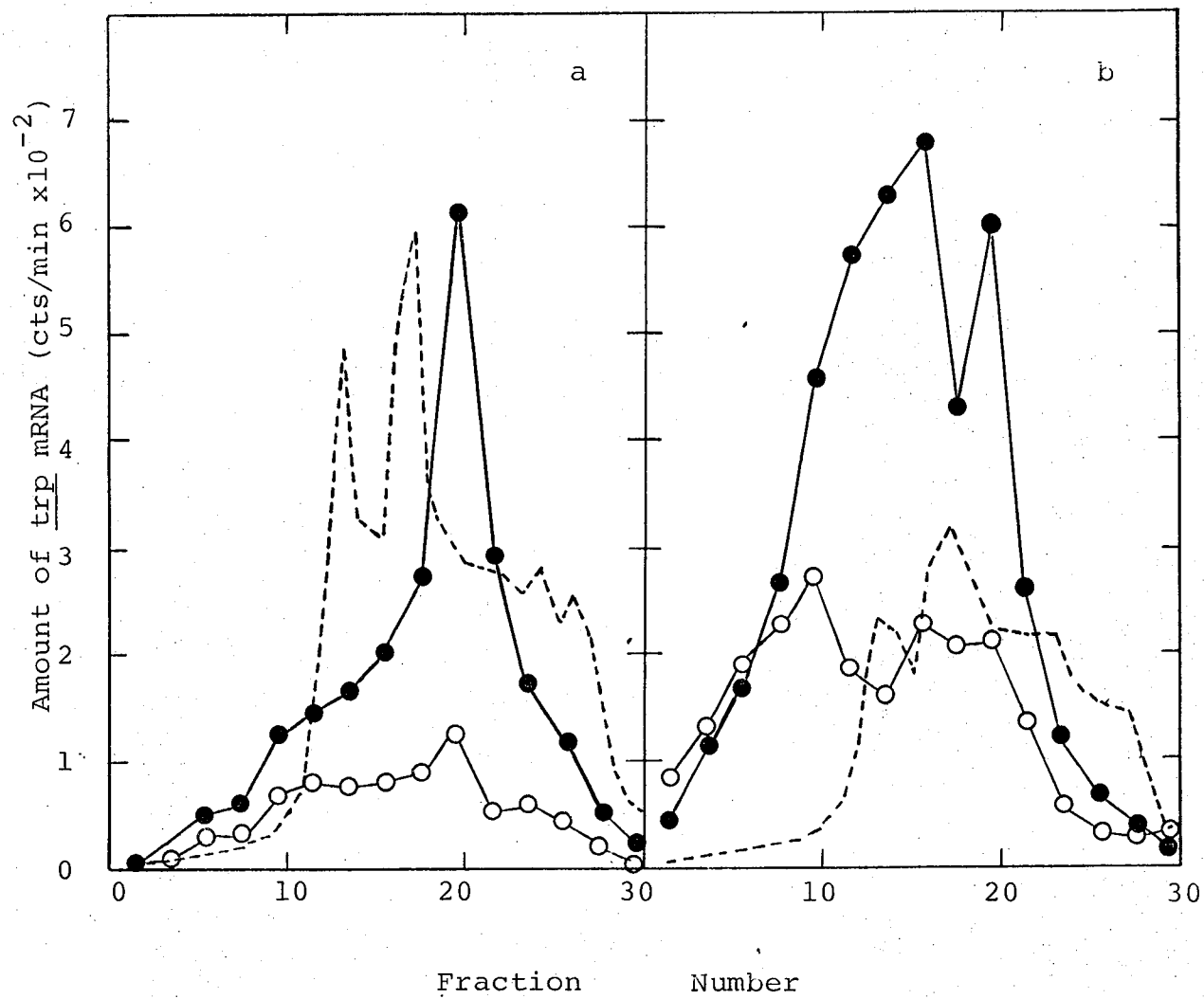
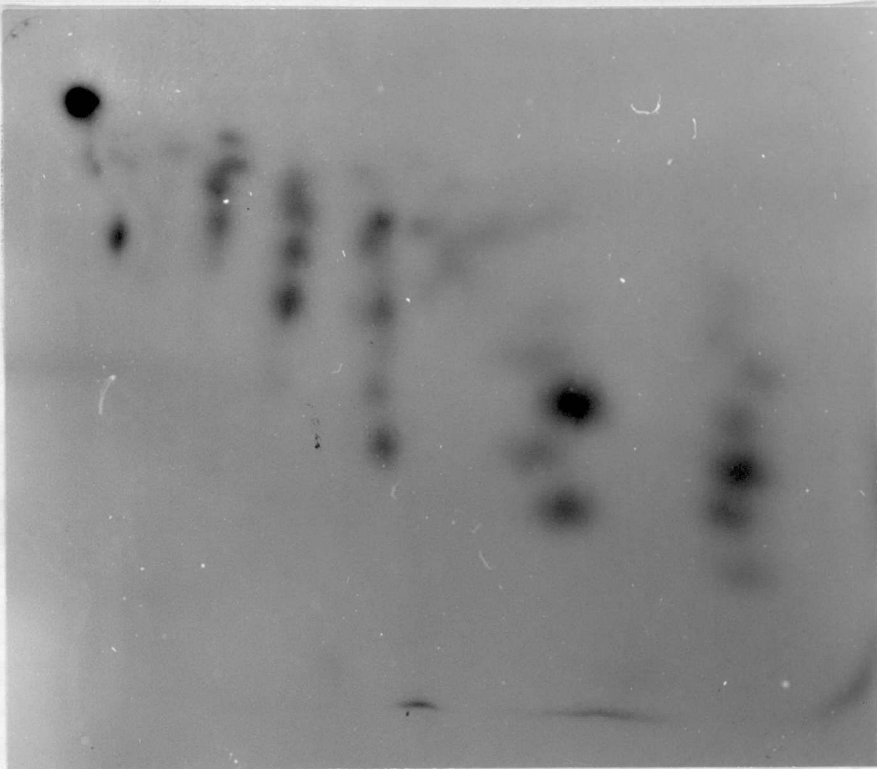
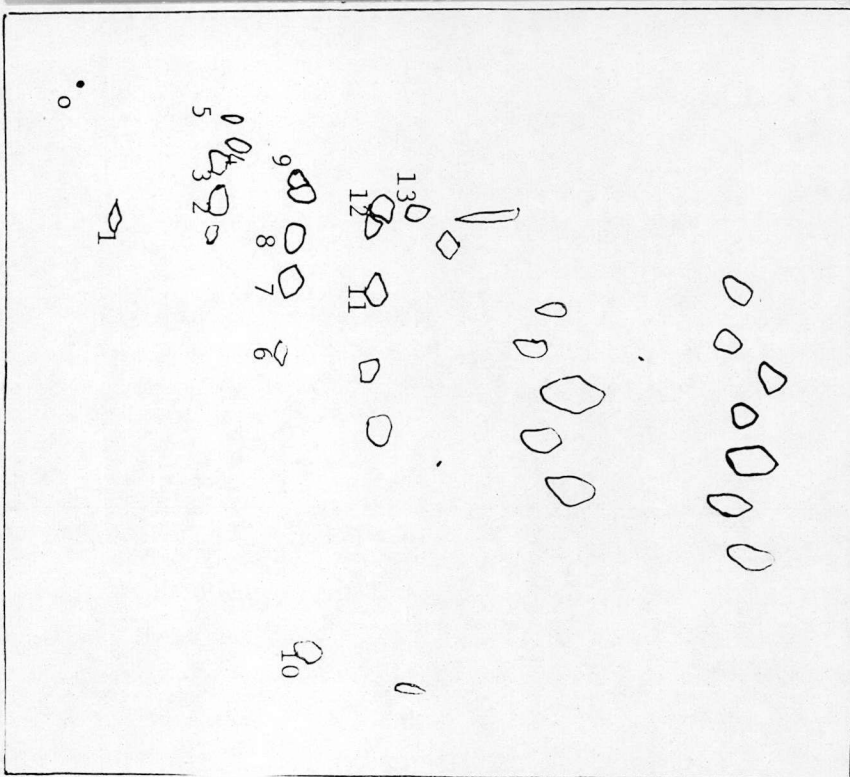




Fig. 6  
a



b



c

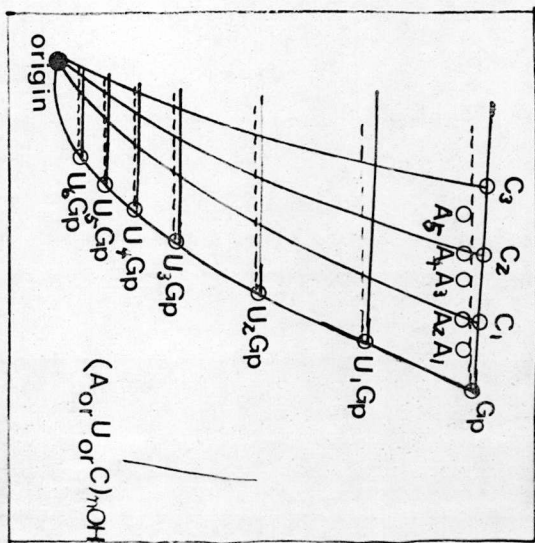
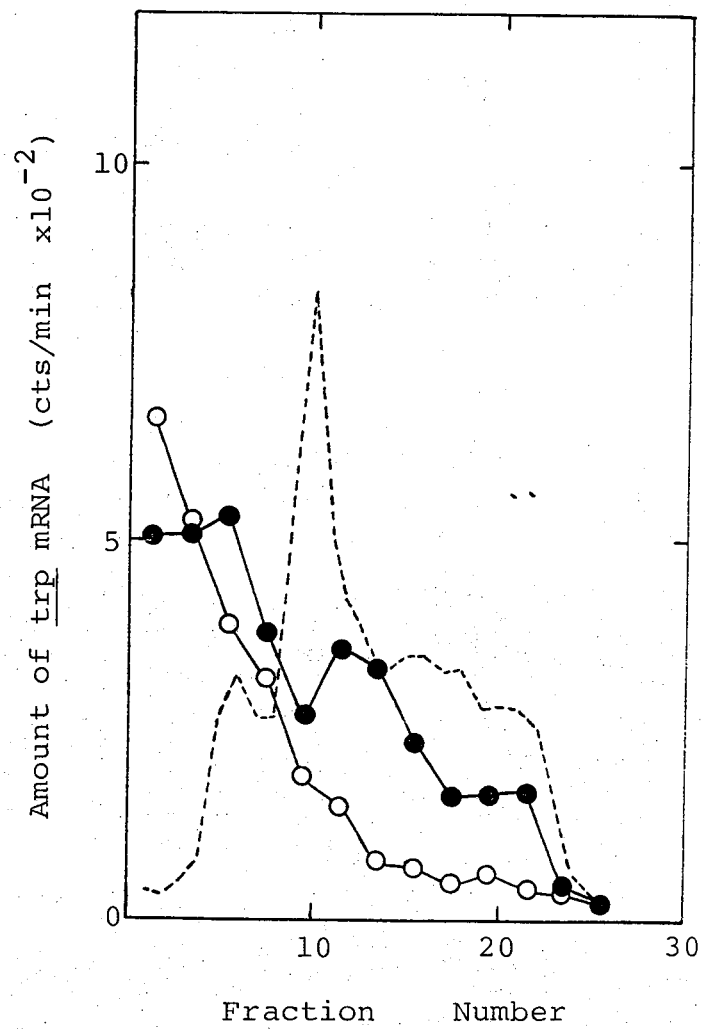


Fig. 7



Part II.

Function of the *tof* Gene Product in Modifying Chemical Stability  
of trp Messenger RNA Synthesized from the  $P_{\underline{L}}$  Promoter of  $\lambda$ trp Phage.

## Summary

The trp operon translocated into the early region of phage  $\lambda$  can be transcribed under the control of two promoters, the authentic trp promoter ( $P_{trp}$  trp mRNA) and the  $P_L$  promoter of the N gene ( $P_L$  trp mRNA) (Imamoto & Tani, 1972; Ihara & Imamoto, 1976a).  $P_L$  trp mRNA has a N message at the 5'-terminal (Tani & Imamoto, 1975) and is chemically stabilized greatly (Yamamoto & Imamoto, 1975), as judged by slow decay to non-hybridizable fragments, of labeled trp mRNA in cultures treated with rifampicin. This stabilization occurs specifically with the  $P_L$  trp mRNA species, but not with  $P_{trp}$  trp mRNA or bulk mRNA species produced by the host bacterial chromosome.

$P_L$  trp mRNA became more stable with time after infection: at early times after infection chemical degradation of  $P_L$  trp mRNA was two-fold slower than for  $P_{trp}$  trp mRNA, while at later times the stabilization of  $P_L$  trp mRNA was almost total. Stabilization of  $P_L$  trp mRNA was markedly reduced when the activity of the tof gene product is low in  $\lambda$  trp carrying a missense (12) mutation of the tof gene. In contrast there is no significant reduction in stabilization when N function is lost in  $\lambda$  trp bearing an amber (53 or 7) mutation of the N gene. The very great difference in stabilization of  $P_L$  trp mRNA produced by normal  $\lambda$  trp and  $\lambda$  trp tof 12 phages was specific for this RNA species: bulk mRNA from the bacterial chromosome decayed at a normal rate in either host cells infected with  $\lambda$  trp and  $\lambda$  trp tof 12 phages.

On the basis of these and other experiments with  $\lambda$  trp tof 12 phage, it is inferred that stabilization of the  $P_L$  trp mRNA is caused by a modification of the decay machinery, most possibly by the protein product of the tof gene. The modified decay machinery could specifically block the signal for initiating mRNA degradation at the 5' end of the  $P_L$  trp mRNA, thereby competing with the normal active decay trigger to the mRNA molecule. Other possible mechanisms are also discussed.

## I. Introduction

Very little is known about the rate-limiting step for chemical degradation of messenger RNA. It is generally accepted that the majority of prokaryotic mRNA is unstable, degraded with an average half-life of a few minutes. However, recent evidence has shown that some species of messenger RNA are rather stable. These may include the messenger species for membrane proteins of E. coli (Hirashima et al., 1973; Lee & Inouye, 1974; Levy, 1975). In fact, at least 10% amount of the RNA molecules that are produced in growing E. coli cells, associate with ribosomes, and are not synthesized under the control of the rel gene product, have been found to be very stable chemically (Silengo et al., to be published). In cells infected with bacteriophages such as T7 (Summers, 1970; Marrs & Yanofsky, 1971), T4 (Craig et al., 1972),  $\phi$ X174 (Hayashi & Hayashi, 1970), M13 (Janish et al., 1970), R17 (Hattman & Hofschneider, 1970) and  $\lambda$  (Gupta & Schlessinger, 1975; Takeda & Kuwano, 1975) phage messengers show greater chemical stability than host mRNA. The differential stability of various mRNA species in a single cell has been directly shown by the demonstration that, in cells infected with  $\lambda$ trp phages, <sup>Y</sup>trp<sub>X</sub>mRNA synthesized as a result of read-through from the P<sub>L</sub> promoter is chemically stable, in contrast to the normal instability of trp mRNA produced originating at the authentic P<sub>trp</sub> promoter (Yamamoto & Imamoto, 1975). In those experiments, it was also found that stability of the trp mRNA from the P<sub>L</sub> promoter produced at 15 minutes after infection is greater than that produced at <sup>X</sup>5 minutes after infection.

Here I show that stabilization of the P<sub>L</sub>trp mRNA molecule is attributable to a modification caused by a phage-coded protein, the product of the tof gene. By comparison, the N gene product, like the tof gene product important in the control of  $\lambda$  mRNA production appears to play little if any role in the stabilization of the trp mRNA. Possible mechanisms of mRNA stabilization by tof gene product are discussed.

## 2. Materials and Methods

### (a) Bacterial and phage strains

The following strains of E. coli K12 were used for infection experiments; W3110 trpR<sup>+</sup>trpAE1, a large deletion mutant covering the whole trp operon (Yanofsky & Ito, 1966) and W3110 trpR<sup>+</sup>trp AE1sul.

The following phages were used for infection experiments; non-transducing phage,  $\lambda$ trp(h <sup>$\lambda$</sup> att<sup>80</sup>trp60-3imm $\lambda$ QSR $\lambda$ ), which replaces the  $\lambda$ genes to the left of N with the whole trp operon of E. coli and in which the trp operon is controlled by two promoters, the trp and the P<sub>L</sub> promoter, and by two operators, the trp and the O<sub>L</sub> operator (Imamoto & Tani, 1972; Ihara & Imamoto, 1976; Segawa & Imamoto, 1974), and its derivatives,  $\lambda$ trpsusN53,  $\lambda$ trptof12 and  $\lambda$ trpsusN7tof12, which were obtained by crosses with  $\lambda$ susN7susN53 or  $\lambda$ tof12 (Takeda et al., 1975). The genetic map of the  $\lambda$ trp phage is shown in Figure 1.

The following phages were used as DNA sources for DNA-RNA hybridization assays; the non-transducing parental phage  $\phi$ 80; non-defective transducing phages  $\phi$ 80trpED and  $\phi$ 80trpC-A. The trp operon segments carried by the  $\phi$ 80trp phages are shown in Figure 1.

### (b) Preparation of $\lambda$ trp phages

$\lambda$ trp phages were prepared by lytic infection of sensitive bacteria C600S (Sato et al., 1968) in the medium containing 10g bacto-tryptone, 2.5g NaCl and one litre water. Phages were concentrated with one cycle of low- and high-speed centrifugation and suspended in <sup>Y</sup> $\lambda$  dilution fluid [ $1 \times 10^{-2}$ M Tris-HCl buffer (pH 7.3) and  $1 \times 10^{-2}$ M MgCl<sub>2</sub>] containing 20  $\mu$ g/ml bovine serum albumin.

### (c) Preparation of phage DNA

Lysates of  $\phi$ 80 and  $\phi$ 80trp phages were prepared by lytic growth on sensitive bacteria E. coli K12 W1485. After low- and high-speed centrifugation, phage

particles were suspended in T1 dilution fluid [ $6 \times 10^{-4} \text{ M MgSO}_4$ ,  $5 \times 10^{-4} \text{ M CaCl}_2$ ,  $1 \times 10^{-3} \%$  gelatin and  $6 \times 10^{-3} \text{ M Tris-HCl}$  buffer (pH 7.3)] and treated with DNAase ( $5 \mu\text{g/ml}$ ). After DNAase treatment, phages were purified by a combination of one cycle of low- and high-speed centrifugation and CsCl density centrifugation. Phages were suspended at a concentration of about  $2 \times 10^{12}/\text{ml}$  in  $0.1 \text{ M}$  sodium phosphate buffer (pH 7.1) containing  $0.1 \text{ M NaCl}$  after dialysis against the buffer. Phage DNAs were extracted by phenol treatment (Kaiser & Hogness, 1960) and dissolved in a saline-citrate solution ( $1 \times \text{SSC}$ :  $0.15 \text{ M NaCl}/0.015 \text{ M}$  sodium citrate) after dialysis against the solution.

(d) Preparation of pulse-labeled RNA

Bacteria were grown with aeration to  $6 \times 10^8$  cells/ml in an enriched medium (L-broth) (Lennox, 1955) supplemented with L-tryptophan ( $50 \mu\text{g/ml}$ ). The cells were collected by centrifugation and washed twice with cold T1-dilution fluid. Cells were suspended in the T1 dilution fluid containing  $2 \times 10^{-3} \text{ M KCN}$  to give a final density of  $2.5 \times 10^9$  cells/ml and were infected with each of the  $\lambda_{\text{trp}}$  phages at a multiplicity of about 5. After incubation for 15 min at  $30^\circ\text{C}$ , unadsorbed phages were removed by centrifugation and infected cells were resuspended in cold minimal medium (Vogel & Bonner, 1956) to give a final concentration of  $6 \times 10^{10}$  cells/ml. The cell suspension was diluted by 30 fold with prewarmed ( $30^\circ\text{C}$ ) minimal medium supplemented with 19 amino acids (each  $0.5 \text{ mM}$ ) with L-tryptophan ( $50 \mu\text{g/ml}$ ) and was shaken vigorously in water bath at  $30^\circ\text{C}$ . At a suitable time during incubation, the cell suspension was pulse-labeled with [ $^3\text{H}$ ]uridine as indicated in the legends to the Figures. A portion ( $6 \times 10^9$  cells) of cell suspension was withdrawn and poured onto 35 ml of crushed frozen medium containing  $1 \times 10^{-2} \text{ M tris-HCl}$  buffer (pH 7.3),  $5 \times 10^{-3} \text{ M MgCl}_2$ ,  $1 \times 10^{-2} \text{ M NaN}_3$  and  $250 \mu\text{g/ml}$  of chloramphenicol. RNA was prepared according to the procedure reported previously (Imamoto, 1969). The [ $^3\text{H}$ ]RNA obtained was dissolved in  $1 \times 10^{-2} \text{ M Tris-HCl}$  buffer (pH 7.3) containing  $0.5 \text{ M}$

KCl and  $1 \times 10^{-3} \text{ M Na}_2\text{EDTA}$ .

(e) DNA-RNA hybridization

The hybridization procedure was as follows: DNA of  $\phi 80$  or  $\phi 80\text{trp}$  phages was diluted to a concentration of  $100 \text{ } \mu\text{g/ml}$  in  $1 \times \text{SSC}$  and heated in boiling water for 10 min followed by rapid cooling in ice water. The RNA was further diluted to a concentration of  $8 \text{ } \mu\text{g/ml}$  in  $3 \times \text{SSC}$ . Five milliliters of the DNA solution was filtered through a Millipore filter (type HA,  $0.45 \text{ } \mu\text{m}$  pore size) of 25 mm diameter. The filter was washed with 40 ml of  $3 \times \text{SSC}$ , cut into 8 pieces and dried at  $80^\circ\text{C}$  for 2 hours. Assay of trp mRNA was carried out by immersing one of these filter bits in  $100 \text{ } \mu\text{l}$  of  $[^3\text{H}]\text{-RNA}$  solution in  $1 \times 10^{-2} \text{ M Tris-HCl}$  buffer (pH 7.3) containing  $0.5 \text{ M KCl}$  and  $1 \times 10^{-3} \text{ M Na}_2\text{EDTA}$  and incubated at  $66^\circ\text{C}$  for 18 hours. Afterwards, the filters were treated with RNAase ( $5 \text{ } \mu\text{g/ml}$ ) in  $1 \times \text{SSC}$  for 30 min at  $37^\circ\text{C}$ , washed with  $1 \times \text{SSC}$ , dried and counted in toluene-based scintillation fluid. Total radioactivity of  $[^3\text{H}]\text{uridine}$  incorporated into RNA (total  $[^3\text{H}]\text{RNA}$ ) was measured as the material precipitable by cold trichloroacetic acid.

(f) Reagents

Tritiated uridine was purchased from the New England Nuclear Company and was used without the addition of carrier. Millipore filters were purchased from the Millipore Filter Company, Bedford, Mass. Chloramphenicol was from Park Davis. DNAase and RNAase were obtained from the Worthington Biochemical Company. RNAase was used after heating at  $80^\circ\text{C}$  for 20 min in  $0.15 \text{ M NaCl}$  to inactivate any contaminating DNAase.



### 3. Results

In order to demonstrate uniquely  $P_L$ -promoted synthesis of trp mRNA, strain trpAE1, a strain with the trp operon deleted but which retains a tryptophan regulator (trpR) gene located near thr on the chromosome of E. coli (Cohen & Jacob, 1959), was infected with  $\lambda$ trp phage in the presence of tryptophan. The trp mRNA synthesized from  $\lambda$ trp was assayed by employing DNA of  $\phi 80$ trp phages carrying various trp gene segments as a DNA complement in DNA-RNA hybridization reaction.

The notion that, in  $\lambda$ trp phages employed here, the  $t_L$  terminator has been deleted is supported by the following lines of evidence: (1) location of the right endpoint of the bacterial substitution in the phage falls well within the imm<sup>21</sup> region, most probably to the right to the  $t_L$  site (Fiandt et al., 1974); (2) the  $P_L$  promoted transcription is permitted to extend from the  $\lambda$  genome into the trp operon, even when synthesis of the N product is blocked by antibiotics (Imamoto & Tani, 1972; Ihara & Imamoto, 1976b) or the product of the N gene loses activity in  $\lambda$ trp phage with an amber mutation in the N gene (Yamamoto et al., to be published); (3) under conditions in which N gene activity diminishes in the E. coli K95 nus host at non-permissive temperature (Friedman et al., 1973), the steady-state level of the synthesis of mRNA for the Q, S and R genes for the  $P_R$  promoter in  $\lambda$ trp phage is severely suppressed, while trp mRNA synthesis from the  $P_L$  promoter is not affected drastically (data not shown).

#### (a) Progressive stabilization of the trp mRNA after infection

To confirm the possibility that stability of the trp mRNA produced from the  $P_L$  promoter increases with time after infection (Yamamoto & Imamoto, 1975) the decay kinetics of stabilization of the  $P_L$  promoted trp mRNA is analyzed in further detail. The result is shown in Figure 2 suggesting that the stabilization might require a modification of decay

machinery, possibly by some phage-specific protein. At the 5th (Fig. 2(a)), 15th (b) or 20th (c) minute of incubation after infecting trpAE1 with  $\lambda$ trp phage in the presence of tryptophan, cells were pulse-labeled with [ $^3$ H]-uridine for 1 min and then chased by the addition of rifampicin and an excess amount of unlabeled uridine to inhibit further incorporation of [ $^3$ H]-uridine into mRNA. Samples were removed to measure the amount of trp mRNA remaining at subsequent times.

The pattern seen in the Fig. 2(a) is consistent with previous observation that, upon addition of rifampicin, initiation of transcription is blocked almost instantaneously and both P<sub>L</sub>trpED mRNA and P<sub>L</sub>trpCBA mRNA decayed chemically at a half-life of 5 to 6 minutes, which was two times slower than for trp mRNA synthesized originating at the P<sub>trp</sub> promoter (Yamamoto & Imamoto, 1975). In contrast to the mode of decay at this early stage of phage infection, P<sub>L</sub>trp mRNA synthesized at a later stage was markedly stabilized. Fifteen minutes after phage infection begins, a part of the trpED mRNA (and sometimes of trpCBA mRNA as well) still decayed, but most of the trp mRNA remained detectable by hybridization with  $\phi$ 80trp DNA for at least 25 minutes after rifampicin addition (Fig. 2(b)). Stabilization of P<sub>L</sub>trp RNA was almost complete if the molecules were labeled with [ $^3$ H]-uridine at the 20th minute after infection and followed one minute later by rifampicin (Fig. 2(c)).

The possibility has been excluded that the stabilization of P<sub>L</sub>trp mRNA observed in the experiment above could be attributable to some physiological change related to phage growth: at the 20th minute after infection of trpAE1 with the  $\lambda$ trp phages in the presence of tryptophan, bulk mRNA from the bacterial chromosome decayed at a normal rate, with a half-life of 3 to 4 minutes at 30°C, as was seen with the mRNA from uninfected cells (data not shown; cf.

Yamamoto & Imamoto, 1975 and Fig. 6). Thereby it is also substantiated that rifampicin shuts off transcription initiation in the later stage of phage infection as rapidly as it does in uninfected bacteria.

As candidates for phage-specific protein factor(s) required possibly for a modification of the decay trigger, I examined the protein products of the tof and N genes of  $\lambda$ trp phage, as described in the following sections.

(b) Effect of  $\text{tof}^-$  and  $\text{N}^-$  mutations on trp transcription of  $\lambda$ trp phage

Before investigating the effect of these mutations on the chemical stability of  $\text{P}_{\text{L}}^{\wedge}\text{trp}$  mRNA, I tested their effects on  $\text{P}_{\text{L}}$ -promoted transcription of the translocated trp operon in  $\lambda$ trp to ensure that the products produced by these mutated genes were <sup>in</sup>active. When trpAE1 was infected with  $\lambda$ trp phage in the presence of tryptophan at 30°C, the rate of synthesis of  $\text{P}_{\text{L}}^{\wedge}\text{trp}$  mRNA reaches a sharp maximum during the first three to seven minutes after infection and then declines until it reaches a steady state. The rate of synthesis remains nearly constant during the period from 12 to at least 20 minutes after infection (Yamamoto & Imamoto, 1975; Ihara & Imamoto, 1976a). The decline in the rate of synthesis seen after several minutes of infection is believed to be caused by the function of the tof gene, whose product acts at the operator of the N gene and acts to reduce transcription from the 1-strand of the "early" region of  $\lambda$  soon after phage development begins (Szybalski et al., 1970).

In Figure 3(a), I show changes in the rate of  $\text{P}_{\text{L}}$ -promoted synthesis of trp mRNA in  $\lambda$ trp phage which has a missense (12) mutation in the tof gene. The synthesis of the trp mRNA by  $\lambda$ trptof12 phage, as expected, now continued at a high, nearly constant rate for at least 24 minutes after infection.

In  $\lambda$ trp phages retaining an amber (53) mutation in the N gene, the rates of trp mRNA synthesis are seemingly much lower than those of normal  $\lambda$ trp phage during incubation for about 23 minutes after infection (Fig. 3(b)).

[Significant production of trp mRNA occurs in this case from only the operator-proximal portion of the trp operon, due to premature arrest of transcription at a specific site in the trpE gene, effected upon a loss of N function, ( see part I of this work ] However, even in the absence of function of the N gene product, reduction in the overall rate of trp mRNA synthesis caused by the tof gene product was also observed.

The synthesis of the trp mRNA by λtrpsusN7tof12 phage exhibited an alleviation of the phenomenon of transcription shutdown, and continued at nearly constant, though lower than normal, rate for at least 24 minutes after infection. Reduction in the overall rate of trp mRNA synthesis from both the operator-proximal and distal region of the operon was partially relieved when an amber mutation in the N gene in λtrpsusN7tof12 phage was suppressed by introduction of sul into host bacteria ( see Fig.2 of part I in this work).

(c) Effect of tof<sup>-</sup> and N<sup>-</sup> mutations on stabilization of trp mRNA

Since the chemical stability of the P<sub>L</sub>trp mRNA becomes even greater as infection progresses, it seems likely that the rate-limiting step to initiate mRNA degradation is somehow modified by phage-specific protein(s). Accordingly, I examined whether the stabilization of the P<sub>L</sub>trp mRNA was altered in λtrp phages retaining an amber (53 or 7) mutation in the N gene, or a missense (12) mutation in the tof gene or both.

In Figure 4, degradation of P<sub>L</sub>trp mRNA in λtrptof12 after inhibition of the synthesis by the addition of rifampicin at the 6th (a), 16th (b) and 21st (c) minute after the phage infection is shown. There is an obvious great delay in stabilization of the P<sub>L</sub>trp mRNA in the mutant phage, when compared with the profiles of P<sub>L</sub>trp mRNA by normal λtrp phage (Fig. 2): the pattern seen in Fig. 4(b) is essentially similar to that of P<sub>L</sub>trp mRNA produced by λtrp at the 5th minute after infection (Fig. 2(a)); and even at the 21st minute after infection of λtrptof12, a considerable fraction (more than 60%

amount) of the P<sub>L</sub>trp mRNA remained unstable (Fig. 4(c)).

The P<sub>L</sub>trp mRNA molecules produced when the activity of the N gene product is lost in λtrpsusN53 phage are found to be stabilized in essentially normal fashion, as seen in Fig. 5(a). However, stabilization of P<sub>L</sub>trp mRNA was apparently prevented or abrogated for λtrpsusN7 phage bearing the tof12 mutation (Fig. 5(b)). [Under these conditions, the trp mRNA sequences assayed corresponded to only the beginning portion of the trpE gene, since the P<sub>L</sub>-promoted transcription was frequently blocked at a site of trpE gene when functional N gene product was deficient (Yamamoto *et al.*, to be published).] The loss of the capacity to stabilize P<sub>L</sub>trp mRNA in λtrp phage retaining the susN7 and tof12 mutations was not significantly relieved even when an amber (7) mutation of the N gene was suppressed by introduction of sul into host bacteria (Fig. 5(c)). Upon suppression of the amber mutation in the N gene, the P<sub>L</sub>-promoted trp mRNA synthesis now extended from the trpE region to the operator-distal trp genes (Yamamoto *et al.*, to be published), thus allowing detection of the mRNA for trpC, trpB and trpA genes.

When the profiles for stability of P<sub>L</sub>trp mRNA from λtrpsusN7tof12 in trpAE1 (Fig. 5(b)) and trpAE1sul (Fig. 5(c)) were compared with those for stability of the mRNA from λtrptof12 (Fig. 4(b) and (c)), P<sub>L</sub>trp mRNA

appears to be somewhat more unstable when both the N and tof gene products are inactive in λtrpsusN7tof12 than when only the tof12<sup>-</sup> lesion is present. However, this seeming difference in the extent of stabilization could be due to a variation of physiological conditions of the host cells, thereby permitting some accumulation, at varied levels, of the protein product of a mutated tof gene, perhaps with some activity (Takeda *et al.*, 1975).

In any event, however, it can be concluded that the stabilization of P<sub>L</sub>trp mRNA is markedly inhibited when the activity of the tof gene product is low, but is not significantly changed in absence of the N gene product.

The remarkable difference in stability of  $P_{L\text{trp}}$  mRNA produced by normal  $\lambda\text{trp}$  and  $\lambda\text{trptofl2}$  phages was specific for this RNA species. Thus bulk mRNA from the bacterial chromosome decayed at a normal rate in either host bacteria infected with  $\lambda\text{trp}$  and  $\lambda\text{trptofl2}$  phages, with a half-life of approximately 3 minutes under both sets of conditions for  $\text{tof}^+$  and  $\text{tof}^-$  (Fig. 6). Therefore, the possibility can be ruled out that the modification of stability of  $P_{L\text{trp}}$  mRNA observed in the experiment presented above might be attributable to some general physiological feature related to phage growth.

#### (4) Discussion

The experiments presented here show that mutation in the tof gene seems to negate or prevent stabilization of trp mRNA produced from the P<sub>L</sub> promoter. In normal  $\lambda$ trp phages, stabilization of P<sub>L</sub>trp mRNA increases with time after phage infection; at the fifth minute after infection, the majority of P<sub>L</sub>trp mRNA still decays chemically, though at an overall rate of two times slower than does the trp mRNA synthesized originating at the authentic trp promoter; while at the 15th minute after phage infection at least half the P<sub>L</sub>trp mRNA is completely stable (Fig. 2). The time course of progressive stabilization of P<sub>L</sub>trp mRNA appears consistent with the onset of the lowered rate of synthesis of P<sub>L</sub>trp mRNA caused by the function of the tof gene product: the level of tof protein becomes high enough to repress the trp mRNA synthesis to a steady-state rate only about 12 minutes of infection (Yamamoto & Imamoto, 1975).

Both functional inactivation and chemical degradation of P<sub>L</sub>trp mRNA are twofold slower at early stages of the phage development (Yamamoto & Imamoto, 1975) <sup>than that of P<sub>trp</sub>trp mRNA</sup>. Slower ~~chemical~~ degradation might somehow be related to the lower frequency of initiation of translation at the 5'-end of the mRNA chains. Some phage specific protein(s) other than the tof and N genes might also play a role in partially stabilizing the P<sub>L</sub>trp mRNA produced at early times after the  $\lambda$ trp infection. Or, alternatively, differences in the nucleotide sequence at the 5' end of the P<sub>L</sub>trp mRNA could make <sup>the</sup>  $\lambda$  molecule less accessible to decay machinery not yet modified by the phage product.

In read-through transcription of the gal operon from the phage's P<sub>L</sub> promoter in cells lysogenic for  $\lambda$ , which is caused upon induction of prophages, it has been suggested that the stabilization of the transcripts may be related to the function of phage protein(s) produced from gene(s) located in the N operon (Adhya et al., Personal communication). In the present

case, however, these factor(s) do not seem to participate in the mechanism for stabilizing the  $P_{L\text{trp}}$  mRNA, since the region of the  $N$  operon between the  $CIII$  and  $att$  has been deleted in  $\lambda\text{trp}$  phage employed: they are replaced with *E. coli* DNA including the  $\text{trp}$  operon. Moreover,  $P_{L\text{trp}}$  mRNA can still be stabilized even in the absence of the  $N$  function (Fig. 5a), under conditions in which the phage genes located downstream from the  $N$  gene or the  $\text{tof}$  gene are not expressed.

In any case, experimental evidence reported here (Fig. 4 & 5) show that the  $\text{tof}$  gene product is critical for the stabilization of  $P_{L\text{trp}}$  mRNA.

The small extent of stabilization of  $P_{L\text{trp}}$  mRNA still observed can reasonably be considered to be due to some leakiness of the missense (12) mutation in the  $\text{tof}$  gene product (Takeda *et al.*, 1975).

Many species of phage messenger RNA in infected cells are relatively stable. In these cases, at least, stabilization of the mRNA might generally be attributable to some modification of the decay machinery of the host bacteria. In addition, degradation of mRNA is believed to be coupled with the translational processes (Schlessinger, 1971; Imamoto & Schlessinger, 1974). Modification by a phage product could therefore occur at the level of the enzymatic machinery associated with ribosomes. Perhaps decay machinery is modified to have a specifically strong affinity for the 5' structure of the  $P_{L\text{trp}}$  mRNA, competitively blocking the accessibility of the mRNA molecule to normally active decay machinery. Alternatively, modification might occur at sensitive sites on the  $\lambda$  portion of the mRNA at which decay is triggered. The structure at the 5' end of  $P_{L\text{trp}}$  mRNA has been so modified by a mechanism involving the function of the  $\text{tof}$  gene product that the molecule is no longer accessible to active decay machinery.



It has recently been reported that trp mRNA synthesis starting from the  $P_L$  promoter in  $\lambda^{\text{trp}}$  phage does not express polarity in vivo (Imamoto & Tani, 1972; Segawa & Imamoto, 1974; Franklin, 1974; Ihara & Imamoto, 1976b).

The mechanism of relaxation of polarity may involve a modification of transcriptional machinery by the action of phage protein(s) (Franklin & Yanofsky, 1976; Adhya et al., 1976; Yamamoto & Imamoto, to be published; Nakamura & Imamoto, to be published), thereby leading to a failure in a normal coupling of transcriptional machinery with translation.

Is the relief of polarity in trp transcripts from  $P_L$  promoter somehow related to the change in function of the mRNA decay machinery as reported here?

Though there is no logical necessity for any connection, in fact, a loss of tof function, which renders mRNA unstable (Fig. 4) also alters the nature of  $P_L$  promoted transcription. In particular, on infecting by  $\lambda^{\text{trp tof12}}$ , trp transcription from the  $P_L$  promoter becomes partially sensitive to blockage of transcription in presence of chloramphenicol, in contrast to insensitivity of transcription in the normal  $\lambda^{\text{trp}}$  phage (though inhibition of transcription by the antibiotic is more severe in the absence of the N product; Nakamura et al., to be published).

All such effect occur subsequent to "triggering" events at the 5'-end of the mRNA, where some indispensable factor(s) required for coupling of RNA polymerase function to translational machinery might also play a role in determining the rate-limiting step of mRNA decay. Both tof and N gene products might first function there.

The metabolism of  $P_L$  trp mRNA cannot fit in a simple, prevailing model where a protein (like rho factor Richardson et al., 1975; Ratner, 1976) arrests transcription upon blocking translation, and where presence of N protein on RNA polymerase counteract this effect.

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## Legends to Figures

Figure 1.

Simplified molecular maps of coliphages.

The relative size of each trp gene carried by  $\phi 80$ trp and  $\lambda$ trp phages is estimated from the molecular weight of the corresponding polypeptides (Imamoto & Yanofsky, 1967). The genetic map of the transducing phage  $\lambda$ trp, is based on the data of Nishimune (1973) and Flandt et al. (1974). Location of the right endpoint of the trp substitution in the phage is represented aligning on a relative scale for  $\lambda$  chromosome length (Davidson & Szybalski, 1971). For the markers describing the maps see Szybalski (1970). Dotted, solid and double lines indicate the region of  $\phi 80$  genome,  $\lambda$  genome and bacterial chromosome including the trp genes, respectively. There are probably still some bacterial genes adjacent to the trpE and trpA.

Figure 2.

Time process of stabilization of trp mRNA synthesized by  $P_L$  promoter in  $\lambda$ trp.

Cells of trpAEl were infected with  $\lambda$ trp and incubated in the presence of L-tryptophan (50  $\mu$ g/ml). Pulse labeling was carried out with 33.0 Ci of [ $^3$ H]uridine/ml of the culture for 1 min at the 5th (a), 15th (b) or 20th (c) minute after infection, and followed by addition of rifampicin (300  $\mu$ g/ml), unlabeled uridine (1 mg/ml) and nalidixic acid (10  $\mu$ g/ml). A portion of the culture ( $6 \times 10^9$  cells) was removed at indicated times during incubation after addition of rifampicin and RNA was extracted. The trp mRNA values are expressed as [ $^3$ H]RNA hybridized/ $\mu$ g <sup>RNA</sup> and normalized to 100% for the maximum value. [ $^3$ H]RNA hybridized specifically with DNA from  $\phi 80$ trpED or  $\phi 80$ trpCBA was designated trpED mRNA or trpCBA mRNA, respectively. The values with  $\phi 80$  DNA background were subtracted from each hybrid value.

Values represented are the average of duplicate determinations. 6.2 to 12.2  $\mu\text{g}$  of [ $^3\text{H}$ ]RNA (spec. act.  $0.7 \times 10^4$  to  $3.9 \times 10^4$  cts/min per  $\mu\text{g}$ ) were used for each hybrid assay. The other conditions are as described in Materials and Methods. (●) trpED mRNA ; (○) trpCBA mRNA

Figure 3.

Time course of transcription of the translocated trp operon in  $\lambda\text{trp}$  in the absence of phage tof or N gene function.

Cells of trpAE1 were infected with  $\lambda\text{trptof12}$  (a) or  $\lambda\text{trpsusN53}$  (b) phages and incubated in the presence of L-tryptophan. The cultures were pulse-labeled with 100 $\mu\text{Ci}$  (a) or 200 $\mu\text{Ci}$  (b) of [ $^3\text{H}$ ]uridine for 1 min at the times indicated. After infection 7.5 to 19.0  $\mu\text{g}$  of [ $^3\text{H}$ ]RNA (spec. act.  $3.4 \times 10^4$  to  $5.0 \times 10^4$  cts/min per  $\mu\text{g}$ ) were used for each hybrid assay. Values represented are the percentage of hybrid RNA relative to the total labeled RNA and the averages of duplicate determinations. Data are plotted in the middle of each period of pulse-labeling. The other conditions are as described in the legend of Fig. 2 and Materials and Methods. (●) trpED mRNA ; (○) trpCBA mRNA.

Figure 4.

Time process of stabilization of trp mRNA synthesized by  $P_L$ -promoter in the absence of tof gene function.

Cells of trpAE1 were infected with  $\lambda\text{trptof12}$  phages and incubated in the presence of L-tryptophan. The cultures were pulse-labeled with [ $^3\text{H}$ ]uridine for 1 min at the 5th (a), 15th (b) or 20th (c) minute after the phage infection. Decay of  $P_L$  trp mRNA was assayed in the condition described in the legend of Fig. 2 and Materials and Methods. 8.8 to 17.6  $\mu\text{g}$  of [ $^3\text{H}$ ]RNA (spec. act.  $0.5 \times 10^4$  to  $2.7 \times 10^4$  cts/min per  $\mu\text{g}$ ) were used for each hybrid assay. (●) trpED mRNA ; (○) trpCBA mRNA

Figure 5.

Effect of N and tof gene function on chemical stability of  $P_L$ -trp mRNA.

In (a) and (b), cells of trpAE1 were infected with  $\lambda$ trpsusN53 and  $\lambda$ trpsusN7tof12, respectively, and, in (c), cells of trpAElsul were infected with  $\lambda$ trpsusN7tof12. After infection cells were cultured in the presence of L-tryptophan and pulse-labeled with [ $^3$ H]uridine for 1 min at the 15th (a and b) and 20th minute (c) after infection. Decay of  $P_L$ -trp mRNA was assayed in the condition described in the legend of Fig. 2 and Materials and Methods. 8.0 to 31.0  $\mu$ g of [ $^3$ H]RNA (spec. act.  $1.4 \times 10^4$  to  $4.3 \times 10^4$  cts/min per  $\mu$ g) were used for each hybrid assay. (●) trpED mRNA ; (○) trpCBA mRNA

Figure 6.

Comparison of chemical stability of bulk mRNA under  $P_L$ -promoted conditions.

Cells of trpAE1 were infected with  $\lambda$ trp (a) and  $\lambda$ trptof12 (b) and then incubated at 30°C at a concentration of  $2 \times 10^8$  cells/ml in the presence of L-tryptophan (50  $\mu$ g/ml). Cells were pulse-labeled with 3  $\mu$ Ci of [ $^3$ H]uridine for 30 seconds at the 15.5th minute of incubation, and followed by the addition of rifampicin (300  $\mu$ g/ml), nalidixic acid (10  $\mu$ g/ml) and unlabeled uridine (1 mg/ml). A portion (0.2ml) of the culture was removed at the indicated times during incubation after addition of rifampicin and poured onto 2 ml of 5% ice-cold trichloroacetic acid. After at least 30 min on ice, each sample was filtered onto a glass fiber filter, washed twice with 5% trichloroacetic acid in 50% ethanol, once with 5 ml of 95% ethanol and once with 0.1 N HCl (Silengo et al., 1974). Dried filters were then counted with a toluene-based scintillator. The total count of RNA unstable during a 20 min incubation of the cultures was set equal to 100%. The fraction of the unstable RNA remaining at each time is plotted against time. The 100% point represents

about  $3.4 \times 10^3$  (a) and  $3.3 \times 10^3$  (b) cts/min. The other conditions are as described in Materials & Methods.

Fig. 1

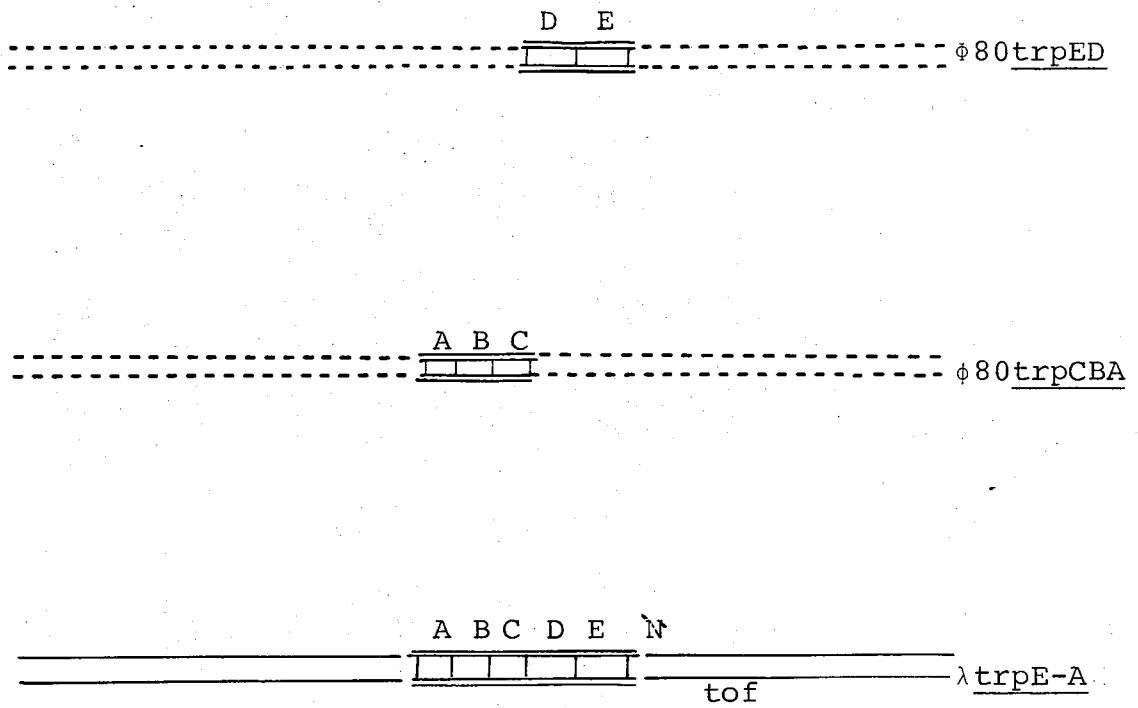




Fig. 2

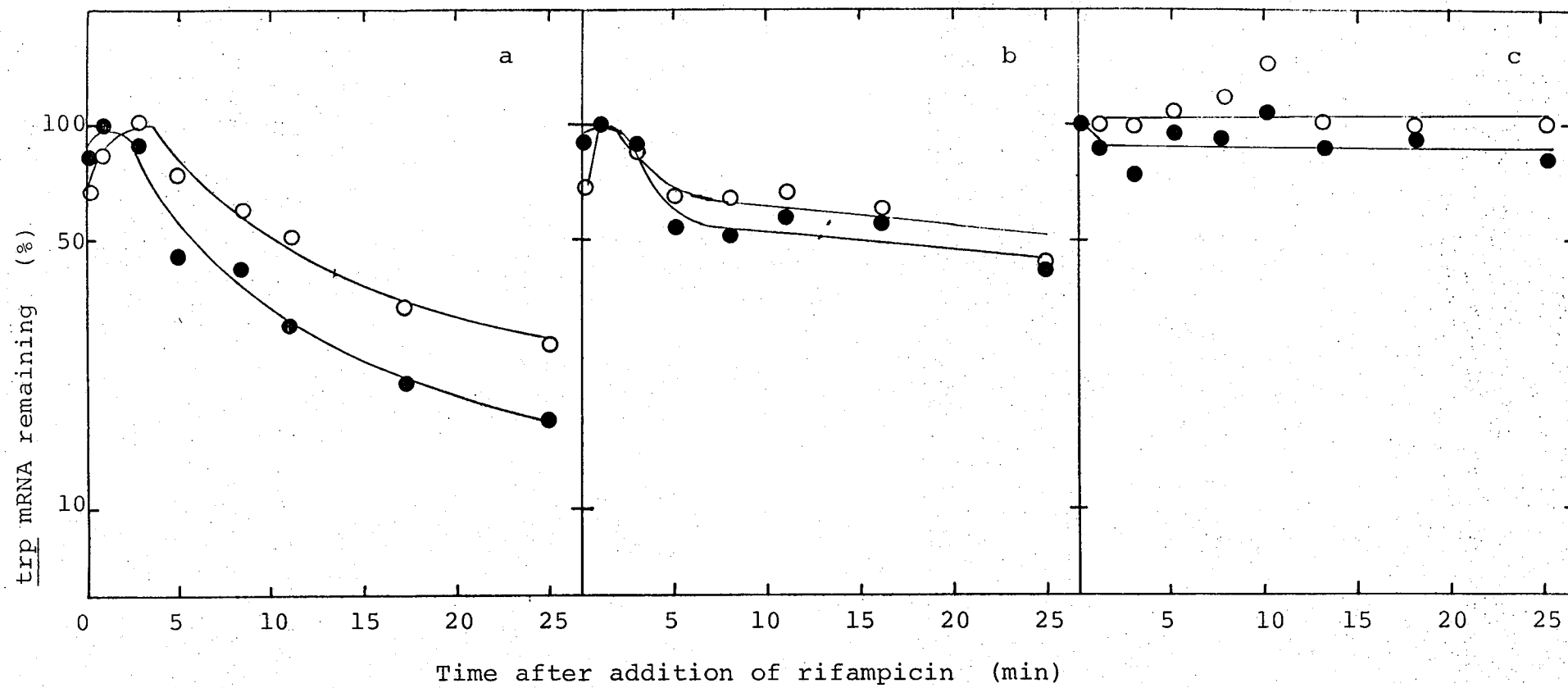


Fig. 3

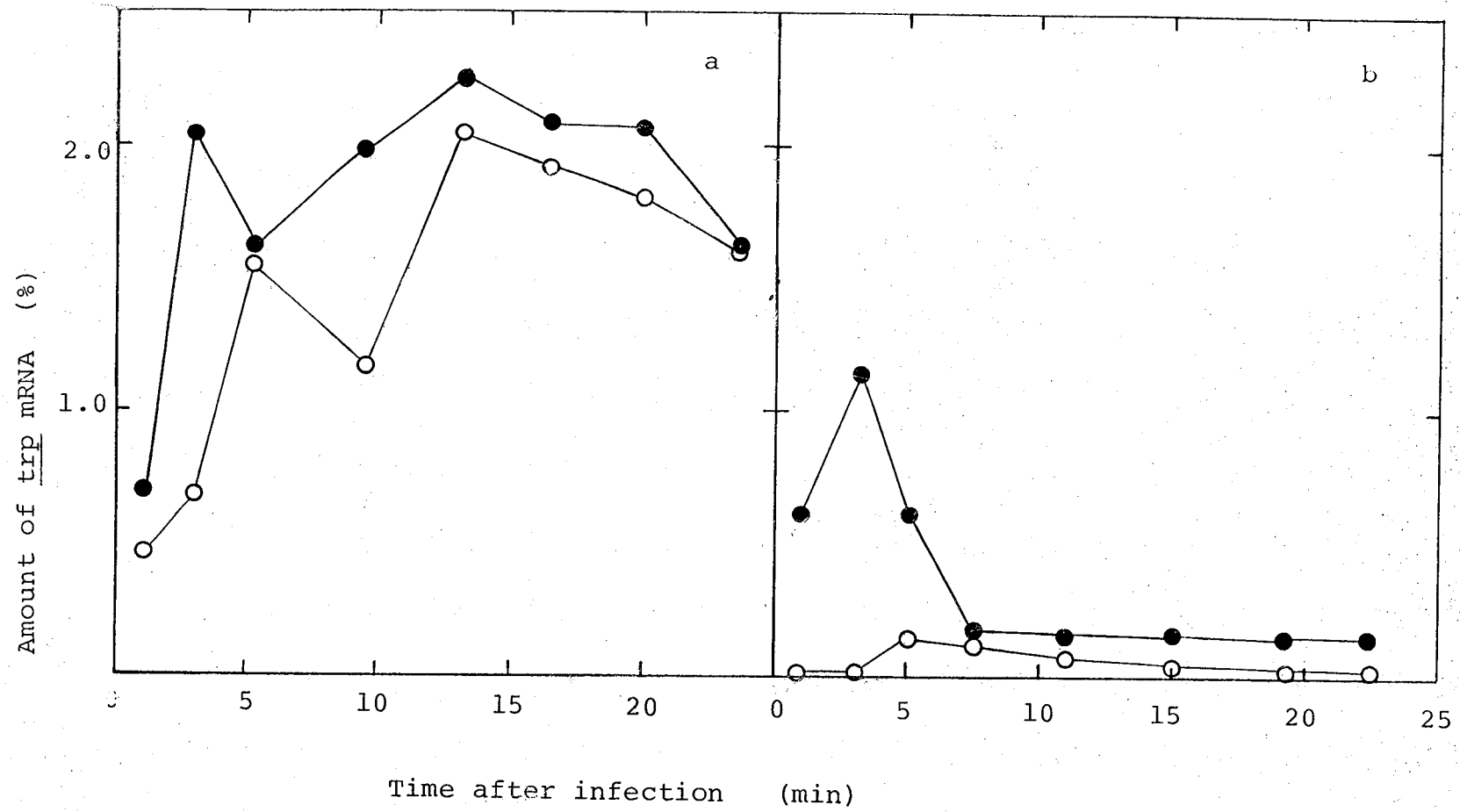


Fig. 4

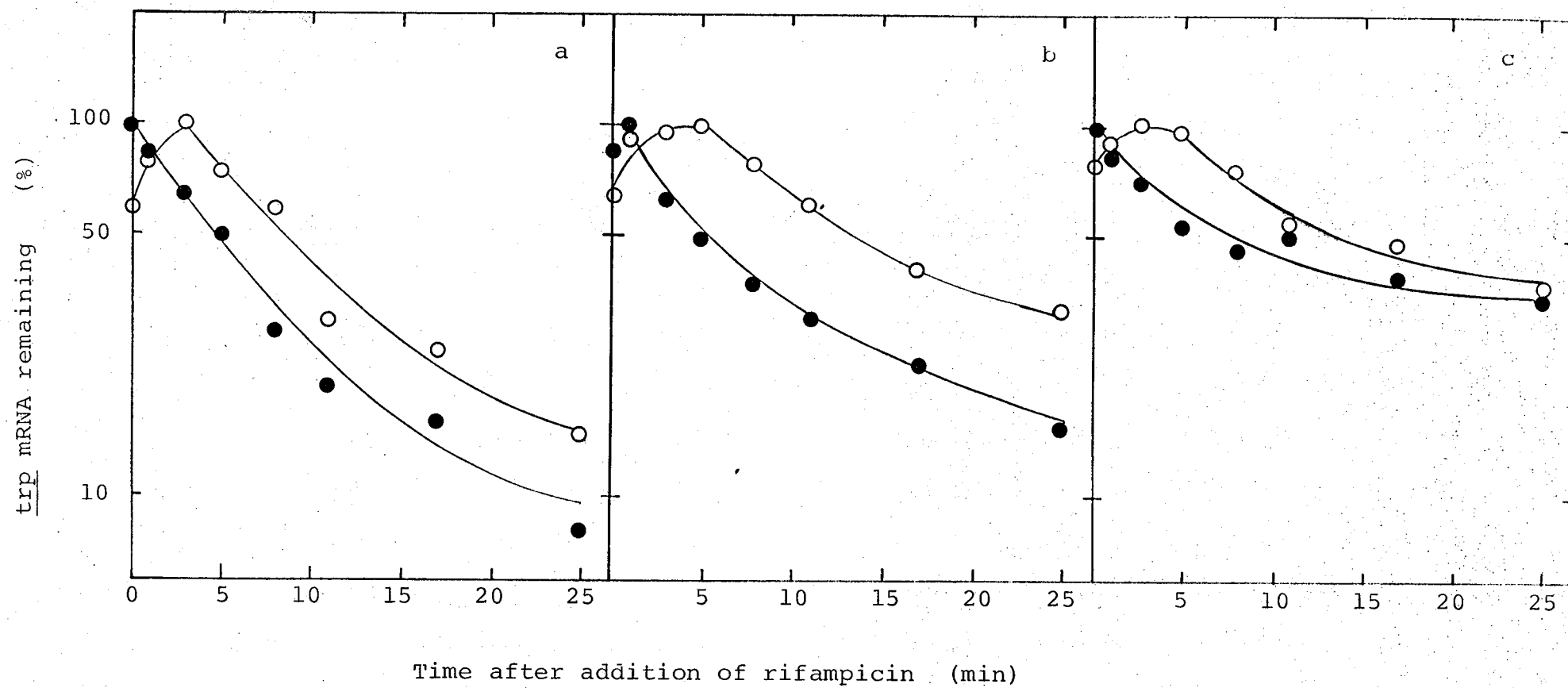


Fig. 5

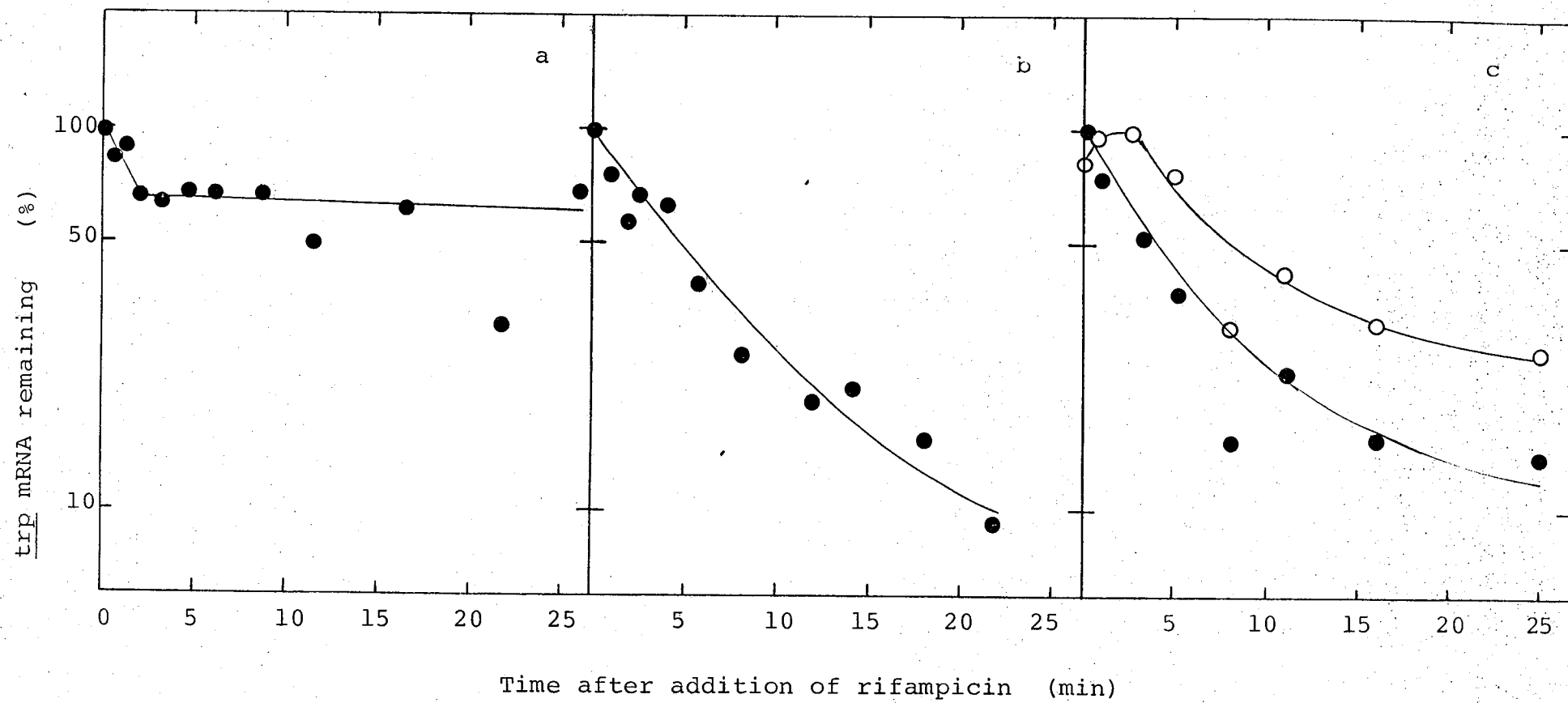
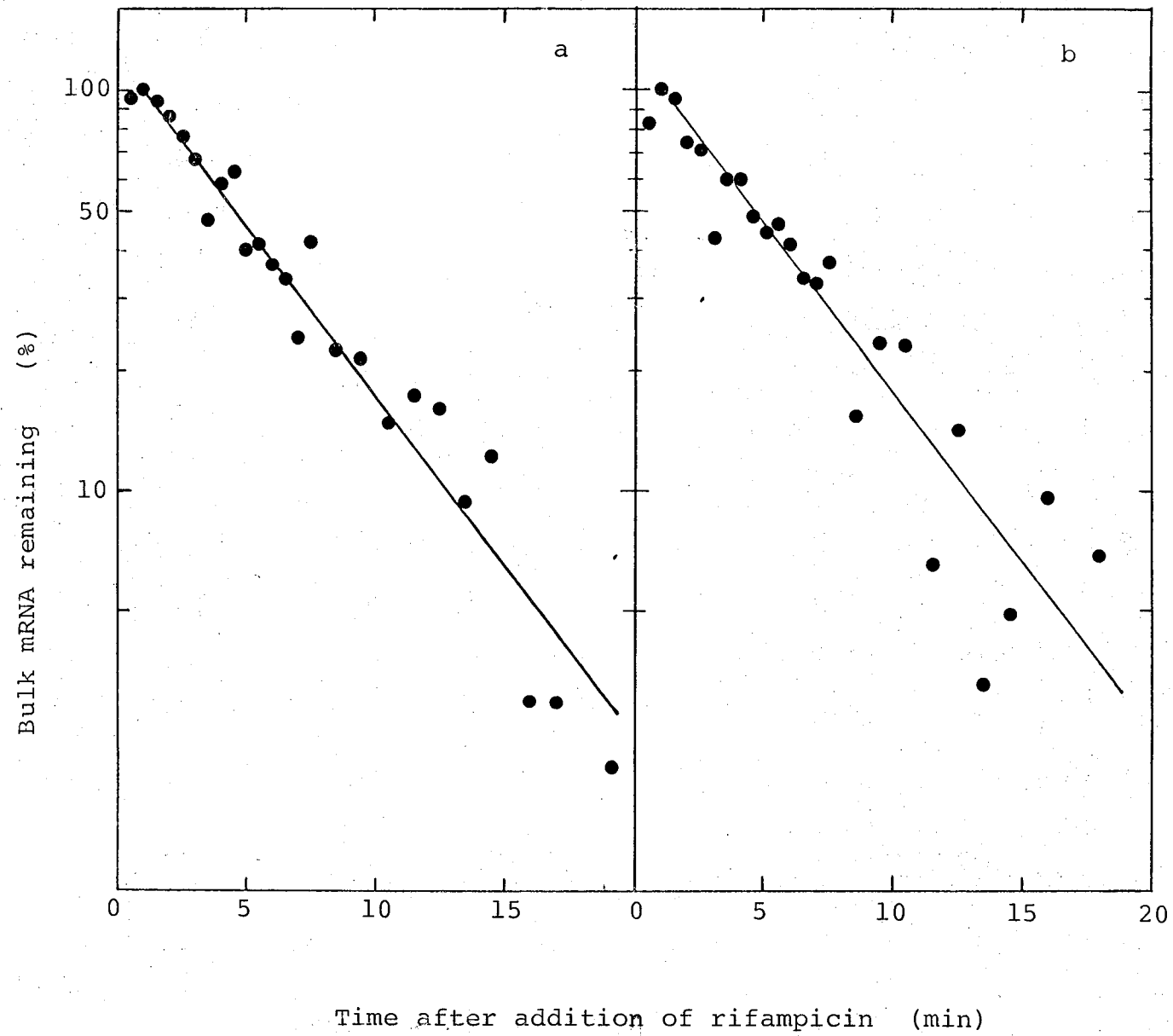


Fig. 6



Part III.

Detection and analysis of stable Messenger RNA in

Escherichia coli

### Summary

Quantitative analysis of hybridization of E. coli bulk mRNA with E. coli DNA or Vibrio DNA (which hybridizes specifically with E. coli rRNA but not with E. coli mRNA) in presence of excess cold rRNA and tRNA showed that about 20% fraction of pulse-labeled E. coli mRNA which can be associated with ribosomes is very stable with a half-life of more than 60 minutes at 30°, whereas the remainder decays exponentially with a half-life of about 4.5 minutes. E. coli strain 10B6 (rel<sup>+</sup>) was examined after being pulse-labeled with [<sup>3</sup>H]uridine and chased at 43° in order to clarify the point that synthesis of these stable mRNA molecular species is not under control of rel gene product.

To examine the possibility of existence of a unique structure at their 5' end of the stable mRNA molecules, E. coli strain trpAE1 was labeled with [<sup>3</sup>H]methyl-methionine and mRNA was extracted followed by digestion with RNAase T<sub>2</sub>. Chromatographic analysis of the digested fragments revealed existence of methylated bases at internal sequences of the stable RNA, but did not demonstrate existence of the unique structure such as m<sup>7</sup>GpppNp at the 5' end of the molecule. However, from a similar type of analysis of <sup>32</sup>P labeled mRNA it was found that at least a part of the stable mRNA molecular species possesses either pppGp or pppAp at their 5' end of the RNA structure. Possible mechanisms of degradation and stabilization of mRNA are discussed.

## Introduction

It has been generally believed that many species of eukaryotic mRNA are stable, while majority of prokaryotic mRNA is labile degrading exponentially with a half-life of a few minutes. Recent studies have revealed the existence of stable mRNA molecular species in E. coli system: the mRNA species for membrane proteins of E. coli (Lee & Inouye, 1974; Levy, 1975) and phage mRNAs, for example, T4 (Craig et al., 1972),  $\phi$ X174 (Hayashi & Hayashi, 1970), M13 (Janish et al., 1970), T7 (Summers, 1970; Marrs & Yanofsky, 1971) R17 mRNA (Hattman & Hofschneider, 1967), show greater chemical stability than the bulk mRNA of E. coli. The greater stability of mRNA may reflect some intrinsic feature of the structure of these mRNA molecules. Using  $\lambda$ trp phage I have shown that the trp mRNA formed by read-through from a  $\lambda$  promoter are far more stable than are the same trp transcripts promoted by the usual trp starting sequence. This suggests that covalent fusion of the 5' end of trp mRNA to the N mRNA sequence leads to ~~X~~stabilization of a trp mRNA sequence (Yamamoto & Imamoto, 1975). This result together with the previous finding that mRNA is broken down from 5' end of the molecule (Morikawa & Imamoto, 1969; Morse et al., 1969) favors the notion that the rate-limiting step to initiate mRNA degradation is determined by a sequence located at or near the 5' end of the messenger RNA.

It has been reported that mRNA in E. coli can be stabilized under the condition of starvation for <sup>u</sup>uracil or amino acids (Forchhammer & Kjeldgaard, 1967; Lindall & Forchhammer, 1969) and blockage of ribosome translocation (Craig, 1972; Cremer et al., 1974). Variation of translation yield of segments along a single mRNA chain result in a change of chemical lifetime of the sequences. The chemical lifetime can be greatly prolonged if mRNA<sup>function</sup> <sub>$\Delta$</sub>  is blocked. This implies that mRNA degradation couples with functional processes of



translation.

Here I show that a fraction of mRNA species is relatively stable in normally growing E. coli cells. These RNA molecules form polyribosomes and are produced under stringent-control. I also present results of experiment in which the structure of the 5' end of stable mRNA molecules is determined. The regulatory mechanisms of mRNA degradation will be discussed.

## 2. Materials and Methods

### (a) Bacterial strains

The following strains of E. coli K12 were used for mRNA preparation; 10B6 rel<sup>+</sup>, a temperature sensitive mutant for valyl tRNA synthetase and W3110 trpR<sup>+</sup> trpAE1, a large deletion mutant covering the whole trp operon (Yanofsky & Ito, 1966). E. coli K12 W3110 and Vibrio metschnikovii were used as DNA sources for DNA-RNA hybridization experiments.

### (b) Preparation of bacterial DNA

E. coli and Vibrio DNA were prepared according to the method of Saito and Miura (1963).

### (c) Pulse-labeling of RNA with <sup>3</sup>H-uridine

Bacteria were grown with aeration to  $6 \times 10^8$  cells/ml in an enriched medium (Lennox, 1955). The cells were collected by centrifugation and washed twice with cold minimal medium (Vogel & Bonner, 1956), and suspended in the same medium to give a final density  $3 \times 10^{10}$  cells/ml.

The cell suspension was diluted 15-fold with prewarmed (30° or 43°) minimal medium supplemented with 20 amino acids (each 0.5 mM) and the suspension was shaken vigorously in a water bath at 30° or 43°. At a suitable time during incubation, cells were pulse labeled with 100-300  $\mu$ Ci/ml. of [<sup>3</sup>H] uridine as indicated in the legends to the figures, followed by addition of rifampicin (300  $\mu$ g/ml), nalidixic acid

(10  $\mu$ g/ml.) and unlabeled uridine (1 mg/ml.). A portion ( $6 \times 10^9$  cells) of cell suspension was withdrawn and poured onto 30 ml of crushed frozen medium containing 0.01 M Tris-HCl buffer (pH 7.3), 0.005 M MgCl<sub>2</sub>, 0.01 M NaN<sub>3</sub> and 150  $\mu$ g/ml of chloramphenicol.

### (d) Long labeling of DNA with L-[methyl <sup>3</sup>H]-methionine or <sup>32</sup>P

Bacteria were grown with aeration to  $6 \times 10^8$  cells/ml in an enriched

medium (L broth) (Lennox, 1955) supplemented with L-tryptophan (50 µg/ml) and L-cystein (50 µg/ml). The cells were collected by centrifugation, washed twice with cold minimal medium or cold buffer free from phosphate [  $5 \times 10^{-2}$  M Tris-HCl buffer (pH 7.3), 0.1 %  $(\text{NH}_4)_2\text{SO}_4$ , 0.05%  $\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2 \text{H}_2\text{O}$  and 0.01%  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  ], for labeling with L-[methyl- $^3\text{H}$ ]-methionine or  $^{32}\text{P}$ , respectively. After washing, cells were suspended in the respective medium to give a final density of about  $5 \times 10^{10}$  cells/ml. About  $10^{10}$  cells of bacteria were transferred to 3 ml of prewarmed ( $30^\circ$ ) minimal medium supplemented with 19 amino acids (each 0.5 mM) excluding L-tryptophan or of prewarmed casamino-acids-broth [1% vitamine-free casamino acids, the phosphorous content of which was reduced to 21 mg/ml by precipitating phosphate as  $\text{NH}_4\text{MgPO}_4$ , 0.25% NaCl, 1mg/l of vitamine B1, 0.04 mM  $\text{KH}_2\text{PO}_4$  and 1 mM  $\text{MgSO}_4$  ] (Ogawa & Tomizawa, 1967), for labeling with L-[methyl- $^3\text{H}$ ]-methionine or  $^{32}\text{P}$ , respectively. The cell suspension was shaken vigorously in a water bath at  $30^\circ$ . Bacteria were labeled for 20 minutes with 0.8 mCi of L-[methyl- $^3\text{H}$ ]-methionine or 1 mCi of carrier free [ $^{32}\text{P}$ ] phosphate, added at the 5th minute of incubation. Labeling was stopped by rapidly pouring the suspension onto 35 ml of crushed frozen medium mentioned above.

#### (e) Preparation of polysomes

Bacterial cultures (  $1 \times 10^{10}$  cells in 6 ml) were pulse-labeled with 300-350 µCi [ $^3\text{H}$ ]uridine at appropriate time of incubation at  $30^\circ$  or  $43^\circ$ . Cells were harvested and mixed with  $2 \times 10^8$  cells prelabeled with 1 µCi [ $^{14}\text{C}$ ]uridine (50 µCi/230µg) for 3 hrs in minimal medium at  $30^\circ$  and lysed according to the procedure of Godson (1967). The lysate was centrifuged on 5 ml of 10 to 30% linear sucrose density gradient containing 10mM Tris-HCl buffer (pH 7.2), 10mM  $\text{MgSO}_4$  and 60 mM KCl for 60 min. at 40,000 rev./min in a SW69 rotor at  $4^\circ$ . A portion of each fraction was put on a filter

disk and counted after drying. Polyribosomal fractions were combined into 6 fractions and [<sup>3</sup>H] RNAs were extracted.

(f) Preparation of RNA

RNA was prepared by the procedure reported previously (Imamoto, 1969). The RNA obtained was filtered through a Millipore filter, precipitated by ethanol and resolved in H<sub>2</sub>O.

(g) DNA-RNA hybridization

The hybridization procedure was as follows: DNA of E. coli or Vibrio was diluted to a concentration of 100 µg/ml in 1XSSC and heated in boiling water for 10 min. followed by rapid cooling in ice water. The DNA was further diluted to a concentration of 16 µg/ml in 3XSSC. 10 ml of the DNA solution was filtered through a Millipore filter (type HA, 0.45 µm pore size) of 25 mm diameter. The filter was washed with 40 ml of 3xSSC and cut into 8 pieces. DNA filter was dried at 80° for 2h. Assay of E. coli mRNA was carried out by immersing one of these pieces of filter in 100 ul of [<sup>3</sup>H] RNA in 4.5xSSC in the presence of excess amount of rRNA and tRNA, and incubating at 66° for 18 hrs.

Vibrio DNA was used for the assay of E. coli rRNA (Pederson & Kjelgaard, 1972). Afterwards, the filters were treated with RNAase (5µg/ml) in 1xSSC at 37° for 30 min., washed with 1xSSC, dried and counted in toluene scintillation fluid.

(h) Sucrose density-gradient analysis of [<sup>3</sup>H] RNA

RNA preparations were sedimented in 5% to 30% linear sucrose gradient containing  $2 \times 10^{-2}$  M Tris-HCl buffer (pH 7.3), 0.1 M NaCl, 0.5% sodium dodecyl sulfate and  $5 \times 10^{-3}$  M EDTA for 110 min. at 64,000 revs./min. at 15° in a SW 65 rotor. After centrifugation the bottom of the tube was punctured and appropriate fractions were collected.

(i) Analysis of 5'-structure of RNA

Procedures of treatment of the labeled RNA with ribonuclease and of column chromatography of the RNA digests were carried out as described by Shimotohno and Miura (1976).

(j) Reagents

All reagents and chemicals were purchased and used as described in Part II. Only the reagents used for base analysis were the same as described by Shimotohno and Miura (1976).

### 3. Results

#### (a) Degradation of mRNA in normally growing cells.

The kinetics of degradation of bulk mRNA (termed here as RNA species other than those of tRNA and rRNA) was analyzed in normally growing cells of E. coli, carrying the temperature-sensitive valyl-tRNA synthetase (Edlin & Stent, 1969). This strain was used to investigate the effect of stringent phenotype at a high temperature because of an inefficient supply of valyl-tRNA (Neidhardt, 1966) on overall rate of degradation of cellular bulk mRNA. The tryptophan derepressed culture of 10B6 rel<sup>+</sup> was exposed to a one minutes pulse of [<sup>3</sup>H]uridine during steady-state transcription at 30°. Further incorporation of [<sup>3</sup>H]uridine into mRNA was prevented by the addition of rifampicin and by diluting the label with an excess of unlabeled uridine. A typical result of these experiments is represented in Fig. 1. Consistent with the previous reports (Imamoto, 1973; Yamamoto & Imamoto, 1975), it was found that trpED mRNA decayed exponentially with a half life of 1 to 1.5 minutes subsequent to instantaneous block of initiation of the synthesis by the addition of rifampicin and trpCBA mRNA disappeared at a slightly slower rate, with a half-life of about 2 minutes after rifampicin addition. The degradation of bulk mRNA hybridizable with E. coli DNA in presence of excess unlabeled rRNA and tRNA was found to exhibit the diphasic pattern of the decay curves. The observation that the pulse-labeled bulk mRNA decayed with an apparent half-life of about 3.5 minutes, which was about half the rate of trp mRNA degradation, suggest the existence of a relatively stable mRNA fraction. A shallow changes in the rate curves of mRNA decay were observed with bulk mRNA compared to that of trp mRNA. This indicates that relatively stable mRNA species

which degrade with a half-life of more than 40 minutes at 30° exist together with unstable mRNA species, such as trp mRNA. Essentially similar observation was also obtained with the experiment in which E. coli strain trpC9941 was employed.

Similar type of the experiment~~x~~ was carried out with E. coli 10B6 rel<sup>+</sup> at 43° under the condition of which transcription~~x~~ of rRNA and tRNA genes was repressed by a stringent control. The result obtained showed that general behavior of synthesis and degradation of bulk mRNA were essentially the same. This suggests that the most of stable mRNA molecules detected under the present conditions are not those species of RNA produced from rRNA and tRNA genes.

(b) Sedimentation analysis of stable bulk mRNA.

I have previously shown that trp mRNA synthesized originating at the P<sub>L</sub> promoter of N gene (P<sub>L</sub>trp mRNA) in λtrp phage in which the trp operon is translocated into the early region of λ phage is greatly stabilized (Yamamoto & Imamoto, 1975). In these experiments, sedimentation analysis showed that a considerable fraction of the mRNA was conserved as large molecules during a period of 18.5 minutes after inhibition of RNA synthesis by rifampicin, though some fragmentation occurred on a part of P<sub>L</sub>trp mRNA fraction, resulting in accumulation of small fragments of the molecules (about 4s).

Essentially similar changes to those in the sedimentation profiles were observed with the stable bulk mRNA. Fig.2 represents the results of experiments with a strain 10B6 rel<sup>+</sup> growing at a permissive temperature (30°). It can be seen in the Figure that a considerable part of the mRNA fraction (about 10% of pulse-labeled mRNA) was conserved as ethanol-

precipitable and E. coli DNA hybridizable materials during incubation for at least 30 minutes after addition of rifampicin. A considerable fraction of stable mRNA was still large molecules after the incubation, though some of these molecules seemed to be endonucleolytically cleaved. Bulk stable mRNA was not hybridized with Vibrio DNA containing the rRNA genes homologous to those of E. coli (Pederson & Kjeldgaard, 1972), thus verifying that these stable RNA species were not those of RNA from rRNA genes, such as spacer RNA species etc.

Figure 2b presents the sedimentation profiles of pulse-labeled RNA in the process of degradation in a strain 10B6 rel<sup>+</sup> cultured at a non-permissive temperature where synthesis of rRNA and tRNA was repressed. General behavior of stable bulk mRNA molecules in the sedimentation profile was essentially similar to that seen in cells growing at a permissive temperature (Fig. 2a), though the rate of disappearance of large molecules was somehow faster at a high temperature.

(c) Stable mRNA recovered in inactive polyribosomal fraction.

Since any significant protein synthesis determined by incorporation of [<sup>14</sup>C]leucine into host-trichloroacetic acid insoluble protein fraction was not observed in E. coli cells after incubation for more than 30 minutes at 30° in the presence of rifampicin (data not shown), I have examined whether stable mRNA might exist as unloaded molecules free from ribosomes or still be associated with ribosomes. Results represented in Figure 3 show profiles of polyribosomes prepared from the strain 10B6 rel<sup>+</sup> pulse-labeled with [<sup>3</sup>H]uridine for one minutes at 30° at a steady-state of transcription and chased the label for 60 minutes at 30° (Fig. 3a) or 30



minutes at 43° (Fig. 5b) after addition of rifampicin. After incubation for these periods at permissive and nonpermissive condition small but discernible amount of large polyribosomes was still found in faster-sedimenting region in the profiles. Sedimented materials were then divided into six subfractions and, after extraction of [<sup>3</sup>H]RNA from each fraction, bulk mRNA were assayed by DNA-RNA hybridization with E. coli DNA or Vibrio DNA in presence of excess of mature rRNA and tRNA.

Results of these experiments are shown in Figure 6. Significant amount of bulk mRNA (about 30 % of [<sup>3</sup>H]RNA remained after incubation with rifampicin), which hybridized with E. coli DNA but not with Vibrio DNA, was detected in large polyribosomal fractions (A, B and C fraction in the Figure 3). This indicates that at least a fraction of stable mRNA molecules exist as inactive polyribosomes even after the long incubation of the cells at either 30° or 43°.

The size of [<sup>3</sup>H] bulk mRNA which was recovered from large polyribosomal fractions (A, B and C fraction of Figure 3) and non-polyribosomal (D, E and F fractions of Figure 3) was analyzed on a linear sucrose-density gradient centrifugation (Fig. 4). Bulk mRNA preparations exhibited a polydispersed pattern in the sedimentation profiles, thus verifying a notion that stable mRNA were conserved as large molecules in polyribosomal fraction after overall activity of protein synthesis in the cells had disappeared during incubation in presence of rifampicin.

(d) Analysis of 5'-end structure of stable mRNA.

The experiments presented in the foregoing sections show existence

of stable mRNA whose synthesis is not controlled by stringent factors. This suggests that the mechanism of degradation of mRNA molecules are diverse. There has been evidence available to support a notion that the rate-limiting step to initiate mRNA degradation is determined by a sequence located at or near the 5' end of the mRNA molecule (Imamoto & Schlessinger, 1974; Yamamoto & Imamoto, 1975). It would, therefore, be an obvious expectation that stable mRNA might possess the unique structure at its 5' end of the molecules. It has been reported that many species of eukaryotic RNA possess a unique structure of  $m^7GpppNp$  at their 5' end of the molecules (for example, Miura *et al.*, 1975), though physiological significance of this structure has still been obscure.

This expectation was examined and the results obtained are presented in Figure 6. Bulk mRNA prepared from *E. coli* strain *trpAEl* labeled with [ $^3H$ -methyl]methionine (Fig. 6a) or  $^{32}P$  (Fig. 6b) was digested with RNAaseT<sub>2</sub> and analyzed by DEAE-cellulose chromatography in presence of 7M urea. In the profile of Figure 6a, any significant radioactivity with net charges of -4.5 to -5.5 should be found if  $m^7GpppNp$  exists. Any other unique bases were not detected, but considerable radioactivity of Nmp was found, which seemed to be derived from internal sequences of RNA molecules. In analysis of  $^{32}P$ -labeled RNA, the radioactivity peaked at a position of net charge of -5. Fractions of the peak region were pooled and reanalyzed by chromatography on a Bio-Rad AG1 column after treatment with Penicillium nuclease P<sub>1</sub>. The elution profile represented in Figure 7 shows that the radioactivities peak in the positions of ATP, GTP and Pi. The distribution of radioactivities for ATP and GTP shows that at least a part of stable mRNA molecules possesses a structure of

pppAp or pppGp at their 5' end of the molecule.

#### 4. Discussion.

It has been reported that in E. coli starved for uracil approximately one-half of the messenger activity of RNA is relatively short-lived, whereas the remaining half shows a much slower decay (Forchhammer & Kjeldgaard, 1967). Later it was pointed out that semi-stable mRNA is only present during starvation of cells for uracil (Lindahl & Forchhammer, 1969). In exponentially growing cells this mRNA fraction is turned over with the half-life characteristic of the short-lived mRNA present in the starved cells. Therefore it can be assumed that slow decay of the mRNA could be due to slow translation which was an indirect result of blocked transcription caused by starvation for uracil. This premise, however, would be inapplicable to the interpretation of results reported here, since the pulse-labeling was followed by dilution of the radioactive precursor with excess unlabeled uridine. The proportion of the fraction of stable mRNA and unstable mRNA was about 1 : 4 in the present condition, but this ratio might be variable according to the physiological conditions of the cells. Recently, mRNA in E. coli for the membrane protein has been reported to be relatively stable both functionally (Lee & Inouye, 1974) and chemically (Takeishi et al., 1976). Since such species of mRNA is considerably small in size (about 5 to 6s), findings reported here suggest that variety of stable mRNA species are obviously present in growing E. coli cells.

The judgement that the most of the stable RNA species presented here are neither RNA produced from rRNA genes nor RNA from tRNA genes is supported by the following observations: these stable mRNA molecules are still synthesized under the stringent control of rel gene product

and major parts of the stable mRNA sediment faster than tRNA in sucrose density gradient analysis (Fig. 2). Experimental results of DNA-RNA hybridization using Vibrio DNA also show that stable mRNA fraction does not possess the spacer sequence of the precursor RNA produced from rRNA genes.

In spite of the greater chemical stability,  $P_L$ trp mRNA loses the capacity to serve as template for enzymes synthesis rapidly as does the normal  $P_{trp}$  mRNA (Yamamoto & Imamoto, 1975). It would be difficult to assume that stable bulk mRNA molecules continue to serve as template for protein synthesis for a long period after the mRNA synthesis has been inhibited by rifampicin, since overall rate of  $^{14}C$ -leucine into proteins becomes undetectable after incubation of the cells for more than 30 minutes in presence of rifampicin. By contraries, functional stability of mRNA for the membrane proteins seems to be greater than that of the many other mRNA species. Factor(s) controlling functional stability of the mRNA template is (are) currently under investigation.

There has been ample supports for a premise that degradation of mRNA is controlled by the event occurring at the 5' proximal sequence of the molecule (Morikawa & Imamoto, 1969; Morse et al., 1969; Yamamoto & Imamoto, 1975). It would be a possibility that the unique structure of  $m^7GpppNp$  might play a role in stabilizing the mRNA, though the function of the cap structure is obscure. Under the present conditions, however, this unique structure was not found with the E. coli stable mRNA. At least a part of the stable mRNA fraction involves those molecules possessing either pppGp or pppAp at their 5' termini. It can be reasonably

speculated that any of activities to degrade mRNA could be restricted at the 5' end of the molecules.

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## Legends to Figures

### Figure.1

Decay kinetics of E. coli bulk mRNA.

Bacteria were pulse-labeled with  $^3\text{H}$ -uridine at a steady state of transcription. One minutes after  $^3\text{H}$ -uridine addition (0 time), rifampicin, nalidixic acid and cold uridine were added to a final concentration of 300, 10 and 1000  $\mu\text{g/ml}$ , respectively. A portion of the culture was removed at indicated times during incubation after addition of rifampicin and RNA was extracted. The trp mRNA and bulk mRNA values are expressed as  $^3\text{H}$ -RNA hybridized/ $\mu\text{g}$  RNA and normalized to 100% for the maximum value.  $^3\text{H}$ -RNA hybridized specifically with DNA from  $\phi 80\text{trpED}$ ,  $\phi 80\text{trpCBA}$  or E. coli DNA was designated trpEDmRNA, trpCBmRNA or E.coli bulk mRNA, respectively. Values represented are the average of duplicate determinations.

(●) trpEDmRNA; (○) trpCBmRNA; (■) E. coli bulk mRNA

### Figure 2

Sucrose density gradient analysis of bulk stable mRNA.

Bulk mRNA was prepared from cultures of 10B6 rel<sup>+</sup> at 30° (a, b, c<sub>g</sub> and d) or at 43° (e, f, g and h). The cultures were pulse-labeled with  $^3\text{H}$ -uridine for 1 minute at a steady state of transcription and followed with the addition of rifampicin, nalidixic acid and cold uridine as described in Fig. 1. Samples were withdrawn from the cultures at 0 (a & e), 7.5 (f), 15 (b & g), (c & h) and 60 (d) minutes after addition of rifampicin. RNA extracted was cosedimented with 10 $\mu\text{g}$  of  $^{14}\text{C}$ -RNA ( $2 \times 10^4$  cts/min per  $\mu\text{g}$  prepared from the rifampicin-sensitive strain as described previously (Imamoto, 1973). Centrifugation was carried out as described in Materials and Methods. After centrifugation

about 30 fractions were collected. Total  $^3\text{H}$  and  $^{14}\text{C}$  as well as E. coli DNA hybridizable and Vibrio DNA hybridizable radioactivity were determined in each fraction.

(●) RNA hybridizable with E. coli DNA; (◻) RNA hybridizable with Vibrio DNA

### Figure 3

Polysomal profiles remaining during incubation after addition of rifampicin

Cultures of strain 10B6 rel<sup>+</sup> was pulse-labeled and chased as described in Fig. 1 and Materials and Methods. After 60 minutes (30°,a) or 30 minutes (43°,b) of incubation after addition of rifampicin,  $^3\text{H}$ -RNA was prepared. Cells are lysed and centrifuged to remove debris. 10 $\mu\text{l}$  of each fraction was put on a filter disk and counted after drying. Arrow in the Figures indicates position of 70S monosome fraction. The other condition is as described in Materials and Methods.

(●)  $^3\text{H}$ -RNA; (○)  $^{14}\text{C}$ -RNA

Divided portions, A to F, in the figures show subfractions from which  $^3\text{H}$ -RNAs were extracted for the experiments of Fig. 4.

### Figure 4

Polyribosomal distribution of bulk stable mRNA

Polyribosomal fractions designated as A-F in Fig. 3 were pooled respectively and  $^3\text{H}$ -RNAs were extracted from each subfraction of A-F. Tritiated RNA from respective subfraction was hybridized with E. coli and Vibrio DNA. Arrows in the Fig. indicate the position of a subfraction which contains 70S

monosome. The other condition and representation are as described in Fig. 3 and Materials and Methods.

(●)  $^3\text{H}$ -RNA hybridized with E.coli DNA; (○)  $^3\text{H}$ -RNA hybridized with Vibrio DNA

#### Figure 5

Sedimentation profile of undegraded mRNA in polyribosomal fraction. The cultures of 10B6 rel<sup>+</sup> were labeled with  $^3\text{H}$ -uridine and incubated in presence of rifampicin as described in Fig. 3. After 30 minutes of chase periods at 43°, cells were lysed and divided into two fractions by centrifugation: polyribosomal fraction (a) and supernatant fraction (b), corresponding to the fractions A,B,C,D and fractions E,F of Fig. 3, respectively. From each fractions RNAs were extracted and analyzed by sucrose density gradient centrifugation. The other conditions or representations are as described in Fig. 2 and Materials and Methods.

(●)  $^3\text{H}$ -RNA hybridizable with E. coli DNA;

(○) Total  $^3\text{H}$ -RNA

#### Figure 6

DEAE-cellulose chromatography of long-labeled E. coli mRNA. The cultures of trpAE1 were labeled with 5mCi of [methyl- $^3\text{H}$ ] (a) or 10mCi of  $^{32}\text{P}$  (b) for 20 minutes during steady state of transcription. Labeled RNA was extracted and the mRNA fraction was applied to DEAE-cellulose chromatography in 7M urea after digestion by RNAaseT<sub>2</sub>. The other conditions are as described elsewhere (Shimotohno & Miura, 1976). Arrow in each figure indicates the elution position of m<sup>7</sup>GpppNp.

Negative numbers represent the net charge of the nucleotides of the position.

(O-O) [methyl-<sup>3</sup>H] radioactivity; (—●—) Absorbance at 260 nm.

#### Figure 7

Chromatography on a Bio-Rad AG1 column of the *Penicillium* nuclease P<sub>1</sub> digest of oligonucleotides in Fig. 6

The oligonucleotides of fraction 80-90 in Fig. 6b were pooled and digested by P<sub>1</sub> nuclease. The digest were applied to the Bio-Rad column.

The other conditions are as described else where (Shimotohno & Miura, 1976).

(O-O) <sup>32</sup>P-radioactivity; (—●—) Absorbance at 260 nm.

Fig.1

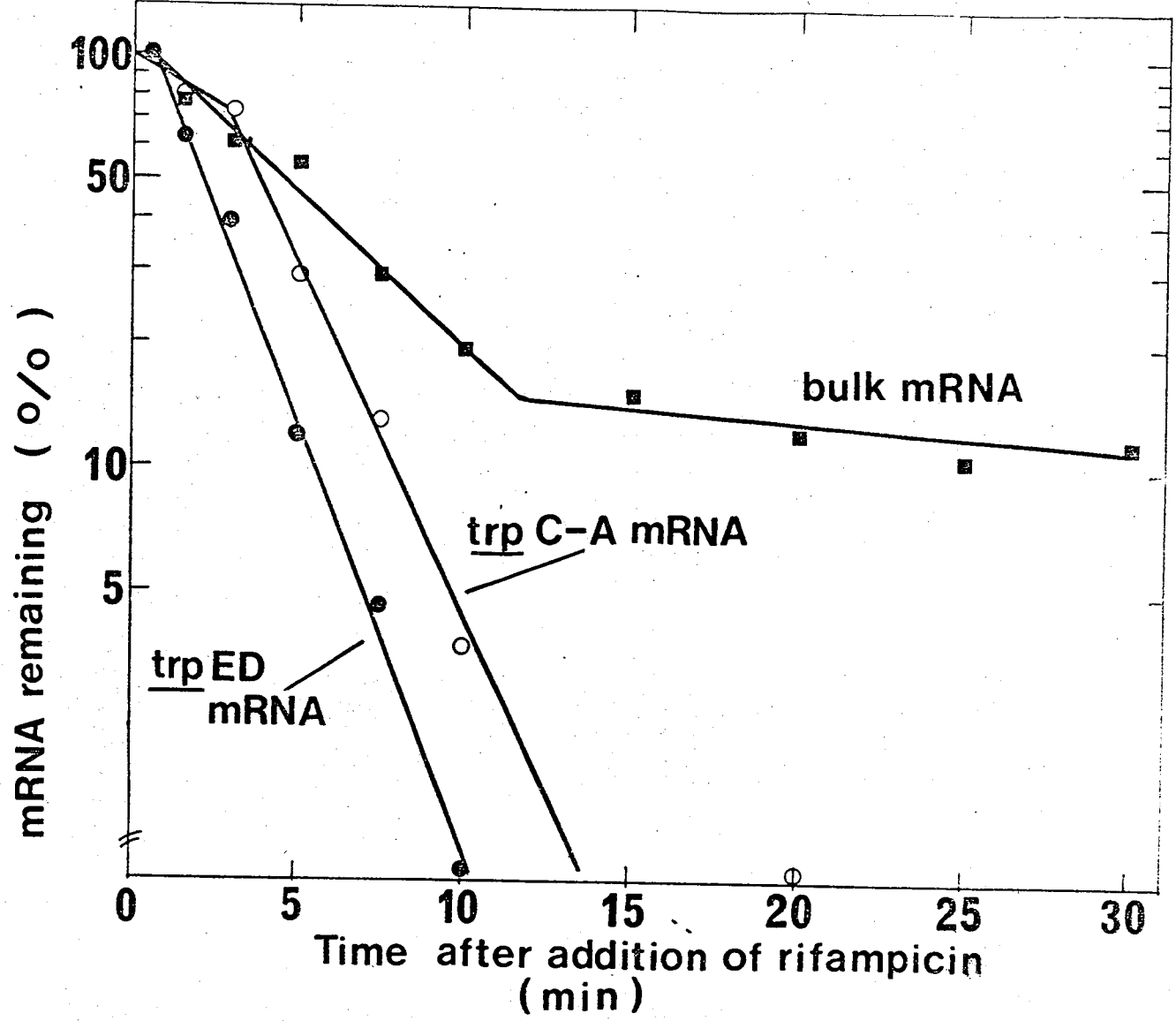


Fig.2

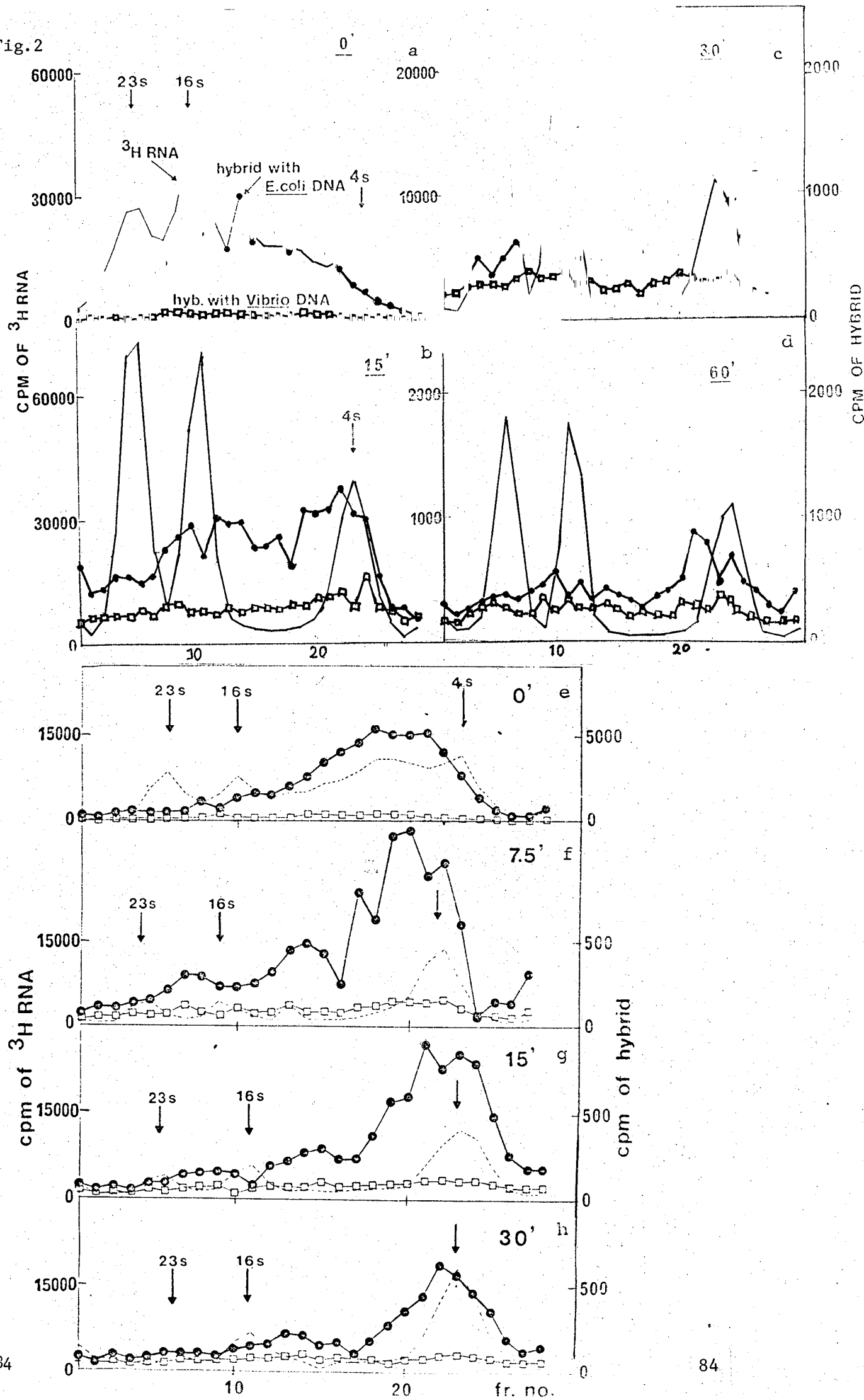


Fig.3

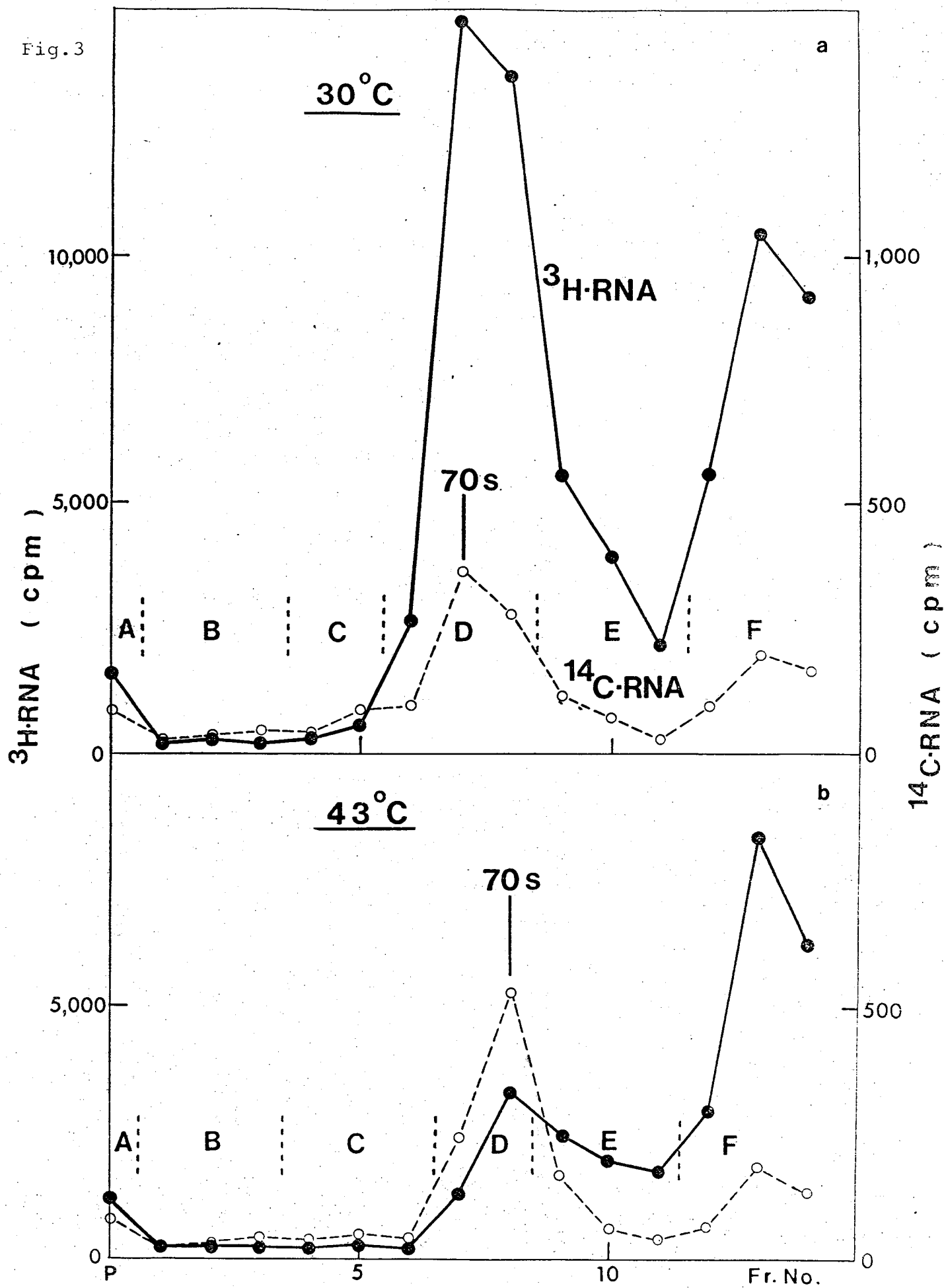


Fig.4

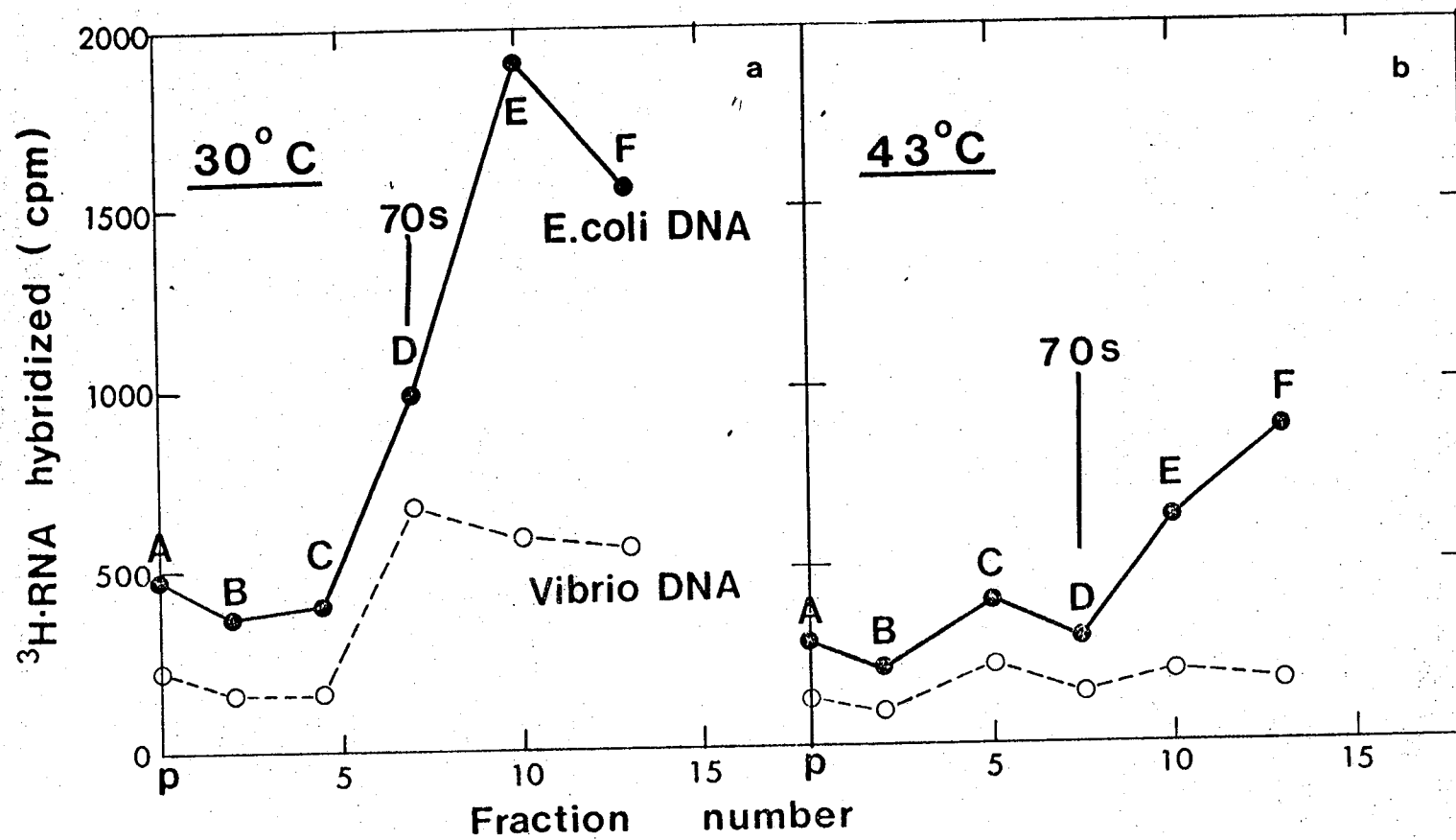




Fig.5

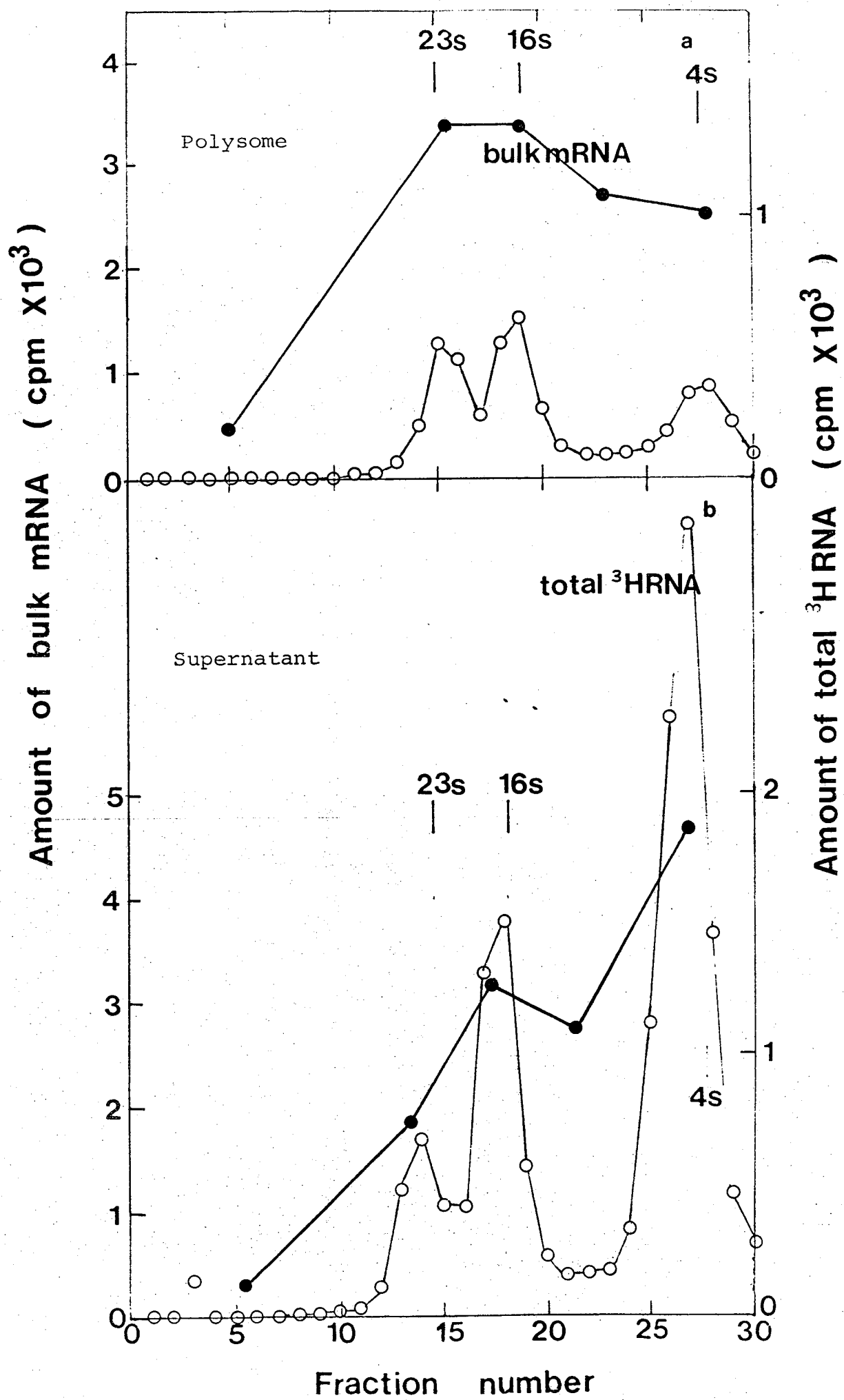


Fig. 6

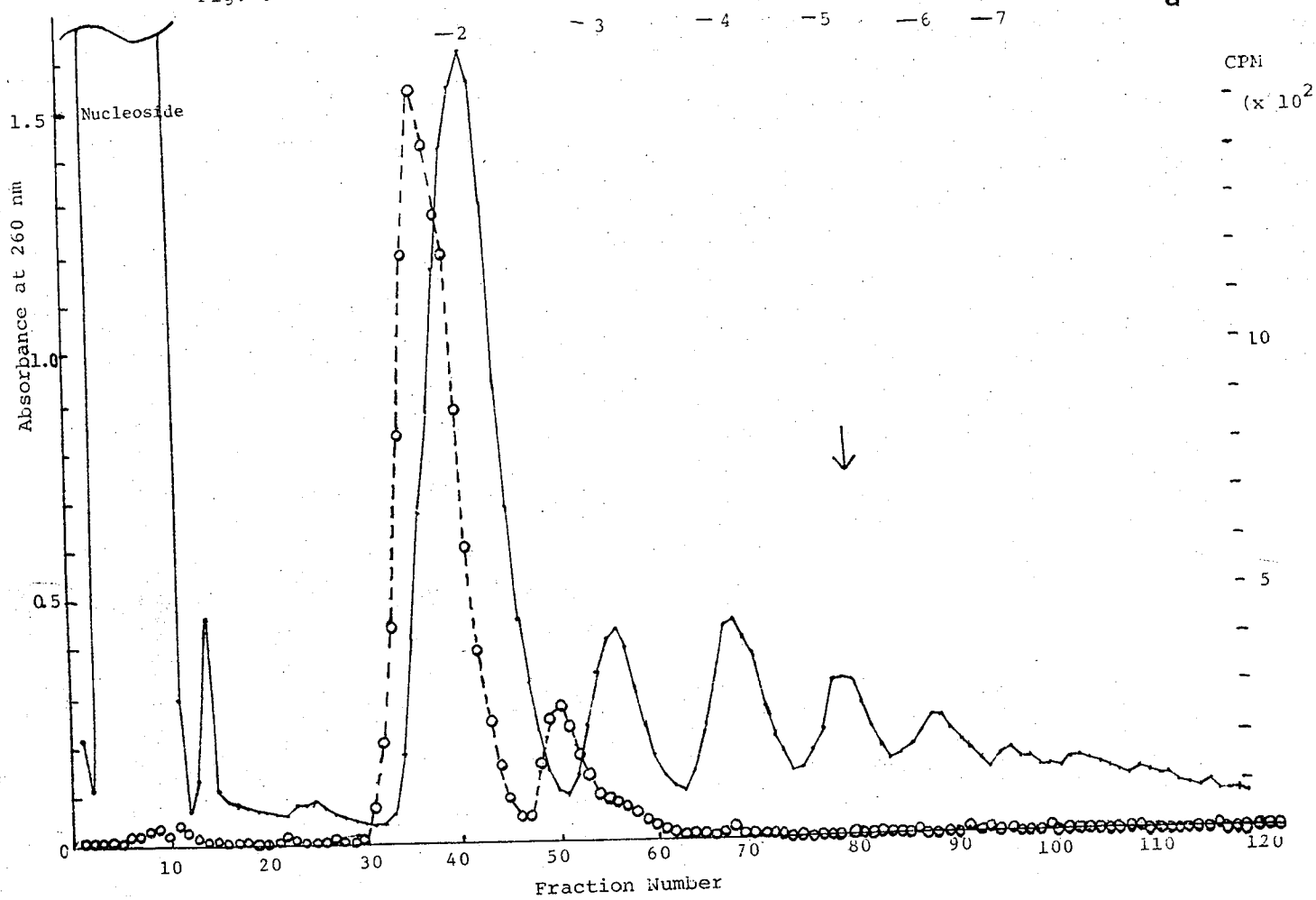


Fig. 6

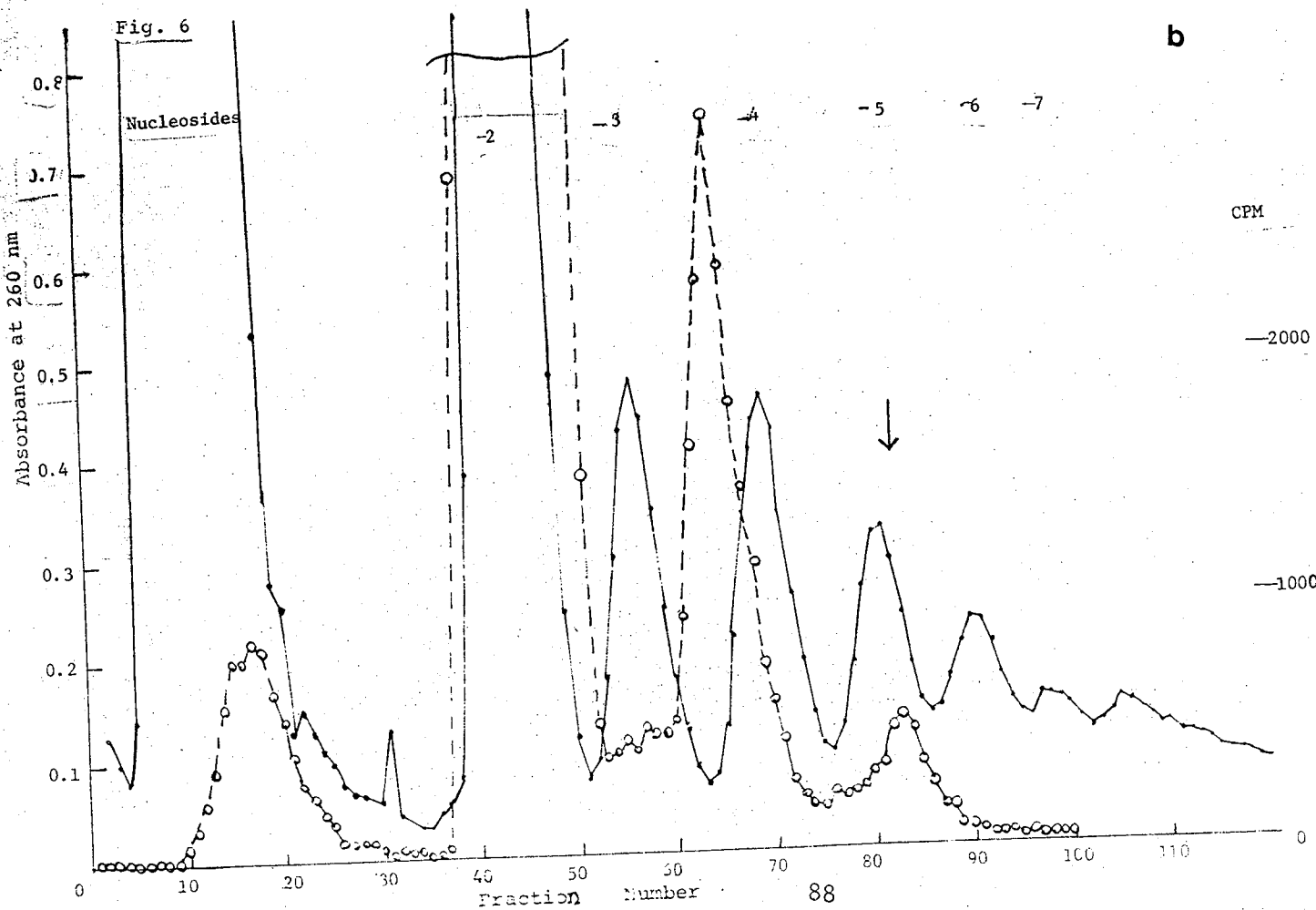
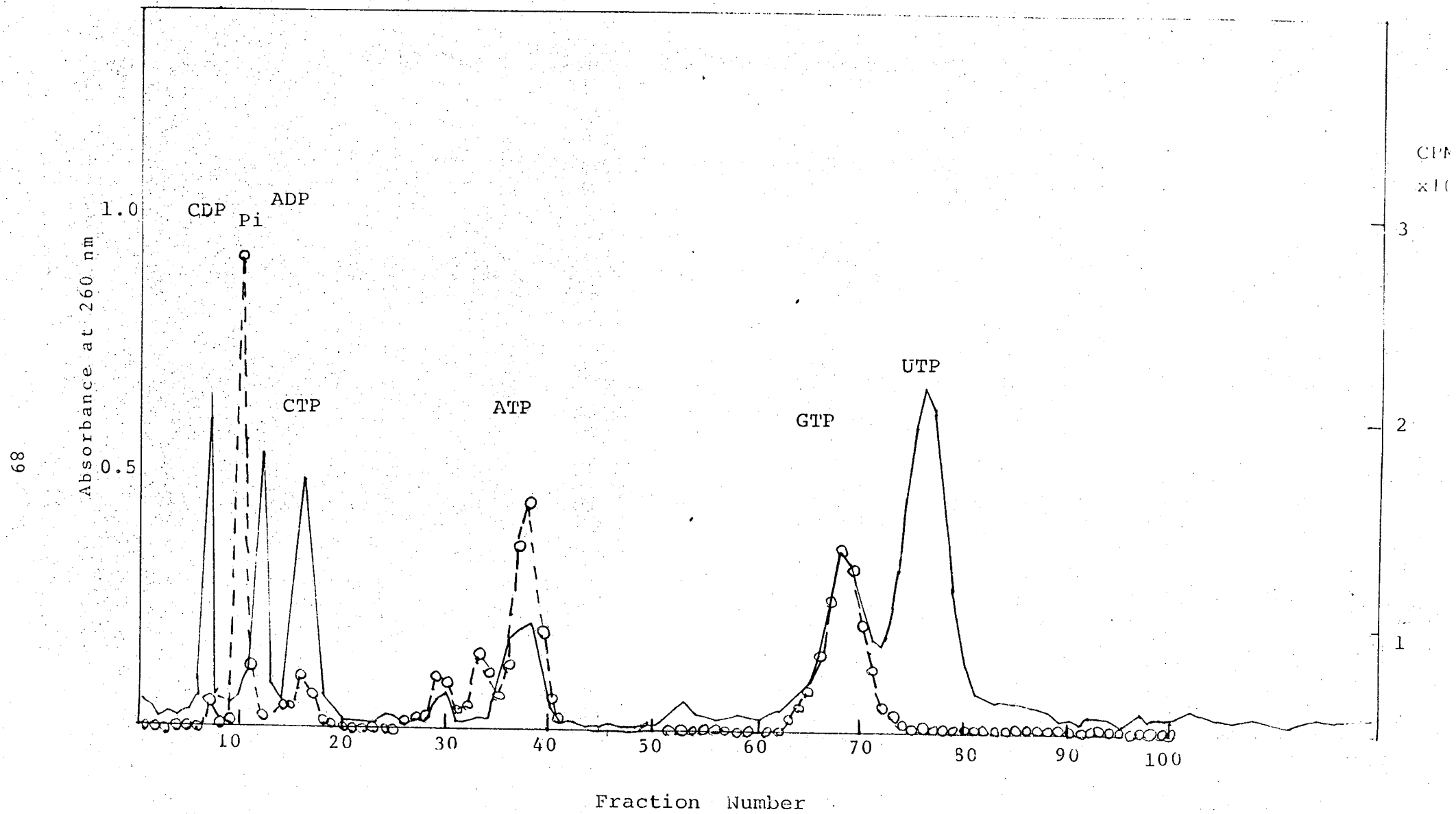


Fig. 7



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