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Determination of Base Ratios of the "Early" and "Late" Messenger RNAs of Bacteriophage T2 under the Different Conditions

by

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I Introduction

It has been well known that the phage specific (messenger) RNAs were separable by a chromatographic column of methylated albumin-hyflosupercel (MAK) with at least three peaks (Ishiham *et al.* '62, Kano-Sueoka & Spiegelman '62), even each one has the same base composition similar to that of the phage DNA, uridylic replacing thymidylic acid (Ishihama *et al.* '62) and also the phage specific proteins (such as deoxycytidylate hydroxymethylase, coat protein and lysozyme, etc.) synthesized upon infection of bacterial cells with phage, were broadly divisible into two categories "early" and "late" according to the time of their appearances. The early proteins are detectable soon after infection and include those enzymes required to initiate phage DNA synthesis (Kornberg *et al.* '59, Wiberg *et al.* '62). The late proteins are detectable from seven to ten minutes after infection and consist of structual proteins and others presumably involved in the maturation of phage particles (Koch & Hershey '59, Epstein *et al.* '63).

Furthermore, many evidences have been reported that the synthesis of those late proteins or the maturation of intact phages was inhibited by irradiating phage particles with ultraviolet (UV) light (Kozloff '53, Watanabe '57, Stahl '59 and Minagawa *et al.* '64a) or treating the culture with chloramphenycol (CM) (Ishihama *et al.* '62, Kano-Sueoka & Spiegelman '62 and Okamoto, Sugino & Nomura '62), amino acid analogues (Ebisuzaki '63), 5-fluorouracil (FU) (Aronson '61) and so on, before or at the early period of phage infection.

In addition, separation pattern of the phage specific RNAs by a MAK column was also affected by those agents: one of the RNA peaks in the chromatogram decreased or absolutely lacked in some cases or increased or accumulated twice or more in other cases, while in both cases formation of complete phages were not accomplished (Minagawa *et al.* '64a, Minagawa '64b

and '65).

The above finding and others suggested a close correlation between separated RNAs and categorically divided two types of protein synthesis (Minagawa *et al.* '64a, Minagawa '64b and '65); that is, the first and the second (hereafter, called "early" peak or m-RNA) eluted at lower concentrations of NaCl from the column corresponded to synthesis of the early proteins and the third, the last peak (hereafter, called "late" peak or m-RNA), eluted at a higher concentration of NaCl corresponded to that of the late proteins. It was also assumed that some early protein was necessary for the synthesis of the late m-RNA (Minagawa *et al.* '64a, Ebisuzaki '63). However, there was no evidence that proved those RNAs were phage-specific.

Attempts have been made to examine whether those are phage specific or not by determining a base ratio of the RNAs separated by the column. Particular interests were focused to the comparison of base ratios between the early and late m-RNAs of T2 phage under the given conditions in which only the early m-RNA was synthesized but the late one was absent or both the early and late m-RNAs were synthesized despite intact phages were not developed.

II Materials and Methods

1) Cells and bacteriophages.

Wild type strain of *Escherichia coli* H, auxotrophic mutants, H. try$^-$ and B. arg$^-$, u$^-$ and bacteriophage T2 H were used. Conditions of cell growth and medium were performed followed to the Minagawa *et al* 's ('64a) previous short communication. Cells of *E. coli* were grown to a final concentration of 5×10^8 cells per ml in an ammonium glucose medium (Hershey '57), harvested, resuspended in the same medium and infected with phage particles at a multiplicity of infection 5 to 10. In all experiments presented the percentage of uninfected cells was less than 0.1% at five minutes after infection.

2) Isolation of $^{32}PO_4$ labeled m-RNA of infected cells.

The culture was labeled with $^{32}PO_4$ for a period of 4 minutes between 10 and 14 minutes after infection. At the termination of labeling, NaN_3 was added to a concentration of 1×10^{-2} M, the incubation mixture was poured onto frozen culture medium containing the same concentration of NaN_3, and the cells were collected by centrifugation. For labeling uninfected cells, the time when the cells were resuspended in the fresh medium was chosen as zero and the culture was labeled with $^{32}PO_4$ for 4 minutes from 10 to 14 minutes. $^{32}PO_4$ used for each experiment was 30 to 40 μc per ml of the medium. The extraction of the RNAs was carried out by the method of Okamoto *et al* ('62),

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Cy, Ad, Ur, Gu and HMC, cytidylic, adenylic, uridylic, guanylic and 5-hydroxymethyl cytidylic acid; U, uracil; Thy, thymine or thymidylic acid; dThy, deoxythymidine; arg and try, arginine and tryptophane; m- or r-RNA, messenger or ribosomal RNA.

using phenol except that dialysis at the final step of the procedure was omitted. The extracted RNAs were dissolved in the solution of 0.02 M acetate buffer (pH 5.2) containing 0.02 M KCl, 0.01 M $MgSO_4$, 0.01 M NaN_3 and applied to the MAK column prepared by a modification (Sueoka & Cheng '62) of the Mandel & Hershey's method ('60). The column was eluted by a linear gradient concentration of NaCl, 0.5 to 1.1 M. Each 4 ml of effluent was collected. One ml of each effluent was dried on a planchet for radio assay to know the nucleic acid synthesized for 4 minutes. The absorbancy of the remainder was measured at 260 m μ to decide position of host ribosomal RNA as a marker.

3) Determination of base ratio of m-RNA.

The RNAs in the effluents around each peak were combined and precipitate by 0.3 M trichloroacetic acid after addition of the yeast RNA as a carrier, and washed with ethanol. The washed RNA was hydrolysed with 0.5 M KOH for 18 to 20 hours at 37°C, and then neutralized by perchloric acid in an ice bath. 50 μl of neutralized fraction was applied to Toyo-Roshi No.50 filter paper-strip (2 cm width, 40 cm length) that had been soaked in an ammonium formate buffer (pH 3.5) and electrophoresized at a voltage gradient of 20 v per cm, for 3 hours at 5°C (ref. the method of Smith '55). The paper-strip was dried at room temperature and 4 nucleotide spots were revealed under UV lamp to be well separated. The ribonucleotide spots marked under UV lamp were cut off from the paper, divided into one cm length, placed in the planchet and counted by G.M. tube or Gas-flow counter. The base ratio was expressed as a percentage of radioactivity of a spot to the sum of that of 4 spots. Same 50 μl of the neutralized RNA was dried on a planchet to test the recovery of the radioactivity of nucleotide spots on the strip. And it was found that the percentage of the recovery was 76.9% at worst but higher than 90% in most experiments.

4) Separation of $UM^{32}P$ (uridylic acid) and $FUM^{32}P$ (fluorouridylic acid).

Percentage replacement of U by FU was determined after re-electrophoresis of eluate from the UMP area on the paper at a voltage gradient of 10 v per cm, for 4 hours in borate buffer (pH 9.2) at 5°C (ref. Gordon & Staehelin's, '59 or Halonberk's, '63 method). FUMP moved faster than UMP.

III Experiments and Results

a) Messenger RNA of normal *E. coli* strain H.

Similar to previous results (Ishihama *et al.* '62, Monier *et al.* '62 and Kano-Sueoka & Spiegelman '62), three peaks of m-RNA of *E. coli* were obtained and clearly separated. Determination of base ratio of each peak was summarized in Table 1. As the reference, base ratios of the DNA and ribosomal RNA of *E. coli* were quoted. Although the percentage of adenylic and guanylic acid were a little bit different when compared to the quoted values, obtained ratio was quite similar to that of the *E. coli* DNA and obviously not likely to that

of r-RNA.

Table 1. Base Ratio of Normal *E. coli* Messenger RNA (%).

Peak \ Nucleotide	Cy	Ad	Gu	Ur	S*	P**	Recovery S/P (%)
I	24.4	24.5	26.0	25.1	2132		
	25.1	25.1	25.4	24.4	1989		
	24.1	24.2	28.7	23.0	1996		
Mean	24.5	24.6	26.7	24.2	2039	2245	89.4
II	23.5	23.6	27.5	25.4	5853		
	23.9	23.5	27.7	24.9	5333		
	22.7	24.0	28.6	24.7	5603		
Mean	23.4	23.7	27.9	25.0	5596	5885	94.9
III	22.8	23.5	27.4	26.3	3674		
	22.9	22.7	27.6	26.8	3408		
	22.7	24.1	26.4	26.8	3573		
Mean	22.8	23.4	27.1	26.7	3552	3760	94.4
E. coli				(Thy)			
r-RNA	(23	23	32	22		Volkin & Astrachan	
	21.5	24.1	31.9	22.8	)		
DNA	25-26	24-25	25-26	24-25		Ishihama *et al.*	

* S: Total counts per minute of 4 nucleotide spots on a paper-strip.
** P: Counts per minute of sample applied onto a paper-strip.

b) Messenger RNA of normal bacteriophage T2.
Three peaks were obtained, the separation pattern was very similar to

Table 2. Base Ratio of Phage T2 H Messenger RNA (%).

Peak \ Nucleotide	Cy	Ad	Gu	Ur	S	P	Recovery S/P (%)
I	20.9	30.0	19.7	29.4	750		
	21.7	29.1	20.0	29.2	763		
	19.9	31.0	20.4	28.9	746		
Mean	20.8	30.0	20.0	29.2	753	789	95.4
II	20.9	30.4	21.8	26.9	2442		
	19.1	28.7	21.6	30.6	2408		
	22.0	30.0	17.9	30.1	1986		
Mean	20.7	29.7	20.4	29.2	2279	2542	89.4
III	20.4	28.8	21.0	29.8	1121		
	18.9	31.6	19.0	30.5	1221		
	19.8	30.6	19.8	29.8	1154		
Mean	19.7	30.3	19.9	30.1	1165	1250	93.1
Phage T2	(HMC)			(Thy)			
DNA	16.8	32.5	18.2	32.5		Sinsheimer	
	17	32	18	32		Ishihama *et al.*	

S and P, both are the same as explained in the foot note of Table 1.

that of uninfected bacteria and also quite comparable to those obtained by previous investigators (Ishihama *et al.* '62, Kano-Sueoka & Spiegelman '62). Results were summarized in Table 2. Base ratio of each m-RNA resembled definitely to that of the phage T2 DNA, but neither to that of the host DNA nor r-RNA.

c) Messenger RNA of UV-irradiated phage T2.

Cells were infected with phage inactivated by UV-irradiation to 2 x 10^{-4} survival. As expected from the report of Minagawa *et al* ('64a), only the early m-RNA (first two peaks) was obtained and the late one was absent, even when the labeling with $^{32}PO_4$ was carried out at 10 to 14 minutes after infection. The same pattern of peaks were reported, when the labeling with $^{32}PO_4$ was done at the early period, between 1 and 5 minutes, after infection using normal phage (Minagawa *et al.* '64a). Base ratio of these two peaks, however, was quite the same each other and both resembled to that of the phage DNA as summarized in Table 3. This means that the UV-damaged phage can transcribe only the early m-RNA but not the late one.

Table 3. Base Composition of UV-Phage Messenger RNA. (%).

Nucleotide	Cy	Ad	Gu	Ur	S	P	Recovery S/P (%)
Peak I	18.4	28.5	21.0	32.1	836	1055	79.2
Peak II	18.7	29.3	20.6	31.4	1632	1777	91.8

Labeling with $^{32}PO_4$ was carried out between 10 and 14 minutes after infection.
Each value presents the mean of determinations from three paper-strips.
S and P are the same as explained in Table 1.

d) Treatment with CM,

CM was added to 75 μg per ml at 2 minutes after infection and the labeling was done with $^{32}PO_4$ between 10 and 14 minutes. The separation pattern of phage m-RNA peaks was shown in Figure 1. Only the early m-RNA (first two peaks, I and II) was obtained and the late one (III) was obscure. In this case, also, base ratio of the 2 peaks resembled to that of the phage DNA as seen in Table 4, and phage particles were not developed.

Table 4. Base Composition of the RNA synthesizez in the Presence of CM. (%)

Nucleotide	Cy	Ad	Gu	Ur	S	P	Recovery S/P (%)
Peak I	17.6	29.2	22.3	30.8	1076	1399	76.9
Peak II	16.9	31.1	19.8	32.2	1601	2015	79.9

CM was employed (75 μg/ml) at 2 minutes after infection and labeling with $^{32}PO_4$ was done at 10 to 14 minutes. Each value presents the mean of thrice determinations and S and P are the same as explained in Table 1.

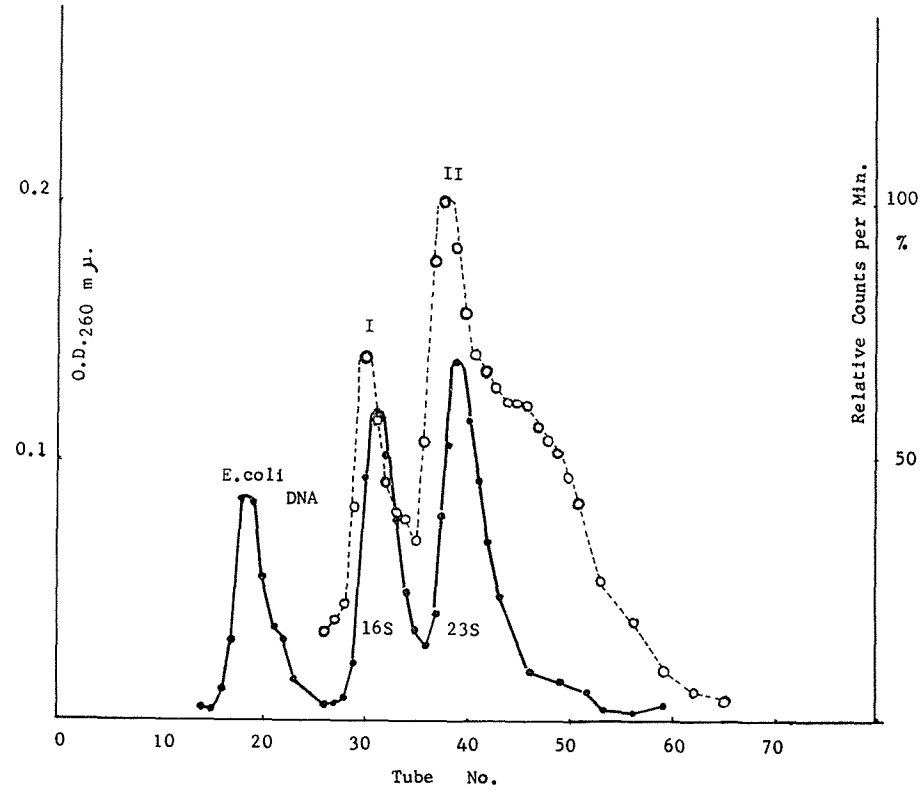

Figure 1. Chromatogram of the RNA synthesized in the Presence of CM.
CM (75 μg/ml)was added at 2 minutes after infection and labeling with $^{32}PO_4$ was done between 10 and 14 minutes. The MAK column was eluted by a linear gradient concentration of NaCl, 0.5-1.1 M from tube No. 1 to 100. Open circles linked with dashed lines shows the radioactivity of each effluent. It was plotted by relative counts/min/ml effluent as the maximum counts of the second peak was 100 %. The other solid lines indicates the absorbancy of each effluent at 260 mμ.
Peak I and II are the early m-RNA of phage T2. The late m-RNA (Peak III) is obscure.

e) Uracil starvation.

Cells of the strain requiring both arginine and uracil grown in the medium supplemented with L-arginine (20 μg/ml) and uracil (20 μg/ml) were harvested, washed, resuspended in the medium supplemented with only arginine and aerated for 10 minutes before infection of phage T2. Subsequent procedures were the same as others. Only the early m-RNA was obtained and base ratio of that was the same as that of the phage DNA as shown in Table 5. Since the late m-RNA was obscure, it seemed superficially to be that prior starvation for 10 minutes was not enough or endogenous pool of uracil was utilized for the transcription of the early m-RNA but all this uracil have been already

exhausted for synthesis of the late m-RNA.

Table 5. Base Composition of the RNA synthesized in the U-deficient Condition. (%)

Nucleotide	Cy	Ad	Gu	Ur	S	P	Recovery S/P (%)
Peak I	17.3	31.0	20.0	31.7	671	767	87.4
Peak II	17.1	31.4	19.9	31.6	703	779	90.3

Cells requiring both arginine and uracil were aerated in the medium containing only L-arginine (20 μg/ml)for 10 minutes before infection. Labeling was done between 10 and 14 minutes after phage infection. Each value presents the mean of determinations from three paper-strips and S and P are the same as explained in the foot note of the Table 1.

f) Uracil replaced with FU.

According to Aronson ('61), phage DNA synthesis was not affected by FU but coat protein synthesis was inhibited by the chemical, if thymine was supplemented. It might be expected that the early m-RNA was normal but the late one should be absent or somewhat irregular, if uracil would be replaced by FU. Washed cells requiring arginine and uracil were resuspended in the medium containing only L-arginine (20 μg/ml) and aerated for 5 minutes, then FU (10 μg/ml) and dThy (20 μg/ml) were added. The culture was aerated further for 10 minutes before infection. Subsequent procedures were the same as the others but at the termination of labeling with $^{32}PO_4$, one half of the culture was ceased by adding NaN_3, the other half chased by phosphate buffer (pH 7. 4) at a final concentration of 0.1 M for 4 minutes and then ceased by adding NaN_3.

Distribution of ^{32}P among $UM^{32}P$ and $FUM^{32}P$ was determined before and after chase. Results are summarized in Table 6-a, b and c.

Contrary to the expectation, both the early and late m-RNA were obtained before and after chase. Base ratio of each peak before chase was all similar to that of the phage DNA as shown in Table 6-a. It was not presented here, base ratio of the early and late RNA after chage was also the same as that of the phage DNA. No accurate comparison on the results, before and after chase, was made since many steps should be passed for the extraction of the synthesized RNAs. It should be noted that, of course, total counts of ^{32}P in the RNA decreased after chase by cold phosphate buffer for 4 minutes but the relative counts of the late RNA increased about 20% after chase (ref. Table 6-b). More interesting fact was that percentage replacement of U by FU rather increased approximately from 30 to 80% in each peak after chase (Table 6-c). The reason for this was not clear yet. Even base ratio of each peak was the same eachother and similar to that of the phage DNA but part of U was replaced by FU (more replaced after chase), so that these m-RNAs could not act normally as expected from previous report (Aronson '61).

Table 6-a. Base Composition of the RNA synthesized in the presence of FU. (%)

Nucleotide	Cy	Ad	Gu	Ur	S	P	Recovery S/P (%)
Peak I	17.8	31.2	19.5	31.5	1151	1372	83.9
Peak II	17.5	29.9	21.1	31.5	1687	1884	89.4
Peak III	17.1	30.6	20.8	31.5	2275	2546	89.2

Cells requiring both arginine and uracil were used. The suspended medium supplemented with only L-arginine (20 μg/ml) was aerated for 5 minutes, then FU(10 μg/ml) and dThy (20 μg/ml) were added. The culture was aerated further for 10 minutes before infection. Labeling was done between 10 and 14 minutes after infection. Each value also presents the mean of thrice determinations. S and P are the same as explained in the Table 1.

Table 6-b. Relative Counts of Each Peak before and after Chase.

	Before chase (counts/min)	relative cts. (%)	After chase (counts/min)	relative cts. (%)
Peak I	1372	23.6	200	5.7
Peak II	1884	32.4	1013	28.8
Peak III	2546	44.0	2319	65.5
Total	5802	100.0	3532	100.0

Each value indicates total counts/min/50 μl of alkaline hydrolysates of each peak RNA.

Table 6-c. Replacement of U by FU in the RNA before and after Chase.

	Before chase UM^{32}P (total counts/min)	FUM^{32}P	FU(%)	After chase UM^{32}P (total counts/min)	FUM^{32}P	FU(%)
Peak I	—	—	—	13	47	78.4
				60	91	60.3
				—	—	—
Peak II	398	196	32.6	—	—	—
	405	200	33.1	70	350	83.2
	344	158	31.5	38	281	88.1
Peak III	672	292	30.3	—	—	—
	596	264	27.5	47	259	84.7
	578	299	34.0	148	679	82.2
Mean			31.5			79.5

FUM^{32}P runs just ahead of 5′-UMP (commercial product, added as a carrier), and UM^{32}P(2′, 3′-P, alkaline hydrolysates) runs behind 5′-UMP on the paperstrip by an electrophoresis. Counting was done using Gas-flow Counter.

g) Replacement with azatryptophane.

If tryptophane were an important site of the early protein necessary for the transcription of the late m-RNA, replacement with its analogue, azatryptophane should cause some effects on the synthesis of the late m-RNA. Cells of H, try⁻ were grown in the medium containing DL-tryptophane (20 μg/ml), collected, resuspended in the same medium without tryptophane and aerated

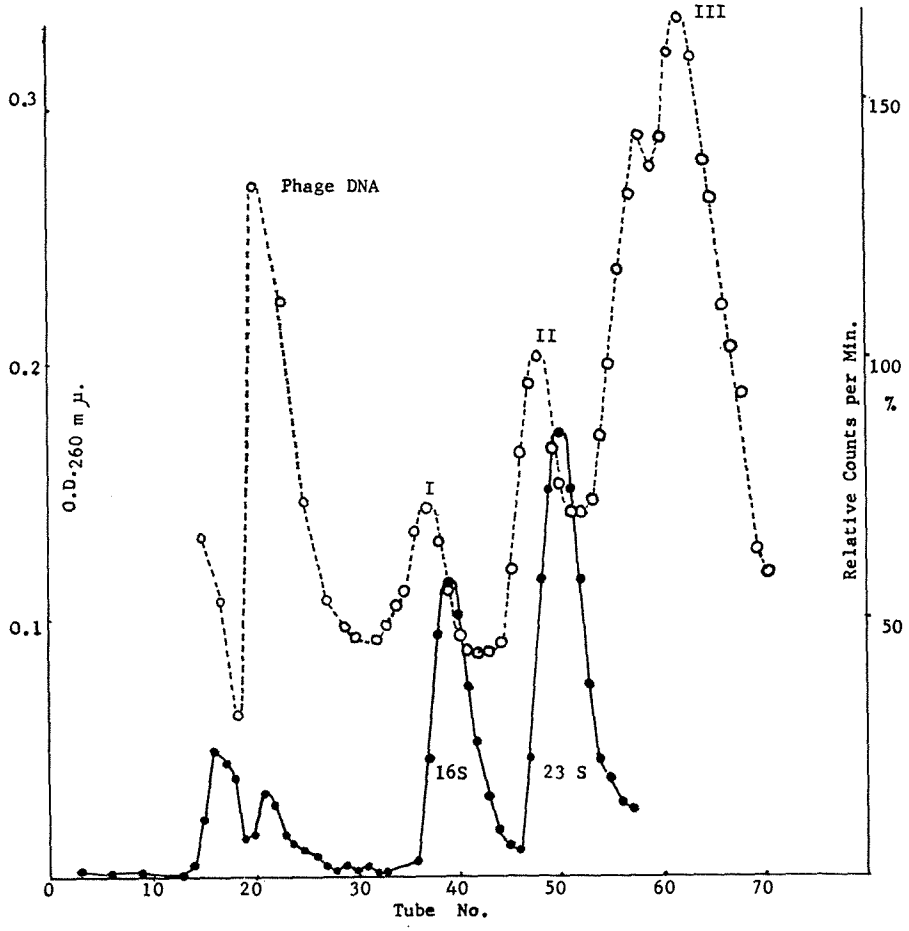

Figure 2. Chromatogram of the DNA and RNA synthesized in the Presence of DL-azatryptophane.

Cells of H. try⁻ were used. At 2 minutes before infection, DL-azatryptophane (20 μg/ml) was added. In this experiment, labeling with $^{32}PO_4$ was performed between 5 and 10 minutes after infection. The method of elution from the column and plotting the results is the same as explained in the Figure 1. Not only the early m-RNA (Peak I and II) but the late one(Peak III) is obtained and this is extremely large compared to normal phage late m-RNA.

for 15 minutes before infection. At 2 minutes before infection, DL-azatryptophane (20 μg/ml) was added. In this case, labeling with $^{32}PO_4$ was performed for a period of 5 minutes between 5 and 10 minutes after infection. At the termination of labeling, the suspension was divided into three parts. One part was stopped by adding NaN_3, the others were chased by adding a final concentration of 0.1M ammonium phosphate (pH 7.4) and DL-tryptophane (500 μg/ml) for both ^{32}P and azatryptophane. One of chasing cultures was ceased by adding NaN_3 after a period of 2 minutes, and the other was done after 6 minutes. Both the early and late m-RNAs were obtained before and after chase, and the late one extremely increased or accumulated as compared to normal (about twice) as shown in Figure 2. Though the chromatogram of the RNAs after chase was not presented, it was quite similar to that of illustrated in the Figure 2. Base ratio of each m-RNA was the same eachother and very similar to that of the phage DNA as summarixed in Table 7-a. Follows should

Table 7-a. Base Composition of the RNA synthesized in the Presence of DL-Azatryptophane. (%)

Nucleotide		Cy	Ad	Gu	Ur	S	P	Recovery S/P (%)
Peak I	(A)	18.6	29.0	19.7	32.7	516	584	88.6
	(B)	18.9	30.1	19.3	31.7	446	530	84.3
	(C)	17.8	30.2	19.0	33.0	581	645	90.2
Peak II	(A)	18.1	29.8	19.1	33.0	989	1042	94.9
	(B)	17.9	29.3	20.4	32.4	911	984	92.4
	(C)	20.1	28.7	20.0	31.2	1294	1360	95.1
Peak III	(A)	17.9	31.5	19.1	31.5	1480	1633	90.6
	(B)	17.0	30.0	21.0	32.0	1043	1149	90.7
	(C)	18.6	31.1	19.0	31.3	1536	1676	91.6

Cells of H, try⁻were used. DL-azatryptophane (20 μg/ml) was added to the medium at 2 minutes before infection. In this experiment, labeling with $^{32}PO_4$ was carried out at 5 to 10 minutes after infection. (A), (B) and (C) present the values of without chase, after chase for 2 minutes and chase for 6 minutes respectively. And each value also indicates the mean from determinations of three paper-strips. S and P are both the same as explained in the foot note of the Table 1.

Table 7-b. Relative Counts of Each Peak before and after Chase.

	Before chase		After chase for 2 min.		After chase for 6 min.	
	(counts/min)	relat. cts. (%)	(counts/min)	relat. cts. (%)	(counts/min)	relat. cts. (%)
Peak I	584	17.7	530	19.9	645	17.5
Peak II	1042	32.0	984	36.7	1360	37.1
Peak III	1633	50.3	1149	43.2	1676	45.4
total	3259	100.0	2663	99.8	3681	100.0

Each value indicates total counts/min/50 μl of alkaline hydrolysates of each RNA peak synthesized in the presence of DL-azatryptophane.

be noted here that the phage DNA was synthesized even in the presence of azatryptophane and relative counts of each peak changed slightly after chase, *i.e.* increased in the second peak of the early m-RNAs and decreased in the late m-RNA after chase both for 2 and 6 minutes (see, Figure 2 and Table 7-b). From these results, it seemed to be that tryptophane was not active amino acid in the early proteins to initiate phage DNA or to read out the late m-RNA, but had a some significant role in the late proteins needed for the structure or probably for maturation of phage particles.

IV Discussion

For the sequential steps of bacteriophage development, an ordered transcription of the phage specific RNAs has been discussed by many investigators (Kano-Sueoka & Spiegelman '62, Minagawa *et al.* '64a, Ebisuzaki '63 and Hall, Nygaard & Green '64). Very recently Edlin ('65) reported that the early protein was not necessary for the transcription of the late m-RNA from his experiments using amber mutant of T4, in the presence of FU and CM. Discussion on the "clock" or "timing" mechanism of a non random reading of the phage specific RNAs is not a purpose of this paper. Further works should be required for this problem.

It has been already pointed out that each nucleic acid peak separated by a MAK column has a different molecular size (Mandel & Hershey '60) and DNA with greater Gu-Cy content was eluted at lower salt concentration fron a MAK column (Sueoka & Cheng '62). If these were applicable to separated RNA peaks, the early m-RNA and the late one should have a dlfferent molecular size and a different Gu-Cy content. And it is recognizable that each m-RNA peak is a summation of similar order RNAs in molecular slze. However, experiments presented here indicated that base ratios of obtained peaks of the phage RNAs were the same each other and absolutely similar to that of the phage DNA, so long as infection with phage was succeeded, even though the numbers of RNA peaks were two or three under the given conditions.

Apart from the problem on molecular sizes, why do both the early and the late m-RNA have the same base composition ? One of answers for this is that the difference of Gu-Cy content between the early and the late m-RNA is so small that it cannot be discriminated by the determing method employed in this paper. The other is that each m-RNA reads out the information from the genome in the way of cyclic permutations of one another along cyclic map of the genes. That is, base sequence in each m-RNA is overlapped in the most part of its molecule. But there is no evidence for this. The last one is simple. They have indeed the same base composition, resembled to that of the phage DNA. If the last is correct, then the next conclusion is conceivable that each m-RNA should have a different base sequence, since it has a same base ratio and a similar molecular size, to transfer each bearing specific information to corresponding protein from the random arrangement of the DNA

nucleotides.

V Summary

Base ratios of peaks of the phage specific (messenger) RNA under the different conditioos separated by a chromatographic MAK column were determined. The conditions were following ; use of partially damaged phages irradiated by UV, treatment with CM at the early period after phage infection, uracil starvation, uracil replacement by FU and tryptophane replacement by its analogue, azatryptophane during the development of phage particles. Base ratios of obtained m-RNAs under the given conditions all resembled to that of the phage DNA. The properties of the phage specific m-RNAs were briefly discussed.

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