ON BACTERIAL AND ØX-174 MESSENGER RNA

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ABSTRACT

General methods for the chromatography of nucleic acids on benzoylated (naphthoylated) DEAE cellulose are described. This procedure results in well-resolved peaks with good recovery and seems to separate nucleic acids on the basis of their secondary structure almost independently of their molecular weight. These methods have proved to be useful in the analysis of the replicative intermediates of MS-2 RNA, ØX RFDNA, and ØX single stranded DNA, for example.

Specific methods for the purification of E. coli. pulse-labeled RNA based on the chromatography on benzoylated DEAE cellulose at pH 7.5 and pH 3.5 were developed. Greater than 60% of the pulse label with less than 4% of the mass label is recovered, and the RNA size distribution in a denaturing solvent (99% dimethyl sulfoxide) after chromatography shows that the mass label closely parallels the pulse label as a function of size; there appears to be no selection or degradation. Pulse-chase experiments indicate that a large fraction of both the pulse and mass labels are chased out, suggesting reasonable purity of the m RNA. These data imply that there is a difference, structural or chemical, between mRNA and other known RNAs that allows all E. Coli. mRNA to act as a group regardless of size

during the purification.

The in vivo ØX mRNA has been studied using the above procedure for purification and analysis. The results show that the ØX mRNA size distribution at early and late times after infection is very broad ranging from a distinct maximum size of 1.7 megadaltons (one genome length of polycistronic mRNA), to a peak in the distribution at 0.2-0.3 megadaltons, and with significant mRNA as small as 10^4 daltons. The only difference observed between the early and late times after infection appears to be in the amount of RNA present: approximately 100 molecules of mRNA per cell are present at 4 min. after infection compared to 1000 molecules per cell at 25 min. after infection. Little if any difference could be found in the size distribution of mRNA made by the strongly polar OP6 ØX mutant or upon infection of the ØX replication-restrictive bacterial mutant rep_3^-. A variety of experimental approaches showed an absence of significant methylation and 5'triphosphate termini in the mRNA.

Attempts were made to ask which RF, parental or progeny, was the template for the transcription of the ØX mRNA. One type of experiment indicated that there was a small amount of pulse labeled RNase-, phenol-, and detergent-resistant RNA specifically attached to the density labeled

parental RF. However, other types of direct experiments (such as analysis of non-RNase treated RF in CsCl density equilibrium gradients) demonstrated that neither type of RF was shifted to higher densities due to attached nascent mRNA; nor could significant pulse labeled RNA be detected in the RF region of the CsCl density gradients. Thus, no unambiguous answer can be given to the template question.

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INTRODUCTION

This introduction on messenger RNA will be limited mainly to a discussion of the mRNA from bacterial (and bacteriophage) systems. MRNAs from eukaryote systems are intrinsically more interesting; however, complications such as redundant DNA patterns (cf. Britten and Davidson, 1969), satellite DNAs (Jones, 1969; Gall, 1970; Hennig and Walker, 1970), mitrochondrial systems (Borst and Kroon, 1969), and nuclear RNA processing (cf. Darnell, 1968; Weinberg and Penman, 1969) preclude an accurate analysis of the mRNA from higher organisms at this time.

A limited amount of evidence does exist suggesting that at least physically the mRNA from eukaryotes does bear some resemblance to bacterial mRNA. The putative mRNA for hemoglobin in Reticulocytes seems to chromatograph on benzoylated DEAE cellulose columns similar to the pattern of E. coli. mRNA (J. Kiger, personal communication; Lyon, 1969). Also, the mRNA for γ-globulin in plasma cell tissue culture behaves in a similar manner (W. Summers, personal communication).

In addition, this introduction will be largely concerned with the myriad of control functions that have been shown to exist in connection with transcription, translation, and mRNA degradation of bacterial mRNA. Clearly, it is the

detailed analysis of these controls that might illuminate some of the molecular mechanisms of differentiation and developement in eukaryote systems.

mRNA Synthesis

It is now clear that the enzyme responsible for the *in vivo* transcription in *E. coli*. is the DNA-dependent RNA polymerase. Most of the evidence comes from data such as the *in vitro* - *in vivo* comparison of rates of synthesis (where the rates are similar if not identical compared to the *in vivo* DNA replication rate - *in vitro* DNA polymerase rate) and DNA strand selection in bacteriophages like ØX or T7. Recently, evidence has been obtained that convincingly proves the point that RNA polymerase is indeed the *in vivo* enzyme. Bacterial mutants with a temperature-sensitive RNA polymerase are temperature-sensitive *in vivo* (with respect to ^{3}H-uridine incorporation into all RNAs) as well as *ts* in the isolated RNA polymerase (Igarashi and Yura, 1969). Similarly, rifampycin blocks RNA synthesis *in vivo* and is a potent inhibitor *in vitro* of RNA polymerase; mutants resistant to the antibiotic also have resistant RNA polymerase (diMauro, Synder, Marino, Lambert, Coppo, and Tocchini-Valintini, 1969).

Major control mechanisms for RNA regulation in *E. coli*. exist at the level of specificity of transcription. Evidence

for this type of major positive control comes from the recent work of Travers (1970), Losick and Sonenshein (1969), and Summers and Siegel (1969) describing RNA polymerase containing a factor, sigma, responsible for the specificity of initiation at a known promotor site. Furthermore, T4, Øe, T7, and other autonomous or mostly autonomous phage systems contain genetic information for the synthesis of new sigma factors to start transcription of whole blocks of temporally controlled late genes; concievably this is the mechanism used to switch transcription from one strand to another (cf. Szybalski, 1969). It appears that a major portion of the RNA polymerase is conserved as if only the factor, sigma, were used to control the specificity of transcription (Geiduschek and Sklar, 1969; Haselkorn, Vogel, and Brown, 1969).

Preliminary evidence seems to suggest that an equally important gross control mechanism exists at the level of termination of transcription as first reported by Roberts (1969). The results suggest that a specific protein is produced whose function is to specifically terminate the transcription reaction - very likely at the end of a polycistronic mRNA. Termination is general, occurring, for example, in T4 infected systems (Schmidt, Mazaitis, Kasai, and Bautz, 1970) and λ infected systems (Roberts, 1969).

Likewise, the existence of an anti-terminator in the T4 system has been postulated (Schmidt et. al., 1970) to explain the ability of the RNA polymerase to read past the boundaries of the pre-early genes into the early genes; anti-terminator proteins which would allow transcription to the left and right of the repressor C_I might be used to explain the function of gene N in the λ system (quoted in Schmidt et. al., 1970).

Fine control is also known for the transcription reaction in E. coli.. This is evidenced by the negative control repressor-operator mechanism (Gilbert and Müller-Hill, 1967; Reznikoff, Miller, Scaife, and Beckwith, 1969) which has clearcut examples in the Tryp operon (Imamoto and Yanofsky, 1967), λ (Dove, 1968; Ptashne and Hopkins, 1968), and the His operon (Venetianer, Berberich and Goldberger, 1968). Positive control of a similar nature exists, and the best example is in the L-Arabinose system (Englesberg,Sheppard, Squires, and Meronk, 1969). Recently, cyclic AMP was implicated in a subtle control function possibly restricted to the catabolite enzyme pathways (Pearlman and Pastan, 1968) in which an effect on lac mRNA and expression in an in vitro system is noted (deCrombrugghe, Varmus, Perlman, and Pastan, 1970; Chambers and Zubay, 1969). In general, fine control by the above mechanisms probably effects only one or two

operons and is capable of responding to subtle small changes very rapidly.

Interestingly, there appears to be increasing evidence to support the notion that mRNA synthesis is not isotropic in the cell, but occurs mainly, if not entirely, on the cell membrane. For example, all (>90-95%) of the pulse-labeled E. Coli. or T4 mRNA and RNA polymerase is found on the membrane-DNA complex (Rouvière, Lederberg, Granboulan, and Gros, 1969; Tremblay, Daniels, and Schaechter, 1969). Minicells and mutants of E. Coli. which lack DNA do not seem to have RNA polymerase (Adler, Fisher, Cohen, and Hardigree, 1967; Hirota, Jacob, Ryter, Buttin, and Nakai, 1968). It is certainly possible that control of transcription by topology in phage or episome systems is an important control mechanism.

The following is an attempt to describe some representative patterns of mRNA in different systems of apparent decreasing complexity. These data are largely based on strand separation of the DNA by ribonucleotide polymer binding studies (cf. Szybalski, 1969) and hybridization studies as a function of time after phage infection.

T4

T4 has a very complicated temporal control of trans-

cription and has been the subject of intensive study using techniques such as hybridization competition (Bolle, Epstein, Salser, and Geiduschek, 1968), deletion hybridization (Kasai and Bautz, 1969), and analysis of the mRNA on acrylamide gels (Adesnik and Levinthal, 1970). In brief, there are two general classes of mRNA as a function of time, with the later class of late mRNA requiring DNA replication and active gene products of genes 55 and 33. The early class is further divided into at least two more classes: the 0-2 min. sub-class which does not require protein synthesis and can be transcribed in vitro by the RNA polymerase (with sigma) (Travers, 1969; Bautz, Bautz and Dunn, 1969) and a second sub-class of early mRNA which requires protein synthesis. It is now thought that one of the gene products of the first subclass is a new sigma factor which then directs the RNA polymerase to start copying the second subclass (Travers, 1970); the first subclass is slowly switched off due to the modified affinity of the new sigma for the promotors of the pre-early genes. Recent evidence suggests that the region between subclass I and subclass II is complicated. Schmidt, et. al. (1970) postulate that there is an additional class of genes, (the lysozyme gene e for example) that is not made during the 0-1 min. period due to the termination

of the mRNA by the host terminator protein (Roberts, 1969), but which is made after 1 min. when an antiterminator protein permits efficient reading beyond and into the lysozyme gene. At late times after infection possibly the gene product of gene 55 (and 33) might be a sigma factor which could switch on many of the true late genes.

The broad overall picture of the T4 mRNA pattern of transcription based on the strand separation-hybridization studies of Szybalski (1969) is shown in Fig. 1 (a.). The switch from early to late is involved in a strand (and direction) switch.

The mRNA pattern from the lysogenic phage λ is a beautiful example of the plethora of transcriptional controls that exist; it is, however, probably less complicated than T4. It is now clear that in the lysogenic state only the C_I region is transcribed, and the gene product of C_I, the repressor (Ptashne, 1968), binds on the DNA in two places and blocks all further transcription. If the repressor is inactivated (by heat, UV, or Mitomycin), transcription proceeds as shown in Fig. 1 (b.). Gene N may be an anti-terminator which allows transcription to proceed both to the left and right of C_I (Roberts, 1969; quoted in Schmidt, 1970). Gene Q very possibly is the λ sigma factor

used to start the transcription of the majority of the λ late genes.

Fig. 1 (b) shows the overall picture of the mRNA, and again strand selection is an obvious control feature. Progress in the study of the sizes of the different mRNA has been made by Kourilsky, Marcaud, Sheldrick, Luzzati, and Gros (1968).

T7

T7 is an attractive system in which to study transcription since it is relatively small, has only 19-20 complementation groups (Studier, 1969), and is transcribed from only one of the two strands of the DNA (Summers and Szybalski, 1968). It appears that in the absence of protein synthesis only gene 1 (or at most a few genes) is transcribed and that the gene 1 product is a new sigma factor which with the host core RNA polymerase copies the rest of the T7 genes (Summers and Siegel, 1969).

Summers (1969) has recently shown that there are 12 distinct (possibly stable) RNA bands in an acrilamide electrophoresis system which account for most of the genetic information of the T7 DNA.

Fig. 1 (c.) represents the status of the mRNA transcribed from the T7 DNA.

Tryp (as a representative operon).

Intensive work on the operon systems such as lac and tryp has revealed major details about the molecular mechanisms involved in transcription and its control; many of these controls are now considered to be fine adjustments of transcription. Most important are the results showing the relationship of the promotor to the operator (Reznikoff et. al., 1969; Imamoto, 1969; Morse and Yanofsky, (1968), the small size of the promotor (Reznikoff et. al., 1969; Morse and Yanofsky, 1969), and subtle complications such as the low efficiency internal promotors (Morse and Yanofsky, 1968).

Studies on the polysome patterns of the lac system (Kiho and Rich, 1965) and the his system (Bagdasarian, Ciesla and Sendecki, 1970), for example, suggest general ideas about polycistronic mRNA products of transcription. The hybridization results of Morse, Mosteller, Baker, and Yanofsky (1969) indicate the basic mechanisms for transcription, translation, and degradation for operons.

<u>ØX</u>

Quite possibly the ØX phage system is the simplest system studied to date with respect to control mechanisms of transcription. Data are presented in Part II of this

thesis to support the conclusion that no new sigma factor is made after infection with no obvious pattern of host mRNA shut-off. The ØX mRNA size distribution is consistent with one polycistronic mRNA translated and degraded by the host mechanisms. The lack of temporal control, transcription from one strand, and the size distribution data suggest that there is only one promotor per ØX RF (cf. discussion Part II).

Translation

It is still undecided whether there is obligative transcription-translation coupling in E. Coli. (for a review see Geiduschek and Haselkorn, 1969). Many studies have shown that, while coupling is not essential (λ and lac repressors function on the DNA not the translation machinery), there is functional coordination. The evidence comes mainly from the work on the tryp operon: de-repression results in immediate synthesis of the operator proximal enzymes proceeding down the operon until all the gene products are synthesized in a coordinate fashion (Morse, Baker and Yanofsky, 1968). Approximately 100 ribosomes per messenger move sequentially before degradation starts from the 5' end (Morse et. al., 1969).

Polarity has been studied in most of the systems listed

above. This phenomenon results in reduced (or absent) translation of genes distal to a mutation near a polypeptide reinitiation point and provides evidence for polycistronic mRNA and the direction of translation (cf. Zipser, 1969). There is evidence that the distal portion of the mRNA (beyond the nonsense mutation) is missing, and a bacterial mutation, SuA, relieves the polarity (Morse and Primakoff, 1970). It is possible that polarity is a general phenomenon normally used as a mechanism to reduce the molar amounts of proteins distal to the operator proximal end.

Recent evidence on the sequence of Qβ and other phage RNAs, although not natural mRNAs (cf. Sedat, Kelly and Sinsheimer, 1968), per se, gives a glimpse of how the structure of the RNA might control local translation of the mRNA. The sequences of twenty-six nucleotides of Qβ (which includes the coat protein initiation region (Hindley and Staples, 1969)) and the three initiation regions for the three known proteins in R-17 (Steitz,1969) have been determined. In both systems the sequenced portions of the RNA were shown to contain the sequence ...pGGUUUGAXXXAUG.. (X = A, G, U, or C). From these data they conclude that there is a portion of the RNA that is not translated into

protein between the end of one cistron and the start of another (AUG codons) and, in the case of the coat protein initiation region, that the AUG is possibly included in a double stranded hydrogen bonded loop which may be the ribosome binding recognition site. Whether the double stranded H-bonded loops are control features is not known yet. Similarly, Nichols (1970) isolated a fragment of R-17 containing the termination region of the coat protein cistron which included the codons UAA and UAG; this fragment also probably exists as a H-bonded double stranded loop. Loops can also be written for the sequence of 57 nucleotides from the interior of the coat protein coding fragment (Adams, Jeppesen, Sanger, Barrell, 1969). Therefore,there is a strong possibility that these loops are ubiquitous for regulation of translation.

Another important translation mechanism could well be the alteration of ribosomes, especially after infection, so that the ribosomes no longer recognize the host mRNA. Indeed this phenomenon has been recently documented by Hsu and Weiss (1969). A protein factor, when combined with the normal ribosomes, confers specificity for selective T4 mRNA translation; this protein is probably coded for by the T4 DNA.

Degradation

M-RNA degradation is still largely an enigma despite intensive efforts to elucidate the mechanism (cf. Geiduschek and Haselkorn, 1969). Functionally, the direction is 5' to 3' (the same direction as translation and transcription), for example, when operons are re-repressed (Ito and Imamoto, 1968). Using hybridization techniques with deletion mutants after re-repression, Morse, Mosteller, Baker and Yanofsky (1969)recently showed that the direction of the mRNA degradation was 5' to 3' in the tryp operon. They also showed that the degradation of the 5' end had occurred before the completion of the operator distal end 3'. Very recently an enzyme, RNase V, that specifically degrades mRNA in this 5' to 3' direction and is an integral part of the 30S ribosome subunit (Kuwano, Kwan, Apirion, and Schlessinger, 1969) has been reported. Enzymatically, it is still possible that an endolytic hit is first placed followed by the 5' to 3' exonuclease action, or alternatively by the well known polynucleotide phosphorylase or even the potassium activated RNase II (cf. Morse et. al., 1969).

Polarity also results in apparent degradation of mRNA distal to the nonsense codon (for a recent review see Morse and Primakoff, 1970) in the tryp operon system. This

polarity (and degradation) can be relieved by the SuA allele, which might make a defective endonuclease specific for the untranslated mRNA in the mutant SuA cell. This locus is not one of the known nucleases. Just how this mechanism is used in the normal cell is not known at this time.

A possible exception to the normal transcription-translation coupled degradation is perhaps the case of T7 infected E. Coli.. Summers (1969) presented evidence showing discrete peaks of T7 mRNA in an acrylamide gel electrophoresis system. Furthermore, the peaks of RNA appeared to be stable. Thus, it is possible that there are variations of the normal degradation schemes, and that these differences could be control mechanisms used to control translation.

This thesis is divided into three parts. Part I is composed of two papers published in the Journal of Molecular Biology, and deals with methods developed to properly analyze, in general, bacterial messenger RNA. Part II is the result of the application of the methods developed in Part I to the *in vivo* ØX mRNA problem (Sedat and Sinsheimer, Submitted for publication.). Part III is an analysis of a few experiments designed to determine which RF, parental or progeny, is the template for ØX transcription.

Figure Legends

Fig. 1

Patterns of transcription for different bacteriophages adapted with permission from Szybalski (1969) (Pergamon of Canada, LTD.)

(a.) The transcription pattern of T4

The strands of T4 were separated by poly-UG binding to the strands and centrifugation in a CsCl density gradient. The strands were then hybridized with mRNA prepared as a function of time after infection, with mRNA from deletion mutants, and with maturation defective mutants (genes 33 and 55), for example. The r-strand is that strand transcribed to the right while the l-strand is transcribed to the left as viewed. The clear arrows are the "early" mRNA; the dark arrows are the "late" mRNAs.

(b.) The transcription pattern of λ

The strands of the λ DNA were separated by poly-UG as above and hybridized with mRNA from deletion mutants as a function of time after infection and with fractionated left and right halves of λ DNA. The mRNA from the lysogenic state is transcribed from the l-strand on the C_1-red region flanked by the repressor molecules bound to each strand. Early mRNA is transcribed immediately

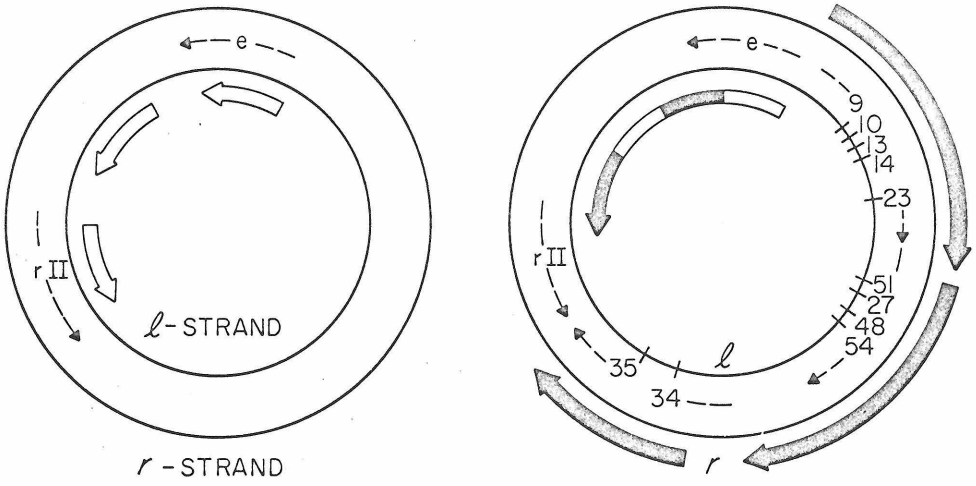
to the right and left of the C_1 region. Late mRNA (with a functional gene Q product) is transcribed off the r-strand. The presumptive poly UG binding sites are the cross lines on the DNA strands.

(c.) The transcription pattern of T7

The separated strands of T7 by the poly UG method are hybridized to mRNA at different times after infection. The early mRNA (from gene 1) starts at the 3' end of the r-strand 0.007 fractional lengths in due to terminal redundancy. Late mRNA is also transcribed from the r-strand.

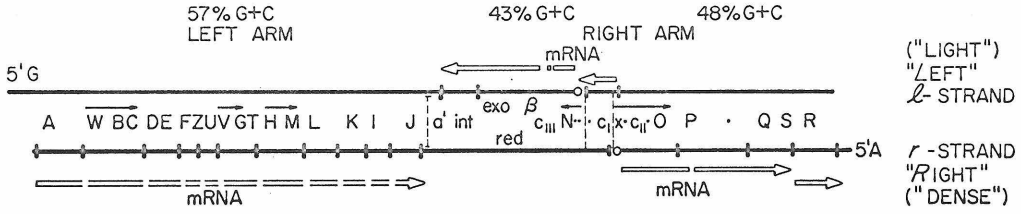
T4

(a)


EARLY (2')
LATE (30')
e
rII
ℓ-STRAND
r-STRAND
9
10
13
14
23
51
27
48
54
35
34
ℓ
r

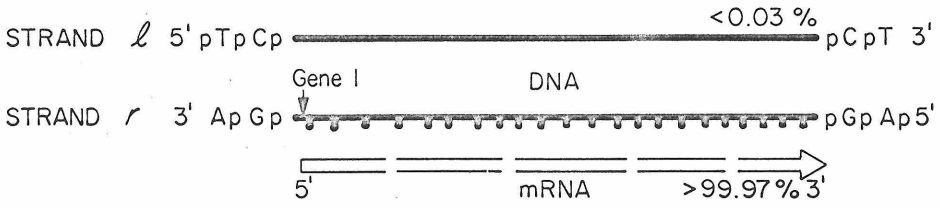
(b)

λ


57% G+C
LEFT ARM
43% G+C
RIGHT ARM
48% G+C
mRNA
5'G
A W BC DE FZUV GT H M L K I J a' int exo β red cIII N cI cx cII O P Q S R
5'A
("LIGHT")
"LEFT"
ℓ- STRAND
r -STRAND
"RIGHT"
("DENSE")
mRNA
mRNA

(c)

T7


STRAND ℓ 5' pTpCp
<0.03 %
pCpT 3'
Gene I
DNA
STRAND r 3' ApGp
pGpAp5'
5'
mRNA
>99.97 %3'

REFERENCES

Adams, J.M., Jeppesen, J.G.N., Sanger, F., and Barrell, B. G. (1969) Nature 223 1009.

Adesnik, M., and Levinthal, C. (1970) J. Mol. Biol. 48 187.

Adler, H.I., Fisher, W.D., Cohen, A., and Hardigree, A.A. (1967) Proc. Natl. Acad. Sci. Wash. 52 321.

Bagdasarian, M., Cieśla, Z. and Sendecki, W. (1970) J. Mol. Biol. 48 53.

Bautz, E.K.F., Bautz, F.D. and Dunn, J.J. (1969) Nature 223 1022.

Bolle, A., Epstein, R.H., Salser, W., and Geiduschek, E.P. (1968) J. Mol. Biol. 33 329.

Borst, P. and Kroon, A.M. (1969) Int'l Rev. of Cytology 26 107.

Britten, R.J. and Davidson, E.H. (1969) Science 159 349.

Chambers, D.A. and Zubay, G. (1969) Proc. Natl. Acad. Sci. Wash. 63 118.

deCrombrugghe, B., Varmus, H.E., Perlman, R.L. and Pastan, I.H. (1970) Biochem. Biophys. Res. Comm. 38 894.

Darnell, J.E., Jr. (1968) Bacteriological Review 32 262.

DiMauro, E., Snyder, L., Marino,P., Lambert, A., Coppo, A. and Tocchini-Valentini, G.P. (1969) Nature 222 533.

Dove, W.F. (1968) Ann. Rev. Genetics 2 305.

Englesberg, E., Sheppard, D., Squires, C., Meronk,F. Jr. (1969) J. Mol. Biol. 43 281.

Gall, J.E. (1970) Nature 225 414.

Geiduschek, E.P. and Haselkorn, R. (1969) Ann. Rev. Biochem. 38 647.

Geiduschek, E.P. and Sklar, J. (1969) Nature 221 833.

Gilbert, W. and Müller-Hill, B. (1968) Proc. Natl. Acad. Sci. Wash. 58 2415.

Haselkorn, R., Vogel, M., and Brown, R.D. (1969) Nature 221 836.

Hennig, W. and Walker, P.M.B. (1970) Nature 225 915.

Hindley, J. and Staples, D.H. (1969) Nature 224 964.

Hirota, Y., Jacob, F., Ryter, A., Buttin, A. and Nakai, T. (1968) J. Mol. Biol. 35 175.

Hsu, W.-T. and Weiss, S. (1969) Proc. Natl. Acad. Sci. Wash. 64 345.

Igarashi, K. and Yura, T. (1969) Biochem. Biophys. Res. Comm. 34 65.

Imamoto, F. (1968) Proc. Natl. Acad. Sci. Wash. 60 305.

Imamoto, F. and Yanofsky, C. (1967) J. Mol. Biol. 28 1.

Ito, T. and Imamoto, F. (1968) Nature 220 441.

Jones, K.W. (1970) Nature 225 952.

Kasai, T. and Bautz, E.K.F. (1969) J. Mol. Biol. 41 401.

Kiho, Y. and Rich, A. (1965) Proc. Natl. Acad. Sci. Wash. 54 1751.

Kourilsky, P., Marcaud, C., Sheldrick, P., Luzzati, P. and Gros, F. (1968) Proc. Natl. Acad. Sci. Wash. 61 1013.

Kuwano, Kwan, Apirion, and Schlessinger, (1969) Proc. Natl. Acad. Sci. Wash. 64 693.

Losick, R. and Sonenshein, A.L. (1969) Nature 224 35.

Lyon, A. (1969) Thesis Calif. Inst. of Technology, Pasadena, California.

Morse, D.E. and Yanofsky, C. (1968) J. Mol. Biol. 38 447.

Morse, D.E. and Yanofsky, C. (1969) J. Mol. Biol. 41 317.

Morse, D.E., Baker, R.F., and Yanofsky, C. (1968) Proc. Natl. Acad. Sci. Wash. 60 1428.

Morse, D.E., Mosteller, R., Baker, R.F. and Yanofsky, C. (1969) Nature 223 40.

Morse, D.E. and Primakoff, P. (1970) Nature 226 28.

Müller-Hill, B., Grapo, L. and Gilbert, W. (1968) Proc. Natl. Acad. Sci. Wash. 59 1259.

Nichols, J.L. (1970) Nature 225 147.

Pearlman, R.L. and Pastan, I. (1968) J. Biol. Chem. 243 5420.

Ptashne, M. (1967) Nature 214 232.

Ptashne, M. and Hopkins, N. (1968) Proc. Natl. Acad. Sci. Wash. 60 1282.

Reznikoff, W.S., Miller, T.H., Scaife, T.G. and Beckwith, J.R. (1969) J. Mol. Biol. 43 201.

Roberts, J.W. (1969) Nature 224 1168.

Rouvière, J., Lederberg, S., Granboulan, P. and Gros, F. (1969) J. Mol. Biol. 46 413.

Schmidt, D.A., Mazaitis, A.J., Kasai, T., and Bautz, E.K.F. (1970) Nature 225 1012.

Sedat, J.W., Kelly, R.B. and Sinsheimer (1967) 26, 537.

Steitz, T.A. (1969) Nature 224 957.

Studier, F.W. (1969) Virology 39 562.

Summers, W.C. (1969) Virology 39 175.

Summers, W.C. and Siegel, R.B. (1969) Nature 223 1111.

Szybalski, W. (1969) Can. Cancer. Conf. 8 183.

Summers,W.C.,&Szybalski,W.(1968) Virology 34,9.

Tremblay, G.Y., Daniels, M.T., and Schaechter, M. (1969) J. Mol. Biol. 40 65.

Travers, A.A. (1969) Nature 223 1107.

Travers, A.A. (1970) Nature 225 1089.

Venetianer, P. Berberich, M.A., and Goldberger, R.F. (1968) Biochim Biophys Acta 166 124.

Weinberg, R.A. and Penman, S. (1969) J. Mol. Biol. 47 169.

Zipser, D. (1969) Nature 221 21.

Part I

Methods For the Analysis of Bacterial Messenger RNA

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LETTERS TO THE EDITOR

Fractionation of Nucleic Acid on Benzoylated-Naphthoylated DEAE Cellulose

Nucleic acids have been fractionated by column chromatography using methylated albumin kieselguhr (Mandell & Hershey, 1960; Sueoka & Cheng, 1962) cellulose CF-11 (Franklin, 1966), agarose (Erikson & Gordon, 1966) and hydroxyapatite (Muench & Berg, 1966). In this communication we describe the fractionation of nucleic acids on columns of benzoylated-naphthoylated DEAE cellulose (Tener, Gilliam, von Tigerstromm, Millward & Wimmer, 1966) (BNC columns) and show that this fractionation is based on the secondary structure of the nucleic acids.

The benzoylated-naphthoylated DEAE cellulose was made essentially according to the procedure of Tener (1966; also personal communication), except that the resin was also washed with anhydrous ether to remove ultraviolet-absorbing material.

The nucleic acids were extracted, using phenol, from sodium dodecylsulfate-lysed *E. coli* C3000 after infection for 20 minutes with MS2 bacteriophage. ^{32}P-labeled λ DNA and ^{3}H-labeled double-stranded RNA (previously isolated from MS2-infected cells and treated with RNase) (Kelly & Sinsheimer, 1964), were added to the infected cell extract as markers before chromatography on the column. Fractions were collected and assayed for ultraviolet absorbance, ^{32}P- and ^{3}H-radioactivity, and infectivity in the *E. coli* spheroplast assay (Strauss, 1964). From Fig. 1 it can be seen that double-stranded DNA and double-stranded RNA are eluted at nearly the same salt concentration. MS2 RNA infectious material, co-incident with a small peak of

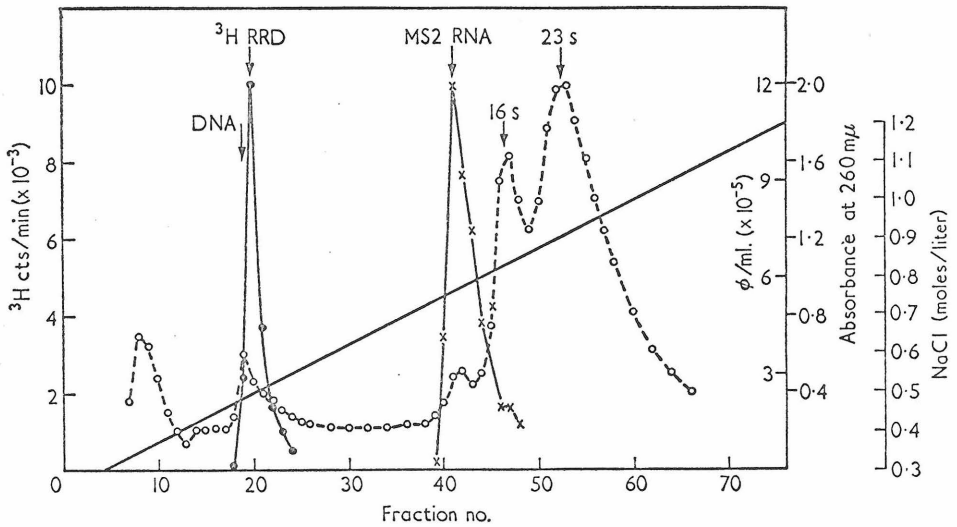

FIG. 1. Chromatography on BNC of the nucleic acids from cells infected with MS2 for 20 min. ^{32}P-labeled DNA from λ phage and ^{3}H-labeled double-stranded RNA from MS2 infected cells (RRD) were added as markers. Fractions were assayed for absorbance at 260 mμ (–○–○–), ^{3}H-radioactivity (—◎——◎—) and RNA infectivity in the spheroplast assay (——×——×——). The position at which the λ DNA is eluted is indicated by an arrow.

ultraviolet absorbance, is eluted before 16 s ribosomal RNA, and the latter before 23 s. The ribosomal RNA's were identified by sucrose density-gradient sedimentation.

The following experiment was performed to obtain a further understanding of the basis of separation. 400 ml. of TPA media (Kelly, Gould & Sinsheimer, 1965) was inoculated with *E. coli* MRE 601 (RNase I$^-$, Heisenberg, manuscript in the press) and grown to 1×10^8 cells/ml., at which time 10 μc [^{3}H]uracil/ml. (2 μg/ml.) were added. When the cell titer increased to 4×10^8/ml., 4 mc ^{32}P ($^{32}PO_4$ carrier-free in neutralized water) were added. After 45 seconds (0·034 generation), sodium azide was added to 0·01 M, and the cells immediately poured over crushed ice and centrifuged. The pellet was suspended in buffer and lysed with lysozyme and sodium dodecylsulfate. The nucleic acids were twice phenol-extracted and precipitated with ethanol.

Figure 2 shows a chromatogram of a portion of this nucleic acid extract. A gradient of dimethylsulfoxide was superimposed on the NaCl elution gradient. The separation is basically similar to that described in Fig. 1. However, there is considerable sharpening

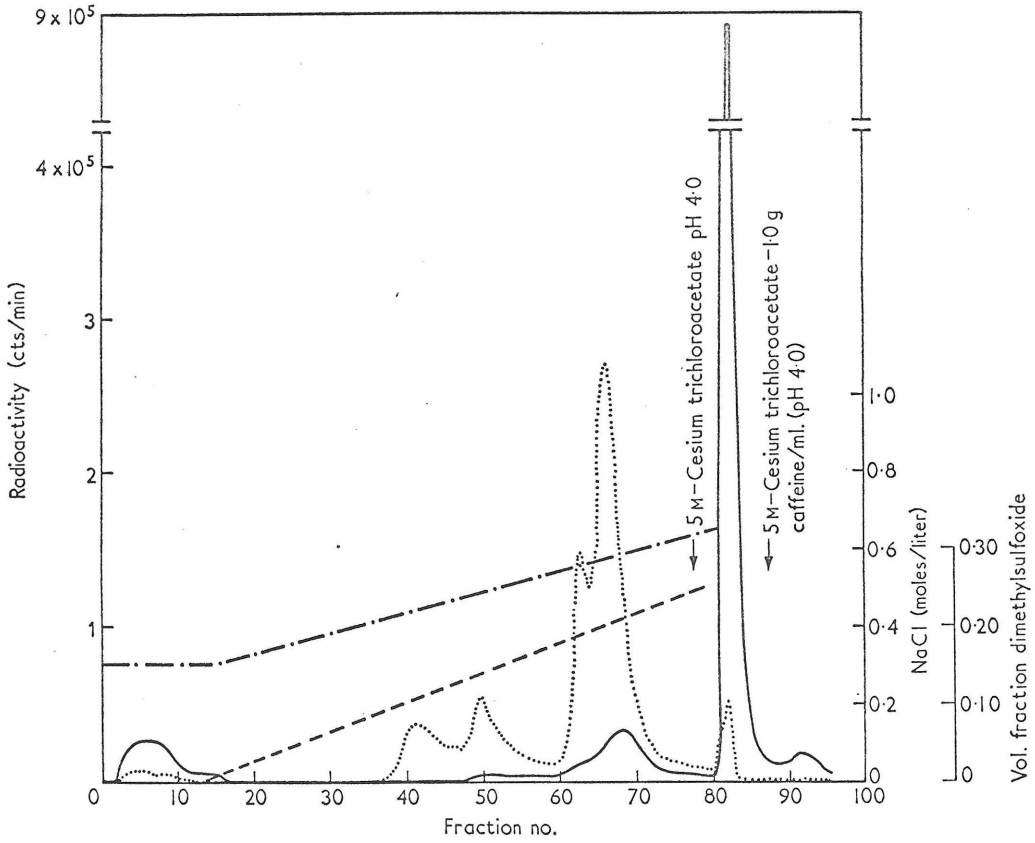

FIG. 2. Fractionation of a ^{32}P pulse-labeled nucleic acid extract on a BNC column. 180 A_{260} units containing $1\cdot0\times10^7$ [^{3}H]uracil counts and $7\cdot2\times10^7$ ^{32}P-counts in 30 ml. of 0·3 M-NaCl, 0·02 M-Tris (pH 7·2) were applied to a 1·5 cm × 3 cm BNC column previously washed with 0·3 M-NaCl (pH 7·2). The column was then eluted with a linear gradient in NaCl, 0·3 M to 0·65 M, concurrent with a gradient in dimethylsulfoxide, 0 to 25 vol. %. All solutions contained 0·02 M-Tris chloride (pH 7·2). Total volume, 150 ml.; flow rate, 0·5 ml./min. Fractions were assayed for ^{3}H radioactivity (.....), ^{32}P radioactivity (———) after correcting for quench by external standardization, and A_{260} (in a Gilford flow monitor).

of the peaks as well as compression of the elution pattern with the addition of the 0 to 0·25 volume fraction gradient of dimethylsulfoxide. The ^{3}H counts (which are coincident with the absorbance pattern) are distributed in the transfer RNA (the second peak), DNA (the third peak), and in the 16 and 23 s ribosomal RNA; the ^{32}P counts from the 45-sec pulse are largely retained on the column. These can be eluted with solvents such as cesium trichloroacetate (pH 4·0). The ratio $^{3}H/^{32}P$ counts in the ribosomal RNA peaks is approximately 10; in the latter peak it is only 0·05. This is consistent with the known properties of messenger RNA (Brenner, Jacob & Meselson, 1961) and suggests that the ^{32}P peak and the further radioactive material eluted with cesium trichloroacetate plus caffeine, contain the bacterial messengers. An additional 1% of the total radioactive material (mostly ^{32}P) applied on the column remains bound even after elution with 5 M-cesium trichloroacetate + 1 g caffeine/ml. (pH 4·0). (Cesium trichloroacetate is used here in analogy with its use as a buoyant denaturing solvent for DNA, D. Clayton & J. Vinograd, personal communication.)

Portions of the two ribosomal RNA peaks and of the pulse-labeled peaks were re-chromatographed under identical conditions as in Fig. 2. The ribosomal RNA absorbance pattern showed only two sharp peaks eluting at the same salt concentrations as before; more than 98% of the total re-run ^{3}H-labeled material eluted without the use of cesium trichloroacetate. In a similar experiment with dialyzed, cesium trichloroacetate-eluted ^{32}P-pulse-labeled material, a small leading peak eluting at the position of 23 s ribosomal RNA was observed; but it represented less than 2% of total ribosomal RNA eluted from the column of Fig. 2.

The eluted ^{32}P-labeled material showed a broad range of sedimentation values in a 5 to 20% sucrose gradient, with a mean of 20 s.

The effects of size and configuration on elution position have been studied with various nucleic acids. $(Ap)_6A$ (Miles Chemical Co. no. 7A31) previously checked for homogeneity by sedimentation equilibrium, was absorbed in 0·1 M-NaCl onto a 0·5 cm × 3 cm BNC column and eluted with a 0·1 to 1·0 M-NaCl (pH 7·2) gradient. The absorbancy pattern at 260 mμ showed a single symmetrical peak eluting at 0·48 M, indicating a rather strong binding for even short single-stranded oligonucleotides. We were not able to elute (at 1·2 M-NaCl, pH 7·2) poly A or poly U, suggesting a very strong binding of both stacked and unstacked regular polynucleotides. Likewise, ϕX174 DNA, a covalent circular single-stranded ring (Fiers & Sinsheimer, 1962) is quantitatively retained even at 4 M-NaCl; it can be eluted at approximately 0·25 M-NaCl with the aid of 20 mg caffeine/ml. (pH 7·4). In contrast, ϕX RF (Burton & Sinsheimer, 1965) is eluted just after the cellular DNA in the normal 0·3 to 1·0 M-NaCl, (pH 7·2) gradient (Komano, manuscript in preparation).

The capacity of this resin seems to be large; a 5 cm × 5 cm column will retain at least 4×10^{3} A_{260} units of nucleic acids from whole-cell extract.

It appears that the fractionation of nucleic acids by this column is related to the proportion of single-stranded regions in each nucleic acid. The role of the aromatic rings of the column may be analogous to the intercalation of purine into model oligonucleotides (Chan, Bangerter & Peter, 1966). With nucleic acids that are mostly double-stranded, there is little fractionation. T-RNA ($\overline{M}_{W} = 26{,}600$) is poorly separated from λ DNA ($\overline{M}_{W} = 3\times10^{7}$), or from the double-stranded ribonuclease-resistant duplex ($\overline{M}_{W} \simeq 2\times10^{6}$). Polynucleotides that are thought to be

combinations of single- and double-stranded configurations, such as ribosomal and MS2 RNA, are actually eluted in reversed order of molecular weight; MS2 ($\overline{M}_w = 1{\cdot}1 \times 10^6$) elutes ahead of 16 s ribosomal RNA ($\overline{M}_w = 0{\cdot}5 \times 10^6$). Probably the secondary structure of MS2 is greater than that of ribosomal RNA (Strauss, unpublished observations). Rabbit reticulocyte ribosomal RNA is not eluted from the column under our salt conditions, although it is only slightly larger than the *E. coli* ribosomal RNA (A. Lyon, personal communication). Less structured single-stranded nucleic acids such as ϕX DNA and pulse-labeled RNA are strongly retained and can only be removed by strong solvents with a preference for solvation of unpaired bases (Hamaguchi & Geiduschek, 1962; Robinson & Grant, 1966).

There is a noticeable effect of ionic strength, such that the elution pattern of polynucleotides with partial secondary structure is affected by the ionic strength in the sample when applied to the column. As an example, MS2 RNA, when applied in 0·1 M-NaCl, is only half as well recovered from the column as when the RNA is applied in 0·3 M-NaCl. In the former case, the elution pattern then shows considerable subfractionation, with shoulders on the main peak. Presumably the binding of the RNA to the column is determined by its initial configuration state(s). An analogous observation has been made with respect to purine binding in the model intercalation systems (Chan & Nelson, manuscript in preparation).

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REFERENCES

Brenner, S., Jacob, F. & Meselson, M. (1961). *Nature*, **190**, 576.
Burton, A. & Sinsheimer, R. L. (1965). *J. Mol. Biol.* **14**, 327.
Chan, S. I., Bangerter, B. W. & Peter, H. H. (1966). *Proc. Nat. Acad. Sci., Wash.* **55**, 720.
Erikson, R. L. & Gordon, J. A. (1966). *Biochem. Biophys. Res. Comm.* **23**, 422.
Fiers, W. & Sinsheimer, R. L. (1962). *J. Mol. Biol.* **5**, 424.
Franklin, R. M. (1966). *Proc. Nat. Acad. Sci., Wash* **55**, 1504.
Hamaguchi, K. & Geiduschek, G. P. (1962). *J. Amer. Chem. Soc.* **84**, 1329.
Kelly, R. B., Gould, J. L. & Sinsheimer, R. L. (1965). *J. Mol. Biol.* **11**, 562.
Kelly, R. B. & Sinsheimer, R. L. (1964). *J. Mol. Biol.* **8**, 602.
Mandell, J. D. & Hershey, A. D. (1960). *Analyt. Biochem.* **1**, 66.
Muench, K. H. & Berg, P. (1966). *Biochemistry*, **5**, 982.
Robinson, D. R. & Grant, M. E. (1966). *J. Biol. Chem.* **241**, 4030.
Sueoka, N. & Cheng, T. Y. (1962). *J. Mol. Biol.* **4**, 161.
Strauss, J. H. (1964). *J. Mol. Biol.* **10**, 422.
Tener, G. M., Gilliam, I., von Tigerstromm, M., Millward, S. & Wimmer, E. (1966). *Fed. Proc.* **25**, 519 (abs. 1854).

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Purification of *Escherichia coli* Pulse-labeled RNA by Benzoylated DEAE-cellulose Chromatography

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The rapidly-labeled RNA fraction of *Escherichia coli* has been purified approximately 15-fold on benzoylated DEAE-cellulose columns. It is metabolically unstable (as shown by a pulse/chase experiment), and is considered to represent messenger RNA. The yield of pulse-labeled RNA is about 70% and comprises 4 to 5% of the RNA of the cell.

The true size distribution of this RNA, determined by sedimentation in a denaturing solvent (99% dimethyl sulfoxide), does not change during purification. This result indicates that neither degradation nor selection for molecules of a particular size has occurred. Upon sedimentation of the final preparation in dimethyl sulfoxide, the distribution of pulse label is the same as that of RNA mass, indicating nearly complete separation from longer-lived RNA components.

1. Introduction

Since the existence of messenger RNA has been established there have been numerous attempts to purify this RNA (for example, by sucrose gradient centrifugation (Attardi, Naono, Rouvière, Jacob & Gros, 1963), methylated albumin kieselguhr column chromatography (Asano, 1965), preparative hybridization (Bautz & Reilly, 1966) and counter-current distribution (Kidson & Kirby, 1964)). However, numerous difficulties have been encountered in attempts to devise a purification scheme that will not be selective in view of the expected heterogeneity of size distribution, base composition and base sequence, and the small fraction of total RNA ($\approx$ 4%) concerned. Other difficulties, such as the lack of any simple relation between sedimentation coefficient and molecular weight of RNA (Boedtker, 1968) and the aggregation of mRNA with rRNA (Asano, 1965; Hayes, Hayes & Guerin, 1966), have complicated the use of sucrose gradients; the problems of hybridization kinetics (Britten & Kohne, 1968) and the difficulty of specific DNA preparation (so that tRNA and rRNA genes are absent) have limited the scope of DNA–RNA hybridization. None of these procedures for the purification of mRNA seems to be generally applicable.

We have approached the problem of mRNA purification by the use of benzoylated (naphthoylated) DEAE-cellulose chromatography (Gillam *et al.*, 1967; Sedat, Kelly & Sinsheimer, 1967). This resin is characterized by high capacity, high recovery, and yields sharp, well-resolved peaks. Resolution on this resin seems to depend upon (at least at pH 7·5) preferential interaction of the single-stranded regions of the nucleic acids with the aromatic rings bound to the cellulose. Chromatography on

BNC† has proved useful in the study of replicating MS2 RNA intermediates (Kelly & Sinsheimer, 1967), the purification and study of intracellular λ DNA (Kiger & Sinsheimer, 1969), and of φX174 intracellular replicating DNA (both single- and double-stranded) (Sinsheimer, Knippers & Komano, 1968; Knippers, Razin, Davis & Sinsheimer, manuscript in preparation). In principle, a structural difference in mRNA might permit it to be separated from the other RNA's of the cell.

We have made use of the pulse-label technique to label selectively the rapidly synthesized mRNA (Gros *et al.*, 1961), with the understanding that this fraction could include some intermediates in rRNA synthesis (Mangiarotti, Apirion, Schlessinger & Silengo, 1968) and will bias the results against any messengers which are not synthesized so rapidly. In most experiments we have made use of a double label technique: $^{32}PO_4^{3-}$ is used as a short pulse-label, and [5-^{3}H]uracil is used to label the culture for a few generations prior to the pulse. The ^{3}H counts are thus a direct measure of the RNA mass (as is also A_{260}), weighted of course by any differences in pyrimidine content per molecule.

We have made use of sedimentation in 99% DMSO (in which RNA is completely denatured at 25°C (Strauss, Kelly & Sinsheimer, 1968)) to attempt to ascertain at each step of the purification whether any degradation or selection has occurred. This procedure is also useful to determine the size distribution of the pulse and mass labels. The distribution of the pulse-label after a chase was studied to determine the metabolic fate of the RNA fraction.

Purified mRNA should be very useful in the study of gene expression, metabolic control, and protein synthesis. There is as yet little information concerning the structure of mRNA or detailed study of its function in *in vitro* systems.

2. Materials and Methods

(a) *Materials*

(i) BNC and BC were prepared essentially according to Gillam *et al.* (1967). However, 5 lb. of anhydrous ether/50 g resin was used to wash the resin further after the benzoylation procedure. The resin was sized through a U.S. sieve no. 80 and then the fines were decanted. Since slight variations in the elution patterns appeared when using various early batches of resin, later batches were stored, moist, at —20°C or suspended in water at 4°C.

(ii) Reagent grade urea (Baker & Adamson or Mallinckrodt) was found to degrade high molecular weight RNA in solution. Figure 1(a) shows an analytical ultracentrifuge pattern of a band sedimentation analysis in 90% deuterium oxide (*vide infra*) of *E. coli* rRNA (previously shown to consist only of 23 S and 16 S components in an estimated mass ratio of 2:1) which had been treated for 10 min at 20°C with 8 M-urea in 0·1 M-CH_3COOH, pH 3·5. Several degradative hits/molecule (1×10^6 avograms) occurred in 10 min. Attempts to purify the urea by passage through an IRC50, Chelex 100 column at pH 5·0 (to remove RNase I and heavy metal ions) were unsuccessful as judged by the above assay method. It was found, however, that Mann high purity urea (Mann Research Laboratories, 136 Liberty St., New York) does not degrade rRNA, as is shown in Fig. 1(b). Ribosomal RNA, treated for 10 hr at 20°C with an 8 M-solution of Mann urea (passed through a 50 mμ Millipore filter to remove particulate impurities) in 0·1 M-CH_3COOH, pH 3·5 (pH adjusted with HCl), and then subjected to analytical band velocity sedimentation through 99% DMSO (*vide infra*), showed, it was estimated, less degradation than 0·1 hit/molecule. Each lot of urea was subjected to the above test before use.

(iii) Earlier lots of DMSO were obtained from J. T. Baker Chemical Co.; later lots were

† Abbreviations used: BNC, benzoylated (naphthoylated) DEAE-cellulose; BC, benzoylated DEAE-cellulose; DMSO, dimethyl sulfoxide.

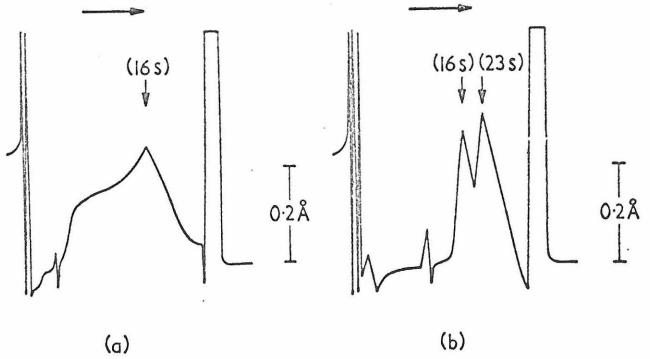

FIG. 1. Analytical velocity sedimentation of rRNA, treated either (a) with 8 M-urea (Baker & Adamson) or (b) 8 M-urea (Mann, passed through VM Millipore filter). Samples of the rRNA peak eluted from a BNC column at pH 7·5, and previously shown to be undegraded by velocity sedimentation in DMSO, were treated (a) with 8 M-urea (Baker & Adamson) in 0·1 M-Tris–Cl, pH 8, for 10 min at 25°C and then sedimented into deuterium oxide, or (b) were treated with 8 M-urea (Mann, passed through VM Millipore filter) in 0·1 M-acetate, pH 3·5, for 10 hr at 25°C and then sedimented in 99% DMSO. Sedimentation is from left to right.

Spectroquality Reagent from Matheson Coleman & Bell, Cincinnati, Ohio. d_6-DMSO (containing 38,000 ^{3}H cts/min/ml.) was obtained from BioRad, Richmond, California.

(iv) RNase-free sucrose was obtained from Schwarz BioResearch, Orangeburg, N.Y.

(v) *E. coli* MRE 601 (RNase I$^-$) was obtained from Dr M. Heisenberg. *E. coli* THU, HF4704, and TPA and TPG media are described in Lindqvist & Sinsheimer (1967). *φXam3* is described in Hutchison & Sinsheimer (1966).

(vi) $^{32}PO_4^{3-}$, carrier-free in water, was obtained from Nuclear Consultants, Glendale, California. [5-^{3}H]uracil (20 to 27 c/m-mole) or [6-^{3}H]uracil (15 to 17 c/m-mole) and [2-^{14}C] (56 mc/m-mole) were obtained from Schwarz BioResearch, Orangeburg, N.Y.

(b) *Methods*

(i) *Preparation of $^{32}PO_4$ pulse–^{3}H uniformly-labeled RNA extracts*

1 liter of TPA medium was inoculated with an overnight culture of *E. coli* MRE 601 and grown to 1×10^8 cells/ml., at which time 2 mc of [5-^{3}H]uracil (2 μg uracil/ml.) were added. After the culture had reached 4 to 6×10^8 cells/ml., 10 mc of neutralized $^{32}PO_4$ were added; 45 sec later (0·035 generation), NaN_3 was added to a concentration of 0·01 M. The cells were then centrifuged, and quickly resuspended in 10 ml. of 0·02 M-Tris-Cl, pH 7·5. One ml. of 1 mg/ml. lysozyme in 0·25 M-Tris–Cl, pH 8·1, plus 1 ml. of 7·5 μg/ml. EDTA were rapidly added and the mixture warmed to room temperature for 3 min, after which sodium dodecyl sulfate was added to 0·5% (final concentration) to lyse the cells. One volume of redistilled phenol, saturated with 0·02 M-Tris–Cl, pH 7·5, was added and the mixture placed on a Vortex mixer for a total mixing time of 3 min (1 min mixing, then 2 to 3 min in an ice bath). After centrifugation, the aqueous layer was removed and re-extracted with 1 vol. of fresh phenol. The phenol layers were re-extracted with 0·5 vol. of 0·01 M-Tris–Cl, pH 7·5; the combined aqueous layers were extracted with cold anhydrous ether 3 times; 0·1 vol. of 3 M-sodium acetate, pH 5·0, plus 2 vol. of 95% ethanol were added to precipitate the nucleic acids. After approximately 10 hr at —20°C, the precipitate was centrifuged, dissolved in 5 ml. of 0·02 M-Tris–Cl, pH 7·5, and stored at —20°C.

(ii) *Preparation of [^{3}H]uracil pulse–[^{14}C]uracil uniformly-labeled, chased RNA extracts*

One liter of TPG+4 μg uracil/ml. + 5 μg thymidine/ml. + 20 μg histidine/ml. was inoculated with *E. coli* THU, and the cells were grown at 37°C to 1×10^8 cells/ml., after which 200 μc of [2-^{14}C]uracil were added. After the culture had reached 3 to 4×10^8 cells/ml., 2 mc of [5-^{3}H]uracil were added for 45 sec; half of the culture was immediately

treated with NaN_3 (0·01 M) and the nucleic acids extracted as in (i). To the other half of the culture was added sterile, unlabeled cytidine plus uridine to 10 mg/ml. each. The cells were then filtered on a large, cold, 142 mm, 0·45 μ HA Millipore filter, and washed with 0·5 l. of ice-cold TPG+10 mg/ml. each cytidine and uridine. Finally the filtered, washed cells were resuspended in 500 ml. of warm TPG + 10 mg/ml. each cytidine and uridine. The culture was grown for one generation (69 min at 37°C) and then the nucleic acids extracted as in (i).

(iii) *Preparation of RNA extracts from ϕXam3-infected cells, pulse-labeled with [^3H]uracil, and from uninfected cells pulse-labeled with $^{32}PO_4$.*

This culture of *E. coli* HF4704 was prepared essentially as in (i). One-half of the culture was infected at 5×10^8 cells/ml. with *ϕXam3* (multiplicity of 5). 12 to 15 min after infection, [6-^{3}H]uracil (2 μg uracil/ml.) was added for 45 sec, after which the cells were treated with NaN_3. The other half of the culture (uninfected) was pulse labeled with 5 mc of $^{32}PO_4$ for 45 sec, and then treated with NaN_3. The centrifuged cells from the two halves of the culture were combined and the nucleic acids extracted as in (i).

(iv) *Fractionation on benzoylated DEAE-cellulose column at pH 7·5*

Approximately half of the nucleic acid from (i) was diluted with 10 vol. of 0·3 M-NaCl, 0·02 M-Tris–Cl (pH 7·5) 0·001 M-EDTA and applied by gravity to a 2·5 cm × 5 cm column of BC, previously washed with 0·002 M-Tris–Cl plus 0·3 M-NaCl buffer. In some cases the extreme viscosity of the sample required stirring up the resin in order to increase the flow rate. The column was then washed with 0·3 M-NaCl buffer until the unadsorbed ^{32}P counts (see Results) were washed out. 200 ml. of a linear gradient of NaCl from 0·3 to 0·7 M and of DMSO from 0 to 30% (v/v) (all solutions contained 0·02 M-Tris–Cl, pH 7·5, plus 0·001 M-EDTA) was applied at a flow rate of approximately 1 ml./min. Absorbance was monitored through a Gilford Absorbance Monitor, and 3-ml. fractions were collected. At the end of the gradient the column was washed with 8 M-urea (see Materials and Methods section (a) (ii)) in 0·1 M-CH_3COOH, pH 3·5, until the pH reached 3·5 and the ^{32}P counts thus eluted were washed out (see Results). 100 ml. of a subsequent linear gradient from 0 to 1·0 M-NH_4Cl in 8 M-urea plus 0·1 M-CH_3COOH, pH 3·5, was applied at a 1 ml./min flow rate; at the termination of this gradient a solution containing 1 M-NH_4Cl plus 2·5% sodium dodecyl sulfate plus 8 M-urea, pH 3·5, was passed through the column to remove additional ^{32}P pulse counts. The individual RNA peaks from both gradients were precipitated by adding 0·1 vol. of 3 M-sodium acetate, pH 5·0, and 2 vol. of 95% ethanol, and stored at least 10 hr at —20°C. After centrifugation the pellets were dissolved in 0·02 M-Tris–Cl, pH 7·5.

(v) *Fractionation on benzoylated DEAE-cellulose columns at pH 3·5*

In order to remove rRNA from the RNA peaks eluted in the prior fractionation at pH 7·5, an additional fractionation was performed entirely at pH 3·5. The RNA from the pH 7·5 fractionation was diluted 10 times with 8 M-urea in 0·1 M-CH_3COOH, pH 3·5, and applied by gravity onto a 1·5 cm × 4 cm BC column, previously washed with the 8 M-urea buffer until A_{260} was low and constant. Additional 8 M-urea buffer was used to wash in the sample; then 100 ml. of a linear gradient of NH_4Cl from 0 to 1 M in 8 M-urea buffer was applied at a flow rate of 1 ml./min. The eluted RNA was precipitated with ethanol as in section (iv).

(vi) *Sedimentation*

Analytical sedimentation in aqueous solvents was performed as described by Strauss & Sinsheimer (1968). Analytical sedimentation through 99% DMSO was also described by Strauss *et al.* (1968), except that when the solution layered onto DMSO had an ionic strength greater than 0·1, a lower speed was used during the establishment of the diffusion gradient. Preparative sedimentation in 99% DMSO was performed as described by Strauss *et al.* (1968), with slight modifications. 5-ml. linear gradients were established between 99% d_6-DMSO (containing 10% sucrose and 0·001 M-EDTA, pH 7·0), and a mixture of 0·1 vol. fraction 99% d_6-DMSO with 0·90 vol. fraction 99% DMSO (containing 0·001 M-EDTA, pH 7·0). Onto this was layered 5 μl. RNA + 90 μl. 99% DMSO + 5 μl. di-

methylformamide. Centrifugation was performed at 64,000 rev./min in a Spinco L2-65B ultracentrifuge for 11 hr at 27°C. Fractions were collected and precipitated with trichloroacetic acid for counting.

(vii) *Miscellaneous*

Hybridization of RNA (from ϕX-infected cells) with ϕX RF DNA was done essentially by the procedure of Gillespie & Spiegelman (1965) in a volume of 0·3 ml. with a 13 mm filter containing 2 to 3 μg of ϕX RF (prepared by the procedure of Komano & Sinsheimer, 1968) at 66°C for 24 hr.

For radioactivity counting, samples were usually dried onto glass filters and counted in a toluene-based scintillation fluid (Liquifluor, Nuclear Chicago, Inc.) in a Beckman LS200 scintillation counter. Readings were subsequently corrected for background and overlap. Some samples, especially those from the sucrose gradients, were counted directly in Bray's scintillation fluid (Bray, 1960).

Several precautions were taken to minimize contamination with RNase. All glassware was heat-sterilized and plastic centrifuge bottles were only used once. Pipettes were disposable (Falcon Plastics) as were the fraction collector tubes. Concentrated buffers were brought to a specified pH with an RNase-free combination electrode, stored cold, and diluted just before use. Great care to use plastic disposable gloves whenever near the RNA or during the procedures was essential.

3. Results

(a) *Chromatography of ^{32}P pulse–^{3}H uniformly-labeled nucleic acid extract on benzoylated DEAE-cellulose*

The chromatography of ^{32}P pulse–[^{3}H]uracil uniformly-labeled nucleic acid extract from RNase I$^-$ *E. coli* 601 on BC at pH 7·5 is shown in Figure 2. A large fraction of the ^{32}P counts applied did not absorb on the BC column; these have been shown to be soluble in trichloroacetic acid and ethanol. The linear gradient (0·3 to 0·7 M-NaCl, 0 to 30% DMSO in 0·02 M-Tris–Cl, pH 7·5) eluted two major ^{3}H-labeled RNA peaks. The first, small one consisted of tRNA (Gillam *et al.*, 1967) and DNA (Sedat *et al.*, 1967). The second ^{3}H peak was largely undegraded rRNA, as determined by analytical band velocity sedimentation. The ^{32}P counts eluted in this gradient were found in the trailing edge of the large rRNA peak.

The additional gradient of 0 to 1 M-NH_4Cl in 8 M-urea plus 0·1 M-CH_3COOH, pH 3·5 (see Materials and Methods section (a) (ii)) eluted a small amount of ^{3}H (mass) label but a larger amount of the ^{32}P label, equalling in some cases the amount of ^{32}P eluted in the first gradient. Analytical band velocity sedimentation in deuterium oxide and DMSO showed that the large rRNA peak from the first gradient and the small peak eluted in the second gradient were undegraded rRNA with the 23 S and 16 S components in a mass ratio of 2:1. We have labeled these fractions $rRNA_I$ and $rRNA_{II}$, respectively.

The additional ^{32}P radioactivity that remained on the column could be removed by the use of 2% sodium dodecyl sulfate in 1 M-NH_4Cl plus 8 M-urea, pH 3·5. This material did not appear to be RNA, although it was precipitated by trichloroacetic acid. It resisted degradation by RNase, DNase, pronase, or alkali (0·3 N). There was no label in this material when the cell culture had been pulse-labeled with [^{3}H]uracil. The nature of this fraction is further complicated by the finding that the ^{32}P counts banded in a CsCl equilibrium gradient at a density of 1·52. These ^{32}P counts may represent an intermediate of lipid or carbohydrate metabolism.

In another experiment in which a pulse of [^{3}H]thymidine replaced the usual $^{32}PO_4$ and [^{3}H]uracil labels, a large peak of ^{3}H counts appeared under the first small

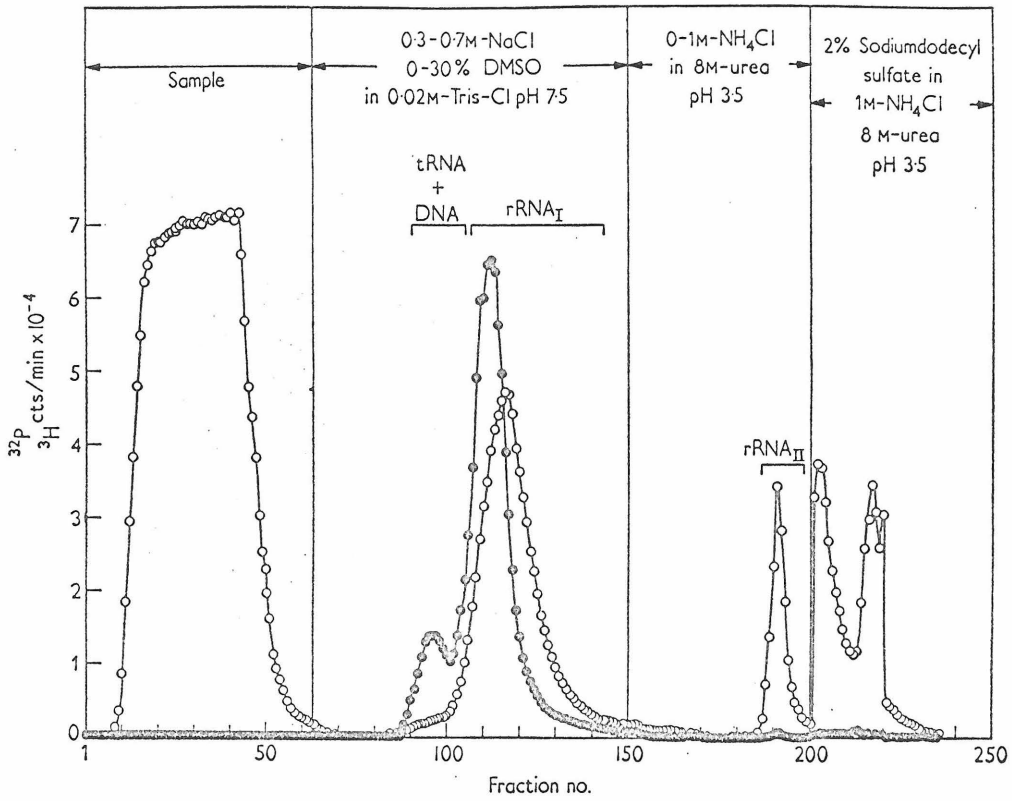

FIG. 2. Fractionation of ^{32}P pulse-labeled–[^{3}H]uracil uniformly-labeled nucleic acid extract on BNC column at pH 7·5. —○——○—, ^{32}P (pulse) cts/min; —●——●—, ^{3}H (mass) cts/min.

peak in Figure 2 in the first gradient; no other ^{3}H counts were eluted until after the peak of $rRNA_{II}$ in the second gradient. ^{3}H label was also eluted when the sodium dodecyl sulfate plus 1 M-NH_4Cl plus 8 M-urea was applied (in this case the ^{3}H counts and A_{260} banded at a density of 1·70). These results are consistent with those obtained by Kiger & Sinsheimer (1969) for λ replicating DNA; Knippers, Whalley & Sinsheimer (manuscript in preparation) for φX RF replicating DNA; and Rohwer (personal communication) for *E. coli* replicating DNA.

Slight variations were occasionally seen in the patterns of Figure 2. Also, early batches of BNC and especially BC showed considerable subfractionation of the tRNA–DNA peak, and the large $rRNA_I$ peak was split into the 16 s and 23 s components (see Fig. 1, Sedat *et al.*, 1967). In addition a large amount of ^{32}P pulse-label (equal in quantity to that eluted with $rRNA_{II}$ in the second gradient), but no ^{3}H (mass) label was eluted from the column when the 8 M-urea, 0·1 M-CH_3COOH, pH 3·5, was used to wash the column and lower the pH. These counts did not precipitate with trichloroacetic acid or ethanol, as in the case of the counts eluted during sample application.

The yield of RNA fractionated on BC at pH 7·5 is summarized in Table 1. A large fraction of the rRNA (83% of the mass) was eluted in the first gradient ($rRNA_I$),

TABLE 1

pH 7·5 Benzoylated (naphthoylated) DEAE-cellulose fractionation

Peak	Fraction of the mass	Fraction of the pulse	Pulse/mass
tRNA +DNA	0·11	0·02	0·20
$rRNA_I$	0·83	0·60	0·85
$rRNA_{II}$	0·03	0·16	4·00
Sodium dodecyl sulfate	0·02	0·23	$\geq$12·50

together with 60% of the pulse (ignoring the unbound ^{32}P counts of the sample eluate). Only 3% of the RNA mass was eluted as $rRNA_{II}$ in the second gradient, together with 16% of the pulse. The fraction of ^{32}P pulse counts eluted with $rRNA_I$ or $rRNA_{II}$ varied; at times the ratio was 1:1. A surprising amount of the $^{32}PO_4$ pulse-label (23%) was eluted with the sodium dodecyl sulfate plus 8 M-urea, although this does not represent nucleic acid. The recovery of the ^3H counts (based on the amount adhering to the column) was approximately 99%, while that of the ^{32}P pulse averaged 90 to 95%.

There are significant ionic effects on the first column fractionation. Substitution of 0·02 M-phosphate, pH 9·6, buffer (see Hayes *et al.*, 1966) for 0·02 M-Tris–Cl, pH 9·6, did not change the elution pattern of the ^3H label but shifted almost all the RNA^{32}P pulse counts to the $rRNA_I$ peak, leaving little if any ^{32}P with $rRNA_{II}$; also, the ^{32}P counts were found coincidentally distributed with the ^3H mass of $rRNA_I$ instead of on the trailing edge.

(b) *Is there any difference between the pulse RNA in $rRNA_I$ and $rRNA_{II}$?*

As is shown in Figure 2, the ^{32}P pulse-label distributed into two fractions both of which contained rRNA. To determine whether this fractionation of the mRNA was meaningful, cells were infected with ϕX174 bacteriophage and the distribution of ϕX mRNA studied by means of hybridization to ϕX RF. A mixed nucleic acid extract from infected cells pulse-labeled with [^3H]uracil and uninfected cells pulse-labeled with $^{32}PO_4$ was chromatographed on BC as in Figure 2, and RNA samples from the $rRNA_I$ and $rRNA_{II}$ components were separately sedimented on sucrose gradients. Each fraction from each gradient was assayed for the ability to hybridize to ϕX RF filters and to ϕX DNA filters. Figure 3 shows the result of this experiment.

The sedimentation distribution of the total pulse-label is the same in $rRNA_I$ and in $rRNA_{II}$ and is the same in infected and uninfected cells. Also, the proportion of the total pulse-label from infected cells which hybridized with ϕX RF and the sedimentation distribution of the hybridizable label were the same (within experimental error) in both $rRNA_I$ and $rRNA_{II}$. This experiment, together with the DMSO sedimentation results presented in section (d), indicated that little, if any, selective fractionation of mRNA had taken place on the BC column.

Furthermore, when $rRNA_I$ and $rRNA_{II}$ were separately re-chromatographed on a BC column at pH 7·5, each was eluted in two peaks similar to those of Figure 2. The fact that in Figure 3 (also see Fig. 15, Sinsheimer, 1968) hybridizable pulse-label appears in the fractions sedimenting faster than does ϕX DNA (21 s) could be a

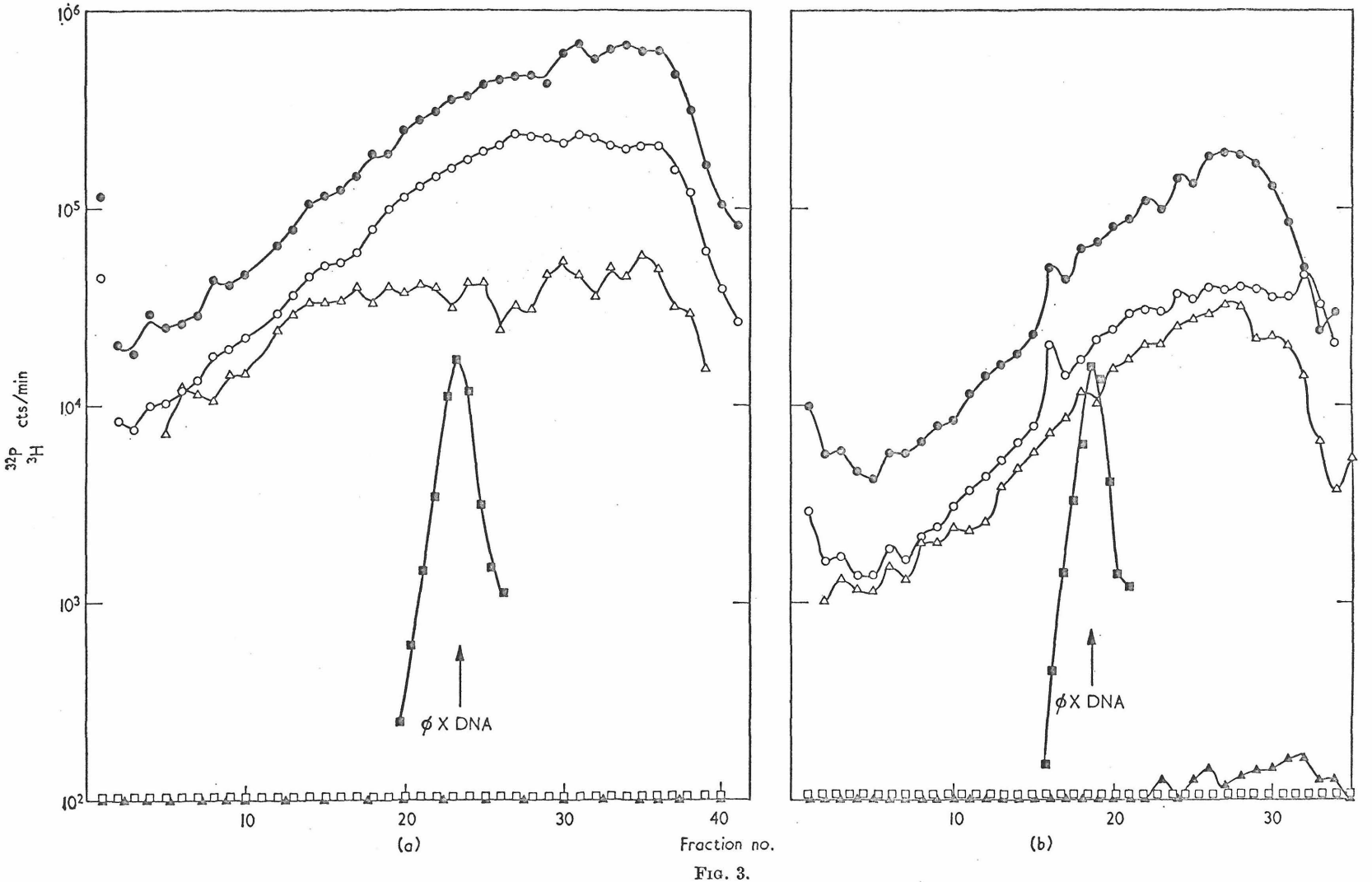
10^6
10^5
10^4
10^3
10^2
^{32}P cts/min
^3H
ϕ X DNA
10
20
30
40
(a)
Fraction no.
ϕ X DNA
10
20
30
(b)

FIG. 3.

consequence of the aggregation of the mRNA to rRNA as reported by Asano (1965), Hayes *et al.* (1966), and Sedat & Sinsheimer (manuscript in preparation).

(c) *Fractionation of pulse-labeled RNA at pH 3·5 on benzoylated DEAE-cellulose*

The results of the ϕX hybridization experiment indicated that there was no apparent selection of mRNA in $rRNA_I$ and $rRNA_{II}$, and analytical and preparative velocity sedimentation of these fractions demonstrated that the major mass components were undegraded 16 s and 23 s rRNA. In order to separate the pulse-label from the rRNA an additional fractionation step on BC entirely at pH 3·5 in 8 M-urea was used. Figure 4 shows the result of chromatography of either $rRNA_I$ or $rRNA_{II}$ in 8 M-urea at pH 3·5. No radioactivity flowed through the column during sample application and a single peak, greatly enriched for the pulse-label, was eluted during the 0 to 1 M-NH_4Cl gradient. Table 2 provides the proportions of the total mass-

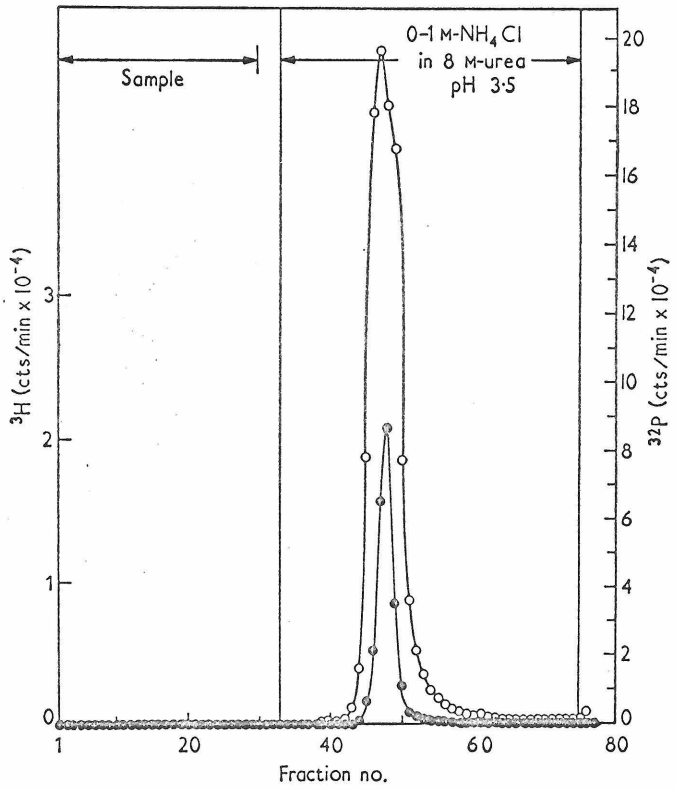

FIG. 4. Chromatography of $rRNA_I$ (isolated from ^{32}P pulse-, ^3H mass-labeled nucleic acid extract on BNC at pH 7·5) on BNC column at pH 3·5 in the presence of 8 M-urea. $rRNA_{II}$ behaves similarly.

—○——○—, ^{32}P (pulse) cts/min; —◎——◎—, ^3H (mass) cts/min.

FIG. 3. Sedimentation in a sucrose gradient of (a) $rRNA_I$ or (b) $rRNA_{II}$ from both $\phi X am3$-infected cells, pulse-labeled with [^3H]uracil for 45 sec at 15 min after infection, and from uninfected cells, pulse-labeled with ^{32}P (phosphate) for 45 sec.

—◎——◎—, Total ^3H cts/min in each fraction; —○——○—, total ^{32}P cts/min in each fraction; —△——△—, ^3H cts/min hybridized to ϕX RF in each fraction; —▲——▲—, ^{32}P cts/min hybridized to ϕX RF in each fraction; —□——□—, ^3H cts/min hybridized to ϕX viral DNA in each fraction; —▣——▣—, position of label in viral ϕX DNA in another sedimentation tube.

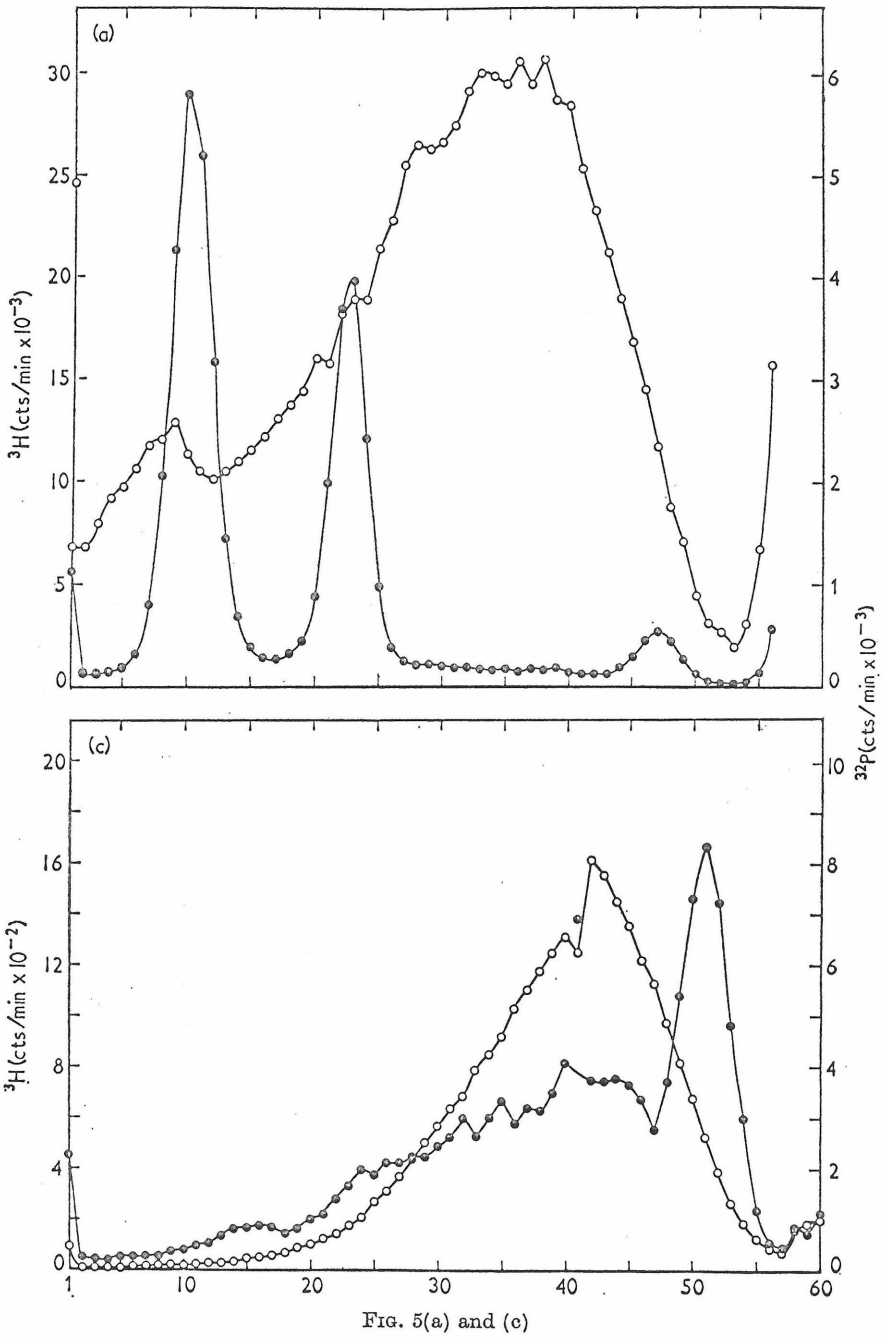

FIG. 5(a) and (c)

FIG. 5. Sedimentation through a sucrose gradient of different RNA fractions eluted from BNC columns. Sedimentation from right to left at 5°C at 25,000 rev./min for 12 hr, through a 5 to 20% sucrose gradient in 0·02 M-Tris–Cl buffer, pH 7·5.

—○——○—, ^{32}P (pulse-label) cts/min; —◉——◉—, ^{3}H (mass-label) cts/min.

(a) $rRNA_I$ from BNC column at pH 7·5.

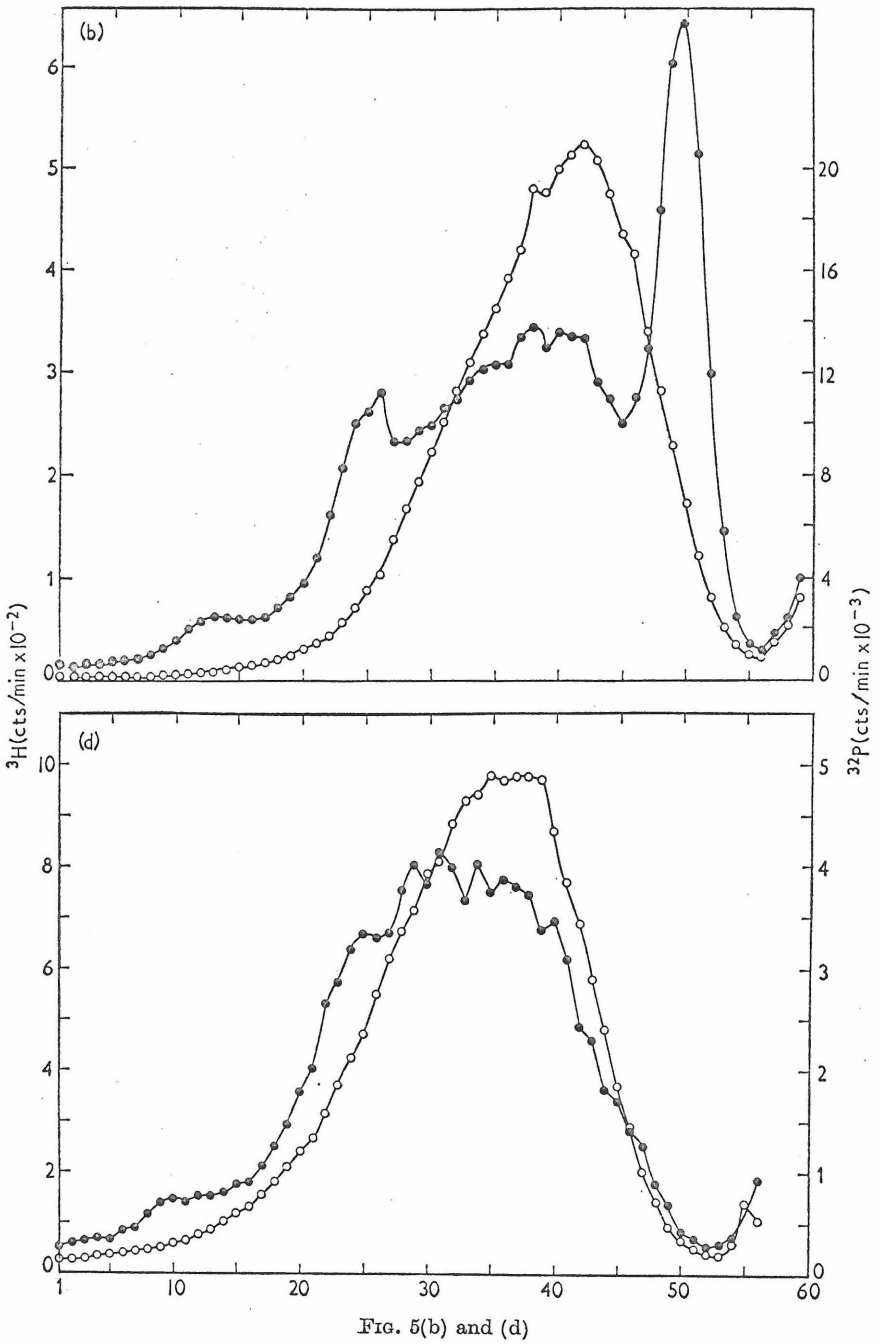

FIG. 5(b) and (d)

(b) The RNA eluted from a BNC column at pH 3·5, after application of $rRNA_I$.
(c) The RNA eluted from a BNC column at pH 3·5, after application of $rRNA_{II}$.
(d) A sample of RNA similar to that used in (b) was rechromatographed on BNC at pH 7·5 and the peak eluted from this column in the second gradient sedimented as above.

TABLE 2

pH 3·5 Benzoylated (naphthoylated) DEAE-cellulose fractionation

RNA applied	Fraction of the mass eluted	Fraction of the pulse eluted	Pulse/Mass
$rRNA_I$	0·10	0·60	5·9
$rRNA_{II}$	0·23	0·80	10·9

and pulse-labels eluted when $rRNA_I$ and $rRNA_{II}$ were chromatographed at pH 3·5 on BC. Only a small fraction of the applied mass was eluted with approximately 60% of the pulse-label (this has been as high as 75% in some pulse experiments). $rRNA_{II}$ was initially enriched for pulse-label, and chromatography of this fraction resulted in an eluate with an increased proportion of the applied mass.

The radioactivity remaining on the column could not be eluted with any of the gradients used with the first column at pH 7·5. The only means yet discovered to dislodge the residual RNA has been partially to degrade it by passage of 8 M-urea (Baker & Adamson only) in 0·1 M-CH_3COOH, pH 3·5, plus 2% sodium dodecyl sulfate, followed by distilled water, which brings off the rRNA largely depleted of ^{32}P pulse-label. As usual with the use of unpurified urea, an estimated five to ten hits/molecule were found in the rRNA. For this chromatographic procedure the pH must be lower than 4·5, NaCl will not substitute for the NH_4Cl, and dimethylurea will not substitute for urea.

The BC resin appears to age on standing for about six months and older batches allowed increasing amounts of rRNA (especially 16 s) to elute with the pulse counts.

The peak eluted at pH 3·5 on BC was RNA as judged by its complete hydrolysis (to trichloroacetic acid-solubility) by RNase. No unusual spots of radioactivity were found after electrophoresis of the nucleotides from a 0·3 N-potassium hydroxide hydrolysate.

Rechromatography of the RNA eluted at pH 3·5 resulted in a pattern identical to that of Figure 4. Greater than 85% of both pulse and mass labels applied was eluted (except for the above-mentioned case when the use of aged resin resulted in elution of some rRNA).

(d) *Velocity sedimentation analysis in sucrose gradients*

The results of preparative velocity sedimentations, in neutral sucrose gradients, of ^{32}P pulse–[^3H]uracil uniformly-labeled, fractionated RNA from the BC columns are shown in Figure 5. Part (a) of this Figure is the velocity pattern of $rRNA_I$; especially noticeable is the large fraction of ^{32}P pulse-label sedimenting faster than the 23 s rRNA. From the pulse experiment in ϕX-infected cells (see Fig. 3) and the analytical sedimentation patterns presented (also see Results, section (d)), both $rRNA_I$ and $rRNA_{II}$ are very similar with respect to distribution of mass- and pulse-label. The sedimentation patterns of the RNA eluted from the BC column in the pH 3·5 fractionation of both $rRNA_I$ and $rRNA_{II}$ appear in (b) and (c), respectively. The 16 s and 23 s rRNA components are largely gone, except for the very small shoulders in the regions where these rRNA components sediment; there is still a small amount of tRNA at 4 s (Gillam *et al.*, 1967). (The presence of tRNA in $rRNA_I$ and $rRNA_{II}$

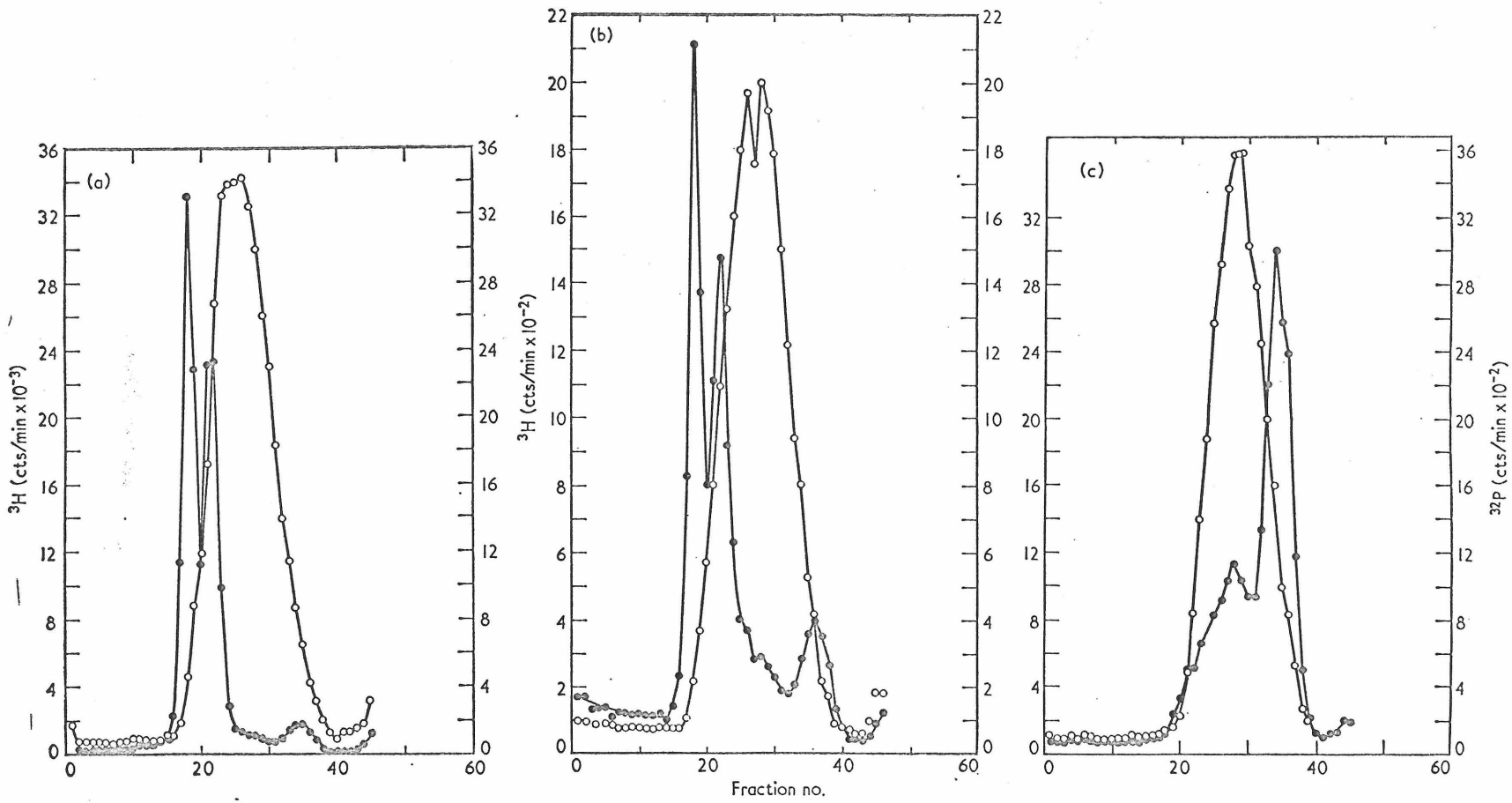

FIG. 6. Sedimentation through 99% DMSO gradients of ^{32}P pulse–labeled–[^{3}H]uracil uniformly-labeled RNA fractions from BNC column. —○——○—, ^{32}P (pulse) cts/min; —◉——◉—, ^{3}H (mass) cts/min.
(a) $rRNA_I$. (b) $rRNA_{II}$. (c) $rRNA_I$ after repeated (2 times) chromatography on BNC column at pH 3·5.

is a consequence in part of trailing from the tRNA peak and in part of retarded elution of acylated tRNA bearing aromatic amino acids.)

The ^3H (mass) label now approximately parallels the pulse-label in both gradients. When the tRNA present in the RNA analyzed in Figure 5 (b) and (c) was removed by rechromatography at pH 7·5 as described in Materials and Methods section (b), (i), and the product sedimented again, the distribution pattern was as shown in Figure 5(d). There is a general correlation of the pulse- and mass-labels; when the sedimentation profile of the original $rRNA_I$ (Fig. 5(a)) is compared to that of the final preparation (Fig. 5(d)), it is seen to be essentially similar except for the disappearance of the pulse counts which initially sedimented ahead of the 23 s rRNA. This result, in addition to the ϕX result, again suggests that the fast pulse-label could be due to mRNA–rRNA aggregation.

(e) *Velocity sedimentation in 99% benzoylated (naphthoylated) DEAE-cellulose*

Such aggregation effects, in addition to the well-established observations that the measured s-values are not simply related to RNA molecular weight, decrease the utility of sucrose gradient analyses of RNA preparations. Sedimentation of RNA through 99% DMSO has been shown to be independent of prior secondary structure and the dependency of *S*-value on molecular weight is regular and known (Strauss *et al.*, 1968). Figure 6 illustrates the result of sedimentation through 99% DMSO of RNA from different stages of the fractionations described here. In Figure 6(a) $rRNA_I$ was used, in Figure 6(b), $rRNA_{II}$, and in Figure 6(c) the RNA eluted from two successive fractionations at pH 3·5 of an $rRNA_I$ fraction. The results of these 99% DMSO gradients indicated that there was no apparent degradation as judged by the mass ratio (2:1) for the 23 s and 16 s rRNA in both $rRNA_I$ and $rRNA_{II}$. The size distribution of the pulse label was the same in the initial (a) as in the final (c) preparation, making it unlikely that selection or degradation had occurred. In (c) there is a good correlation of the pulse- and mass-labels, disregarding the slower-sedimenting tRNA component. Comparison of the distribution of pulse label on the sucrose gradients and in the DMSO gradients indicates a good agreement except for the pulse-label sedimenting ahead of the 23 s marker; since this component is absent in DMSO, it probably represents aggregation as suggested above.

(f) *Fate of the [^3H]uracil pulse- and [^{14}C]uracil uniformly-labeled RNA during a "chase"*

The experiments described above were performed with a ^{32}P pulse- and ^3H uniform-label and the good correlation of the pulse- and mass-counts in the final preparation suggested that the mRNA had been purified to its actual mass (approx. 3 to 5% of the total RNA mass of the cell). As a further test of this conclusion, a culture of *E. coli* was labeled with [^{14}C]uracil for one generation and then with a 45-second

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FIG. 7. Sedimentation of RNA eluted from BNC column at pH 3·5 ($rRNA_I$ applied). The RNA of the cells was uniformly labeled with [^{14}C]uracil and pulse-labeled (45 sec) with [^{3}H]uracil. At the end of the pulse, one-half of the culture was chilled on ice and the other half incubated in the absence of label for one generation of growth (chase).

Equal amounts (A_{260}) of the RNA, extracted from the two halves of the culture and chromatographed, were sedimented at 10°C for 8 hr at 40,000 rev./min through a 5 to 20% sucrose gradient in 0·02 M-Tris–Cl buffer.

—○——○—, A_{260}; —◉——◉—, ^{3}H (pulse) cts/min; —▲——▲—, ^{14}C (mass) cts/min; △, ratio $^{14}C/^3H$ in each fraction.

(a) Immediately at end of 45-sec pulse. (b) After chase for one generation of growth.

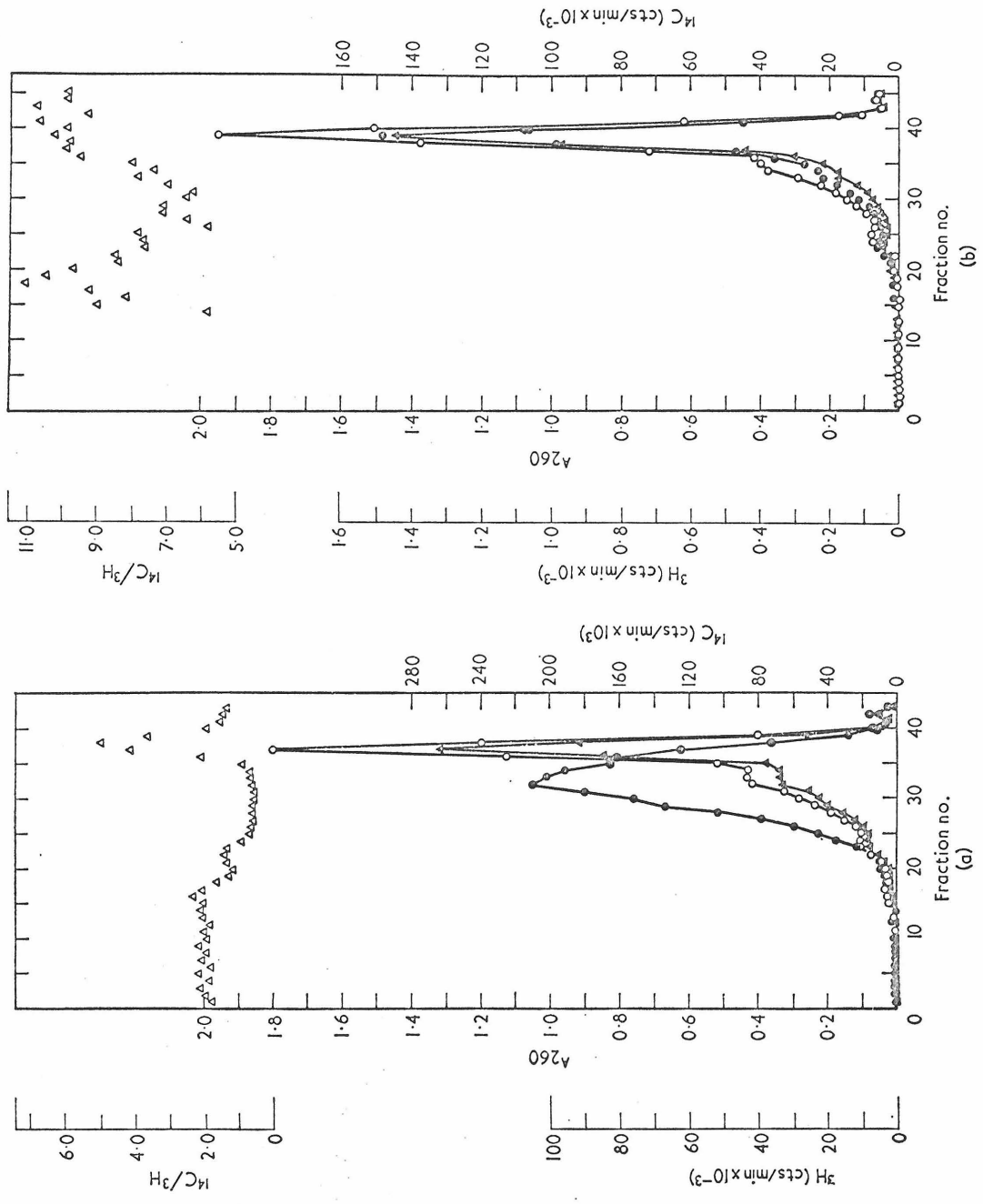
3H (cts/min x 10⁻³)
A260
14C (cts/min x 10³)
14C/3H
Fraction no.
(a)
3H (cts/min x 10⁻³)
A260
14C (cts/min x 10⁻³)
14C/3H
Fraction no.
(b)

FIG. 7.

pulse of [5-^{3}H]uracil. The culture was then split; one-half was killed with NaN_3, while the label in the other half was chased by adding a large excess of unlabeled uridine and cytidine, filtering off the cells, and resuspending them for one generation of growth in excess unlabeled uridine and cytidine. The nucleic acids were then extracted, and fractionated on columns of BC at pH 7·5 and pH 3·5. Successful change of medium was indicated by the lack of further incorporation of the ^{14}C or ^{3}H labels; also, the specific activity of the ^{14}C decreased by one-half during the chase, indicating uninterrupted cell growth. Equivalent amounts of RNA (A_{260}) from the two half-cultures were sedimented on sucrose gradients (Fig. 7). In Figure 7(a) the RNA from the ^{3}H pulse–^{14}C uniformly-labeled control half of the culture shows the usual heterogeneous sedimentation distribution of ^{3}H pulse-label with corresponding A_{260} and ^{14}C label. The sedimentation distribution of the RNA from the chased half of the culture is seen in Figure 7(b). The ^{3}H label has largely disappeared, indicating success of the chase. The ^{14}C counts have also decreased; however the ratio of ^{14}C/^{3}H as compared to that of the control indicates there is substantial ^{14}C label that does not chase or does so very slowly. Much of this is probably tRNA but it is surprising that the distribution of persistent ^{14}C label sedimenting ahead of tRNA is very similar to that of the control.

4. Discussion

(a) *The basis for fractionation on benzoylated DEAE-cellulose*

At pH 7·5 the BC and BNC columns appear to separate nucleic acids on the basis of their secondary structure (see Sedat *et al.*, 1967). It is, however, unexpected that such chromatography fractionates *E. coli* pulse-labeled RNA into two peaks (in 0·3 M-NaCl, 0·02 M-Tris–Cl, pH 7·5). It was thought that this result might be explained by the aggregation of mRNA to rRNA, but even after two successive fractionations at pH 3·5 to remove rRNA, rechromatography at pH 7·5 distributes the pulse-label (both in position and amount) identically as in Figure 2. Whatever the basis of this fractionation of the pulse-label, it is very sensitive to a change in buffer, as to 0·02 M-PO_4, pH 9·6, in 0·3 M-NaCl†.

The fractionation of mRNA from rRNA at pH 3·5 is not well understood. The requirements for urea (and not dimethylurea), for NH_4Cl (and not NaCl), and that the pH $\leq$ 4·5 seem to have no obvious basis: equally bizarre is the ability to bring off the rRNA with water (after the sodium dodecyl sulfate wash in the presence of a degrading 8 M-urea).

There are several observations to suggest that the interaction of the column with nucleic acid is not an equilibrium reaction as in most ion-exchange chromatographic procedures. First, the separations and resolution are not significantly improved by an increase in height of the column (even by a factor of 20), and no change is noted by mixing up the column bed during the loading process (the capacity is $\geq$ 100 A_{260}/ml. bed volume at pH 7·5). Second, we find that the fractionation with respect to position and peak appearance is very sensitive to the conditions of application, suggesting that the RNA molecules are fixed on the BC column (decreasing their degrees of freedom) in a state dependent on the solvent in which they were applied and that this state determines the necessary ionic conditions for elution.

† It should be mentioned that reticulocyte rRNA is not completely eluted during BC chromatography unless a 0 to 4 M-urea gradient is substituted for the 0 to 30% DMSO gradient (Lyon, unpublished observations).

We find no evidence that there is any selectivity for different messengers across peaks eluted from BC, at any pH, as evidenced by the similar sedimentation patterns of different fractions and the constant ratio of ϕX ^{3}H pulse-label to ^{32}P uninfected pulse-label.

(b) *Proof that the pulse-label represents mRNA*

The pulse-label (in $rRNA_I$ and $rRNA_{II}$) from ϕX-infected cells (and not from uninfected cells) contains messenger RNA that hybridizes specifically to ϕX RF. (That it does not hybridize to ϕX DNA indicates that the complementary strand of the RF is the site for transcription (with a preference better than 10^6:1) in agreement with previous work on ϕX mRNA (Hayashi, Hayashi & Spiegelman, 1963). The sedimentation distributions of the purified pulse-labeled RNA are in good agreement with many previous studies of the sedimentation distribution for the heterogeneous *E. coli* mRNA (cf. Bremer & Yuan, 1968). The total mass of the final pulse-labeled RNA preparation (mass-label now coincident with pulse-label distribution) is in general agreement with recent estimates for the mass of *E. coli* mRNA: 3% (Salser, Janin & Levinthal, 1968); 1·5 to 3% (Leive, 1965); 3% (Mangiarotti & Schlessinger, 1967); 2·2% (Mueller & Bremer, 1968). The chase experiment indicates that the pulse-label is chased out, as expected, in accord with other observations (see the discussion by Salser *et al.*, 1968). It is also of interest that the pulse-labeled RNA isolated on BNC in this way from phage-infected cells contains messenger RNA fully active in the stimulation, in cell-free systems, of the synthesis of specific, biologically active proteins of the phage T4 (T. Young, personal communication).

(c) *Possible selection of mRNA fractions*

It is very difficult to ascertain if there has been selective enhancement of any mRNA fraction during the preparation of the total heterogeneous mRNA. One major uncontrolled process wherein selection could arise is in the lysis and extraction with phenol. It is known that more of the single-stranded replicating DNA is retained in the phenol layer (Ganesan & Lederberg, 1965; Hanawalt & Ray, 1964), and pulsed RNA or DNA is found bound to the interface of phenol layers in extracts of animal cells (Kidson, Kirby & Ralph, 1963). However, the high yields of ϕX mRNA, tryptophan operon mRNA, *lac* operon mRNA and lambda phage mRNA by the phenol extraction procedure indicate that this is probably not a significant objection. Another step in which unwanted selection could arise is during the BNC fractionation. Since the recovery of ^{3}H-mass counts is $> 95\%$ (Fig. 2), it is unlikely that selection of this RNA is significant; the recovery of the ^{32}P pulse-label is over 90%. Much of the firmly bound ^{32}P pulse-label is in a non-nucleic acid component, which can be eluted with sodium dodecyl sulfate. No selection of pulse-label into $rRNA_I$ or $rRNA_{II}$ is evident in the profiles of ϕX RF hybridizable-RNA. Rechromatography of previously fractionated (see Fig. 5(a)) pulse-label, at pH 3·5 or pH 7·5 on BNC, results in a firm binding of approximately 10% of the ^{32}P pulse; however, from DMSO velocity gradients there appears to be no selection of a particular component. All the fractionation steps seem to have very similar or identical leading edges in DMSO gradients which may be the best evidence that the BNC chromatography is not selective.

(d) *Problems of RNA degradation*

If it is accepted that a mass ratio of rRNA components < 2:1 indicates degradation during the preparation, then from the DMSO sedimentation (where masked hits

are unfolded) little degradation is evident. Of course, even with the use of RNase I$^-$ mutants it is still possible that there might be polysome degradation (but not ribosome) during the lysozyme–EDTA lysis before addition of sodium dodecyl sulfate and phenol.

The degradation of RNA by 8 M-urea was unexpected, requiring the use of a highly purified reagent.

(e) *Purity of mRNA*

One of the best indications of purity of the mRNA preparation is the observation in DMSO (or sucrose) gradients that the mass counts closely parallel the pulse counts as a function of the mRNA size (in DMSO). It is extremely unlikely that this is a coincidence but more probably reflects the purification to the actual mass of the mRNA. This mass, allowing for losses on the columns and correction for the tRNA contaminant, agrees with the reported value of 3%. Another argument for purity of this fraction comes from the observation that the ratio of pulse- to mass-label in the sedimentation region between rRNA and tRNA (near the mean molecular weight of mRNA) in the $rRNA_I$ sedimentation in DMSO (Fig. 6(a), tube 30) is the same as in the final purification (Fig. 6(c), tube 30). This observation is also true for $rRNA_{II}$.

There are, however, some indications that some impurities may exist in the final preparation. First, the ratio of pulse to mass in $rRNA_I$ is approximately one-half of that in RNA_{II}, and this is still true for the RNA eluted at pH 3·5 (after removal of ribosomal RNA) from both of these fractions (see tube 30 in Fig. 6(a), (b) and (c); tube 35 in Fig. 5(b) and (c)). This is not understood but it might represent an impurity. Second, it is possible to see small shoulders in the mass counts in Figure 5(b), (c) and (d) in the region where one would expect rRNA; these are reduced on rechromatography at pH 3·5 or pH 7·5 but not eliminated.

The most important indication of impurity comes, however, from the pulse/chase experiment. The pulse/chase labeling sequence results in the loss of approximately 97% of the pulse-label from the final mRNA preparation, but only about 68% of the mass-label. (The contribution of tRNA contaminating these fractions was eliminated by comparing the values for individual points in the DMSO gradients of the final preparations. These points were taken from the peak of the pulse-label distribution, well ahead of the tRNA peak.) An excess of mass-label implies that the molecules containing it were synthesized before addition of the pulse-label. Three ways in which such molecules might appear in the final mRNA preparation have been discerned. First they might be some minor RNA component, perhaps a degradation product of rRNA, which happens to co-elute with mRNA. The second possibility is that some fraction of the total mRNA synthesized by the cell is metabolically stable. Long-lived mRNA synthesized before addition of pulse-label would contribute to the mass-label, but not to the pulse-label, and would not chase. However, the fraction of total mRNA which would have to be postulated to be stable ($\geq$ 0·3) in order to account for the observed effect seems too large. A third possibility is that the unchasable mass-label comes from cells which have died during the initial period of labeling with mass-label. (It is of course then necessary to assume that all mRNA in dead cells remains undegraded.) It should be noted that the cultures were filtered, which may have caused some cell death. However, general experience with the strains used indicates that the procedures used cause very little loss of viability. It is

unlikely that the observed effect stems solely from any one of these hypothetical mechanisms; it is perhaps attributable to a combination of all three operating simultaneously.

It appears therefore that it is in general difficult to decide the purity of the mRNA. Though it has been purified 15-fold, more work will have to be done to decide this point.

(f) *Implications of this research*

The finding that the mRNA pulse-label elutes as a single peak at the final pH, 3·5, resolved from rRNA, but with a size distribution identical to that present in the starting RNA, implies that there is some common feature, structural or chemical, in mRNA molecules permitting them to act as a group during BNC fractionation.

Our sedimentation velocity patterns in DMSO imply that very little (if any) mRNA is larger than 1×10^6 avograms, enough RNA for at most ten cistrons. The peak of the mRNA distribution is at an S-value corresponding to a molecular weight of about 72,000. (The corresponding mol. wt. for ϕX messenger RNA is 116,000.)

These low average values of messenger size raise the possibility of a selective degradation of messenger RNA (and not of ribosomal RNA) during isolation. However the isolation, as previously mentioned, of functional messenger RNA from infected cells by these methods argues qualitatively against the likelihood that extensive degradation has occurred.

Our results on the mechanisms of fractionation on BNC or BC suggest that a new chromatographic effect is operative such that the elution (recovery and position) of the nucleic acid is strongly influenced by the conditions that prevailed when it was applied on the column; it is as if the degrees of freedom are fixed once the nucleic acid is bound to the column and can only be changed by elution when the ionic conditions are right.

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REFERENCES

Asano, K. (1965). *J. Mol. Biol.* **14**, 71.

Attardi, G., Naono, S., Rouvière, J., Jacob, F. & Gros, F. (1963). *Cold Spr. Harb. Symp. Quant. Biol.* **28**, 363.

Bautz, E. K. F. & Reilly, E. (1966). *Science*, **151**, 328.

Boedtker, H. (1968). *J. Mol. Biol.* **35**, 61.

Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.

Bremer, H. & Yuan, D. (1968). *J. Mol. Biol.* **34**, 527.

Britten, R. J. & Kohne, D. E. (1968). *Science*, **161**, 529.

Ganesan, A. T. & Lederberg, J. (1965). *Biochem. Biophys. Res. Comm.* **18**, 824.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E. & Tener, G. M. (1967). *Biochemistry*, **6**, 3043.

Gillespie, D. & Spiegelman, S. (1965). *J. Mol. Biol.* **12**, 829.

Gros, F., Gilbert, W., Hiatt, H., Attardi, G., Spahr, P. F. & Watson, J. D. (1961). *Cold Spr. Harb. Symp. Quant. Biol.* **26**, 11.

Hanawalt, P. L. & Ray, P. S. (1964). *Proc. Nat. Acad. Sci., Wash.* **52**, 125.

Hayashi, M., Hayashi, M. N. & Spiegelman, S. (1963). *Proc. Nat. Acad. Sci., Wash.* **50**, 664.

Hayes, P. H., Hayes, F. & Guerin, M. F. (1966). *J. Mol. Biol.* **18**, 494.

Hutchison, C. A., III & Sinsheimer, R. L. (1966). *J. Mol. Biol.* **18**, 429.
Kelly, R. B. & Sinsheimer, R. L. (1967). *J. Mol. Biol.* **29**, 229.
Kidson, C. & Kirby, K. S. (1964). *J. Mol. Biol.* **10**, 187.
Kidson, C., Kirby, K. S. & Ralph, R. K. (1963). *J. Mol. Biol.* **7**, 312.
Kiger, J. & Sinsheimer, R. L. (1969). *J. Mol. Biol.* **40**, 467.
Komano, T. & Sinsheimer, R. L. (1968). *Biochim. biophys. Acta*, **155**, 295.
Leive, L. (1965). *J. Mol. Biol.* **13**, 862.
Lindqvist, B. & Sinsheimer, R. L. (1967). *J. Mol. Biol.* **28**, 87.
Mangiarotti, G., Apirion, D., Schlessinger, D. & Silengo, L. (1968). *Biochemistry*, **7**, 456.
Mangiarotti, G. & Schlessinger, D. (1967). *J. Mol. Biol.* **29**, 395.
Mueller, K. & Bremer, H. (1968). *J. Mol. Biol.* **38**, 329.
Salser, W., Janin, J. & Levinthal, C. (1968). *J. Mol. Biol.* **31**, 237.
Sedat, J. W., Kelly, R. B. & Sinsheimer, R. L. (1967). *J. Mol. Biol.* **27**, 537.
Sinsheimer, R. L. (1968). *Prog. Nucleic Acid Res. Mol. Biol.* **8**, 115.
Sinsheimer, R. L., Knippers, R. & Komano, T. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 443.
Strauss, J. H., Jr., Kelly, R. B. & Sinsheimer, R. L. (1968). *Biopolymers*, **6**, 793.
Strauss, J. H., Jr. & Sinsheimer, R. L. (1968). *J. Mol. Biol.* **34**, 453.

PART II

THE PROCESS OF INFECTION WITH BACTERIOPHAGE ØX174.

The Process of Infection with Bacteriophage ØX174. XXXVII. The *in vivo* ØX mRNA.*

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Running title: *in vivo* ØX mRNA

*The previous paper in this series was Mayol and Sinsheimer (submitted for publication).

Summary

The size of the *in vivo* ØX mRNA molecules has been studied by sedimentation of pulse- and uniformly-labeled RNA (from ØX-infected cells) through 99% dimethyl sulfoxide gradients and subsequent hybridization of fractions to ØX RF; the resultant profiles indicate a broadly heterogeneous size distribution of both pulse and mass labels with a distinct maximum size of 1.7 megadaltons and a maximum frequency at 0.2 megadaltons. This mRNA size distribution does not change with time after infection. Little or no RNA capable of hybridization with RF is found in uninfected cells; no RNA capable of hybridization with ØX DNA is found in infected cells at early or later times.

The RNA distributions from infection with the strongly polar ØX mutant *op* 6 or in the bacterial ØX-replication mutant rep_3^- show little change from the RNA pattern of the normal infection. No methylated bases or 5' triphosphate termini could be detected in mRNA by a variety of techniques. The implication of these results on mRNA metabolism and on current ideas of the ØX infection cycle are discussed.

1. Introduction

In recent years extensive studies have been made concerning the replication of ØX RF (Knippers, Whalley & Sinsheimer, 1969), ØX viral DNA (Knippers, Razin, Davis & Sinsheimer, 1969), and the ØX specific proteins synthesized during infection. The latter has been analyzed by SDS polyacrylamide gel electrophoresis of ØX proteins in UV-irradiated hosts (Burgess & Denhardt, 1969; Gelfand and Hayashi, 1969), more directly by double-label techniques in infected and uninfected cells (Mayol & Sinsheimer, submitted for publication), by chromatographic procedures on extracts from chloramphenicol-treated cells (Levine & Sinsheimer, 1969a), and by serum blocking techniques (Krane, 1965; Rohwer & Sinsheimer, unpublished). In contrast, ØX mRNA has received scant experimental attention since the initial observation of Hayashi, Hayashi,& Spiegelman,(1963) that the <u>in vivo</u> ØX mRNA was transcribed off the complementary strand of the RF and the report of an <u>in vivo</u> pulse labeled RNase-, phenol-, and detergent-resistant RNA-RF complex (Hayashi & Hayashi, 1966).

The processes of replication, transcription,and protein synthesis would seem to be intricately related in this infection since the parental RF must be transcribed before

replication and especially since the association of RF with a replicative site (Yarus & Sinsheimer, 1967) may be a requirement for participation in genetic complementation (Sinsheimer, 1968). Transcription in polycistronic messages is implicated by the observation of polarity (Tessman, Kumar & Tessman, 1967; Hutchison, 1969; Benbow, Mayol & Sinsheimer, in preparation). That the ØX proteins are synthesized in highly varied numbers of copies at early and late times (Burgess & Denhardt, 1969; Gelfand & Hayashi, 1969; Mayol & Sinsheimer, submitted for publication), and the observation of asymmetric complementation (Hutchison, 1969; Tessman, 1966) are at present unexplained. An analysis of the size and amounts, in tempore, of mRNA should give a partial elucidation of some of these integrative cellular-virus processes.

In this study of ØX mRNA we have made use of double label techniques; usually a short pulse of one isotope is given to infected cells that have been previously labeled for two generations with another isotope. Thus it is possible to study both the rapidly labeled (pulse) and mass components of the mRNA, and eventually to calculate the molecules per cell of a given size. This procedure was broadly verified in the previous study of the purification of E. coli pulse labeled mRNA (Sedat, Lyon & Sinsheimer, 1969).

We continue to use, in some cases, benzoylated DEAE cellulose chromatography to selectively purify the total pulse labeled mRNA (Sedat et. al., 1969). This procedure removes the rRNA without detectable selection or degradation of the mRNA, and allows increased quantities of RNA to be analyzed on the capacity limited 99% DMSO gradients.

All mRNA size distributions are studied by sedimentation through 99% DMSO gradients (in which RNA is completely denatured at 25°C (Strauss, Kelly & Sinsheimer, 1968)). With this procedure a distinct maximum size is observed for ØX mRNA. Analysis by sedimentation in sucrose gradients, in contrast, provides equivocal results with mRNA; no maximum size for ØX can be found in sucrose gradients as judged by the finding of RF-hybridizable RNA all the way to the bottom of the tube (See Fig. 15, Sinsheimer, 1968; Fig. 3, Sedat et al., 1969; Warnaar, De Mol, Mulder, Abrahams & Cohen, 1970). This is not a consequence of concentration-dependent sedimentation or salt concentration, and no procedure such as benzoylated DEAE cellulose chromatography or prior denaturation seems to eliminate this artifact. This phenomenon is probably a consequence of aggregation of rRNA with mRNA as described by Asano (1965) and Hayes, Hayes & Guerin (1966). This aggregation seems perfectly general with other mRNA systems (tryptophan operon mRNA, Morikawa

& Imamoto, 1969; Imamoto & Yanofsky, 1967; λ mRNA, Kourilsky, Marcaud, Sheldrick, Luzzati & Gros, 1968; lactose operon, Attardi, Naono, Rouvière, Jacob & Gros, 1963; M13 mRNA, Jacob & Hofschneider, 1969; T4 lysozyme mRNA, Salser, Gestland & Ricard, 1969), thus precluding use of sucrose gradients in the study of mRNA size.

A procedure for DNA-RNA hybridization in DMSO selected for maximal specificity of the hybrid is described. (This procedure was independently developed by T. Young and H. Goodman, personal communication). The DMSO system has many ideal properties: little RNase degradation takes place in DMSO (Strauss et al., 1968); good recovery of RNA infectivity (Strauss et al., 1968) (and of the ability to direct the synthesis of a specific protein, T4 lysozyme (Ted Young, personal communication)) is indicative of few thermal "hits" in the RNA during hybridization at 37°C.

2. Materials and Methods

Most of the materials and methods in this paper are described in Sedat *et al.* (1969). The following are additional.

(a) Materials

(i) *E. coli* C-416 (met^- $purine^-$) and *E. coli* C are strains from the collection of Dr. R. L. Sinsheimer. C-1415 (F^-rha^- his^- rep_3^- ϕX^S P_2^S λ^S rec^+) is a strain obtained from Dr. R. Calendar in which the Denhardt rep_3^- locus has been transduced into a genetically defined *E. coli* C strain.

(ii) *op6* is an opal (UGA) mutant of ØX in gene VII very strongly polar for genes VII, III, II isolated by Dr. Clyde Hutchison III and described by Benbow, Mayol & Sinsheimer (in preparation). Stocks of this phage were a gift of Mr. Benbow.

(iii) [2-^{14}C] uracil (59 mC/mM), [8-^{3}H] adenine (19-22 c/mM), [8-^{14}C] adenine (50.5 mC/mM), [methyl-^{3}H] L-methionine (3.3-6 C/mM) and [8-^{3}H] guanine (10.2 C/mM) were obtained from Schwarz Bioresearch, Inc., Orangeburg, N.Y.

(iv) DNase I (RNase free) DPFF-9ED was obtained from Worthington Biochemical, Freehold, N.J.

RNase I (5X crystallized) was a product of Sigma Chemical Co., St. Louis, Mo.

(v) All the methylated bases were obtained from Cyclo Chemical, Los Angeles, Calif.

(vi) Benzoylated DEAE cellulose (Lot. No. 6902, 50-100 mesh) was purchased from Schwarz Bioresearch, Orangeburg, N.J. DEAE cellulose (DE52 preswollen microgranular), a Whatman product, was obtained from Reeve Angel, Clifton, N.J.

(vii) Thin layer chromatograms (6064, cellulose, without fluorescent indicator) was an Eastman Kodak, Rochester, N.Y., product.

(viii) Large cellulose nitrate sheets (Cat. No. HAWP 00010) were obtained from the Millipore Corp., Bedford, Mass.

(ix) Medical X-ray film for autoradiography was Kodak Royal Blue RB-54; the scintillation image intensifier was Omnispray TM (NEF 932), a product of the New England Nuclear Corp., Boston, Mass.

(x) The small conical vials used for small samples, digestions, and concentrations were obtained from Pierce Chemical Co., Rockford, Ill. The small 2 ml. plastic disposable beakers with polyethylene caps used for hybrid formation were obtained from Scientific Products, Los Angeles, Calif.

(xi) Trifluoroacetic acid (anhydrous) was obtained from Pierce Chemical, Rockford, Ill.

(b) Methods

(i) Preparation of pulse and uniformly labeled RNA extracts.

Most of the explicit details for the preparation of RNA extracts are described in Sedat et al. (1969): however the labeling details for each of the different kinds of experiments in the Results are described below.

(A) ^{32}P-pulse (pulse labeled at 4 and 25 min after infection), ^{3}H-uniformly labeled, ØX-infected RNA extracts. 500 ml. of HF4704 were grown to 1 x 10^8 cells/ml. in TPA media, and then split into two 200-ml. cultures into which 5 mC [5-^{3}H] -uracil were added. At 2.35 x 10^8 cells/ml. (viable) both cultures were infected with ØX am3 ($m \leq 12$); then at 4.0 min after infection one culture was pulse labeled with 12.5 mC $^{32}PO_4^{3-}$ (45 sec) at 25 min after infection.

(B) ^{32}P-pulse, ^{3}H-uniformly labeled, uninfected and ØX-infected RNA extracts; [methyl-^{3}H] labeled, uninfected and ØX-infected RNA extracts; 450 ml. of C-416 in TPA (met^-) + 6 µg/ml. L-met + 10 µg/ml. adenine + 10 µg/ml. guanosine were grown to 1 x 10^8 cells/ml. and then split into four 100-ml. cultures. 2.5 mC [5-^{3}H] uracil was added to each of two of the cultures; at 5 x 10^8 cells/ml. one culture was infected with am3 ($m \leq 7$) and then pulsed with 10 mC $^{32}PO_4^{3-}$ (45 sec) at 25 min after infection; the other culture was

not infected and was similarly pulsed. The other two cultures were grown to 5 x 10^8 cells/ml. after which one was infected with am3 (m $\leq$ 7) and both were immediately labeled by addition of 5.5 mC [methyl-^{3}H] L-methionine. At 25 min after infection both cultures were terminated with sodium azide (0.01 M).

(C) ^{3}H-adenine pulse-, and ^{14}C-uracil uniformly-labeled, ØX-infected RNA extract; ^{3}H guanine pulse-, ^{14}C-adenine uniformly-labeled, ØX-infected RNA extract. Two 200 ml. cultures of HF4704 in TPA medium were grown to 1 x 10^8 cells/ml. and 400 μC [2-^{14}C] -uracil added to one culture. 500 μC of [8-^{14}C] -adenine was added to the other. At 5 x 10^8 cells/ml. am3 (m $\leq$ 8) was added to both cultures, and at 25 min after infection 10 mC [8-^{3}H]-adenine pulsed for 45 sec into the ^{14}C-uracil culture; 5 mC [8-^{3}H] -guanine was pulsed for 45 sec into the ^{14}C-adenine culture.

(D) ^{32}P pulse-, ^{3}H uniformly-labeled, ØX op6 infected RNA extract. 200 ml. of E. coli C in TPA medium were grown to 1 x 10^8 cells/ml., and then 4 mC [6-^{3}H] -uracil added. When the cells reached 5 x 10^8/ml. op6 (rev frequency = 2 x 10^{-5}) (m $\leq$ 5) was added, and at 15 min after infection the culture was pulse-labeled for 45 sec with 12.5 mC $^{32}PO_4^{3-}$.

(E) ^{32}P pulse, ^{3}H uniformly-labeled, ØX am3 C-1415

(rep_3^-) RNA extract. When 200 ml. of TPA grown C-1415 (shown to be rep_3^- and $\emptyset X^S$ by the absence of intracellular $\emptyset X$ at m = 15 and the eclipse of infective am3, respectively) reached 1 x 10^8 cells/ml., 5 mC [6-^{3}H]-uracil was added. At 5 x 10^8 cells/ml. the culture was infected with am3 ($m \leq 20$), and then pulsed-labeled at 25 min after infection with 12.5 mC $^{32}PO_4^{3-}$ for 45 sec.

(ii) DNase treatment of RNA extracts. The RNA extract from (i-A) was treated with DNase to digest the DNA present. The nucleic acid pellet from the ethanol precipitation was dissolved in 20 ml. of 0.02 M Tris-HCl, pH 8 and reprecipitated with ethanol at 2°C for 12 hr. The resulting pellet was dissolved in 10 ml. of 0.1 M Tris acetate pH 7.5 + 0.01 M $MgSO_4$ + 250 μg DNase I (RNase free) and then incubated at 22°C for 20 min. The solution was finally ethanol-precipitated and the pellet dissolved in 6 ml of 0.02 M Tris-HCl pH 8.

(iii) Fractionation of total RNA extracts on Benzoylated DEAE cellulose columns at pH 3.5. The entire nucleic acid extract prepared as in Sedat et al. (1969--see (i-v) p. 417-418) was freed of rRNA and DNA by adsorption and elution from BC at pH 3.5. The nucleic acid extract from the ethanol precipitation step was diluted 10 times with 8 M urea

in 0.1 M CH_3COOH pH 3.5 and applied to a 1.5 x 3 cm BC column previously washed with 8 M urea pH 3.5. The column was further washed with 8 M urea pH 3.5 until the ^{32}P counts were low and constant, and then the RNA was eluted with 8 M urea + 1 M NH_4Cl + 0.1 M CH_3COOH pH 3.5.

(iv) Sedimentation through 99% DMSO gradients. 5 ml. linear 99% DMSO gradients are established between 2.50 ml of 99% d_6 DMSO (containing 10% w/v sucrose and 1% v/v 0.1 M EDTA pH 7.0) and 2.65 ml. of a mixture of 0.1 vol fraction 99% d_6 DMSO with 0.9 vol fraction 99% DMSO (containing 1% w/v 0.1 M EDTA pH 7.0) by means of a plexiglas gradient device and pumping through a Solvaflex tubing (selected to pump 15 min/gradient) with a Technicon pump (both from Technicon Corp., Chauncey, N.Y.) to the bottom of a polyallomer ultracentrifuge tube (in this case the gradient starts with the lowest density solution); pumping is continued until the solution is within 4 mm of the top of the tube. A mixture of 5-20 µl of RNA in $\leq$ 0.01 M salt (containing less than 1-2 A_{260} units of RNA (M_w= 1 x 10^6 daltons)) plus 90 µl 99% DMSO plus 10 µl dimethylformamide are carefully layered on the above gradient and centrifuged in a Beckman L2-65B for 11 hr at 64,000 rev./min at 27°C (in a SW 65 rotor) or 14 hr at 50,000 rev/min at 27°C (in a SW 50.1 rotor).

16-drop fractions from a Büchler drop collector are collected into small 2 ml. clean disposable plastic beakers resulting in 45 reproducible 120-125 ul fractions. The positions in the centrifuged gradient of 23S (1.1×10^6 daltons), and 16S (0.5×10^6 daltons) rRNA, and tRNA (26,600 daltons), at all times of centrifugation, fall on a straight line if plotted as the log (M_w) vs. fraction number. Thus the M_w can be determined at any point in the DMSO gradient.

(v) Preparation of filters for hybridization with ØX am3 RF DNA. ØX am3 RF prepared essentially according to Komano & Sinsheimer (1968) in 0.1 M Tris-HCl pH 8 was heated to 100°C for 5-15 min in order to introduce breaks ($\approx$ 2 hits/molecule) in the closed circular duplex (this result was monitored by analytical band sedimentation through 3 M CsCl 0.1 M KOH in the Model E ultracentrifuge). The quickly ice-cooled RF solution was further denatured by dilution into 10 ml. of 0.1 M KOH and 1.5 ml. of this alkaline RF solution (containing 1.25 mg of RF) mixed with 250 ml. of 6X SSC (the resulting pH is 7). This was passed through a 6X SSC-soaked large 12.5 x 15 cm HAWP Millipore filter, and further washed with 250 ml. of 6X SSC, dried overnight at room temperature, and then dried in a vacuum oven at 80°C for 2 hr. 11-mm filters were punched out with a stainless

steel punch on a clear Teflon surface resulting in 140-145 filters for each large sheet (=9-10 μg RF/filter). Blank filters were made in a similar manner except no DNA was added. Additional experiments using the above conditions plus a small amount of ^{3}H- or ^{32}P-ØX DNA showed approximately 95-98% adsorption of the denatured DNA.

(vi) Preparation of filters for hybridization with am3 ØX DNA. These filters were prepared analogously as in (v) except 0.5 mg ØX DNA (95% circles as judged by analytical band centrifugation) was adsorbed on one large Millipore filter sheet. These filters contain approximately 3.5 μg ØX DNA/filter.

(vii) Hybridization in 40% DMSO plus 2X SSC at 37°C. Conditions were selected to maximize the specificity of ØX mRNA hybridization to RF consistent with reasonable hybridization kinetics. The conditions chosen are incubation in 40% (v/v) DMSO 2X SSC (with or without 0.05% SDS) at 37°C for 24-60 hr. After a 5-10 μl sample is removed for assay of total counts from each fraction from a 99% DMSO gradient (as in [iv]) 165-180 μl of 3.33X SSC is added to make each 2X SSC and 40% DMSO. A blank filter is added followed by a filter containing ØX or RF DNA, and the beaker capped with a polyethylene cap, then placed in a 37°C incubator for a

stated period of time. The samples are gently shaken every 4-8 hr.

At the termination of the reaction each set of two filters is removed with a clean forceps and placed in a clean sterile test tube with 10 ml. 2X SSC. The tubes are next Vortexed and the fluid contents aspirated off. An additional 10 ml. fresh 2X SSC is added. Vortexing and aspiration are repeated. Next 2 ml. of 30 μg/ml. RNase I (heat treated to inactivate DNase) is added and the tubes incubated at 24°C for 30-40 min (the background on the blank filters is 10-50 times higher without RNase treatment). The RNase solution is then aspirated off. 10 ml. 2X SSC are added, the tube is Vortexed, and the fluid is aspirated off. The filters are removed and each placed in a scintillation vial, then dried at 70°C for 40 min.

Preparative hybridization was performed in a similar manner; use of as concentrated RNA solutions as possible was essential to achieve reasonable hybridization kinetics.

(viii) Elution of hybridized RNA off hybridization filters. Elution of hybridized RNA from filters that had been counted in toluene scintillation fluid was accomplished by first washing such filters in reagent grade toluene (3 times) followed by air evaporation of the residual toluene. These

filters (or filters not counted in toluene) were then transferred to disposable plastic tubes, placed on ice, and washed with 2 changes of deionized, distilled, ice-cold water, resulting in a removal of 10% of the counts in the case of the counted filters and 1% in the case of the uncounted filter samples. 1-2 ml. of freshly prepared 50% DMSO-deionized distilled H_2O is added to each tube after which they are placed in a 42°C incubator for 30 min. The solution is drawn off, saved, and an additional 1-2 ml. 50% DMSO added to the filters, which are then incubated at 42°C for 30 min. This solution is combined with the first eluate and the DMSO is removed by lyophilization. Less than 1% of the initial counts remain bound to the filters.

(ix) Chromatographic procedures. To samples for digestion were added two A_{260} units of E. coli tRNA, or in the case of digestion to the free purine bases, 5 µg each of the following methylated bases: M_2^6 ade, M^1 cyt, M^5 cyt, M^6 ade, thy, cyt, M^2 ade, m^1 ade, m^7 ade, m_2^6 gua, ade, m^1 gua, Hm ura, m^3 gua, m^7 gua, and gua, and then concentrated to small volumes in the conical Reactivials™.

Digestion to the free purine bases and pyrimidine mononucleotides (also pyrimidine ribose 2'O methyl-dinucleotides) was accomplished by treatment of the RNA with 1 N HCl at 100°C for 30 min (the Reactivials™ were sealed tightly

with a Teflon-lined lid) or by anhydrous TFA at 175°C (in a sealed ampule) for 30 min (in this case the sample is first exhaustively lyophilized). The HCl or TFA is then removed in a vacuum desiccator over solid KOH. Digestion to mononucleotides (and ribose 2'O methyl-dinucleotides) was accomplished by treatment of the RNA with 0.3 N KOH at 37°C for 18-24 hr. The KOH was removed by acidification to pH 3 with HCl or CH_3COOH, adsorption to activated charcoal, washing with H_2O, and elution with ethanol-H_2O-NH_4OH (50:40:10) or by passage through a small column of Dowex 50-X8 (NH_4^+ form) followed by washing with H_2O; the effluent and washings are combined and concentrated to small volumes.

Digested (and desalted--vide supra) samples ($\leq$ 50 µl) are spotted on cellulose thin layer chromatograms, and developed in either of two chromatography solvent systems. System I is adapted from Isaksson & Phillips (1968), and is used for the separation of free purines. The first dimension is n-butanol-water (86:14) with NH_3 in the vapor phase; the second dimension is 2-propanol-conc. HCl-H_2O (171:41, H_2O to 250 ml.). System II is adapted from Hayashi, Osawa & Miura (1966) and is used to separate mononucleotides (especially the methylated mononucleotides). The first dimension is isobutyric acid-0.5 N NH_4OH (50:30). The second dimension

is 2-propanol 6N HCl (65:35).

The TLC are carefully air-dried after each dimension (for at least 12 hr after the second dimension). The UV visible spots are marked and the TLC is sprayed with the autoradiographic scintillator Omnispray™ and further dried for 1 hr. Radioactivity is then detected by exposure of the TLC plate to Kodak Royal Blue X-ray film at -70°C for varied periods of time. The limits of detection by this method are ≈ 50 cts./min (10 wks) for ^{14}C and ≈ 1000 cts./min ^{3}H (10 wks). Alternatively the TLC plates are scanned in a 2π two-dimensional windowless counter (Varian/Aerograph); this system can detect ≈100 cts./min ^{14}C and ≈5000 cts./min ^{3}H in 48 hr.

(x) DEAE cellulose chromatography. Essentially the chain length separation procedure of Jorgensen, Buch & Nierlich (1969) was used to search for 5' triphosphate ribonucleoside 3' monophosphates in an alkaline digest of ØX mRNA. Approximately 4 x 10^7 ^{32}P cts./min RF-hybridized, RNase-treated, and eluted mRNA was mixed with two A_{260} units of E. coli tRNA; this was digested with KOH, desalted, and concentrated as in (ix). The products of this digestion were then mixed with an RNase I-treated E. coli tRNA digest (prepared by treatment of 200 µl of 142 A_{260}/ml. E. coli tRNA + 10 µl 2 M

Tris acetate pH 7.5 with 20 µl 10 mg/ml. RNase I at 37°C for 30 min), diluted with 3 ml. 8 M urea, 0.0025 M Tris-HCl pH 7.5, 0.01 M NaCl, and adsorbed to 0.2 x 25 cm micro-granular DEAE cellulose (previously washed with 4 M NaCl, the H_2O, then equilibrated in 8 M urea 0.01 M NaCl 0.0025 M Tris acetate pH 7.5). After the sample had been washed into the column, a linear gradient from 0.01 M NaCl (150 ml.) to 0.25 M NaCl (150 ml.) (both containing 8 M urea 0.0025 M Tris acetate pH 7.5) with a flow rate of 0.075 ml./min was used to elute the oligonucleotides of increasing chain lengths. The A_{260} was monitored in a Gilford absorbancy monitor with a 2 mm path flow cell; approximately 1 ml. fractions were collected.

(xi) Counting procedures. Samples from DMSO gradients are TCA-precipitated on Whatman 3 mm filters essentially as described by Stone (1970) and counted in a Liquifluor (New England Nuclear-160 ml./8 pints)- toluene scintillation fluid; these filters are then counted in a Beckman LS200 counter. Filters from hybridization reactions are washed as in (viii) and counted under the same conditions. In all cases the blank cts./min are subtracted from the RF or ØX DNA filters. They usually average less than 0.1% the counts on the DNA filters.

^{32}P, ^{14}C, and ^{3}H overlap corrections were calculated by spotting high molecular weight labeled DNA or RNA on both types of filters and counting as above.

Spots from TLC plates were counted in the scintillation counter by first scraping the spots into a scintillation vial and adding 1 ml. H_2O or 10% NH_4OH, heating to 80°C for 10 min, then adding 10 ml. Bray's (Bray, 1960) scintillation fluid.

3. Results

(a) The size distribution of ØX mRNA

The size distribution of *in vivo* ØX mRNA as determined by sedimentation through 99% DMSO gradients of total *am*3-infected DNase-treated RNA (^{32}P pulsed at 4 or 25 min after infection-^{3}H uniformly labeled) is shown in Figure 1. The total pulse and mass size distributions are quite similar to those described in Sedat *et al*. (1969) for total RNA sedimentation through DMSO gradients. The ØX mRNA size distribution (pulse label) as determined by RF hybridization of each fraction is the same at both times after infection; however, the amount of mRNA in the 25 min sample is approximately ten times that of the 4 min sample. Both samples show a broad heterogeneous size distribution with a peak frequency at 3×10^5 daltons, a sharp upper size limit to

hybridizable RNA at 1.5-1.7 megadaltons, and significant hybridizable RNA to as small as 10^4 daltons. The hybridizable uniform-RNA radioactivity is only evident in the 25 min sample; although showing some scatter, it seems to approximate the pulse-label size distribution; this point is much clearer in other experiments (vide infra).

In a previous paper (Sedat et al., 1969) it was shown that the pulse-labeled mRNA could be fractionated (without degradation or selection) away from the 16 and 23S rRNA by use of benzoylated DEAE cellulose column chromatography at pH 7.5 and pH 3.5. This procedure has now been extended to show that very similar results can be obtained by a single chromatography (on benzoylated DEAE cellulose at pH 3.5) of the entire nucleic acid extract. The result of sedimentation through 99% DMSO gradients and subsequent RF hybridization of BC chromatographed ^{32}P-pulsed (at 4 and 25 min after infection), ^{3}H-uniformly labeled am3-infected RNA is shown in Figure 2.

The total pulse label is distributed similarly (neglecting the tRNA region) in the 4 and 25 min samples, and the size of the host mRNA is such that very little is larger than 2 megadaltons. The molecular weight of the most abundant species is approximately 10^5 daltons. If the total

pulse size distribution in Figure 2 is compared to the total size distribution in Figure 1, the former is shifted toward smaller molecular weight pieces. This difference seems to represent nascent rRNA which is selected out by the BC chromatography; it is more noticeable in longer pulses (E. coli HF4704 seems to have larger quantities of nascent rRNA in a 45 sec pulse than other cell strains used, such as E. coli MRE601 (cf. Sedat et al., 1969, Figure 6 (a) and (c)).

A large fraction (≈10%) of the pulse counts hybridize to the RF at 25 min after infection; approximately 1% of the total pulse counts hybridize at 4 min. The hybridized RNA in both samples distributes in a broad and continuous quite symmetrical distribution slightly ahead of the total pulse (host) distribution. There is an upper size limit to hybridized RNA in both cases at 1.7 megadaltons, with a maximum in the distribution at 2.6-2.7 x 10^5 daltons. The hybridized pulse counts in Figure 1 and Figure 2 compare very favorably, indicating further confidence in the purification of mRNA by BC chromatography.

The profile of the uniform label in the 4 and 25 min samples is indicated in Figure 2 (b) and (d). The total mass counts show a size distribution very similar to the total pulse size distribution in both cases (ignoring the

tRNA contributions). The hybridizable mass RNA counts distribute in both the 4 and 25 min samples from 1.7 megadaltons to 10^4 daltons parallel to the size distribution of the hybridizable pulse RNA. From the observed specific activity (over-all ratio of ^3H counts/A_{260}), the number of cells corresponding to the RNA on each gradient (assuming complete recovery of mRNA and complete hybridization of the ØX mRNA), and assuming that each point on the DMSO gradient corresponds to a unique size class, an approximate calculation of the number of ØX mRNA molecules per cell can be made. The 4 min RNA samples seems to have approximately 100 molecules/cell (equivalent in total mass to 6.1 rounds of transcription) while the 25 min RNA has approximately 1000 molecules/cell.

In an attempt to determine whether the size distribution of RF hybridizable mRNA might reflect the sum of many more discrete sizes of varying base compositions, the following experiment was done. One part of an am3-infected culture was pulse labeled with ^3H-guanine and uniformly labeled with ^{14}C-adenine (labeling equally the purines); the other half of the infected culture was pulse labeled with ^3H-adenine and uniformly labeled with ^{14}C-uracil (labeling equally the pyrimidines). Figures 3(a) and (b) are the results of

sedimentation through 99% DMSO of the ^{3}H-adenine pulse-^{14}C-uracil uniformly labeled RNA, and Figures 3(c) and (d) are the sedimentation results of the ^{3}H-guanine pulse-^{14}C-adenine uniformaly labeled RNA. The total pulse and hybridizable pulse size distributions are essentially identical to the distributions in Figure 1 and 2 with respect to maximum length, peak of hybridizable RNA, and the spread of the distribution. Similarly, the mass profiles show little difference from the mass size distribution in Figures 1 and 2; the same can be said for the calculated molecules/cell in both of these differently labeled RNA samples. These data are taken to mean that there is no obvious bias in the distribution for purine or pyrimidine base compositions.

Two representative mutants have been selected to study the size distribution under abnormal conditions. The first mutant studied was the ØX mutant op6, a very strongly polar mutant in Gene VII polar for Genes III and II (Benbow, Mayol & Sinsheimer, in preparation). The sedimentation of op6-infected ^{32}P pulse-^{3}H uniformly labeled BC chromatographed RNA is shown in Figures 4(a) and (b). Essentially no difference, except for slightly reduced amounts of hybridized pulse counts, is seen when compared to the normal am3 distributions.

Analogously, mRNA made upon infection of the bacterial mutant rep_3^-, restrictive for ØX and P2 replication (Denhardt, Dressler & Hathaway, 1967; Denhardt, Larison & Burgess, submitted for publication; Calendar, Lindqvist, Sironi & Clark, 1970) was studied. Figures4(c) and (d) represent the sedimentation results of the rep_3^- RNA (^{32}P pulse-^3H- uniformly labeled, BC chromatographed). Again, the size distribution pattern is identical to the am3 distribution; a slight reduction in the fraction of the pulse hybridized is the only difference seen.

(b) Controls

Most of the experiments described in section (a) of the Results rely on the assumption that RF-RNA hybridization is ØX infection specific and the mRNA hybridizes to the complementary strand of the RF. Figure 5 shows the sedimentation profile of RNA from uninfected and am3-infected (at 25 min after infection) ^{32}P pulse-, ^3H uniformly-labeled. There is little difference between the total pulse and mass size distributions of the two gradients, but a large difference between the hybridized pulse and mass distributions. The uninfected samples show that a small fraction of the total pulse counts hybridize to the RF; this is found around the 20-30,000 dalton size trailing off to larger sizes. However,

the (no DNA) blank filters show a similar increased background hybridization in the same size range; the hybridized pulse counts in Figure 5 represent only two or three times the background on the blank filters.

No mass counts from uninfected cells hybridize to the RF. The infected control shows results identical to that shown in Figures 1,2 and 3 and represents at least two orders of magnitude more counts hybridized than in the uninfected sample.

It was also desirable to test whether the label was hybridizing specifically to the complementary strand of the RF since previous work had shown that the complementary strand of the RF was the template for transcription (Hayashi, Hayashi & Spiegelman, 1963; Sedat et al., 1969). This has been studied at early and late times after infection by hybridizing am3-infected ^{32}P pulse-(at 4 and 25 min after infection), ^{3}H uniformly-labeled, BC chromatographed, and 99% DMSO sedimented RNA to ØX DNA filters. The results shown in Figure 6 demonstrate that a small fraction of the pulse counts hybridize to the ØX DNA in a pattern similar to that obtained for the uninfected RNA sample in Figure 5. Here also the blank filters adsorb nonspecifically the pulse counts (the ØX DNA filters have twice the counts of the

blanks) in the size range of 20-30,000 daltons. This result, taken with the uninfected cell experiment in Figure 5, demonstrates adequate hybridization specificity.

Another assumption upon which the validity of the ØX mRNA size distribution rests is that the heterogeneous distribution of sedimentation is composed of discrete size species of mRNA. Therefore, selected fractions from different regions of the distribution were ethanol precipitated and sedimented through fresh 99% DMSO gradients. Figures 7 (a) and (c) are large and small sized fractions from a ^{32}P pulse-, ^{3}H uniformly-labeled mRNA distribution described in Figure 2, while Figure 7(b) was the resedimentation result of a fraction taken from the peak of the distribution in Figure 3(c). The selected fractions (both pulse and mass) largely reran as in the original distributions indicating meaningful size distribution using the 99% DMSO system. Great care not to overload the DMSO gradient must be taken, however; concentration-dependent sedimentation results in DMSO gradients with slower moving distributions and a sizable fraction of the pulse counts remain under the large tRNA peak.

All 99% DMSO gradients show a small but detectable fraction ($\leq$5%) of the hybridizable (to RF) counts sedimenting ahead of the main peak; these are thought to be an

ill-understood artifact for the following reasons. First, this is not a centrifugal artifact such as an instability in the density gradient because reducing the time of centrifugation does not remove or reduce these counts; also, recovery of these counts and recentrifugation results in the counts resedimenting to the original fractions. Secondly, RNA from the main peak, upon resedimentation, results in a new fraction of rapidly sedimenting counts (see Fig. 7 (a), (b) and (c)). Thirdly, centrifugation of even alkali- or RNase-treated RNA in DMSO results in a fraction of the counts throughout the entire gradient. No procedure tested has removed or reduced these counts, and since they represent such a small fraction of the total counts, they have been neglected.

Appropriate controls have been performed to demonstrate that there is no DNA contamination of the RNA samples. No difference is seen in the size distribution of total and hybridizable pulse and mass counts of DNase-treated RNA or untreated RNA through DMSO gradients. There are no peaks (neglecting the top) of counts throughout the DMSO gradients if the RNA samples have been KOH- or RNase-treated. Furthermore, no optical absorbance (at 265 mμ) could be found in a CsCl equilibrium density gradient (mean density = 1.71) in

the analytical Model E ultracentrifuge. Less than 0.1% of the KOH-digested, hybridized and eluted RNA could be found in the origin on thin layer chromatograms.

Another critical assumption upon which all the size distributions rely is that the hybridization kinetics in DMSO are not a strong function of molecular size. In principle the observed size distribution could merely reflect a preference in the hybrid reaction for the smaller sizes of RNA. This assumption has been tested in the following way: An initial set of RF filters were placed in the RNA fractions from a DMSO gradient of Figure 3 and hybridized in 40% DMSO-2X SSC at 37°C for 48 hr (only 10% additional counts hybridize between 24 and 48 hr); the filters were removed and counted, and fresh RF filters added to the same vials and the hybridization reaction continued for an additional 60 hr. The results are shown in the left panel of Figure 3. Less than 30% of the initial counts hybridized in the first 48 hr hybridize during the second period. The pattern of size distribution is quite similar, with a slight preference the first time for the smaller sizes. In most cases the mass ratio of RF to RNA hybridized is greater than 100. We conclude that the variation of hybridization kinetics as a function of size does not significantly affect the size

distribution.

(c) Methylation

The data of Moore (1966) indicate that as a first approximation mRNA does not contain methylated bases. However, several methylation studies, using [^{3}H-methyl] or [^{14}C-methyl] L-methionine as a methyl donor in which the RNA was purified by BC chromatography indicated that methyl counts sedimented in parallel to the pulse or mass counts in a DMSO gradient. A short pulse of [^{3}H-methyl] methionine (3 min) resulted in a substantial, heterogeneous peak of radioactivity in the putative mRNA region of a DMSO gradient. Therefore, experiments were undertaken to ascertain whether ØX mRNA contained methyl groups. Figure 8 shows the results of sedimentation of [^{3}H-methyl] -labeled RNA from uninfected and am3-infected cells through 99% DMSO gradients, after isolation by BC chromatography. In both the uninfected and infected RNA samples [^{3}H-methyl] counts sediment in the region of mRNA; in the infected case [^{3}H-methyl] counts hybridize to the RF. The size distribution is quite similar to the other distributions of Figures 1-3.

In order to identify the methylated base (or ribose 2'-O-methyldinucleotides) the hybridized methyl-labeled RNA from the DMSO gradient of Figure 8 was eluted from the DNA

and hydrolyzed with acid. Figure 9 shows the result of thin layer two-dimensional chromatography of the acid hydrolysate. The majority (over 85%) of the methyl counts chromatograph as the pyrimidine mononucleotides, while another 5% is present in the purine bases. However, m^5 cytidylic acid or ribose 2'-O-methyl-dinucleotides would not separate clearly in this system.

In a second experiment, KOH hydrolysis of the fractions contained in the tRNA and mRNA regions of the gradient of Figure 8 followed by separation in a two-dimensional TLC chromatographic system that would separate all methylated nucleotides, again revealed substantial methyl label only in the pyrimidine rings. Thus no significant methyl counts in the hybridized RNA could be found in the methylated bases or nucleotides (or ribose 2'-O-methyl-dinucleotides). The sensitivity of the assay is such that a limit of methylation of one or two per molecule cannot be excluded.

In yet another attempt to find methylated bases in ØX mRNA, the pyrimidine- and purine-labeled mRNA hybridized to RF in Figure 3 was eluted and hydrolyzed with KOH as described in Materials and Methods. The products were separated in the TLC system II which separates well all the methylated nucleotides (and ribose 2'-O-methyl-dinucleotides)

from the four major nucleotides. No minor spots were found even though 0.1% could have been detected. Results using large amounts of ^{32}P-labeled, KOH-hydrolyzed, mRNA eluted from RF hybridizations indicate the same results.

Therefore, we conclude that ØX mRNA is not detectably methylated.

(d) Search for 5' end group

The 5' end groups of *in vivo* total nascent RNA have been shown to be pppGp and pppAp (Jorgensen *et al.*, 1969). It would be of interest to see if after KOH hydrolysis the ØX mRNA eluted from RF hybridization contained ^{32}P pulse radioactivity chromatographing with carrier tetranucleotides. The sensitivity of detection can be maximized in a short ^{32}P pulse since the radioactivity is preferentially located in the β and γ positions of the 5' triphosphates (Bolton & Roberts, 1964).

4×10^7 ^{32}P counts/min of such eluted ØX mRNA was combined with carrier tRNA, hydrolyzed with KOH, desalted on a small Dowex 50W-X8 column, and combined with carrier oligonucleotides (prepared by RNase I digestion of tRNA). The mixture was then chromatographed on a DEAE cellulose column in 8 M urea as described in Materials and Methods. Figure 10(a) is the result of such a chromatographic separation. Approximately 0.5-0.6% of the applied ^{32}P counts

chromatograph as dinucleotides or larger oligonucleotides. However, the ^{32}P counts do not parallel the carrier A_{260} but rather form a broad trailing elution pattern. The fractions containing the dinucleotides through the pentanucleotides were pooled, concentrated on a small DEAE cellulose column, and desalted by elution with triethylammonium bicarbonate as described in Razin, Sedat & Sinsheimer (submitted for publication). These fractions were rechromatographed as described above. An essentially identical elution pattern resulted as shown in Figure 10(b). Less than 0.01% of the counts appear in the tetranucleotides (peak IV) and can be counted as 5' termini. The ^{32}P counts in peaks II, III, and IV were pooled, desalted by adsorption on to charcoal and elution, and chromatographed in System II on TLC plates (which should separate pXp, ppXp, pppXp; X= Pu or PY). Many different spots appeared, none of which chromatographed where the 5' triphosphate nucleotide 3' monophosphate should have migrated. All the ^{32}P in the pooled material was sensitive to treatment with alkaline phosphatase, indicating absence of ribose 2'-O-methyl-oligonucleotides.

An alternative method to search for 5' termini was to hydrolyze ØX mRNA, heavily ^{32}P pulse-labeled, that had been

hybridized and eluted twice, with KOH, and then to chromatograph the digest on TLC in System II directly. Figure 11 is the result of such an experiment. No obvious spot of radioactivity in the region of the 5' triphosphate nucleoside 3' monophosphate is seen in the 24 hr exposure. (One 5'-triphosphate terminal per 100 mRNA molecules of the mean mRNA molecular weight could have been detected).

We do not have an explanation of the ^{32}P components which chromatograph as oligonucleotides; it is puzzling that there appear to be discrete components, some of which parallel the carrier peaks. Conceivably, some of these represent 5' monophosphate nucleotide 3' monophosphate terminal groups which are a result of the mRNA 5' → 3' degradation system (Morse, Mosteller, Baker & Yanofsky, 1969).

The results of these experiments suggest that the in vivo ØX mRNA molecules very possibly do not have 5' triphosphate termini. However, a definitive answer to this question will have to wait until in vivo and in vitro ØX mRNA can be compared in two-dimensional finger-print nucleotide maps (Sanger, Brownlee & Barrell, 1965).

4. Discussion

(a) The size of the ØX mRNA

The experiments presented in the Results section on

controls indicate that the experimental conditions are sufficiently rigorous to provide a valid mRNA size distribution. Sedimentation through 99% DMSO results in a straight line plot of the log of the molecular weight vs. fraction number of the gradient, and selected points in a distribution resediment in a fresh 99% DMSO gradient in their original molecular weight position; this is taken to mean that sedimentation through 99% DMSO is adequate to define the size of the mRNA. The hybridization technique in 40% DMSO - 2X SSC (37°C) has adequate specificity as indicated by the observations that the amount of mRNA hybridizing to RF is at least two orders of magnitude greater from am3-infected cells than from uninfected cells, or than the amount of mRNA hybridizing to ØX viral DNA. The hybridization reaction is not a strong function of the size of mRNA, and most of the added mRNA is hybridized to the RF filters at the termination of the reaction.

The mean size distribution of uninfected cell, ØX-infected cell, and ØX-specific mRNA in DMSO gradients appears to be rather small. The peak of the E. coli mRNA distribution is at $\tilde{\sim}$ 320 nucleotides while that of the corresponding ØX mRNA is at 950 nucleotides. There is, of course, a distinct possibility of selective mRNA (but not rRNA) degradation

during the lysis of the cell. If selective degradation is occurring it must be severe (multiple hits) since all the mRNA distributions show a smooth decrease in concentration as the size reaches the maximum length. The following lines of evidence support the hypothesis that selective degradation is not a significant factor: (1) the use of RNase I^- mutants does not change the size distributions nor does the addition during lysis of agents that bind nucleases, such as bentonite; (2) upon the addition of purified rRNA (23 S) to the cells before lysis and analysis of the RNA extract by acrylamide gel electrophoresis no degradation of the added marker could be observed (D. McMahon, personal communication); (c) a large fraction ($\geq$ 50%) of the functional (T4 lysozyme) specific mRNA (Salser, Gesteland & Bolle, 1967) can be recovered by these methods. These data suggest that in a qualitative sense selective degradation of mRNA is not severe; however it cannot be rigorously excluded.

A more attractive explanation for the size distribution is the coupled transcription and degradation of mRNA as proposed by Morse et al.(1969) to explain degradation of the 5' end of the tryptophan mRNA before the completion of the 3' end; this model proposes that very little, if any, full length mRNA should be present in the cell. Qualitatively,

the data on the size distribution of ØX mRNA fit the model. The mechanisms that lead to such degradation must be present at 4 min after infection and act similarly at 25 min after infection. Since the shapes of the total E. coli mRNA distribution are identical to the distribution shapes of the ØX mRNA, this could mean that the degradation mechanism that acts on the E. coli acts similarly on the ØX mRNA.

Quantitatively, the data suggest that there must be a probability rather than a certainty of degradation to explain the finding of a few full length molecules of ØX mRNA.

Proof by hybridization for the transcription-coupled model of ØX mRNA degradation is precluded due to the small amounts of mRNA present in the DMSO gradient, making competition or saturation experiments very difficult. However, the base composition of the ØX mRNA does not vary across the DMSO gradient.

The maximal length of ØX mRNA found is 1.7 megadaltons which represents within experimental error (at early and late times after infection) one full genome length polycistronic mRNA. No conclusions can be drawn as to whether a small portion of the ØX RF is not transcribed.

(b) ØX temporal control

The similarity of the size distributions at early and

late times after ØX infection seems to indicate that there is no temporal control at the transcriptive level. This is further substantiated by the analysis of viral-specific protein synthesis in ØX-infected cells (Mayol & Sinsheimer, submitted for publication); all ØX proteins are made in the same ratios at early and late times after infection. The large fraction of the total mRNA specific for ØX ($\approx$ 10%) at 15-25 min is also in agreement with the analyses of ØX-specific protein synthesis. The absence of temporal control or the shut-off of host mRNA is taken to mean that there probably is no de novo synthesis of a viral "sigma factor" for the E. coli RNA polymerase (Burgess, Travers, Dunn & Bautz, 1969) such as occurs with T7 (Summers & Siegel, 1969) and T4 (Bautz, Bautz & Dunn, 1969; Travers, 1969).

At 4 min after infection the maximum length of ØX mRNA is already 1.7 megadaltons, as large as the maximum length RNA at 25 min after infection. This implies that the rate of transcription at 37°C is at least 19.3 nucleotides/sec (5500 nucleotides [one complete mRNA] /4.75 min). This is consistent with the propagation rate of the Tryp operon (18-20 nucleotides/sec at 37°C, Imamoto, 1968; Baker & Yanofsky, 1968), T4 "early" mRNA (28 nucleotides/sec at 37°C, Bremer & Yuan, 1968) and uninfected cells (45 nucleotides/sec

at 37°C, Manor, Goodman & Stent, 1969).

At 4 min of the ØX infection cycle, only one (or at most a few) RF molecule exists (Sinsheimer, 1968), and from the work of Knippers & Sinsheimer (1968) and Salivar & Sinsheimer (1969) this resides on the membrane fraction of the cell. Since the ØX mRNA is made at this time, the parental RF must serve as the template for transcription. Transcription of this template must be configurationally complicated because the parental RF(s) is undergoing simultaneous replication (Knippers & Sinsheimer, 1968), and there is very likely a 20-fold difference in the specific rate of DNA synthesis as compared to the rate of transcription.

Conceivably, there is an alternation of replication and transcription, and such an alternation might be used to explain the increased transcription at 15-25 min concomitant with the decrease of RF replication (Lindqvist & Sinsheimer, 1967; Stone, 1970). Similarly, ØX or S13 mutants in Gene VI which do not replicate beyond the formation of the parental RF (hence do not compete with transcription) make normal or increased amounts of ØX proteins (in the same proportions as wild type ØX) (Burgess & Denhardt, 1969; Gelfand & Hayashi, 1969; Schleser, Tessman & Casaday, 1969; Mayol & Sinsheimer, submitted for publication). Likewise, the ØX replication

bacterial mutant rep_3^- has only one RF/cell (Denhardt et al., 1967) yet has from the data in the Results a near normal mRNA size distribution and yield. However, we cannot say, as yet, whether the parental RF is the sole template in normal ØX infection.

(c) The methylation of ØX mRNA

The results of the methylation studies indicated that no detectable methylated bases (or ribose 2'-O-methyl-dinucleotide) could be found in the ØX mRNA either by direct labeling with [^{3}H-methyl] methionine or by examination of minor spots in a two-dimensional thin layer chromatographic system that separated methylated nucleotides or bases from the parent heavily labeled nucleotides or bases. The absence of ribose 2'-O-methyl-dinucleotides was confirmed by the complete hydrolysis of ^{32}P counts by alkaline phosphomonesterase after KOH digestion of the ØX mRNA. We cannot eliminate the possibility that there are one or two methyl groups per molecule in the ØX mRNA.

(d) Modulation of translation

The intracellular proteins of ØX seem to be present in nonstoichiometric amounts with the gene products of cistrons V and VII present in the largest molar amounts (Mayol & Sinsheimer, in preparation). If the ØX mRNA is transcribed

as a polycistronic message, then some form of translation modulation must take place, possibly analogous to the translation of MS2 RNA (Cf. Kaernen, 1969). In contrast, the gene products of the tryp operon (Morse, Baker & Yanofsky, 1968), lac operon (Kepes, 1967; Brown, Brown & Zabin, 1967), gal operon (Michaelis & Starlinger, 1967; Wilson & Hogness, 1969) and his operon (Whitefield, Gutnick, Margolies, Martin, Rechler & Voll, 1970) seem to be made in stoichiometric amounts except for some cases when the first gene product is made in slightly larger amounts, (see Whitefield et al., 1970). This could be a reflection of internal promoters analogous to the tryp operon (Morse & Yanofsky, 1968), or a basic difference between phage and E. coli mRNA structure or metabolism.

(e) The mRNA from the ØX polar op6

The infection of E. coli with the strong polar ØX mutant op6 leads to the identical mRNA size distribution and yield as obtained in the am3 infection. This result is in contrast to that obtained from strong polar amber, ochre, and frameshift mutations in the tryp operon (Cf. Zipser, 1969; Morse & Primakoff, 1970), but is consistent with current data on the lack of polar effect on mRNA size in T4 (D. McMahon, personal communication). Thus these results might reflect

differences between phage and E. coli mRNA or differences between the translation of opal, ochre, and amber nonsense codons.

(f) The mRNA from rep_3^- cells

The molecular size distribution of ØX mRNA upon infection of the E. coli rep_3^- is identical to that in E. coli C indicating that all ØX genes are transcribed in agreement with the recent protein results of Burgess et al. (submitted for publication). The fraction of the total pulse counts hybridized is only one-tenth ($\approx$ 1%) that usually hybridized in the am3 case ($\approx$ 10%); however, from the hybridization of mass label the ØX mRNA molecules per cell are approximately equal to result attained with am3; we have no ready explanation for this difference. Thus the block is probably not related to transcription or translation of the single RF.

(g) The purity of mRNA isolated by benzoylated DEAE cellulose chromatography

In a previous paper (Sedat et al., 1969) data were presented which indicated that BC chromatography of a nucleic acid extract resulted in specific purification of the E. coli pulse-labeled RNA and that there was neither selection nor degradation during the procedure. The data in this paper extend this observation for a specific messenger RNA. It

appears that no selection in size or yield takes place during the BC chromatography for ØX mRNA.

That ØX mRNA elutes together with the E. coli mRNA without apparent selection implies that both mRNAs have a common feature, structural or chemical, which permits them to act as a group during fractionation. ØX mRNA might be a good representative messenger with which to study the basis of this difference of mRNA's.

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Footnote

Abbreviations used: BC (benzoylated DEAE-cellulose); DMSO (dimethyl sulfoxide); SDS (sodium dodecyl sulfate); RF (replicative form); m_2^6Ade (dimethyladenine); m^1Cyt (1-methylcytosine); m^5Cyt (5-methyl-cytosine); m^6Ade (6-methyl-adenine); Thy (thymine); Cyt (cytosine); m^2Ade (2-methyl-adenine); m^1Ade (1-methyladenine); m^7Ade (7-methyladenine); m_2^6Gua (dimethylguanine); Ade (adenine); Gua (1-methylguanine); Hm Ura (hydroxymethyluracil); m^3Gua (3-methylguanine); m^7gua (7-methylguanine); Gua (guanine); TFA (trifluoro-acetic acid); SSC (sodium chloride (0.15 M)- sodium citrate (0.015 M)).

References

Asano, K. (1965). J. Mol. Biol. 14, 71.

Attardi, G., Naono, S., Rouvière, J., Jacob, F. & Gros, F. (1963). Cold Spring Harbor Symp. Quant. Biol. 28,363.

Baker, R.F. & Yanofsky, C. (1968). Proc. Nat. Acad. Wash. 60, 313.

Bautz, E.K.F., Bautz, F.A. & Dunn, J.J. (1969). Nature 223, 1022.

Bolton, E.T. & Roberts, R.B. (1964). In Studies of Macromolecular Biosynthesis, ed. R.B. Roberts (Carnegie Inst. of Wash. Publ. 624, 61.

Bray, G.A. (1960). Analyt. Biochem. 1, 279.

Bremer, H. & Yuan,D. (1969). J. Mol. Biol. 38, 162.

Brown, J.C., Brown, D.M. & Zabin, I. (1967). J. Biol. Chem. 242,4254.

Burgess, A.B. & Denhardt, D.T. (1969). J. Mol. Biol. 44,377.

Burgess, R.R., Travers, A.A., Dunn, J.J. & Bautz, E.K.F. (1969). Nature 221, 43.

Calendar, R., Lindqvist, B.H., Sironi, G. & Clark, A.T. (1970). Virology 40,72.

Denhardt, D.T., Dressler, D.H. & Hathaway, A. (1967). Proc. Nat. Acad. Wash. 57,813.

Gelfand, D.H. & Hayashi, M. (1969). J. Mol. Biol. 44,501.

Hayashi, M.N. & Hayashi, M. (1966). Proc. Nat. Acad. Sci. Wash. 55, 635.

Hayashi, M.N., Hayashi, M. & Spiegelman, S. (1963). Proc. Nat. Acad. Sci. Wash. 50,664.

Hayashi, Y., Osawa, S. & Miura, K. (1966). Biochim. Biophys. Acta 129, 519.

Hayes, P.H., Hayes, F. & Guerin, M.F. (1966). J. Mol. Biol. 18, 494.

Hutchison, C.A. (III). (1969). Thesis. Calif. Inst. of Tech., Pasadena, Calif.

Imamoto, F. (1968). Proc. Nat. Acad. Sci. Wash. 60,305.

Imamoto, F. & Yanofsky, C. (1967). J. Mol. Biol. 28,1.

Isakson, L.A. & Phillips, J.M. (1968). Biochim. Biophys. Acta 155, 63.

Jacob, E. & Hofschneider, P.H. (1969). J. Mol. Biol. 46,359.

Jorgensen, S.E., Buch, L.B. & Nierlich, D.P. (1969) Science 164, 1067.

Kepes, A. (1967). Biochim. Biophys. Acta 138,107.

Komano, T. & Sinsheimer, R.L. (1968). Biochim. Biophys. Acta 155,295.

Knippers, R. & Sinsheimer, R.L. (1968). J. Mol. Biol. 34,17.

Knippers, R., Razin, A., Davis, R. & Sinsheimer, R.L. (1969). J. Mol. Biol. 45, 237.

Knippers, R., Whalley, M. & Sinsheimer, R.L. (1969). Proc. Nat. Acad. Sci. Wash. 64, 275.

Kaerner, H.C. (1969). J. Mol. Biol. 42, 259.

Kourilsky, P., Marcaud, C., Sheldrick, P., Luzzati, D. & Gros, F. (1968). Proc. Nat. Acad. Sci. Wash. 61,1013.

Krane, S.G. (1965). Thesis. Calif. Inst. of Tech., Pasadena, Calif.

Levine, A.J. & Sinsheimer, R.L. (1969). J. Mol. Biol. 35, 655.

Lindqvist, B.H. & Sinsheimer, R.L. (1967). J. Mol. Biol. 28, 87.

Manor, H., Goodman, D. & Stent, G.S. (1969) J. Mol. Biol. 39, 1.

Michaelis, G. & Starlinger, P. (1967). Mol. Gen. Genet. 100,210.

Moore, P.B. (1966). J. Mol. Biol. 18, 38.

Morikawa, N. & Imamoto, F. (1969). Nature 223, 37.

Morse, D.E., Baker, R.F. & Yanofsky, C. (1968). Proc. Nat. Acad. Sci. Wash. 60, 1428.

Morse, D.E. & Yanofsky, C. (1968). J. Mol. Biol. 38, 447.

Morse, D.E., Mosteller, R., Baker, R.F. & Yanofsky, C. (1969). Nature 223, 40.

Morse, D.E. & Primakoff, P. (1970). Nature 226, 28.

Salivar, W.D. & Sinsheimer, R.L. (1969). J. Mol. Biol. 48, 39.

Salser, W., Gestland, R.F. & Bolle, A. (1967). Nature 215, 588.

Salser, W., Gestland, R.F. & Ricard, B. (1969). Cold Spr. Harb. Symp. Quant. Biol. 34, 771.

Sanger, F., Brownlee, G.G. & Barrell, B.G. (1965). J. Mol. Biol. 13, 373.

Schleser, R., Tessman, E.S. & Casaday, G. (1969). Virology 38, 166.

Sedat, J., Lyon, A. & Sinsheimer, R.L. (1969). J. Mol. Biol. 44, 415.

Sinsheimer, R.L. (1968). Prog. Nuc. Acid Res. and Mol. Biol. 8, 115.

Stone, A.B. (1970). J. Mol. Biol. 47, 215.

Strauss, J.H., Jr., Kelly, R.B. & Sinsheimer, R.L. (1968). Biopolymers 6, 793.

Summers, W.C. & Siegel, R.B. (1969). Nature 223, 1111.

Tessman, I., Kumar, S. & Tessman, E.S. (1967). Science 158. 267.

Tessman, E.S. (1966). J. Mol. Biol. 17, 218.

Travers, A.A. (1969). Nature 223, 1107.

Warnaar, S.O., DeMol, A., Mulder, G., Abrahams, P.U. & Cohen, J.A. (1970). Biochim. Biophys. Acta 199, 340.

Whitefield, H.J., Jr., Gutnick, D.L., Margolies, M.N., Martin, R.G., Rechler, M.M., & Voll, M.J. (1970). J. Mol. Biol. 49, 245.

Wilson, D.B. & Hogness, D.S. (1969). J. Biol. Chem. 244, 2143.

Yarus, M. & Sinsheimer, R.L. (1967). J. Virology 1, 135.

Zipser, D. (1969). Nature 221, 21.

Figure Legends

Fig. 1. The sedimentation of ^{32}P pulse- (at 4 and 24 min after ØX infection), ^{3}H uniformly-labeled, DNase-treated RNA extracts through 99% DMSO. The upper panels represent the pulse components; the bottom panels represent the mass components.

●---● ^{32}P (pulse) cts/min

o---o ^{3}H (mass) cts/min

x---x ^{32}P (pulse_ cts/min hybridized to RF

▲---▲ ^{3}H (mass) cts/min hybridized to RF

Fig. 2. The sedimentation of ^{32}P pulse- (at 4 and 25 min after ØX infection), ^{3}H uniformly-labeled (DNase-treated and chromatographed on BC) RNA extracts through 99% DMSO. The upper panels represent the pulse components; the bottom panels represent the mass components.

●---● ^{32}P (total pulse) cts/min

o---o ^{3}H (total mass) cts/min

□---□ the calculated molecules/cell

x---x ^{32}P (pulse) cts/min hybridized to RF

Δ---Δ ^{3}H (mass) cts/min hybridized to RF

Fig. 3. The sedimentation of ^{3}H adenine pulse-, ^{14}C uracil uniformly-labeled and ^{3}H guanine pulse-, ^{14}C adenine uniformly-labeled (chromatographed on BC) RNA through 99% DMSO.

The left side represents the ^{3}H adenine pulse-, ^{14}C uracil uniformly-labeled RNA, and the right side represents the ^{3}H guanine pulse-, ^{14}C adenine uniformly-labeled RNA. The upper panels represent the pulse components/the bottom panels represent the mass components.

●---● ^{3}H (total pulse) cts/min

o---o ^{14}C (total mass) cts/min

□---□ the calculated molecules/cell

▣---▣ ^{3}H (pulse) cts/min hybridized to RF during a second exposure

⊗---⊗ ^{3}H (mass) cts/min hybridized to RF during a second exposure

x---x ^{3}H (pulse) cts/min hybridized to RF

Δ---Δ ^{14}C (mass) cts/min hybridized to RF

Fig. 4. The sedimentation of ^{32}P pulse-, ^{3}H uniformly-labeled op6 and rep_3^- (BC chromatographed) RNA through 99% DMSO. The left side (A and B) represent the op6 RNA. The right side (C and D) represent the rep_3^- RNA.

●---● ^{32}P (total pulse) cts/min

o---o ^{3}H (total mass) cts/min

□---□ the calculated molecules/cell

x---x ^{32}P (pulse) cts/min hybridized to RF

Δ---Δ ^{3}H (mass) cts/min hybridized to RF

Fig. 5. The sedimentation of ^{32}P pulse-, ^{3}H uniformly-labeled uninfected and ØX infected (pulsed at 25 min after infection) (chromatographed on BC) RNA through 99% DMSO. The upper panels represent the pulse components while the bottom panels represent the mass components.

●---● ^{32}P (total pulse) cts/min

o---o ^{3}H (total mass) cts/min

x---x ^{32}P (pulse) cts/min hybridized to RF

Δ---Δ ^{3}H (mass) cts/min hybridized to RF

Fig. 6. The sedimentation of ^{32}P pulse- (at 4 and 25 min after ØX infection), ^{3}H uniformly-labeled (DNase treated and BC chromatographed) RNA through 99% DMSO and subsequent hybridization to ØX DNA. The upper panels represent the pulse components while the bottom panels represent the mass components.

●---● ^{32}P (total pulse) cts/min

o---o ^{3}H (total mass) cts/min

x---x ^{32}P (pulse) hybridized to ØX DNA

Δ---Δ ^{3}H (mass) hybridized to ØX DNA

Fig. 7. The resedimentation of selected size fractions from previous 99% DMSO gradients. RNA fractions from previous DMSO gradients were ethanol-precipitated and applied to fresh 99% DMSO gradients.

(a) Fraction 17 (representing RNA 1 megadalton in size) was taken from the DMSO gradient in Fig. 2 (C and D).

(b) Fraction 25 (representing RNA 10^5 daltons in size) was taken from the DMSO gradient in Fig. 3 (A and B).

(c) Fraction 35 (representing RNA 10^4 daltons in size) was taken from the DMSO gradient in Fig. 2 (C and D).

●---● ^{32}P (pulse) cts/min

o---o ^{3}H (mass) cts/min

Fig. 8. The sedimentation of [^{3}H-methyl] uniformly-labeled uninfected and ØX infected (chromatographed on BC) RNA through 99% DMSO.

●---● ^{3}H (total) cts/min

x---x ^{3}H cts/min hybridized to RF

Fig. 9. Two dimensional thin layer separation of methylated bases from a TFA hydrolysis of [^{3}H-methyl] labeled ØX mRNA. The TLC plate was developed in System I as described in the Materials and Methods.

Fig. 10. The chromatography on DEAE cellulose of KOH hydrolyzed ØX mRNA (^{32}P pulse-, ^{3}H uniformly-labeled) as described in the Materials and Methods.

o---o A_{260}

●---● ^{32}P cts/min -100 ul.

Fig. 11. Two dimensional thin layer separation of KOH digested, ^{32}P pulse-, ^{3}H uniformly-labeled ØX mRNA that had been twice hybridized to RF and eluted. The TLC plate was developed in System II as described in the Materials and Methods. 5'-triphosphate termini would be in the left 1/3 of the TLC plate.

Figure 1

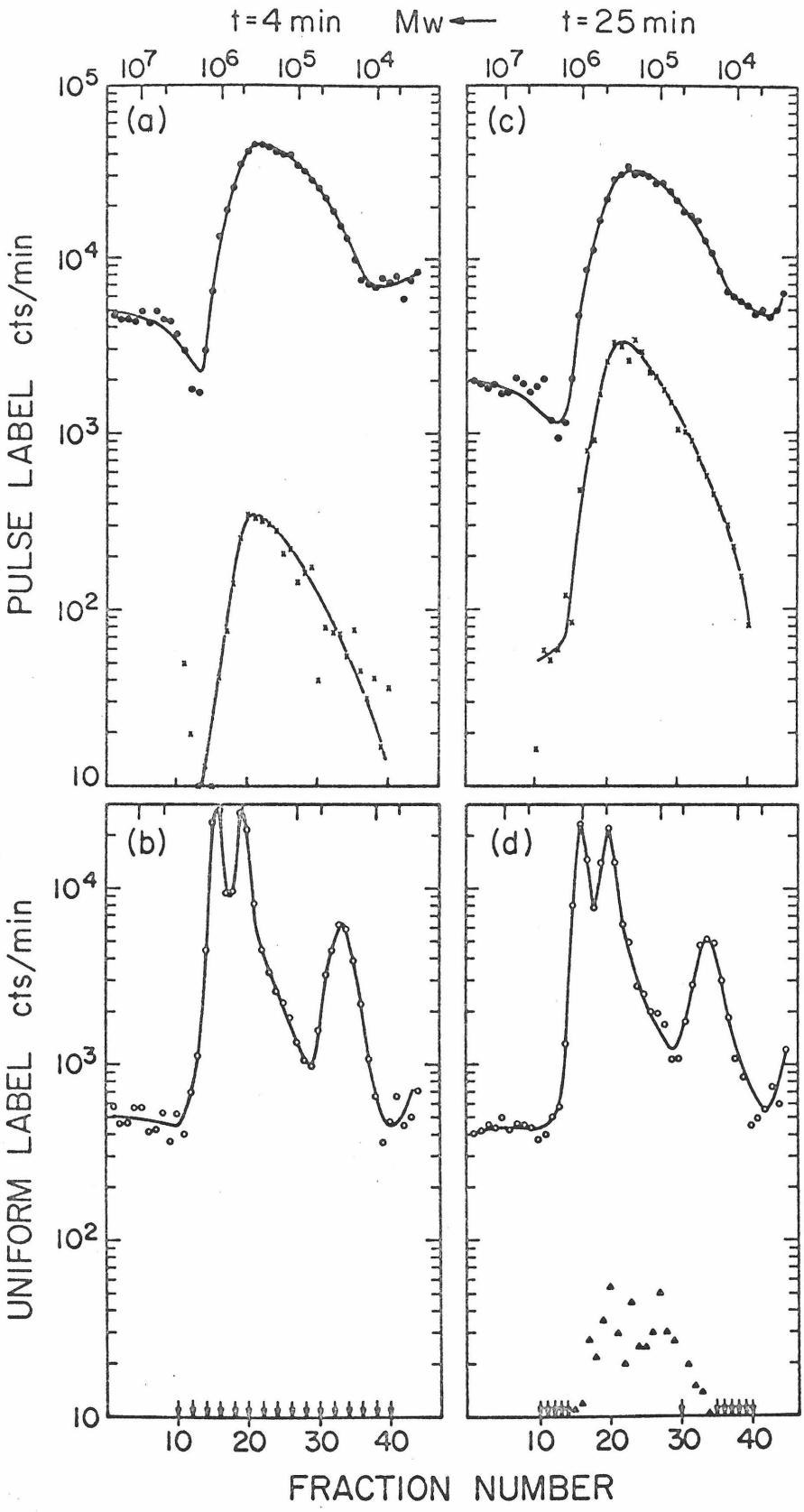
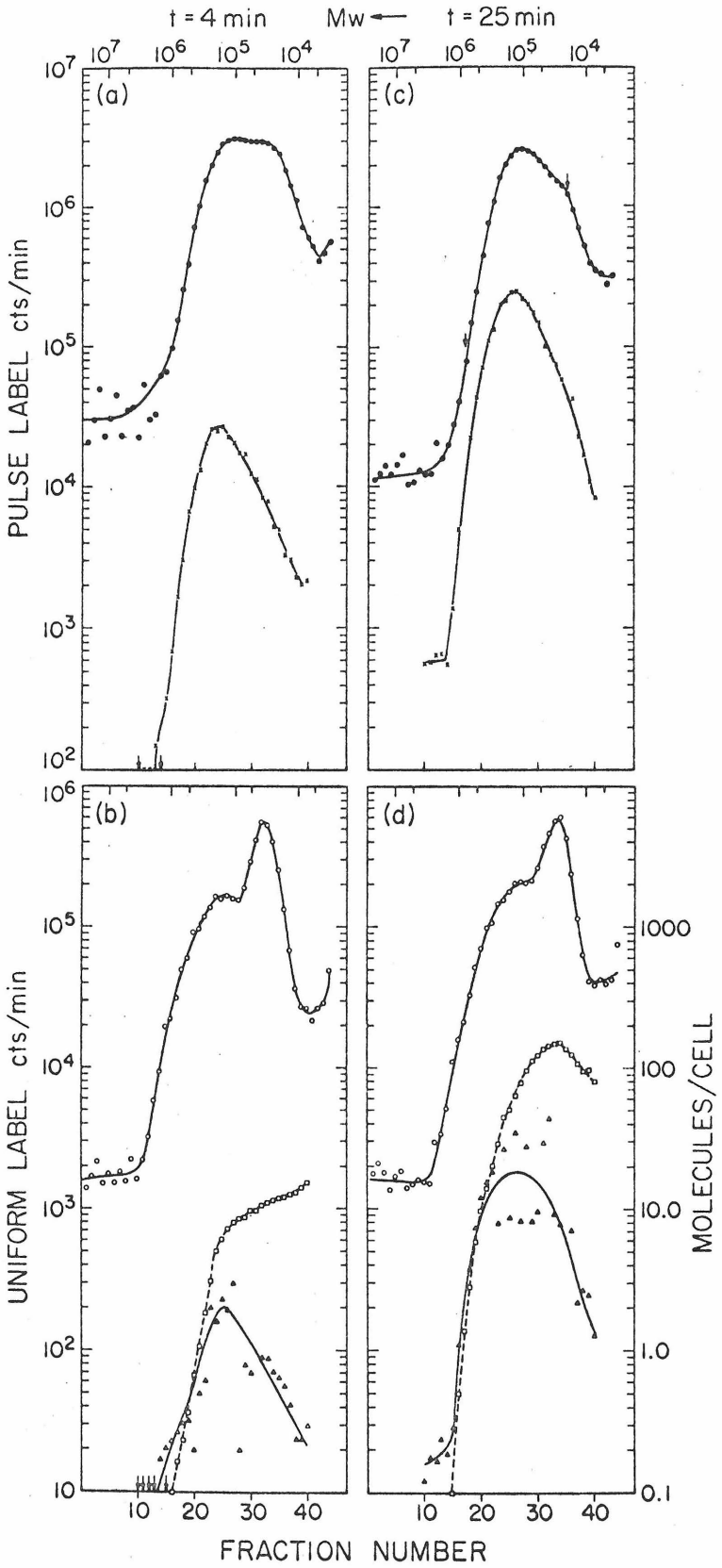

t=4 min
Mw
t=25 min
10^7
10^6
10^5
10^4
(a)
(c)
(b)
(d)
PULSE LABEL cts/min
UNIFORM LABEL cts/min
10^5
10^4
10^3
10^2
10
10
20
30
40
FRACTION NUMBER

Figure 2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Mw ←
t = 25 min
10^7
10^6
10^5
10^4
(a)
(c)
(b)
(d)
PULSE LABEL cts/min
UNIFORM LABEL cts/min
MOLECULES/CELL
1000
100
10.0
1.0
0.1
10
20
30
40
FRACTION NUMBER

Figure 3

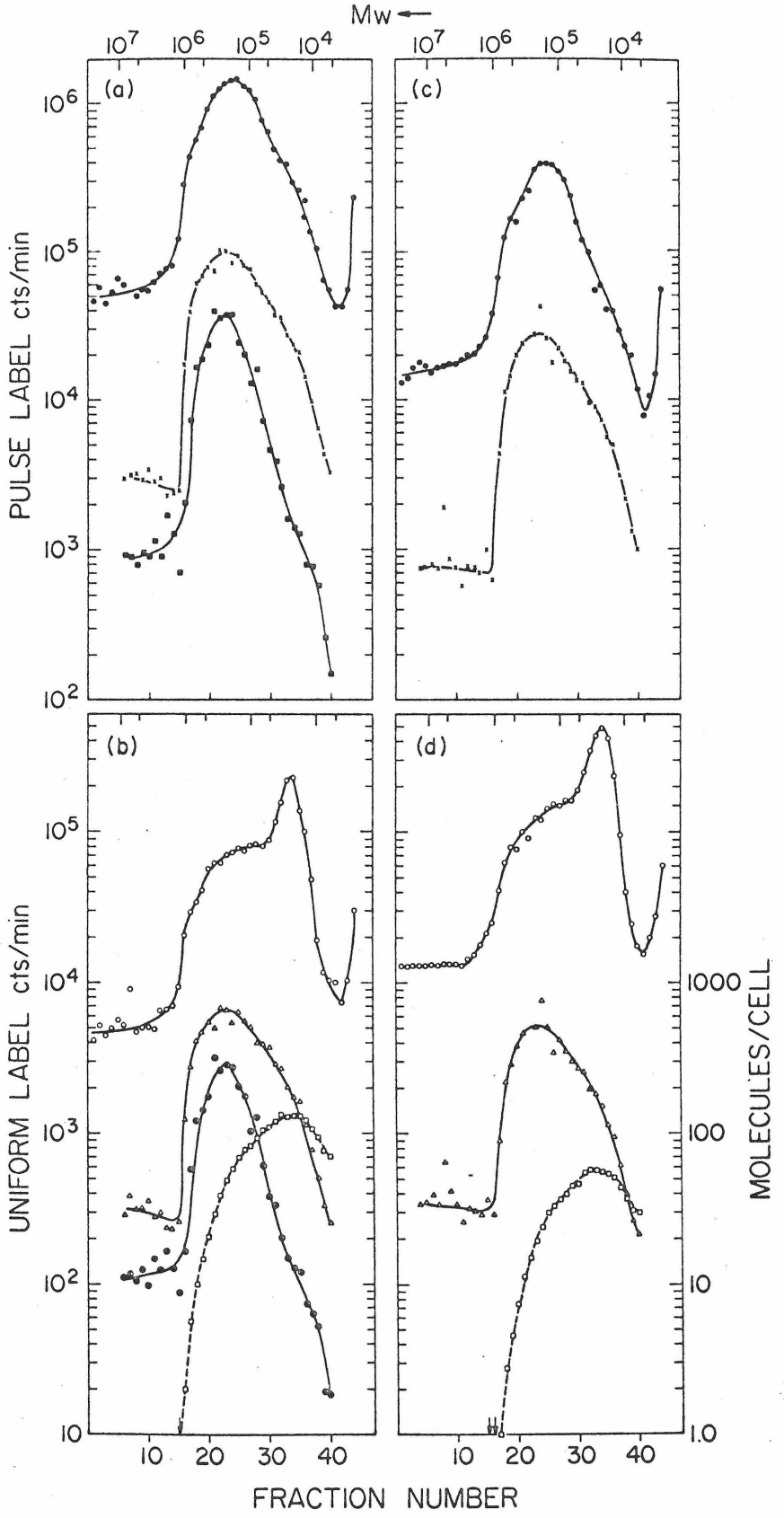

Mw ←
10^7 10^6 10^5 10^4
(a)
(c)
(b)
(d)
PULSE LABEL cts/min
UNIFORM LABEL cts/min
10^6 10^5 10^4 10^3 10^2
10^5 10^4 10^3 10^2 10
1000 100 10 1.0
MOLECULES/CELL
10 20 30 40
FRACTION NUMBER

Figure 4

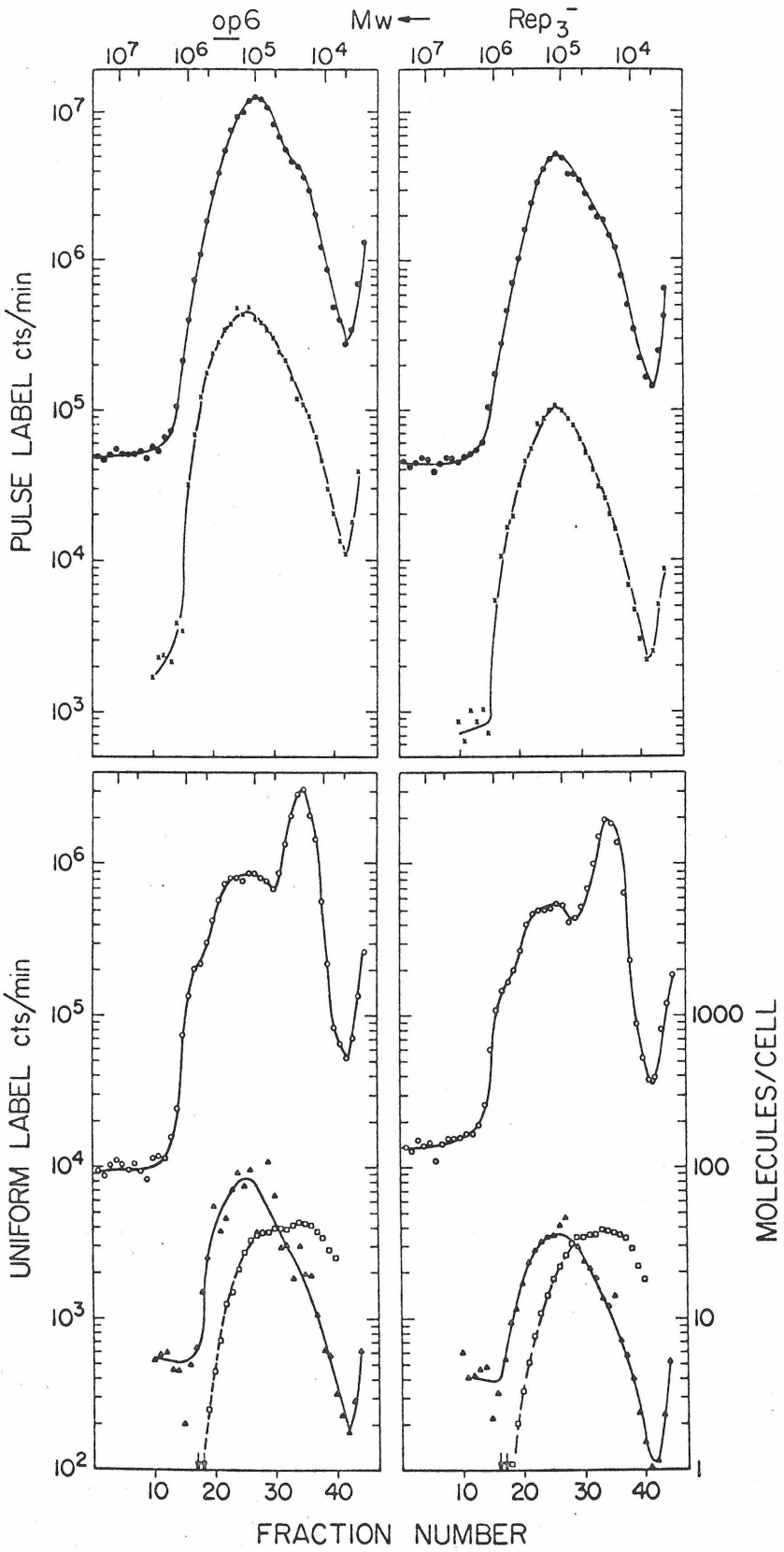

op6
Mw ←
Rep3⁻
10⁷ 10⁶ 10⁵ 10⁴
PULSE LABEL cts/min
UNIFORM LABEL cts/min
MOLECULES/CELL
1000
100
10
1
10 20 30 40
FRACTION NUMBER

Figure 5

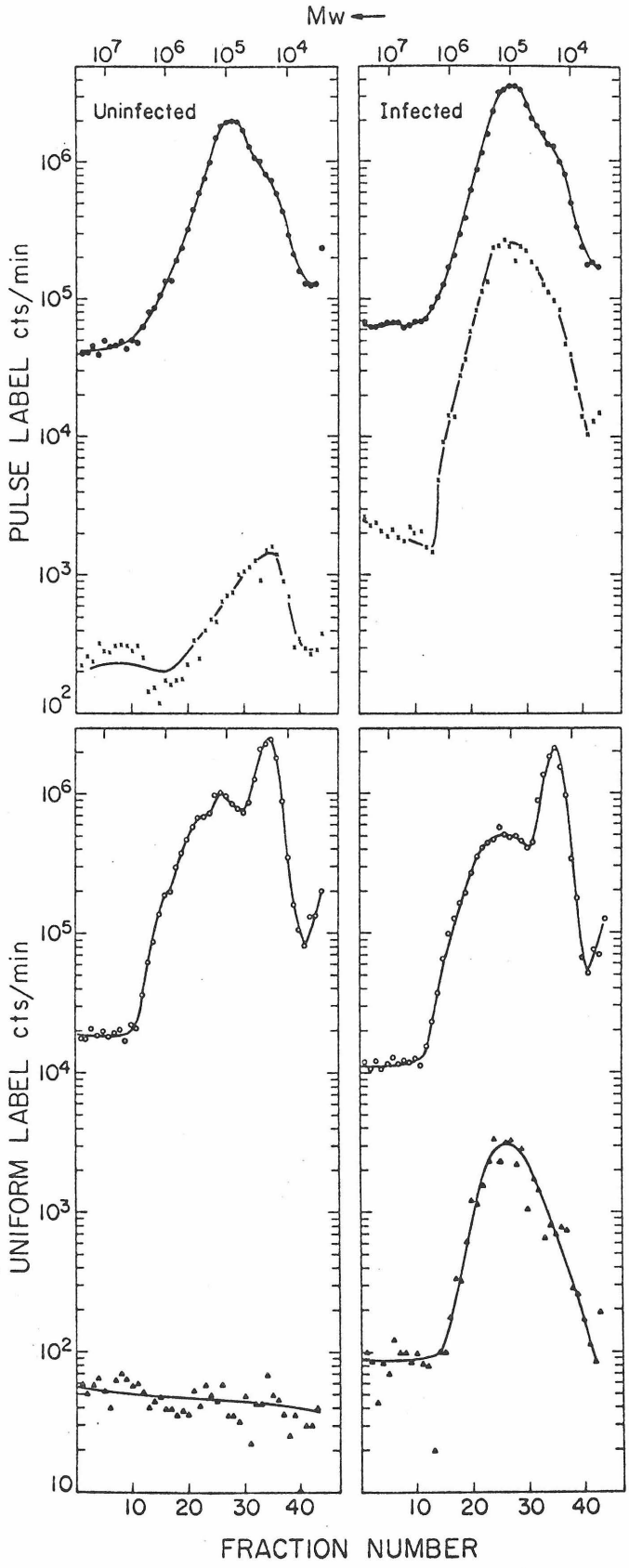

Mw ←
10^7 10^6 10^5 10^4
Uninfected
Infected
PULSE LABEL cts/min
UNIFORM LABEL cts/min
10^6
10^5
10^4
10^3
10^2
10
10 20 30 40
FRACTION NUMBER

Figure 6

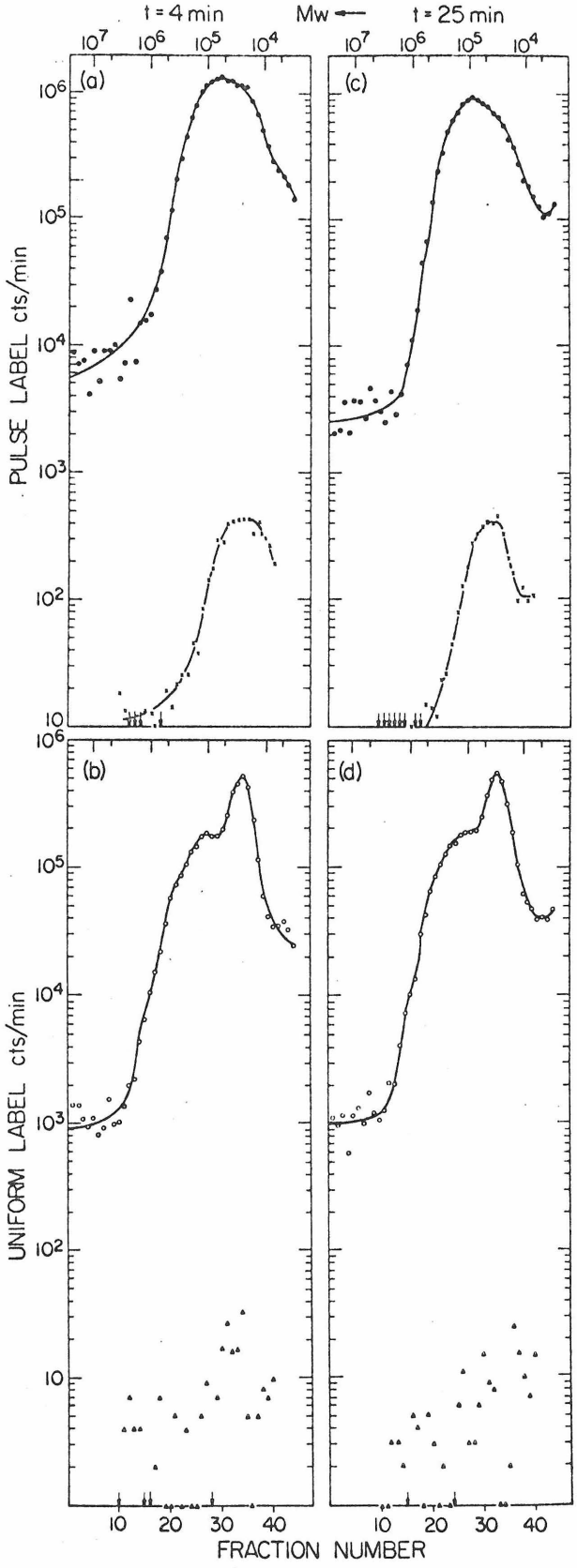

t = 4 min
Mw ←
t = 25 min
10^7
10^6
10^5
10^4
(a)
(c)
(b)
(d)
PULSE LABEL cts/min
UNIFORM LABEL cts/min
10^6
10^5
10^4
10^3
10^2
10
10
20
30
40
FRACTION NUMBER

Figure 7

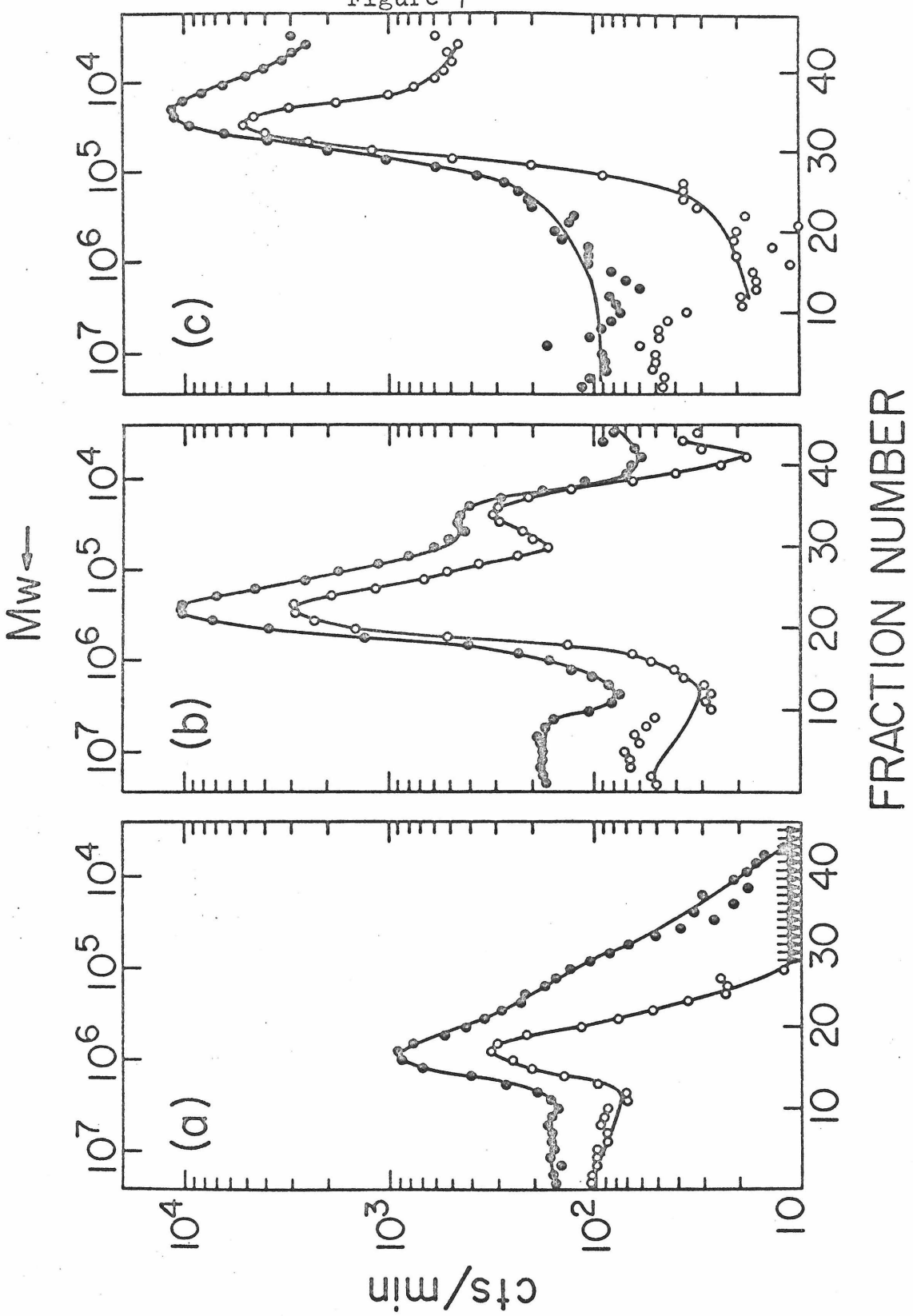


Figure 8

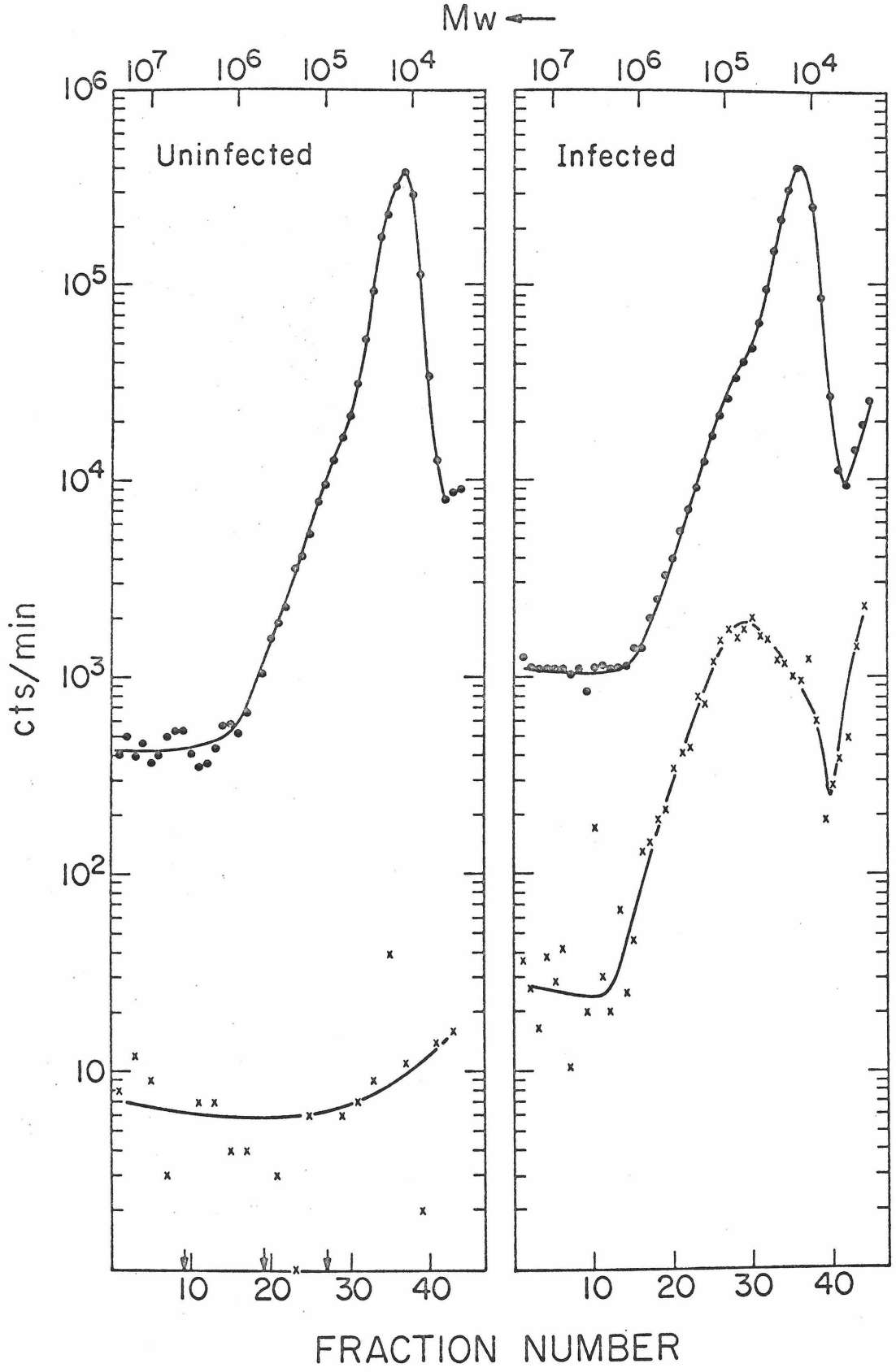

Mw
10^7 10^6 10^5 10^4
Uninfected
Infected
cts/min
10^6
10^5
10^4
10^3
10^2
10
10 20 30 40
FRACTION NUMBER

Figure 9

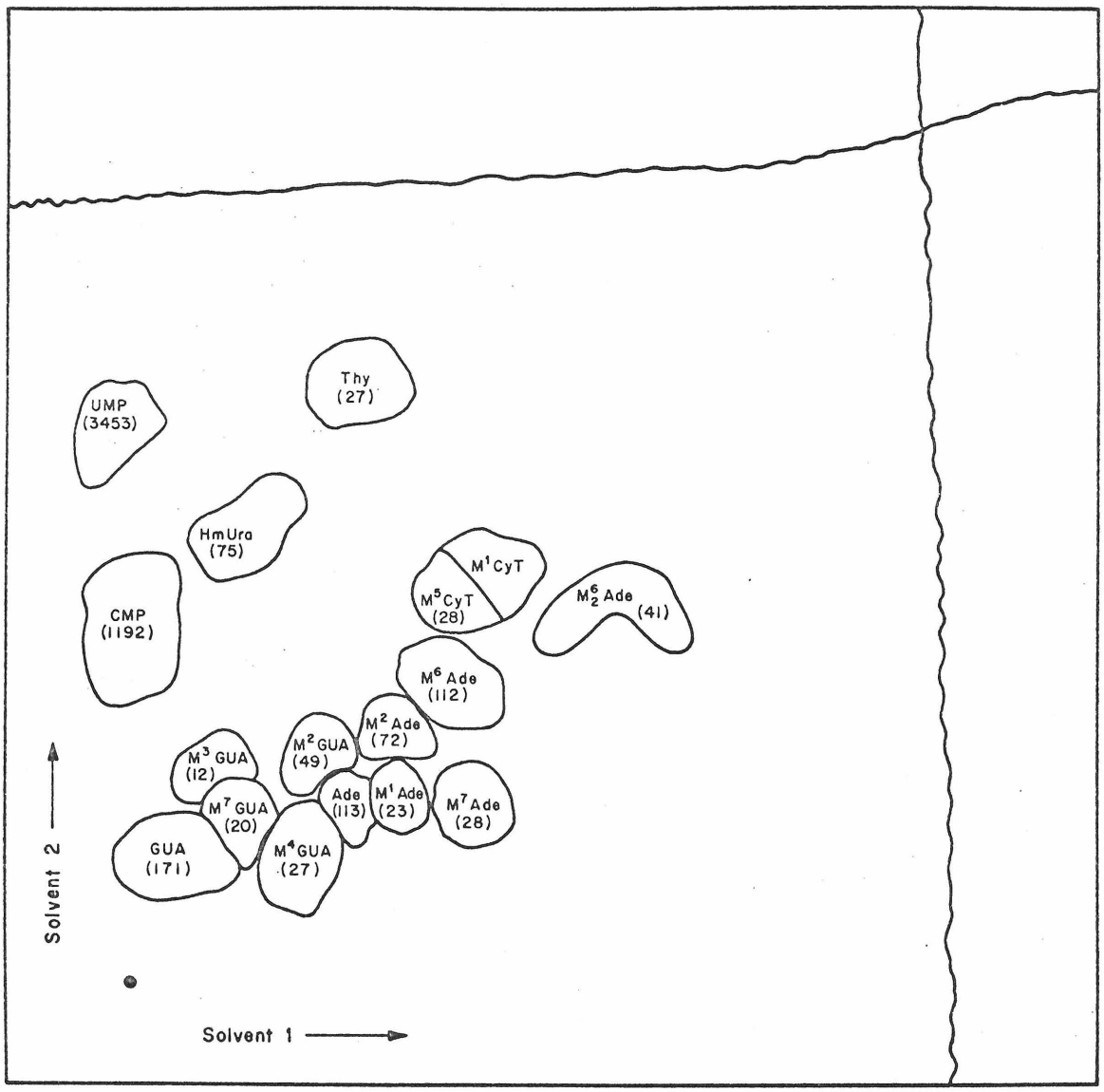

UMP
(3453)
Thy
(27)
HmUra
(75)
CMP
(1192)
M¹CyT
M⁵CyT
(28)
M²₂Ade
(41)
M⁶Ade
(112)
M²Ade
(72)
M²GUA
(49)
M³GUA
(12)
M⁷GUA
(20)
Ade
(113)
M¹Ade
(23)
M⁷Ade
(28)
GUA
(171)
M⁴GUA
(27)
Solvent 2
Solvent 1

Figure 10

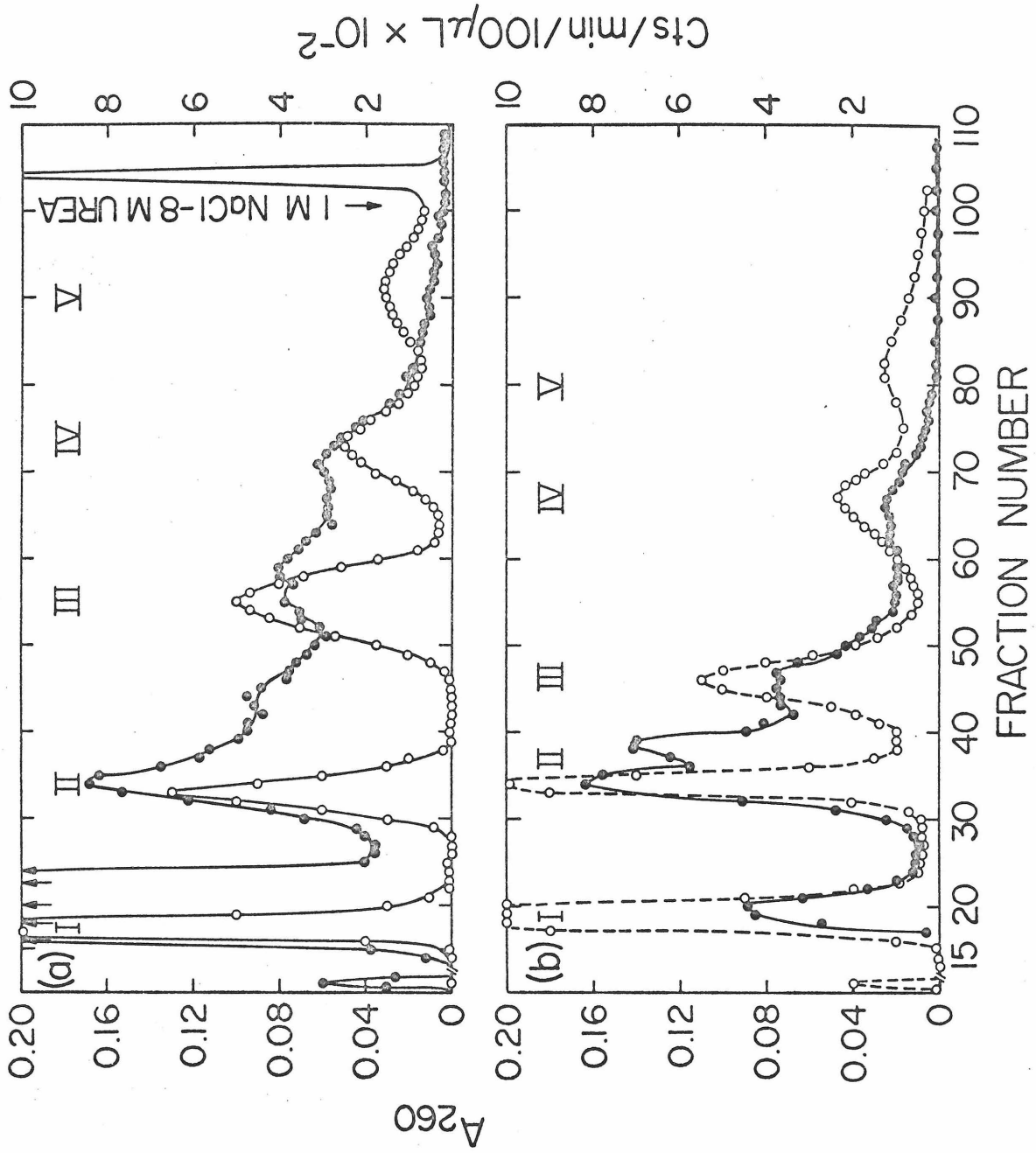
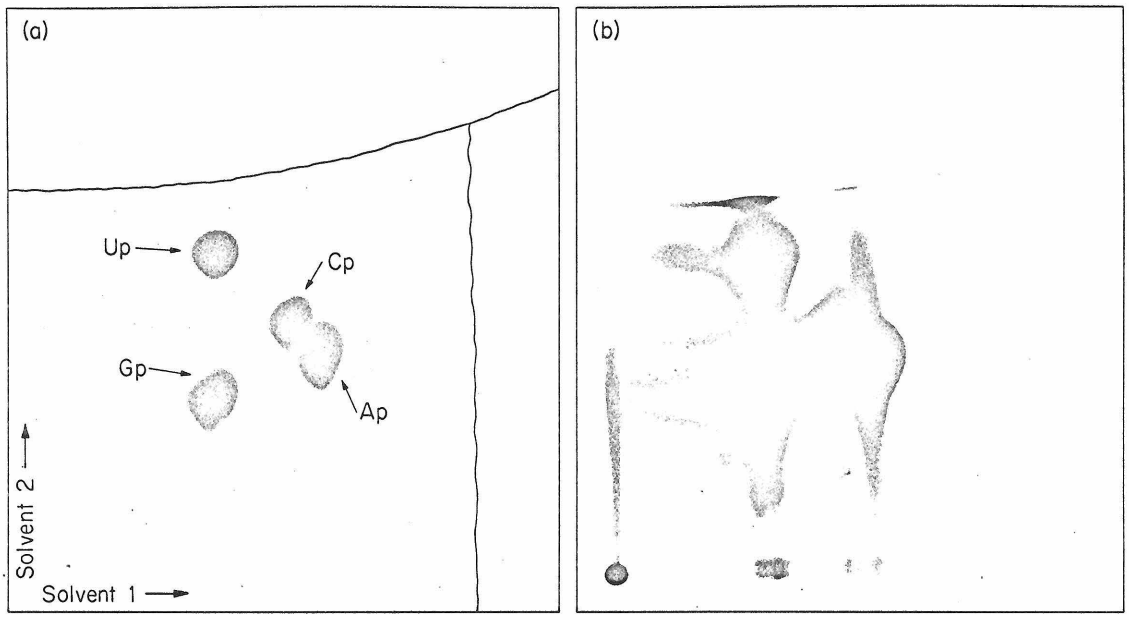

A260
Cts/min/100μL × 10⁻²
FRACTION NUMBER
(a)
(b)
I
II
III
IV
V
1 M NaCl-8 M UREA

Figure 11



Part III

The Template for ØX Transcription

Introduction

There has been considerable attention drawn recently to the topographical site in the bacterial cell for DNA transcription. In all cases studied so far this appears to be the membrane-DNA fraction of the cell (Rouvière, Lederberg, Granboulan and Gros, 1969; Tremblay, Daniels, and Schaechter, 1969). Mutants of E. Coli. that are DNA-less because of defects in cell division can be shown to be lacking RNA polymerase (Hirota, Jacob, Ryter, Buttin, and Nakai, 1968; Adler, Fisher, Cohen, and Hardigree, 1967); the RNA polymerase activity is largely if not entirely associated with the DNA-membrane fraction (Tremblay, et. al., 1969).

Similarly, there is abundant evidence that the membrane fraction is equally important for parental ØX RF replication (Knippers and Sinsheimer, 1968; Salivar and Sinsheimer, 1969), and from the evidence presented in Part II the parental RF on the membrane is involved in transcription and possibly is the only site for the transcription of the ØX DNA. Therefore, a study was undertaken to find out which RF, parental or progeny, was the template for transcription.

As a first approach to the problem of a transcriptional template, use was made of an in vivo RF - pulse labeled RNA hybrid in which the attached RNA was RNase, phenol, and detergent-resistant (Hayashi and Hayashi, 1966). Later

work (Hayashi and Hayashi, 1968) showed that this intermediate was attached to the complementary strand of the RF, could be partially chased out in a typical pulse-chase experiment, and both the parental and progeny RFs were associated with the proposed 3' nascent piece of ØX mRNA. Hence, infection of E. Coli. with a density labeled ^{32}P ØX am3, pulse labeling with [^{3}H-5]uracil at 10-15 min after infection (both parental and progeny RF are present at this time), and isolation of the RNase-treated RF in a CsCl density gradient should provide an answer to the question of transcription template.

Alternatively, more direct experiments were done in which RF molecules were isolated without treatment with RNase. First, an attempt was made to see if any RF could be shifted to higher density due to attached RNA. Secondly, an attempt was made to find ^{32}P RNA attached to the RF in a CsCl density gradient in a massive ^{32}P pulse experiment.

2. Materials and Methods

Most of the materials and methods are described in Parts I and II of this thesis. The exceptions follow:

(i) Preparation of $^{13}C^{15}N^{32}P$ am3 ØX

The preparation of this isotopically labeled ØX is described in Salivar and Sinsheimer (1969) and was a gift of Dr. R. Knippers.

(ii) Preparation of $^{13}C^{15}N^{32}P$ (heavy-light) am3 parental RF-$^{12}C^{14}N$ (light-light) progeny RF pulse labeled with [^{3}H-5] Uracil

10 ml of $^{13}C^{15}N$ TPG media was innoculated with heavy media adapted E. Coli THU and grown to 3 x 10^8 cells/ml. The cells were centrifuged, washed once with fresh TPG (no glucose), or centrifuged and resuspended in 10 ml fresh TPG (no glucose or NH_4Cl). The cells were incubated with aeration for 30 min at 37°C, followed by centrifugation and washing with fresh media. Finally, the cells were suspended in 10 ml prewarmed TPG media plus ^{12}C glucose and NH_4Cl plus 1 µg/ml uracil and infected with $^{13}C^{15}N^{32}P$ am3 (m= 20). After 10 min. 1 MC [^{3}H-5] uracil (27 C/mM) was added for 45 sec., followed by addition of NaN_3 (0.01 M.).

Lysis was accomplished by the procedure outlined in Part I.

The nucleic acid precipitate from the ethanol precipitation step was dissolved in 1 ml. of 2 X SSC plus 30 µg/ml RNase I (previously heat treated at 90°C for 10 min. to inactivate DNase) and incubated for 30 min. at 22°C. The RNase digestion was followed by an ethanol precipitation step and then sedimentation

through a 5 - 20% sucrose (in 0.2 M NaCl, 0.02 M Tris-HCl pH 7.5) gradient to select the RF molecules. The pooled RF region was then dialyzed and banded to equilibrum in a neutral CsCl (mean density = 1.71) density gradient.

(iii) The preparation of ^{32}P parental, ^{3}H progeny RF without RNase treatment)

30 ml. of HF 4704 in TPA media was grown to 5 x 10^8 cells per ml., and then 40 µg.ml. Mitomycin C was added for 30 min. at 37°C. The cells were infected with ^{32}P am3 (m = 5), and at 6 min. after infection 1.5 MC [CH_3-^{3}H] Thymine (27 C/mM) was added. At 18 min. after NaN_3 was added to 0.01 M., and the cells lysis was accomplished as described in Part I.

The nucleic acid pellet from the ethanol precipitation step was dissolved in 0.02 M. Tris-HCl pH 7.5, 0.2 M NaCl, and an aliquot was banded to equilibrum in a CsCl (mean density = 1.71) density gradient with a dense florocarbon cushion (density = 1.97; fluid no. 9 Pierce Chemicals, Rockford, Ill.).

3. Results

(a.) The indirect approach

The distribution of [^{3}H-5] uracil pulse-labeled RNase,

phenol, and detergent-resistant RF-attached radioactivity in a CsCl density gradient (in which the parental RF is density labeled) as prepared in Materials and Methods (ii) is shown in Fig. 1. The ^{32}P from the infecting ØX is distributed into two peaks; the most dense peak is most likely $^{13}C^{15}N^{32}P$ single stranded ØX DNA from uneclipsed phage particles, and the second peak is, from its position relative to the Yh DNA marker, the parental hybrid density RF. The unlabeled progeny RF molecules would band still lighter in density. The ^{3}H pulse-labeled material is clearly seen only in the position of the parental RF.

Two lines of evidence indicate that the ^{3}H radioactivity is RNA. First, in subsequent experiments the ^{3}H counts, although low, were alkali-labile. Secondly, the precursor, [^{3}H-5] uracil would not be expected to label DNA. A breakdown product of the label might label DNA, however, but these counts should also appear in the region of the light-light progeny RF; this is not the case.

Thus, assuming that the RF attached ^{3}H radioactivity is the nascent mRNA-F intermediate analogous to that found by Hayashi and Hayashi (1968), the above experiment would suggest that the parental RF is the template for transcription under these conditions.

(B) Direct approaches

A more direct approach to determining which RF is the template for transcription can be made by CsCl density analysis of the parental and progeny RF molecules without RNase treatment. Data presented in Part II showed that at 4 min. after infection approximately 100 molecules of mRNA per cell were present, and at 25 min. after infection at least 10 times this number was present. If the Hayashi model of the mRNA-RF intermediate is correct, and the Morse, Mosteller, Baker, and Yanofsky (1969) transcription coupled degradation ideas are also correct, these mRNAs should be attached by the 3' end to the RF(s). Hence the density of the RF(s) should be shifted to higher density in a CsCl density gradient due to the attached mRNA (if one molecule of ØX mRNA of size 300,000 daltons were attached to an RF, the density would be shifted to $\rho \cong 1.72$). Simple experiments, such as infection with ^{32}P ØX and ^{3}H thymine labeling followed by extraction and banding in a CsCl density gradient, should indicate which RF has the attached mRNA and is the template for transcription.

Fig. 2 shows the result of such an experiment. ^{32}P am3 ØX was used to infect HF 4704 previously treated with Mitomycin to suppress host DNA synthesis and $\left[{}^{3}H\text{-}CH_3\right]$

thymine labeled at 6-18 min after infection. The cells were lysed, phenol extracted, and banded in a CsCl density gradient with a dense florocarbon cushion on the bottom. The result shows that approximately 25% of the ^{32}P radioactivity and 1% of the ^{3}H radioactivity band against the florocarbon cushion (density $\geq$ 1.8), while the rest of the ^{32}P and ^{3}H radioactivity band at an apparently unshifted normal density for RF (there is probably little single strand synthesis at this time). Controls were done to show that the initial sample sustained little if any degradation, as judged by the ratio of the 23S to the 16S (2:1) rRNA components after band sedimentation in the analytical model E, and that free RNA banded against the florocarbon cushion under these conditions.

The ^{32}P and ^{3}H radioactivity banding on the cushion was then analyzed to see if it was sensitive to RNase or alkali. All the ^{32}P and ^{3}H was found to be sensitive to RNase or alkali; this is consistent with the notion that some of the ^{32}P (and less of the ^{3}H) was degraded to the nucleotide precursor level and reincorporated into RNA. Thus, the conclusion of this experiment is that little if any of the RF, parental or progeny, is shifted to increased density due to attached mRNA.

A similar experiment supporting the above conclusion has been done recently. E. Coli. HF 4704 was infected with $^{13}C^{15}N^{3}H$ am3 ØX, and at 25 min. after infection a massive ^{32}P (15-20 sec.) pulse was given to heavily label the mRNA. The cell lysate was then separated into a membrane-DNA pellet and supernatant fractions according to the procedure of Komano and Sinsheimer (1968). Greater than 90% of the ^{32}P pulse was associated with the membrane fraction. Both the supernatant and membrane fractions were phenol extracted and banded in a CsCl density gradient with a florocarbon cushion. Most of the ^{32}P banded on the cushion, and upon RNase treatment of the ^{32}P radioactivity on the cushion (30 μg/ml. in 2X SSC for 30 min at 22°C), no ^{32}P or ^{3}H (from the infecting phage) could be found to sediment in a CsCl velocity gradient as RF. Little if any ^{32}P (approximately 1-2% of the total pulse) banding in the initial CsCl equilibrium gradients as RF was digestible with RNase, suggesting freedom of the RF from appreciable RNA.

The above experiments show that under conditions where no RNase is used to maximize the probability of finding an RF-mRNA intermediate little if any RNA is attached to the RF molecule.

4. Discussion

The results presented in the previous section are confusing with respect to the question of whether the parental or progeny RF is the template for transcription. The experiment based on the Hayashi intermediate would suggest that the parental RF is the template. However, in view of the other more direct experiments which show that essentially no RNA is attached to the RF molecules, no conclusion as to the template for transcription can be drawn at this time.

It is interesting to note that the pulse labeled RNA is consistently found in the membrane fraction analogous to the results of Rouvière et. al. (1969) and Tremblay, et. al. (1969). The parental RF is closely associated with the membrane fraction (Knippers and Sinsheimer, 1968; Salivar and Sinsheimer, 1969), and the progeny RFs (and single strand replication) seem to be in the cytoplasm (Knippers, Razin, Davis, and Sinsheimer, 1969). The evidence is indirect, but points in the direction of the parental RF occupying the role as template for transcription (cf. Part II, Discussion).

Most if not all the pulse labeled mRNA is not associated with any RF and is probably dissociated by the treatment with phenol and detergents in agreement with the results obtained by Bremer and Conrad (1964). No adequate

explanation can be offered to explain the [^{3}H-5]uracil counts in the Hayashi intermediate or why the intermediate is associated with the parental RF. It is interesting to note that the in vivo Hayashi intermediate does not chase out completely, leaving approximately 25% of the initial RNA still attached (Hayashi and Hayashi, 1968). Conceivably, this material represents some control mechanism involved in starting or stopping transcription. Alternatively, this may be some form of aberrant cell function particularly since it seems to be present in increased quantities under the Chloramphenicol conditions used by Hayashi and Hayashi (1968). Further work will have to be done to determine what function this RNA serves.

Figure Legends

Fig. 1. The location of RF bound RNase resistant [^{3}H-5] uracil pulse labeled RNA on parental ^{13}C-^{12}C, ^{15}N-^{14}N ^{32}P (heavy-light) RF or ^{12}C ^{14}N (fully light) progeny RF in a CsCl equilibrum density gradient. The purified RF fraction as prepared in the materials and methods was added 1.3 g/ml. CsCl (ρ = 1.71; in 0,02 M Tris Cl pH 7.5) plus ϒh ØX DNA as a density marker and centrifuged to equilibrum (60 hr) at 37 K rev.min at 15°C in a SW 50.1 rotor. Increasing density, is from right to left.

o---o ^{32}P cts/min

⊙---⊙ ^{3}H RNase resistant cts/min

x---x ϒh DNA infectivity

Fig. 2. The CsCl equilibrum density dentrifugation of ^{32}P parental-^{3}H progeny RF with a dense (ρ =1.97) florocarbon cushion. The entire RF-Host nucleic acids without RNase treatment was mixed with 1.3 g/ml. CsCl (ρ=1.71 in 0.02 M Tris Cl pH 7.5) plus 0.3 ml Fluid No. 9 (ρ=1.97), and centrifuged for 60 min at 35 K rev/min at 15°C in a SW50.1 rotor. Increasing density is from right to left.

⊙---⊙ ^{3}H cts/min (Progeny RF)

x---x ^{32}P cts/min (Parental RF)

Figure 1

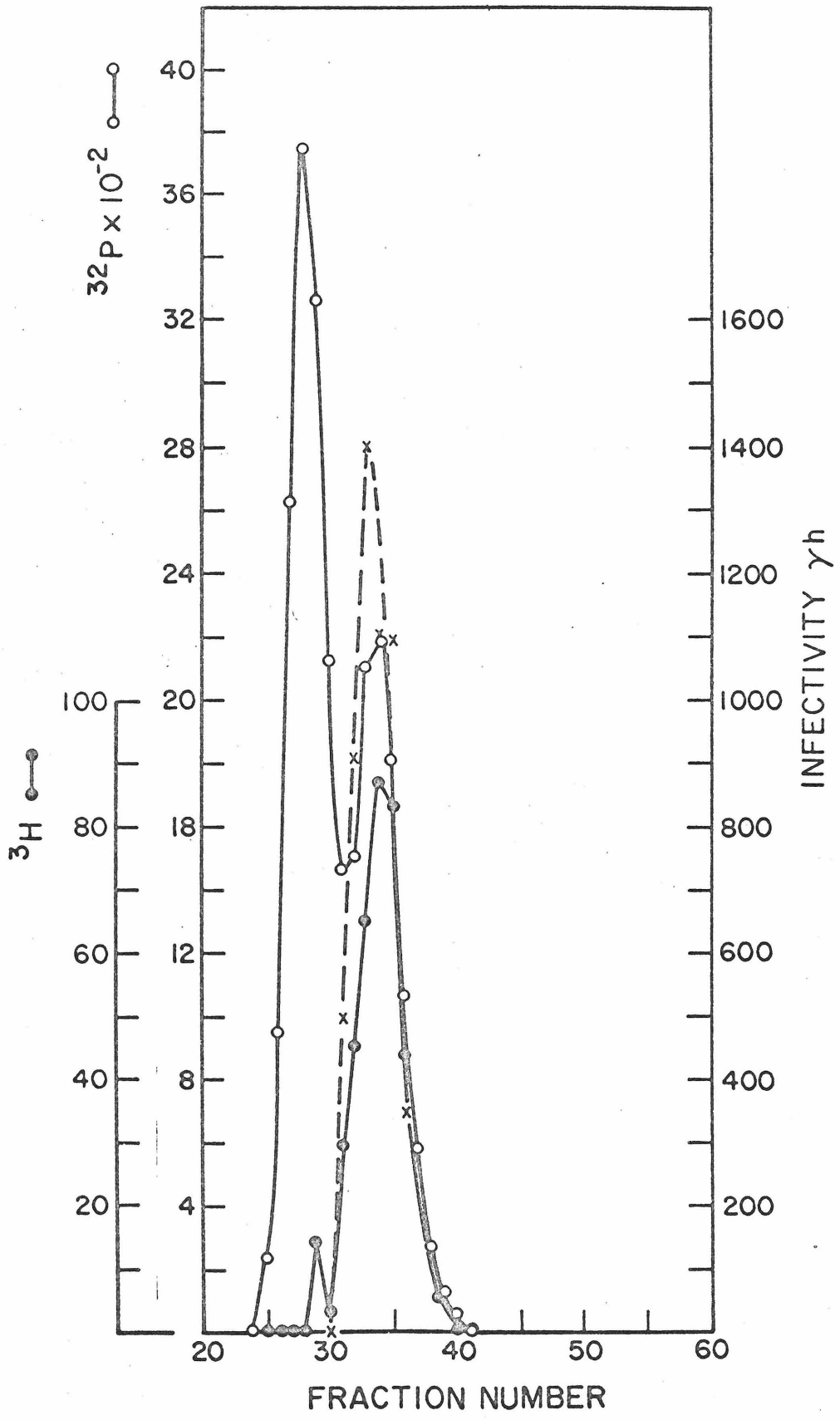
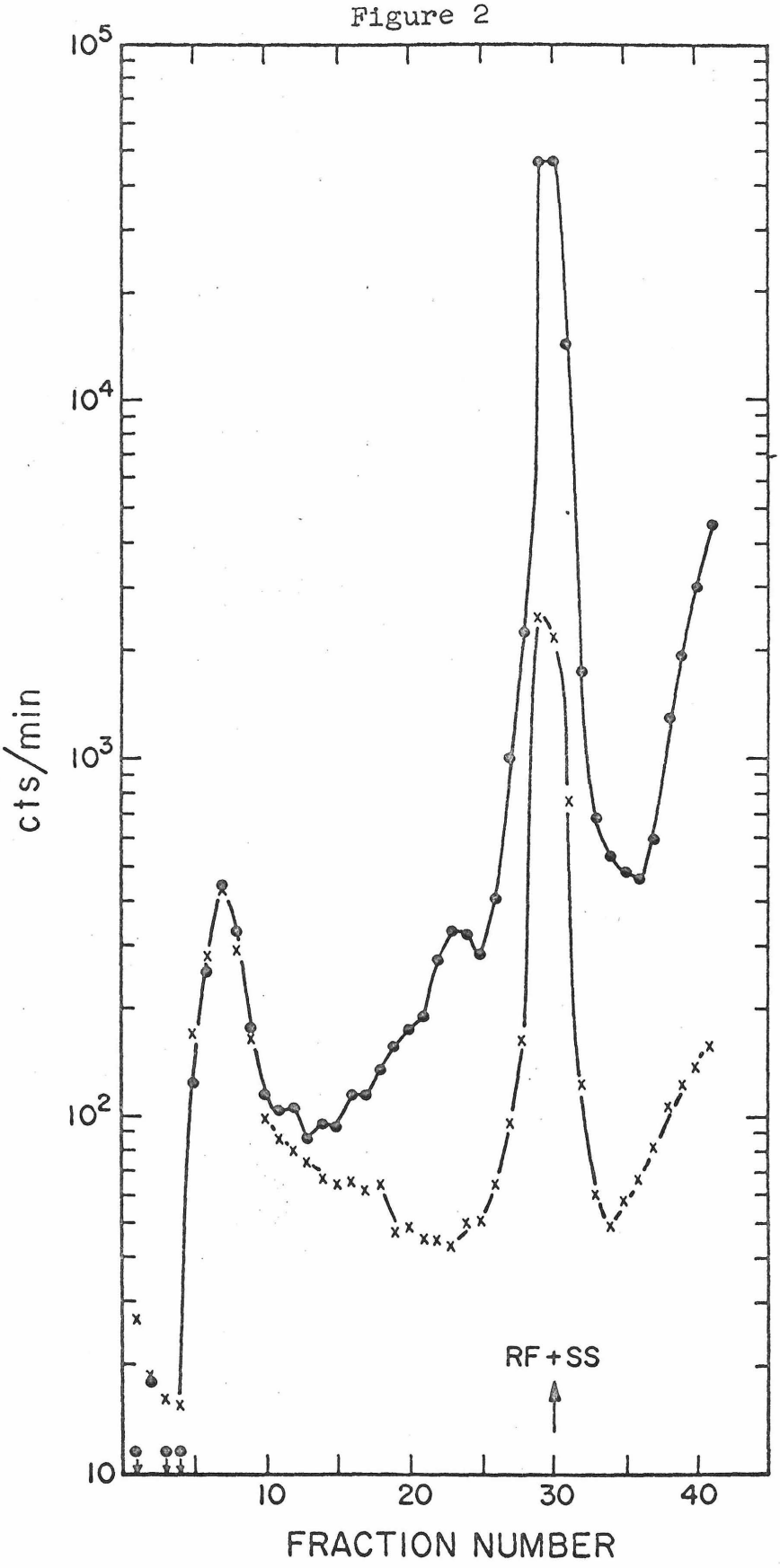

$^{32}P \times 10^{-2}$
40
36
32
28
24
20
18
12
8
4
^{3}H
100
80
60
40
20
INFECTIVITY γh
1600
1400
1200
1000
800
600
400
200
20
30
40
50
60
FRACTION NUMBER

Figure 2



References

Adler, H.I., Fisher, W.D., Cohen, A., & Hardigree, A.A. (1967). Proc. Natl. Acad. Sci., Wash. 57, 321.

Bremer, H., & Konrad, M.W. (1964). Proc. Natl. Acad. Sci., Wash. 51, 801.

Hayashi, M.E., & Hayashi, M. (1966) Proc. Natl. Acad. Sci., Wash. 55,635.

Hayashi, M.E., & Hayashi, M. (1968) Proc. Natl. Acad. Sci., Wash. 61,1107.

Hirota, Y., Jacob, F., Ryter, A., Buttin, G., & Nakai, T. (1968) J. Mol. Biol. 35, 175.

Knippers, R., & Sinsheimer, R.L. (1968). J. Mol. Biol. 34, 17.

Knippers, R., Razin, A., Davis, R., & Sinsheimer, R.L. (1969) J. Mol. Biol. 45, 237.

Komano, T. & Sinsheimer, R.L. (1968) Biochim. Biophys. Acta 155, 295.

Rouvière, T., Lederberg, S., Granboulan, P., and Gros, F. (1969) J. Mol. Biol. 46, 413.

Salivar, W.O. and Sinsheimer, R.L. (1969) J. Mol. Biol. 41, 39.

Tremblay, G.Y., Daniels, M.J. & Schaechter, M. (1969) J. Mol. Biol. 40, 65.