Characterization of Lysozyme Messenger and Lysozyme Synthesized in Vitro

WINSTON SALSER[1,3], RAYMOND F. GESTELAND[2], AND BERNICE RICARD[1]

Department of Zoology and Molecular Biology Institute, University of California at Los Angeles[1]; Cold Spring Harbor Laboratory for Quantitative Biology[2]; and Institut de Biologie Physico-Chimique, Paris, France[3].

In vitro systems for protein synthesis have been in wide use for about 10 years. In most of the early work protein synthesis was measured by following the incorporation of radioactive amino acids into acid precipitable material. This test cannot distinguish between the synthesis of complete, active proteins and various sorts of aberrant products which might result from incorrect initiation, translation in the wrong reading frame, amino acid substitutions, improper termination, or other defects in in vitro translation. The first unambiguous demonstrations of biologically meaningful in vitro protein synthesis were obtained with hemoglobin and the coat proteins of the RNA bacteriophages, where it was possible to show good correspondence between the peptide patterns from in vitro and natural products. These results were very encouraging but did not establish whether the fidelity of the in vitro translation process could be great enough to permit the synthesis of active enzymes in vitro. That this is possible was suggested by DeVries and Zubay (1967), when they synthesized in vitro a fragment of β-galactosidase which could restore enzymatic activity to the complementary fragment made in vivo. Salser, Gesteland, and Bolle (1967) demonstrated in vitro synthesis of active molecules of bacteriophage lysozyme and were able to show that the ratio of enzyme activity synthesized to total protein synthesized was similar to that obtained in intact cells.

During the past two years in vitro synthesis of active lysozyme has become routinely useful and it is currently being used as an assay for biologically meaningful in vitro protein synthesis in many laboratories. The best demonstration of the convenience of the technique is that in vitro lysozyme synthesis has been successfully used as a student exercise in courses given in Gottingen, Geneva, and Los Angeles. In at least the latter course the students themselves made all of the components of the system and were almost uniformly successful in obtaining active preparations. Further, the ease, flexibility, and sensitivity of the technique have been increased considerably by a number of minor alterations, described in the legend for Fig. 1. These simplifications make lysozyme synthesis an even more convenient assay for biologically meaningful translation.

Purification of the Lysozyme Made in Vitro

Salser et al. (1967) and Gesteland et al., (1967) showed that the lysozyme activity synthesized in vitro is a result of the *de novo* translation of the *e* gene messenger of T4. Perhaps the most important evidence supporting this conclusion was the demonstration that amber mutations in the *e* gene for the phage lysozyme prevented the appearance of lysozyme activity in extracts from an Su^- (non-permissive) host. This was clearly not due to some trivial inactivation of the messenger since addition of purified tRNA from bacteria carrying genetic suppressors restored in vitro lysozyme synthesis by suppressing the nonsense mutation in vivo. These experiments could not reveal, however, whether the lysozyme made in vitro was identical to the natural product or whether it might differ in various subtle ways, as was found when RNA phage coat proteins synthesized in vitro were compared with the natural products (e.g. Gussin et al., 1966).

As a preliminary approach to the establishment of the identity of in vitro and in vivo products we have compared the chromatographic behavior of the two products on columns of amberlite IRC 50 resin. Tsugita et al., (1968) showed that T4 lysozyme, a strongly basic protein, absorbs to IRC 50 resin in low salt buffer and can be eluted from it by 0.3–0.4 M salt pH 6.5. Considerable purification is obtained since most other proteins are eluted at lower salt concentrations or do not absorb to the column. For preliminary experiments we used a batchwise purification on IRC 50 resin as shown in Table 1. Clearly most of the lysozyme made in vitro (but only a small proportion of the total radioactivity incorporated into proteins) was recovered by eluting the resin with high salt (Sample *A*). Since lysozyme inhibitors present in the crude extract (S-30) are removed by this procedure, the recovery appears to be greater than 100%. When lysozyme synthesized in vivo is mixed with a blank (chloramphenical inhibited) in vitro incubation, similar results are obtained

TABLE 1. PURIFICATION OF LYSOZYME MADE IN VITRO OR IN VIVO BY BATCH ELUTION FROM AMBERLITE IRC50 RESIN

	Sample *A*		Sample *B*	Sample *C*
	in vitro radioactivity	in vitro lysozyme activity	CM inhibited incubation + in vivo lysozyme activity	in vivo lysozyme activity alone
Sample before absorption	100%	100%	100%	100%
Unabsorbed	71%	~21%	16%	16%
Removed in wash	22%	≤30%	30%	27%
Eluted with NaCl	6.6%	159%*	160%*	98%

* The recoveries appear to exceed 100% because of the removal of a lysozyme inhibitor present in the S-30 extract. This inhibitor is lost in the unabsorbed material and the wash.

Samples containing lysozyme and radioactive proteins made in vitro (Sample *A*), in vivo lysozyme mixed with a chloramphenicol inhibited in vitro reaction mixture (Sample *B*), or in vivo lysozyme alone (Sample *C*); in a volume of 1.8 ml were absorbed onto amberlite IRC50 resin (0.1 ml packed volume) by agitating 5 times on a vortex mixer during several minutes at 37°C. Subsequent reactions were carried out in the cold. The resin was washed twice with 2 ml aliquots of 10^{-3} M $MgSO_4$, and 0.1 M phosphate pH 7.0 and eluted with 0.35 ml of 10^{-3} M $MgSO_4$, 0.5 M NaCl, and 0.1 M phosphate pH 7.0.

The original samples each contained 0.9 ml 5×10^{-4} M $MgSO_4$ and 0.05 M phosphate pH 5.75 plus the following:

Sample *A*: 0.9 ml incubation mixture containing radioactive proteins and lysozyme made in vitro.

Sample *B*: 0.05 ml of a lysozyme standard extract of infected cells described earlier (Salser et al., 1967) plus 0.9 ml of a chloramphenicol (CM) inhibited in vitro incubation mixture.

Sample *C*: 0.05 ml lysozyme standard plus 0.9 ml of 1.5×10^{-4} M $MgSO_4$ and 0.15 M phosphate pH 5.75.

Radioactivity was determined as hot TCA precipitable counts. Lysozyme activity was assayed as described by Salser et al. (1967).

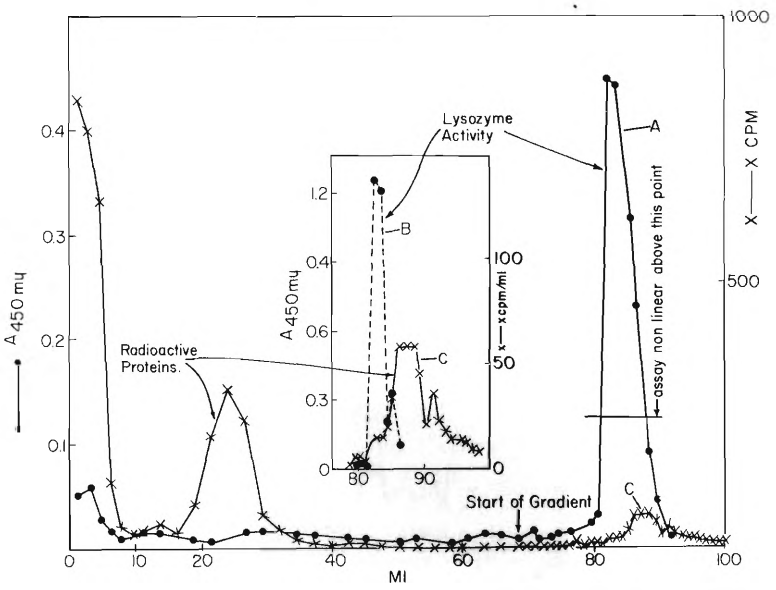
(Sample *B*). The purification of lysozyme in the presence of the large amounts of protein present in the S-30 does not seem to be radically different from that in phosphate buffer alone (Sample *C*). Since large numbers of samples can be purified rapidly on IRC 50 resin this method potentially provides a convenient way of increasing the sensitivity of the assay for in vitro synthesis of active lysozyme. We have not used it extensively however, because the activities normally obtained without purification (20- to 80-fold above background) have been adequate for most work. The results shown in Table 1 confirmed the results of Tsugita et al. (1968) and suggested that the lysozyme made in vitro is a strongly basic protein like the natural product.

A more detailed study of the behavior of the enzyme made in vitro is shown in Fig. 1. An incubation mixture containing ^{14}C-valine labeled proteins and lysozyme synthesized in vitro was absorbed onto a column of IRC 50, washed with neutral phosphate buffer, and eluted with an exponential salt gradient. As before, most of the labeled proteins do not absorb to the column or are removed in the wash. In this case some of the lysozyme activity did not absorb, probably due to overloading of the column. Most of the lysozyme activity recovered is eluted in one peak corresponding to a shoulder on one of the major peaks of radioactive protein synthesized in vitro. If the active lysozyme molecules synthesized contained many random amino acid substitutions in noncritical regions of the enzyme (without destroying the activity) then we might have expected a broad peak reflecting the heterogeneous structure of the molecules. Instead we see that the activity appears in a very narrow peak.

There remained the possibility that the lysozyme made in vitro might differ from the natural product in some systematic way so that it would elute as a sharp peak, but at a position different from the natural product. To test this hypothesis we took advantage of the fact that the lysozyme made in vitro elutes just ahead of a prominent peak of radioactive protein made in vitro (Fig. 1). We can use this peak as a marker and ask whether lysozyme activity made in vivo will elute in the same or a different position relative to the in vitro radioactivity when the two are co-chromatographed. To avoid confusion due to the enzyme activity made in vitro we mixed the two preparations in a ratio so that over 98% of the enzyme activity was due to the in vivo enzyme (Fig. 2). The in vitro proteins were labeled with ^{35}S-methionine and those synthesized in vivo with ^{3}H-methionine. Only the NaCl gradient portion of the elution is shown. The enzyme activity made in vivo does elute just before the ^{35}S labeled in vitro marker peak, just as was seen with the in vitro enzyme. The peak has a fairly complicated structure however, possibly due to the fact that the salt gradient was made much shallower here than in Fig. 1 in an attempt to achieve better resolution. This does not represent a separation of in vitro and in vivo activities: the in vitro activity is undetectable since it represents less than 2% of the total. Congruence of the elution profiles of radioactive proteins labeled in vitro and in vivo with each other and with the lysozyme activity (samples 95 to 103) also suggests that the natural and in vitro products behave in a similar manner.

We have used the major radioactive peak eluted at about 0.38 M NaCl as an internal marker. This protein is synthesized both in vitro and in vivo, as

FIGURE 1. *Chromatography of lysozyme made in vitro on a column of amberlite IRC50 resin.*



Lysozyme labeled with ^{14}C-valine was synthesized in vitro using RNA extracted 25 min after infection (25 min RNA) and a 2.8 ml sample of the in vitro incubation was adjusted to pH 5.8 with acetic acid and diluted with water to give a volume of 6 ml. This was centrifuged to remove material which might clog the column and applied to a column with 1.2 ml bed volume amberlite IRC50. The column was run in the cold at 1.2 ml/hr, washed with 60 ml of S buffer (0.001 M $MgSO_4$ and 0.1 M PO_4, pH 6.5), and then eluted with an exponential salt gradient: 20 ml S buffer in the mixing chamber, and S buffer plus 0.4 M NaCl in the reservoir.

Curve *A*: Lysozyme was assayed by reading the turbidity following a 10 min incubation to find the general location of the lysozyme. This assay is very sensitive but is extremely overloaded in the peak region so that the peak appears much broader than it actually is. Curve *B*: The true shape of the peak was determined by reassaying the peak fractions using the standard assay conditions (Salser et al., 1967) which are *linear* in this range of lysozyme concentrations. Curve *C*: Acid precipitable radioactivity (^{14}C valine incorporated into proteins in vitro).

The following alterations have been made in our basic techniques reported in earlier papers (Salser et al., 1967, Gesteland et al., 1967):

1. Messenger RNA (mRNA) is extracted from cells infected for 25 min or infected with superinfection to prevent lysis for 35 min. Such preparations are about 1.5 or 2.1 times as active as 20 min RNA in directing in vitro lysozyme synthesis.

2. The time spent in assaying the lysozyme synthesized in vitro has been halved by the discovery of conditions which permit us to freeze and store the lysozyme substrate preparations without loss of sensitivity. This substrate was used routinely for all of the experiments carried out in Paris and Los Angeles. To prepare the substrate, cells of *E. coli* B^E are grown to saturation in medium M9 (Bolle et al., 1968a) supplemented with glucose and casamino acids and diluted 50-fold into the same medium (37°C). When the cells reach an $OD_{630} = 0.75$ (Zeisse PMQ II spectrophotometer) they are chilled and centrifuged in the Sorval GSA rotor. All centrifugations are for the minimum time necessary to pellet the cells. The cells from one liter of culture are resuspended in 50 ml of 0.05 M Tris buffer pH 7.8 saturated at room temperature with chloroform. A few ml of chloroform are added and the cells are incubated at room temperature for 20 min, shaking gently 3 or 4 times during the interval.

The cells are then chilled in ice and poured into centrifuge tubes, being careful to exclude any droplets of chloroform. The tubes must be nylon or polypropylene, as polycarbonate tubes yield completely inactive preparations, probably due to traces of chloroform dissolving the plastic. The chloroform treated cells are twice centrifuged and resuspended in 25 ml of 0.05 M Tris buffer pH 7.8 with no chloroform, centrifuged a third time, and resuspended in 3 ml of the same buffer, again keeping centrifugation times minimal to avoid damaging the cells.

This preparation can be used fresh, diluting with Tris buffer to give the desired OD_{540} (about 100-fold for $OD_{540} = 0.4$), or it can be divided in small aliquots and frozen in liquid nitrogen and stored until needed. Degassed buffer should be used for diluting the cells to avoid bubbles on cuvette windows. The diluted substrates are held on ice, the fresh preparation remaining useful for 2 days, the frozen preparations for one day only.

3. The in vitro incubations themselves can be done more simply and reliably by the obvious stratagem of preparing large mixes of the chemicals which are added to S-30 to give the preincubation mixture and those (except for the mRNA) which need to be added to the preincubation mixture to give the incubation mixture. Such frozen mixtures seem to be almost as active as those freshly made. The conditions are otherwise as described by Salser et al., 1967, except for the use of additional Cleland's reagent (Gesteland et al., 1967) and the final magnesium concentration experimentally found to give optimal lysozyme synthesis with each S-30 preparation.

4. Three changes have been made in the procedure for extracting mRNA. The cells harvested at late times are fragile and lyse in the Sharples centrifuge pellet. Consequently it is necessary to use a tissue homogenizer with a teflon pestle to dissociate the viscous pellet adequately. No egg white lysozyme is used to open the cells unless they have been infected for less than 20 min or with a lysozyme defective mutant. Because of the larger amounts of DNA present later in infection we lyse the cells in a larger volume (200 ml of 0.01 M KCl, 0.005 M MgCl, 0.01 M Tris buffer pH 7.3 per 6×10^{12} cells) to avoid an intractable jelly.

5. Previously all S-30 extracts were prepared with alumina (Salser, et al., 1967). Alternatively we have found that it is entirely successful to break the cells open in a French Pressure Cell with 4000 to 8000 lb. pressure. In this procedure the cells are broken open immediately rather than being frozen as a pellet prior to alumina grinding. All other details are the same.

It is important to follow the standard procedure for dialysis and preincubation of the S-30 (Capecchi, 1966, Salser et al., 1967). Haselkorn and Wilhelm (personal communication) have shown that preparations made by the method of Nirenberg and Matthaei (1961) are much less active in lysozyme synthesis. However, as described by Brawerman et al., (1969), the crude system may be fractionated considerably (into washed ribosomes, S-100 and the three purified initiation factors) while still retaining the ability to synthesize active lysozyme.

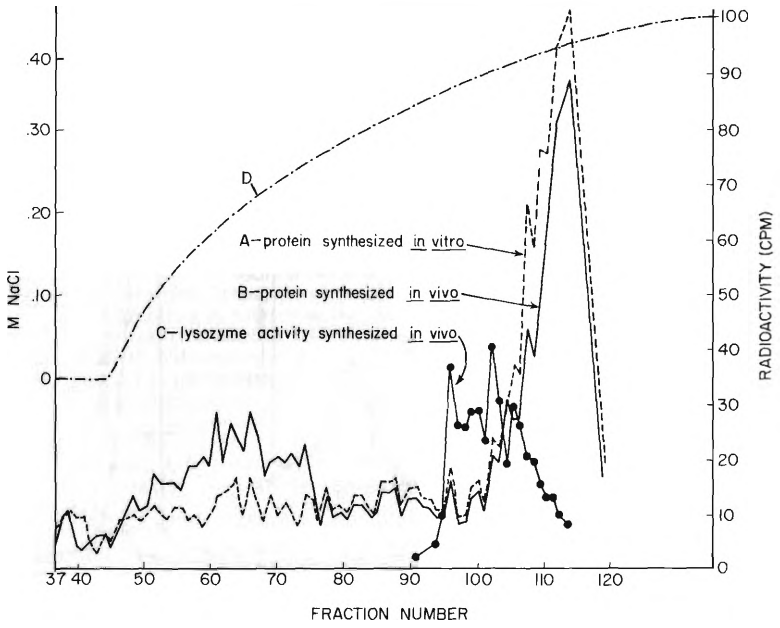


FIGURE 2. *Co-chromatography of ^{35}S-methionine labeled proteins made in vitro and ^{3}H-methionine labeled proteins made in vivo.*

Curve *A*: protein synthesized in vitro (labeled with ^{35}S-methionine); Curve *B*: proteins labeled late in infection (34 to 90 min in a superinfected culture) with ^{3}H-methionine; Curve *C*: lysozyme activity and; Curve *D*: NaCl concentration.

Samples (0.4 ml) of our in vitro incubation, programmed with T4 25 min RNA were added to 5 ml of a lysate labeled in vivo with ^{3}H-methionine. The mixture was chromatographed on an amberlite IRC50 column as described in Fig. 1 with the following modifications: the bed volume was 2.5 ml, the lysate was treated with DNase (2 μg/ml), flow rate was 3–4 ml/hr, the S buffer wash was 150 ml, and the gradient elution was made very shallow by using a 40 ml mixing chamber and introducing first S buffer plus 0.33 M NaCl and then, after fraction 95, changing to S buffer plus 0.4 M NaCl.

is shown by the good agreement in the ^{3}H and ^{35}S radioactivity profiles in Fig. 2. The most important result is that both the lysozyme activity made in vitro (Fig. 1) and that made in vivo (Fig. 2) elute immediately before this major peak. Thus we are not able to distinguish the lysozyme made in vitro from the natural product on the basis of their chromatographic behavior on IRC 50 resin. This suggests that the in vitro product does not differ from lysozyme made in vivo in any very dramatic way such as would have been the case, for instance, if failure of chain termination had resulted in the synthesis of an abnormally long protein molecule or a protein molecule with a transfer RNA (tRNA) molecule still attached to the C-terminal amino acid. A more likely difference would result from failure of the deformylating enzyme (Adams, 1968) or other enzymes to remove part of the N-terminal amino acid sequence. Deformylation of the methionine which is the N-terminal amino acid of the natural product would result in the gain of one charged group. Unfortunately we cannot say whether a one charge difference would have resulted in a detectable change in the chromatographic behavior on IRC 50. Experiments in progress may enable us to establish the amino acid sequence of the in vitro synthesized product.

TIME COURSE OF mRNA SYNTHESIS

Salser et al. (1967) showed that RNA extracted 5 min after infection at 30°C was not active in programming the synthesis of phage lysozyme in vitro. This is the result which might be expected since there is no in vivo synthesis of lysozyme at this time, but Bautz had shown by RNA-DNA hybridization techniques that lysozyme messenger is made early in infection (Bautz et al. 1966, Kasai and Bautz, 1969). The amount of lysozyme messenger made during this early burst of synthesis appears to be comparable to the amount made late in infection and Bautz et al. postulated that there was a block preventing the translation of the lysozyme messenger at early times. To fit the model proposed by Bautz et al. (1966) and Kasai and Bautz (1969) it is necessary to imagine that the translational block is of what we will call the trans-dominant type. By this we mean that the block is a property of the protein synthesizing system and not a property of the mRNA (this would be cis-dominant). According to this type of model we should have been able to extract functional lysozyme messenger from cells harvested after 5 min of infection at 30°C. Although we were unable to do so, it was possible to imagine that 5 min was not an appropriate time to obtain the early lysozyme message under our conditions of growth, so we isolated RNA at 2 min intervals during the early part of the infection and tested them for their ability to synthesize lysozyme in vitro (Fig. 3). Experiments carried out independently in Cold Spring Harbor and Paris gave the same result: no active lysozyme messenger could be found in the cells before about 11 min after infection (Gesteland and Salser, 1969).

The result is not sufficient to rule out trans-dominant translational control models because it is possible to imagine that messenger is rapidly degraded when translation is blocked. Degradation

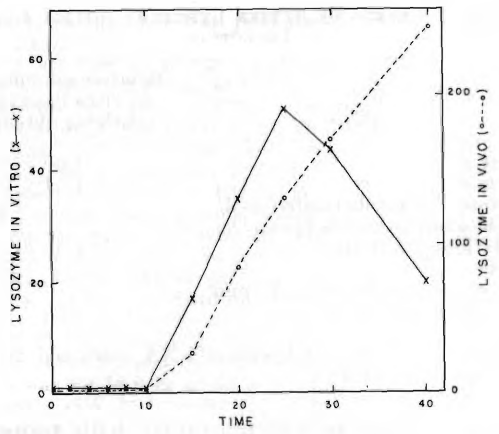


FIGURE 3. *Lysozyme and lysozyme mRNA synthesized during T4 infection (Gesteland and Salser, 1969).*

At time zero the culture (at 30°C) was infected with T4 at a multiplicity of 5. The in vivo enzyme activity (○) is the enzyme produced in the cells during the course of infection. The points represent the activity in 20 ml of sonicated aliquots of the culture. The in vitro activity (×) measures the lysozyme mRNA extracted at each time and represents the activity produced by in vitro protein synthesis in a 100 μl reaction mixture supplemented with 80 μg of each total RNA sample. The lysozyme activities are calculated from the maximum slope of the change in turbidity (Δ OD_{450}) and are expressed as the per cent decrease in 10 min.

limited to a few endonucleolytic cleavages would inactivate the messenger without interfering with its detection by the RNA-DNA hybridization methods used by Bautz and his co-workers. Gesteland and Salser (in prep.) have tested this hypothesis by trying to translate functional lysozyme messenger in crude extracts of cells infected for 5 and 20 min (the 20 min extract being prepared from cells infected with the lysozyme amber H26 in order to prevent in vivo lysozyme synthesis which would interfere with the assay). If there were a trans-dominant block we would have expected to obtain good lysozyme synthesis in the 20 min extract but none in the 5 min extract. Instead we found that lysozyme synthesis programmed by 20 min RNA was equally efficient in the 5 and 20 min extracts. To avoid the objection that late tRNA molecules supplied in the 20 min RNA preparation might be essential for translation of the lysozyme mRNA, we repeated the experiment with mRNA from which tRNA had been removed by chromatography on Sephadex G100 and the same results were obtained.

It is clear that such experiments cannot unambiguously rule out trans-dominant translational control mechanisms—one can always postulate that the control is inactivated when the cell is broken open so that we do not see it in our extracts. Still, it is worthwhile to consider alternative models involving cis-dominant translational control in which the lysozyme messenger seen early in infection fails to function because of something inherent in its structure rather than because of an outside block. Two possibilities are especially interesting:

In the first model, which is suggested by the work of Kasai and Bautz (1969), lysozyme messengers present early and late in infection are synthesized using different initiation points for the RNA polymerase. This might happen if the early initiation point served the early genes known to lie to the right of lysozyme but, because of a failure to terminate, the polymerase continued on into the lysozyme gene. The inactivity of this early messenger in lysozyme synthesis is then explained by saying that the messenger is folded in such a way as to mask the ribosomal attachment site for lysozyme. Late in infection modifications occur which permit the polymerase to attach at new sites (cf. Bolle et al., 1968 a, b) one of which is located prior to the *e* gene permitting synthesis of an active messenger.

In the second model we postulate that lysozyme messenger is always inactive as made. Something present late in infection modifies this messenger in such a way as to activate it, for instance, by cleaving off a fragment to expose the ribosomal attachment site. An analogous model has been proposed by Lodish and Robertson (this volume) to explain why the inhibition of synthesis of the RNA polymerase of phage *f*2 by polar mutants of the coat protein can be reversed by disrupting the secondary structure of the mRNA.

This model is easily testable. "Late" RNA should direct lysozyme synthesis in extracts of uninfected cells or cells harvested early in infection because it has already been activated. Messengers taken early in infection should not, because the activating agent is not present in uninfected cells or cells harvested early in infection. But "early" lysozyme messenger should *become activated and synthesize active lysozyme when put into extracts of cells harvested late in infection.*

Finally, we would like to point out that there does seem to be a trans-dominant control of lysozyme mRNA translation during the late phase of infection. Lysozyme is synthesized at a practically linear rate during the late period of infection (Sekiguchi and Cohen, 1964; Gesteland and Salser, 1969). In the absence of translational control one would expect that the amount of active lysozyme messenger should be proportional to the rate of synthesis of the enzyme and thus constant during this period. Instead there is a threefold increase in the level of active messenger between 15 and 25 min (Gesteland and Salser, 1969). In cells which have not been superinfected the amount

of recoverable active lysozyme messenger decreases after 25 min. But in superinfected cells a still further increase in lysozyme messenger activity occurs (Table 2). The superinfection may increase the recovery by preventing lysis of the cells or it may prevent a shutdown of RNA synthesis which naturally occurs prior to lysis. In any case it is clear from Fig. 3 and Table 2 that the amount of active lysozyme messenger present changes 3- or 4-fold (depending upon whether superinfection is used) during a period (15–25 or 15–37 min after infection) when the rate of synthesis of the enzyme is remaining nearly constant.

It is somewhat distressing to think that the simple linear synthesis of an enzyme may turn out to be the result of a complicated interplay of translational and transcriptional controls. Sometimes it would seem that bacteriophage T4, which has been called a classical phage, more aptly deserves the term baroque. However, the seeming inefficiency due to production of an inactive lysozyme messenger early in infection or of using the late lysozyme messenger with variable efficiency should not be a matter of great concern. As pointed out by Salser, Janin, and Levinthal (1968), of the total energy spent on protein synthesis, the part devoted to the synthesis of the mRNA is very small indeed. For *E. coli* the energy devoted to mRNA turnover was estimated as less than 5% of the total energy for protein synthesis. Using new estimates of the number of copies of proteins made per messenger (Morse et al., 1968) and assuming that mRNA breaks down to nucleotide diphosphates rather than monophosphates, it appears that this should be revised downward to about $\frac{1}{2}$% for the synthesis of a specific protein (tryptophan synthetase). When such a small fraction of the total energy of the cell is devoted to mRNA synthesis it is clear that a great deal of this messenger could be "wasted" without very seriously lowering the overall efficiency of the cell. If, for instance, creation of a new class of polymerase recognition sites led to better control of some early proteins and, also *happened* to result in the synthesis of an inactive lysozyme messenger early in infection, this messenger would represent a sort of evolutionary debris. How rapidly the phage dealt with this "debris" would depend on the selectional advantage to be gained (which might be very small as pointed out above) and the difficulty involved in doing this by mutation (which might be great if altering the offending initiating sequence necessarily disrupted other processes).

A very simple explanation of the increase in active lysozyme messenger late in infection is suggested by the finding of Morse, Mosteller, and Yanofsky (this volume) that degradation of the message proceeds concomittantly with transcription and translation. According to this finding, intact messengers for the tryptophan operon are very rare. A greater proportion of the messenger for the lysozyme operon may be complete since it is probably less than $\frac{1}{3}$ the size of the tryptophan operon. Nevertheless, the fraction of the mRNA extracted from the cell that is intact may be very small, and it is probable that incomplete or partially degraded messengers will be inactive in vitro. Therefore, our demonstration of a 4-fold increase in the amount of lysozyme messenger active in vitro need not be interpreted as demonstrating a similar change in the total amount of lysozyme messenger present in the cell. We can postulate that our result is partly or entirely due to a change in the *fraction* of lysozyme messenger found in intact molecules, rather than to changes in the total amounts of lysozyme messenger.

TABLE 2. LEVELS OF ACTIVE LYSOZYME mRNA LATE IN INFECTION

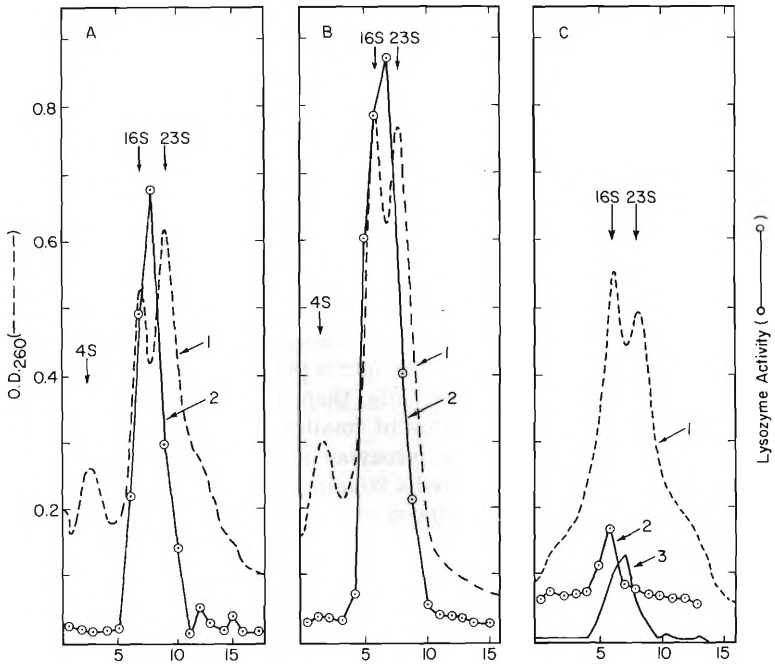
RNA	Relative amounts of in vitro lysozyme synthesis obtained
20 min	1.00
25 min	1.54
25 min, from cells treated with chloramphenicol between the 18th and 25th min.	1.48
37 min (cells superinfected at 12 min to prevent lysis).	2.1

Twenty minute RNA means RNA extracted 20 min after infection at 30°C.

The data of Table 2 also demonstrate a separate point, that active lysozyme messenger can be made in the absence of protein synthesis. Chloramphenical has no effect on the increase in the amount of active lysozyme messenger between 18 and 25 min. It had already been shown (Gesteland, Salser, and Bolle, 1967) that the messenger containing amber mutations is made normally in Su^- cells where it cannot be translated.

THE SIZE OF THE LYSOZYME MESSENGER

Lysozyme is a relatively small protein containing 164 amino acids (Tsugita and Inouye, 1968). Therefore the minimum size of the messenger is about 500 nucleotides, which would correspond to a sedimentation rate of 9.3 S if the molecule behaved like ribosomal RNA (rRNA) (Boedtker, 1968). We have tested this directly by fractionation on sucrose gradients. A preliminary result is shown in Fig. 4*A*. The active lysozyme messenger runs in a sharp peak just ahead of 16 S rRNA. This main peak appears to be almost monodisperse as judged by comparing its peak width at half height with that of the 4 S, 16 S, and 23 S RNA species. We cannot



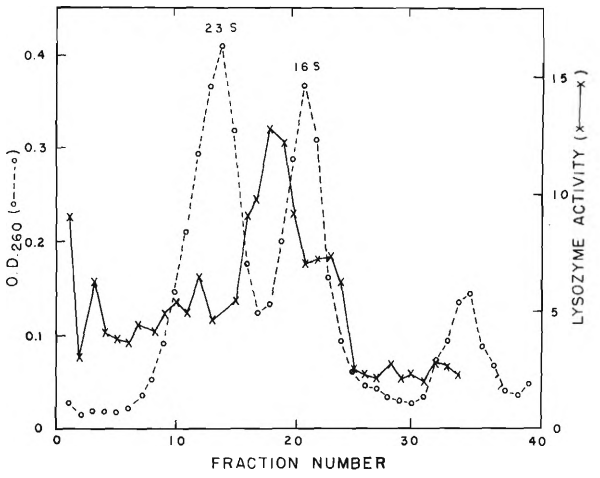


FIGURE 4. *Sucrose gradient fractionation of T4 mRNA.*

In Fig. 4*A* through 4*C* curve 1 shows the UV absorbing material and curve 2 the active lysozyme mRNA. (*A*) Centrifugation is from left to right. The RNA was extracted from cells infected 25 min as described in Fig. 1. RNA (0.5 mg) in 0.01 M EDTA and 0.01 M Tris pH 7.4 at a concentration of 0.8 mg/ml was mixed without heat treatment with an equal volume of 0.1 M NH_4Cl, 0.01 M NH_4Ac pH 4.6, and layered on a 5–20% gradient made with autoclaved sucrose in the same buffer. The autoclaved sucrose is responsible for some of the UV absorption. Centrifugation was for 14.6 hr at 25,000 rpm in a Spinco SW 25.1 rotor. The fractions were precipitated with 3 volumes of cold ethanol, the precipitated RNA pelleted in the clinical centrifuge and the pellets drained for 1–2 hr at 4°C. The pellets were then dissolved in 50 μl of distilled water and 25 μl were taken for assay of lysozyme messenger activity. (*B*) The RNA (0.5 mg) at a concentration of 0.8 mg/ml in 0.01 M EDTA and 0.01 M Tris pH 7.4 was heated to 80°C for 12 min and then chilled with ice to destroy aggregates before mixing with an equal volume of gradient buffer and layering on the gradient. Otherwise conditions are exactly as described in *A*. (*C*) Procedure exactly as in Fig. 4*A* except that the amount of RNA was reduced twofold and the RNA sample used had been purified on Sephadex G 100 to remove tRNA 8 months prior to this experiment. It had become badly inactivated during storage, perhaps as a result of nuclease inadvertently introduced during the fractionation on Sephadex. Curve 3 shows the distribution of active lysozyme messenger from Fig. 3*A* scaled down for comparison with this data. (*D*) Data of Gesteland and Salser (1969). The RNA was heated for 4 min in 1 mM sodium acetate pH 5.0, at a concentration of 4 mg/ml. It was then diluted to 1 mg/ml and adjusted to 0.1 M potassium chloride, 0.01 M Tris pH 7.5, and 1 ml was layered onto a 37.5 ml 5–20% sucrose gradient, made with Mann RNase free sucrose in the same buffer and centrifuged in a Spinco SW27 rotor for 19 hr at 27,000 rpm and 4°C. Centrifugation is from right to left. Other details are as described by Gesteland and Salser, 1969.

take this result as a dependable indication of the size of the lysozyme messenger however, because there is clearly some aggregated RNA on the gradient (shoulder at roughly 30 S with traces of lysozyme messenger activity). Such aggregation is a common result of hot phenol extraction and it seemed possible that the lysozyme messenger could be monocistronic but aggregated with other messengers or with rRNA so that the sedimentation value did not reflect the actual size.

To test this we have attempted to disaggregate the RNA by heating it to 80°C for 12 min in low salt and EDTA and then fast cooling. The rationale behind this is that all secondary structure should be destroyed at this temperature and that once the molecules are apart, the strong electrostatic repulsion between the charged phosphate groups at this low salt concentration should prevent reaggregations during the rapid cooling. This treatment does successfully disaggregate the RNA as seen by the disappearance of the 30 S shoulder in Fig. 4*B*. At the same time the lysozyme messenger

activity sedimenting at greater than 23 S disappears. In parallel experiments we have found that heating in this way actually increases lysozyme messenger activity, suggesting that messenger in aggregates is inactive. The activation due to heating seems to be maximal (about 25%) after 2 min of heating under the conditions used in Paris (80°C) and is followed by a slow loss of messenger activity upon further heating. Most striking, however, is that the main peak of lysozyme messenger activity remains at 17–18 S. Essentially no active lysozyme messenger (less than 4% of the peak value) is found at 9.3 S where we would expect to find monocistronic lysozyme messenger.

If the active lysozyme messenger is polycistronic, as is suggested by these results, then it might be possible to obtain fragments of smaller size which are still able to code for lysozyme synthesis. To test this hypothesis we used a 20 min mRNA preparation which had been stored at −20°C for eight months. During this time its ability to program the incorporation of radioactive amino acids and its ability to code for active lysozyme synthesis had dropped about 3-fold and 10-fold respectively (this is not typical—some preparations remain completely active after long storage). This messenger was sedimented on a sucrose gradient as shown in Fig. 4*C* and it was found that the small amount of lysozyme messenger activity remaining was in molecules which sediment at about 14 S. It is possible that this represents the true size of the messenger and the 17–18 S material is in aggregates which are not destroyed by our heat treatment in low ionic strength buffer. We consider it more likely however, that the 17–18 S material is the intact (polycistronic?) messenger and that the 14 S material represents fragments which contain an intact lysozyme transcript and can program lysozyme synthesis albeit possibly at a much slower rate.

At about the same time these experiments were being performed in Paris, exactly the same conclusions were reached as a result of similar but completely independent experiments carried out at Cold Spring Harbor. Figure 4*D* shows that here again the major peak of active lysozyme messenger is at 17–18 S after disaggregation by heating under somewhat different conditions. Some slower sedimenting activity, with a peak at about 14 S, possibly represents partially degraded messenger. Again no lysozyme messenger sediments at 12 S or slower (fraction 25).

We do not feel that the size of the lysozyme messenger is unequivocally established by these data. There still remains the possibility that the high sedimentation velocity of the active lysozyme messenger is due to the reformation of aggregates with 16 S RNA or other mRNAs under the salt

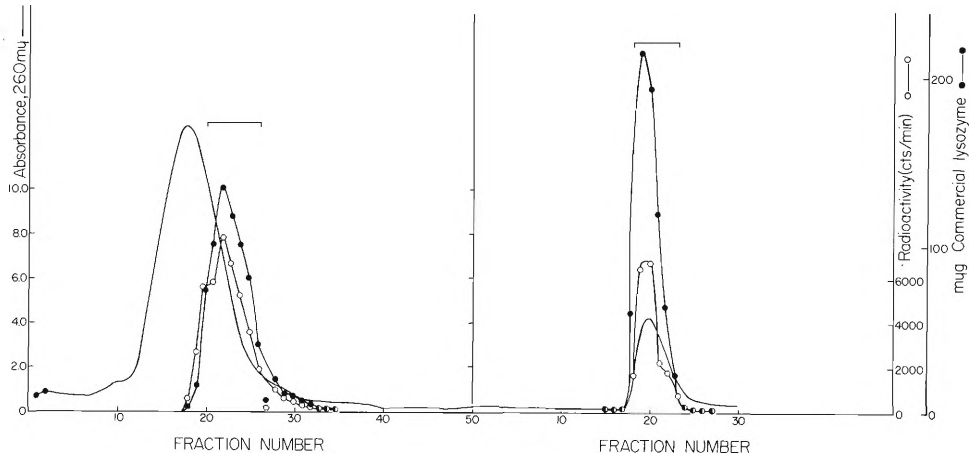


FIGURE 5. *Fractionation of T4 mRNA on BD cellulose columns.*

Bulk RNA (25–40 mg) was diluted with 10 volumes of 0.3 M NaCl, 0.02 M Tris-Cl, pH 7.5, and 0.001 M EDTA, and loaded onto a 2.5 cm × 5.0 cm column of benzoylated DEAE-cellulose, previously washed with the same buffer. The column was then washed with starting buffer until the optical density was zero. Two hundred ml of a linear gradient of NaCl from 0.3 M to 0.7 M and of dimethylsulfoxide from 0 to 30% (v/v) was applied at a flow rate of 1 ml/min. Four ml fractions were collected. At the end of the gradient the column was washed with 8 M urea in 0.1 M HAc pH 3.5 until the pH reached 3.5. A linear gradient of NH_4Cl (100 ml) from 0 to 1.0 M was then applied at a 1 ml/min flow rate (second part of graph). Again, 4 ml fractions were collected.

Optical density was read in a Gilford Spectrophotometer and each fraction precipitated with 2 volumes cold EtOH and $\frac{1}{10}$ volume 3 M NaAc, pH 5.0. After storing at least 20 hr at −20°C, the fractions were centrifuged and the pellets dissolved in sterile glass-distilled H_2O. The fractions were analyzed for capacity to make lysozyme in an in vitro protein synthesizing system and for incorporation of ^{14}C-valine into acid precipitable material. The amount of lysozyme made in vitro is expressed in mμg equivalents of a CalBiochem T4 lysozyme preparation.

conditions used on the gradient. These questions will not be resolved until we determine the sedimentation behavior of the active messenger in denaturing solvents or after purification. Work on both approaches is in progress.

Purification by Column Chromatography on Benzoylated DEAE Cellulose

Several purification procedures are available for fractionation of RNA on the basis of molecular weight. One of the most promising techniques which may provide a complementary method is chromatography on benzoylated DEAE (BD) cellulose. Here we report our preliminary results with this technique. RNA was prepared in the usual manner, including heat treatment to break down aggregates, and absorbed on columns of BD cellulose. The columns were then eluted following a technique developed by Sedat and associates, (1969) for the separation of the rapidly labeled RNA fraction of *E. coli* from longer-lived RNA components. The RNA is first absorbed onto a 2.5 × 5 cm column and then eluted, first with a gradient of increasing NaCl and dimethylsulfoxide concentrations in 0.02 M Tris-Cl, pH 7.5, 0.001 M EDTA, and then with a second gradient of increasing NH_4Cl concentrations in 0.1 M HAc, pH 3.5, 8 M urea (Fig. 5). The bulk of the rRNA is eluted at a concentration of about 0.51 M NaCl, 13% dimethylsulfoxide. A sharp peak of active lysozyme messenger (routinely about half of the total activity recovered) closely follows this peak. During the second gradient another peak of UV absorbing material is seen, which is much smaller than the first, and coincides with another peak of active lysozyme message usually comparable in amount to the first. The recoveries of UV absorbing material are essentially complete. The recovery of active lysozyme messenger is strongly dependent upon the source of the urea (Mann ultra high purity urea is satisfactory) and care in handling materials, but can approach 100%. In the case shown, 60% of the original activity was recovered in the two peaks.

Further purification is achieved by pooling the active fractions and re-running each of the two peaks on a BD cellulose column developed with a urea gradient as shown in Fig. 6. When the first peak was rerun the recovery of UV absorbing material was about 11% and the recovery of lysozyme mRNA activity about 93% (Fig. 6*A*) The purification of this material was 1.5-fold after the first step and 10-fold after the second (computed as the ratio of lysozyme messenger activity to UV-absorbing material). When the second peak from Fig. 5 was rerun (Fig. 6*B*) there was again about a 12% recovery of UV-absorbing material but also a low recovery of active lysozyme messenger (42%), so that the total overall purification achieved in the two steps is again 10-fold.

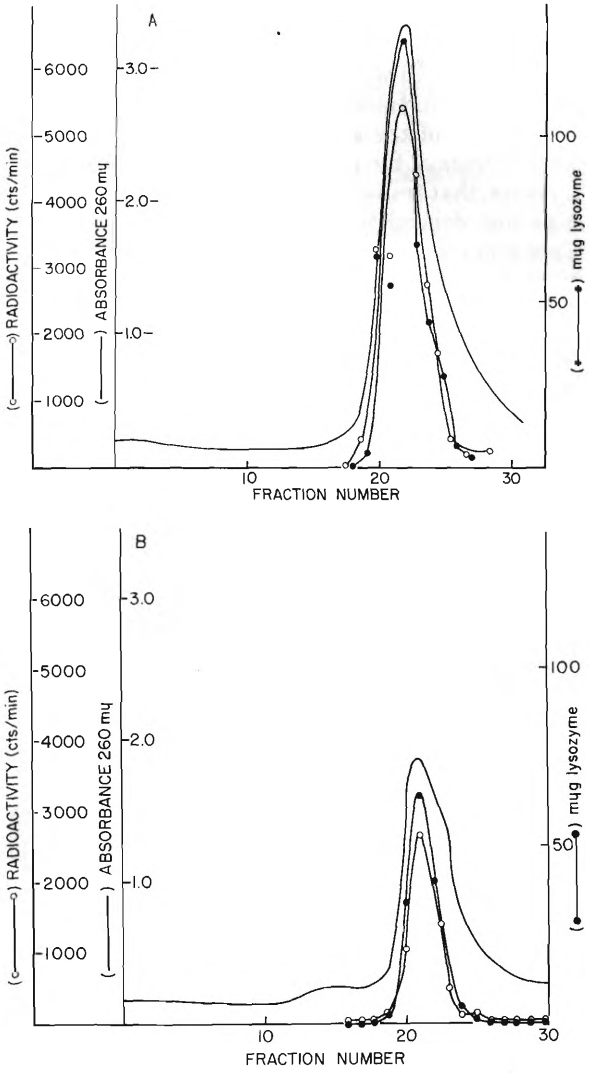


Figure 6. *Rechromatography on BD cellulose in urea at pH 3.5.*

(*A*) Fractions 20–26 of the NaCl, dimethylsulfoxide gradient in Fig. 4 were pooled, diluted with 10 volumes 8 M urea in 0.1 M HAc pH 3.5, and layered onto a 2.5 cm × 5 cm column of BD previously washed with the same buffer until the optical density of the effluent was low and constant. A 100 ml linear gradient from 0 to 1.0 M NH_4Cl in 8 M urea and 0.1 M CH_3COOH pH 3.5 was then applied at a 1 ml/min flow rate. Fractions of 4 ml were collected, ethanol precipitated, and analyzed in an in vitro protein synthesizing system, as in Fig. 4. (*B*) Fractions 18–23 of the NH_4Cl gradient in Fig. 4 were pooled and treated as in Fig. 5, left.

Sedat et al. (1969) has shown that the total pulse labeled RNA from *E. coli* is separated into two peaks coincident with our two peaks of active lysozyme messenger. He postulated that this did not necessarily represent fractionation into two groups of *different* messengers. The fact that we

find active lysozyme messenger in both positions agrees with this interpretation of his data.

There have been many reports of changes in activating enzymes, changes in existing tRNA species and of the synthesis of new tRNA species after infection by phage T4. Our results suggest, however, that translation of the lysozyme messenger does not depend upon any of these factors. We have removed the tRNA from messenger preparations by sedimentation on sucrose gradients, by chromatography on BD cellulose and by chromatography on Sephadex G-100 (Gesteland and Salser, in prep.). In all cases the lysozyme messenger obtained is fully active in extracts of uninfected cells, showing that the phage modified or phage synthesized tRNAs are not essential for translating any of the codons in lysozyme messenger. The results of Schweiger and Gold (this volume) suggest a similar conclusion for α-glucosidase and β-glucosidase.

Acknowledgments

We wish to thank Dr. Francois Gros for his interest in this work, for the support of his laboratory, and for his gracious hospitality during a pleasant year spent by one of us (WS) in Paris; John Sedat for advice on the BD cellulose chromatography, and Drs. Clifford Brunk and John Urey for a critical reading of the manuscript. This work has been aided by the excellent technical assistance of Miss Sabine Döhring, Mrs. J. Micklebank, and Mrs. Ruth Mazner. The research has been supported by a grant from the National Science Foundation to R.F.G., grants from the Fonds de Developpment de la Recherche Scientifique et Technique, the Centre National de la Recherche Scientifique, and the Ligue Nationale Francaise Contre le Cancer to Francois Gros, and grants from the National Institutes of Health (GM 10578-01) and the Cancer Research Coordinating Committee of the University of California to W.S. W.S. was a postdoctoral fellow of the Helen Hay Whitney Foundation (1967–68). R.F.G. is the recipient of a Public Health Service Research Career Development Award (Number 1-K4-GM-18, 029-01). B.R. was supported by training grant GM 1531 of the Institute of General Medical Sciences National Institutes of Health, US Public Health Service.

REFERENCES

Adams. J. M. On the release of the formyl group from nascent protein. J. Mol. Biol. *33:* 571.

Bautz, E. K. F., T. Kasai, E. Reilly, and F. A. Bautz. 1966. Gene-specific m-RNA. II. Regulation of m-RNA synthesis in *E. coli* after infection with bacteriophage T4. Proc. Nat. Acad. Sci. *55:* 1081.

Boedtker, H. 1968. Molecular weight and conformation of RNA, p. 429–450. *In* L. Grossman and K. Moldave [ed.] Methods in enzymology, article 129, vol. 12B. Academic Press, New York.

Bolle, A., R. H. Epstein, W. Salser, and E. Geiduschek. 1968a. Transcription during bacteriophage T4 development. Synthesis and relative stability of early and late RNA. J. Mol. Biol. *31:* 325.

——, ——, ——, ——. 1968b. Transcription during bacteriophage T4 development: Requirements for late messenger synthesis. J. Mol. Biol. *33:* 339.

Brawerman, G., M. Revel, W. Salser, and F. Gros. 1969. Initiation factor requirements for the *in vitro* synthesis of T4 lysozyme. Nature (in press).

Capecchi, M. R. 1966. Cell-free protein synthesis programmed with R17 RNA: Identification of two phage proteins. J. Mol. Biol. *21:* 173.

DeVries, J. K., and G. Zubay. 1967. DNA directed peptide synthesis. II. The synthesis of the α-fragment of the enzyme β-galactosidase. Proc. Nat. Acad. Sci. *57:* 1010.

Gesteland, R. F., W. Salser, and A. Bolle. 1967. *In vitro* synthesis of T4 lysozyme by suppression of amber mutations. Proc. Nat. Acad. Sci. *58:* 2031.

Gesteland, R. F., and W. Salser. 1969. Bacteriophage T4 lysozyme m-RNA. Genetics *61:* suppl. *1:* 429.

Gussin, G. N., M. R. Capecchi, J. M. Adams, J. E. Argetsinger, J. Tooze, K. Weber, and J. D. Watson. 1966. Protein synthesis directed by RNA phage messengers. Cold Spring Harbor Symp. Quant. Biol. *31:* 257.

Kasai, T., and E. K. F. Bautz. 1969. Regulation of gene-specific RNA synthesis in bacteriophage T4. J. Mol. Biol. *41:* 401.

Morse, D. E., R. F. Baker, and C. Yanofsky. 1968. Translation of tryptophan messenger RNA of *Escherichia coli*. Proc. Nat. Acad. Sci. *60:* 1428.

Nirenberg, M. W., and J. H. Matthaei. 1961. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. Proc. Nat. Acad. Sci. *47:* 1588.

Salser, W., R. F. Gesteland, and A. Bolle. 1967. *In vitro* synthesis of bacteriophage lysozyme. Nature *215:* 558.

Salser, W., J. Janin, and C. Levinthal. 1968. Measurement of the unstable RNA in exponentially growing cultures of *Bacillus subtilis* and *Escherichia coli*. J. Mol. Biol. *31:* 237.

Sedat, J., A. Lyon, and R. L. Sinsheimer. 1969. The purification of *E. coli* pulse labeled RNA by benzoylated DEAE-cellulose chromatography. J. Mol. Biol. *44:* 415.

Sekiguchi, M., and S. S. Cohen. 1964. The synthesis of messenger RNA without protein synthesis. II. Synthesis of phage induced RNA and sequential enzyme production. J. Mol. Biol. *8:* 638.

Tsugita, A., and M. Inouye. 1968. Complete primary structure of phage lysozyme from *E. coli* T4. J. Mol. Biol. *37:* 201.

Tsugita, A., M. Inouye, E. Terzaghi, and G. Streisinger. 1968. Purification of bacteriophage T4 lysozyme. J. Biol. Chem. *243:* 391.