[3] Swift, H. H., and R. Kleinfeld, *Physiol. Zool.*, **26**, 301 (1953).
[4] Wimber, D., and W. Prensky, *Genetics*, **48**, 1731 (1963).
[5] Abbadessa, R., and A. B. Burdick, *Drosophila Information Service*, **37**, 54 (1963).
[6] King, R. C., A. C. Rubinson, and R. F. Smith, *Growth*, **20**, 121 (1956).
[7] Brown, E. H., and R. C. King, *Growth*, **28**, 41 (1964).
[8] Jacob, J., and J. L. Sirlin, *Chromosoma*, **10**, 210 (1959).
[9] Grell, R. F., these PROCEEDINGS, **48**, 165 (1962).
[10] Rhoades, M. M., *The Cell*, ed. J. Brachet and A. E. Mirsky (New York and London: Academic Press, 1961).
[11] Maquire, M. P., *Genetics*, **51**, 23 (1965).
[12] Moens, P. B., *Chromosoma*, **15**, 231 (1964).
[13] Grell, R. F., *Symposium on Genes and Chromosomes*, J. National Cancer Institute Monograph 1965, in press.

---

*STUDIES ON MICROBIAL RNA, III. FORMATION OF SUBMETHYLATED sRNA IN SACCHAROMYCES CEREVISIAE**

BY KERSTIN KJELLIN-STRÅBY AND HANS G. BOMAN

MOLECULAR BIOLOGY GROUP, INSTITUTE OF BIOCHEMISTRY, UNIVERSITY OF UPPSALA, SWEDEN

Communicated by Arne Tiselius, April 14, 1965

The formation of methylated bases in sRNA has been studied in several laboratories. It has been established that the methyl groups in these bases originate from methionine, that S-adenosylmethionine can function as an intermediate, and that the reaction only occurs on a polynucleotide level (see refs. 1–3). With bacterial sRNA as substrate, RNA methylases have been demonstrated in a wide variety of species. However, sRNA from the same cells is normally a rather poor substrate,[4] presumably because it already contains its characteristic set of methylated bases. For bacteria, a few special conditions are known to produce an RNA which serves as substrate for enzymes from the same species (referred to as submethylated RNA). In *Escherichia coli* with relaxed control of RNA synthesis, submethylated RNA can be produced during methionine starvation.[1] In our group, we have recently found that chloramphenicol treatment of *E. coli* can give rise to the formation of submethylated RNA regardless of the presence of exogenous methionine.[5]

We have previously described a yeast enzyme which could methylate sRNA from log phase *E. coli*.[2] In the search for a submethylated yeast sRNA, it was natural to start with conditions resembling those which had produced submethylated RNA in *E. coli*. We have therefore investigated the result of methionine withdrawal from a methionine-requiring mutant of *Saccharomyces cerevisiae*. In such a strain, submethylated sRNA was found to be produced during a defined state of methionine depletion. The possible significance this may have for the control of RNA and protein synthesis will be discussed. We have also treated this strain with the antibiotic actidione which has been reported to produce effects in yeast comparable to chloramphenicol effects in bacteria.[6, 7]

Materials and Methods.—Organisms and media: Saccharomyces cerevisiae strain D84 was used except when otherwise stated. The strain was isolated and kindly donated by Dr. M. Grenson. It is haploid and heterothallic and requires methionine, histidine, and uracil. One liter basal medium[8] contained 0.7 gm of $MgSO_4$ (7 H_2O), 1 gm of KH_2PO_4, 0.4 gm of $CaCl_2$, 0.5 gm of NaCl, 1.2 gm of $(NH_4)_2SO_4$, 10.5 gm of citric acid, and 1 ml of "solution 59" (trace elements). One liter

of "solution 59" contained 500 mg of H_3BO_3, 40 mg of $CuSO_4(5\ H_2O)$, 100 mg of KI, 5 gm of $FeCl_3(6\ H_2O)$, 400 mg of $MnSO_4\ (H_2O)$, 200 mg of $Na_2MoO_4\ (2\ H_2O)$, 720 mg of $ZnSO_4(7\ H_2O)$, and 10 gm of citric acid.[8] Before sterilization of the basal medium, pH was adjusted to 6.1 (with about 16 ml of 10 *M* KOH) and the final volume was brought up to 1 liter. The basal medium was supplemented with 1% of a vitamin solution,[9] 3% glucose, 100 μg/ml of DL-methionine, 20 μg/ml L-histidine, and 100 μg/ml of uracil.[8] In this medium, strain D84 had a generation time of 140 min. Growth was measured with a Klett-Summerson photometer (filter no. 66, 13-mm light path). Daily transfers maintained the strain actively growing on agar slants with 2% of glucose and 0.5% of yeast extract.[8] For overnight cultures in minimal medium, inoculation from slants was estimated to give exponentially growing cultures, which next morning had a density of 60–120 Klett units. When a culture of D84 was depleted of a required metabolite, the cells were filtered (CO5, Membrane filter, Göttingen, Germany), washed 2–3 times on the filter with 0.5% of NaCl, and then resuspended in the desired medium. All solutions were prewarmed to the growth temperature, 30°C. After each experiment the strain was tested for its requirements. No reversions occurred.

In vivo incorporation of metabolites: The incorporation of uracil and phenylalanine into cold-acid-insoluble material was taken as measurements on RNA and protein synthesis.[10] Samples withdrawn from cultures (normally 150 μl) were pipetted down into 3 ml of ice-cold 5% TCA containing "cold" uracil and phenylalanine. After thorough mixing, the samples were left overnight at 0°C. Next day, the insoluble material was collected on CO5 filters, washed with 0.05 *N* HCl, dried at 60°C, and counted in a Nuclear-Chicago liquid scintillation counter (system 723). Heat-inactivated cells incubated with the same concentration of labeled metabolites were used as blanks. For uracil these blank values were rather high, but within the range of studies independent of the cell density. Tritium-labeled uracil was tried but gave too high blanks for double labeling experiments. All values given have been corrected for appropriate blanks (given in the legends).

Preparation of methylase and sRNA: The methylase preparation was from *S. cerevisiae* C836 grown in large scale as described before.[2] The purification procedure consisted of streptomycin and ethanol precipitations from a particle-free extract of homogenized cells (Björk and Svensson, to be published).

Cultures and aliquots for the preparation of sRNA were rapidly chilled, washed 3 times with cold 0.5% NaCl, and frozen. Total RNA was prepared as described elsewhere.[11] From this material sRNA was extracted by 1 *M* NaCl, precipitated with 70% ethanol, redissolved in 0.02 *M* tris-HCl, pH 7.3, and stored frozen.

Assay for the incorporation of methyl groups into sRNA: The reaction mixture had pH 8.0 and the concentrations of the reagents were: 100 mM tris-HCl, 10 mM $MgSO_4$, 20 mM NH_4Cl, 0.1 mM EDTA, 2 mM GSH, 0.01 mM C^{14}-methyl-labeled S-adenosylmethionine (39.4 mC/mM, New England Nuclear Corp., Boston, Mass.), 7.75 mg methylase (protein) per ml reaction volume, and varying amounts of sRNA. Reaction mixtures were made up in ice bath, and the incubations were carried out at 37°C. The reaction was terminated at different times by transferring of aliquots into 2 ml of cold 0.02 *M* $La(NO_3)_3$ in 0.5 *M* $HClO_4$. Addition of carrier, washing of precipitate, and counting with gas-flow counter were as described previously.[2]

Results.—RNA synthesis after methionine deprivation: Yeasts are known to have rather large pools of amino acids and other metabolites.[12] We therefore first decided to study RNA and protein synthesis during the emptying of the pool of a required amino acid. An exponentially growing culture of strain D84 was washed and divided into three equally diluted samples with minimal medium minus methionine. One sample was used to follow growth as recorded by a Klett photometer. Another sample was supplemented with C^{14}-uracil, and another with C^{14}-phenylalanine. All three samples were incubated on a rotary shaker at 30°C, and at different times aliquots were withdrawn from the tracer cultures. The results from these three methionine-depleted subcultures are shown in Figure 1. The emptying of the amino acid pool caused growth to decline in three distinct phases. The first one

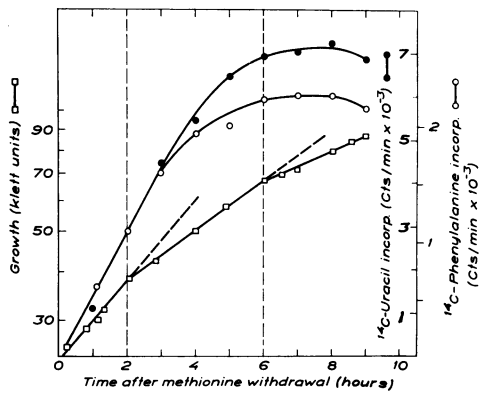

FIG. 1.—Growth (□) and incorporation of uracil (●) and phenylalanine (○) for the methionine-requiring yeast D84 during depletion of methionine. The incubation temperature was 30°C. An exponentially growing culture was filtered, washed with 0.5% NaCl, resuspended in methionine-free medium, and at zero time divided into three subcultures, one for each variable. The radioactivity added per ml culture was 3.8 μC of uracil and 0.06 μC of phenylalanine. Incorporation, estimated as cold-TCA-insoluble radioactivity per 150 μl aliquot, was corrected for blanks with heat-treated cells (820 cpm for uracil and 60 cpm for phenylalanine).

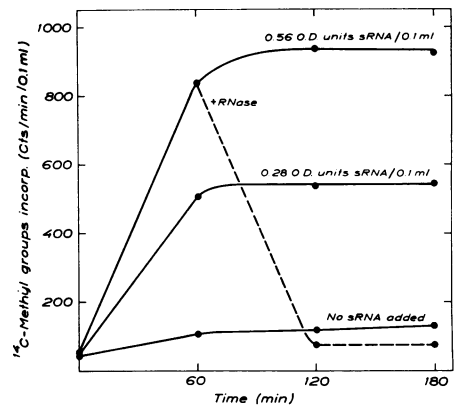

FIG. 2.—Time curves for the *in vitro* incorporation of methyl groups into submethylated sRNA for D84. The enzyme was a partly purified sRNA methylase from yeast, and S-adenosylmethionine served as methyl donor (see *Materials and Methods*). The incubation temperature was 37°C. The submethylated sRNA was from D84 which had been depleted for methionine during 9 hr at 30°C. Crystalline pancreatic RNase (Sigma Chemical Co., St. Louis, Missouri) was used in the final concentration of 50 μg/ml.

lasted for about 2 hr with a doubling time slightly lower than the normal 140 min. The second phase lasted for about 4 hr, and the third phase began 6 hr after methionine withdrawal. Uracil and phenylalanine incorporation continued at decreasing rates during the first two phases, but no incorporation was obtained during the third phase. Growth curves with breaks as in Figure 1 were also obtained during depletion of histidine or uracil. The duration of each of the two first phases was found to vary from one experiment to another. The third phase usually started 6–8 hr after withdrawal of the required metabolite.

Since RNA synthesis continued during methionine depletion, it was possible that this newly formed RNA could be devoid of its methylated bases. To test this proposition, sRNA was prepared from strain D84 in the third phase. The sRNA was assayed with a partly purified sRNA methylase from *S. cerevisiae* strain C836 and S-adenosylmethionine as methyl donor. The time curves in Figure 2 show that the sRNA had methyl acceptor activity, that the incorporation was proportional to the added amounts of sRNA, and that the reaction product was sensitive to RNase. The time course for the formation of submethylated sRNA was studied in a larger-scale experiment. From 1-liter cultures, 200-ml samples were withdrawn at different times during the three growth phases. sRNA was prepared from each sample and assayed as in Figure 2. The specific activities for sRNA from cells harvested at different times are shown in Figure 3. sRNA from cells in the first phase did not accept methyl groups, and the same result was obtained with sRNA from cells in balanced growth in minimal medium (cf. also Table 2). During the second phase submethylated sRNA began to appear and the methyl acceptor

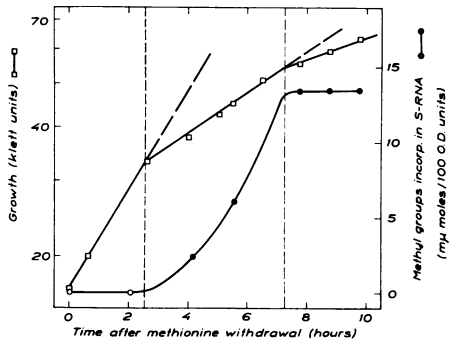

FIG. 3.—Growth (□) and the formation of submethylated sRNA (circles) during a methionine depletion experiment with large cultures of strain D84. The two first points (○) and the five following (●) were from two parallel subcultures. Each value for the methyl group incorporation into sRNA was obtained from the plateau values of two time curves with different amounts of sRNA.

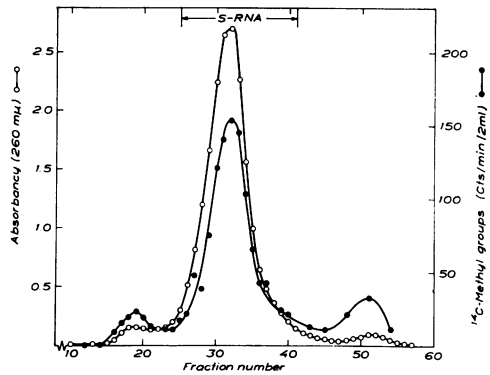

FIG. 4.—Gel chromatogram of sRNA reisolated after *in vitro* incorporation of methyl groups into submethylated sRNA (●). Carrier yeast sRNA was added to increase the absorbancy at 260 mμ (○). Column (1.5 × 55 cm) with Sephadex G 200 in equilibrium with 0.05 *M* TEA-HAc buffer, pH 5.4. Fraction volume about 2 ml. Aliquots (200 μl) removed for estimation of radioactivity were counted for 1000 counts.

activity increased until a constant value was reached at the beginning of the third phase.

Characterization of in vitro methylated sRNA: The following experiment was performed in order to ascertain that the methyl group acceptor was sRNA and not a contaminating RNA of a larger molecular weight. sRNA from the third growth phase was methylated as in Figure 2. The reaction was terminated by the addition of phenol, carrier yeast sRNA was added, and total RNA was reisolated as described previously. The labeled RNA obtained was examined by gel chromatography during conditions known to separate rRNA and sRNA.[13] The results in Figure 4 show that most of the radioactivity was recovered in the sRNA region. Minor peaks were obtained in the rRNA region (maximum in tube 19) and in the region for low-molecular-weight compounds (maximum in tube 51).

The methylated sRNA from Figure 4 was recovered and lyophilized. It was dissolved in 1 *M* KOH and left at 37°C for 18 hr. The neutralized hydrolyzate was examined by paper electrophoresis. Figure 5 shows that the main band of radioactivity traveled slightly slower than the band of uridine phosphate. The methylated compound is therefore tentatively identified as thymine ribotide. In addition the hydrolyzate contained smaller amounts of a methylated guanosine phosphate.

The fact that thymine ribotide was the main methylated nucleotide obtained during *in vitro* methylation of sRNA does not necessarily imply that *in vivo* the formation of thymine was blocked selectively. It is possible that the enzyme fraction we have used was a partly purified uridyl-5-methylase or that the reaction conditions used could have favored the formation of thymine. The previous lack of a homologous substrate has prevented the characterization of the enzyme preparation.

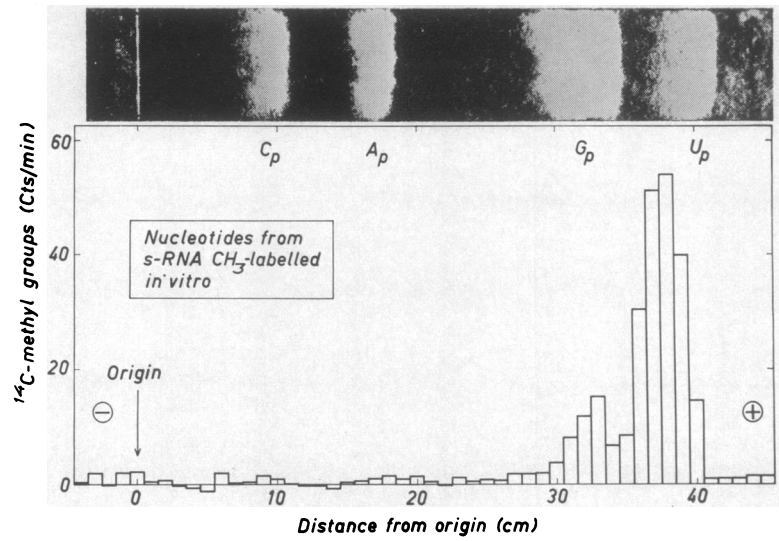

FIG. 5.—Paper electrophoresis (2 hr at 40 v/cm) of alkaline hydrolyzate obtained from the sRNA peak in Fig. 4. Buffer 0.05 *M* NH_4COOH pH 3.5. Ultraviolet print (top part) and other conditions as described.[14] The paper was cut into 1-cm strips and counted in a liquid scintillation counter (lower part).

In vivo effects of actidione: Yeast strains have been found to differ considerably in their responses to actidione. We therefore first examined the influence of different concentrations of the antibiotic on exponentially growing as well as methionine-depleted cultures of strain D84. As in Figure 1, RNA and protein synthesis were followed through the incorporation of uracil and phenylalanine. Table 1 shows that protein synthesis was strongly inhibited by 0.5 μg of actidione/ml. Also, RNA synthesis was inhibited but a residual incorporation of about 25 per cent was left even after 2 hr with 5 μg actidione/ml. These effects were comparable to the differential inhibition reported previously and indicated that the primary action of actidione was the blocking of protein synthesis.[6] An actidione concentration of 5 μg/ml was chosen for treatment of two cultures of D84, one in exponential growth phase, the other in the third phase obtained after methionine withdrawal. After the incubation with actidione, sRNA was isolated and tested for *in vitro* incorporation of methyl groups. The results in Table 2 show that the submethylated sRNA

TABLE 1

In vivo EFFECTS OF ACTIDIONE ON RNA AND PROTEIN SYNTHESIS

Growth state	Actidione conc. (μg/ml)	Incorporation (cpm) Uracil 30 min	Uracil 120 min	Phenylalanine 30 min	Phenylalanine 120 min
Log phase	0	659	5017	699	3090
"	0.5	598	1375	129	327
"	5	550	1348	14	42
"	25	357	999	8	26
Prestarved	0	8	329*	101	221*
"	5	31	407*	—	—

* Incubation time, 150 min.

Cultures of D84 were incubated at 30°C with C^{14}-uracil (1.6 μC/ml) or C^{14}-phenylalanine (0.06 or 0.09 μC/ml). The prestarvation was 7 hr incubation at 30°C in methionine-free medium. Both tracers and actidione were added at zero time. Incorporation, estimated as cold-TCA-insoluble material in 200-μl aliquots, was corrected for blanks with heat-treated cells (460 cpm for uracil and 82 or 113 cpm for phenylalanine).

TABLE 2

In vitro METHYLATION OF sRNA FROM YEAST TREATED WITH ACTIDIONE

Growth state	Actidione conc. (μg/ml)	Methyl groups incorporated (mμmoles/100 O.D. units sRNA)
Log phase	0	<0.1
Log phase	5	<0.1
Prestarved	0	34.5
Prestarved	5	3.5

An exponentially growing culture of D84 was divided into two cultures with complete medium and two others with methionine-free medium. After 7.5 hr incubation at 30°C, when the methionine-depleted cultures entered the third phase (cell density, 70 Klett units), one of them was supplemented with actidione. Actidione treatment of the log phase culture was started at a cell density of 70 Klett units. All cultures were harvested after 2 hr additional incubation at 30°C with or without actidione. sRNA was prepared and tested for its methyl group acceptor activity with a partly purified methylase from yeast (see *Materials and Methods*). Each value for the acceptor activity given in the table was obtained from determinations with two different amounts of the same sRNA preparation.

formed during the methionine depletion is changed to methylated sRNA by actidione treatment. Since actidione did not block the methylation of sRNA, the proposed similarity in action between chloramphenicol and actidione[6, 7] does not fully hold for the influence on the *in vivo* methylation of RNA.

Discussion.—Our results presented here indicate that the formation of submethylated RNA may be somewhat different in bacteria and yeast. The growth curves shown in Figures 1 and 3 were drawn in a log scale in spite of the fact that a linear plotting for the two first phases would fit the data almost equally well. However, the curve for the formation of submethylated RNA (Fig. 3) showed three distinct phases with transitions simultaneously to the breaks in the exponentially plotted growth curve. Additional evidence for a transition from a second to a third phase was obtained from the fact that the incorporation of uracil and of phenylalanine, and also viable counts, reached plateau levels at this stage. The precise nature of the three phases is not yet clear, but it seems likely that the third phase represents a true starvation which starts when the pool of the withdrawn amino acid is fully exhausted.

In a "stringent" bacterial strain, RNA and protein synthesis are both stopped as soon as a required amino acid is withdrawn. In the yeast strain D84, RNA and protein synthesis were stopped simultaneously at the end of the second phase (see Fig. 1). If this concomitant control is the essential criterion, it will be most reasonable to consider strain D84 as "stringent." The fact that in D84 submethylated sRNA was produced during the second phase can be explained simply as a competition for the last portion of the methionine pool. No competition of this type has yet been observed in bacteria where the pool is smaller and can be removed by washing of the cells.[15] However, in yeast a methyl competition could have a function since the absence of some of the methylated bases in sRNA could serve as a regulatory device for the stopping of RNA and protein synthesis. It has previously been suggested that in bacteria catalytic amounts of free amino acids together with sRNA regulate RNA synthesis.[16–20] The fact that actidione treatment caused submethylated sRNA to disappear from methionine-depleted cells is most easily explained by assuming that methionine was released from breakdown of protein. This would mean that catalytic amounts of free amino acids were produced without a concomitant release of RNA synthesis. The argument can, however, be invalid if a slow primary effect of actidione could stop RNA synthesis. If not, in *S. cerevisiae* both the control of RNA synthesis and the cause of the formation of submethylated

RNA may differ from the corresponding mechanism operating in *E. coli*. This is consistent with the species specificity observed for the *in vitro* methylation of sRNA from these species.

Summary.—For a methionine-requiring yeast mutant, growth conditions are described which result in the formation of submethylated sRNA. This sRNA was assayed with S-adenosylmethionine and a partly purified methylase from yeast. The reaction product was characterized, and thymine was the main methylated base formed. Actidione treatment of methionine-starved yeast caused the submethylated sRNA to disappear.

We should like to thank Dr. M. Grenson for the yeast mutant used and for valuable advice on its growth and maintenance. Mr. G. Björk and Dr. I. Svensson kindly provided us with a partly purified preparation of sRNA methylase. A sample of actidione was donated by the Upjohn Company, Kalamazoo, Michigan. The work was supported by grant Dnr 2453 from the Swedish Natural Science Research Council. K. K.-S. was the holder of a Sven and Lilly Lawski fellowship.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; O.D. unit, the amount of material which in 1 ml gives $A_{260} = 1.00$; sRNA, soluble RNA; rRNA, ribosomal RNA; TCA, trichloroacetic acid; TEA-HAc, triethylamine-acetic acid.

* Part I in this series is ref. 2; part II is ref. 11.

[1] Srinivasan, P. R., and E. Borek, *Science*, **145**, 548 (1964).

[2] Svensson, I., H. G. Boman, K. G. Eriksson, and K. Kjellin, *J. Mol. Biol.*, **7**, 254 (1963).

[3] Hurwitz, J., M. Gold, and M. Anders, *J. Biol. Chem.*, **239**, 3462 and 3474 (1964).

[4] Gold, M., J. Hurwitz, and M. Anders, these PROCEEDINGS, **50**, 164 (1963).

[5] Gordon, J., H. G. Boman, and L. A. Isaksson, *J. Mol. Biol.*, **9**, 831 (1964).

[6] Kerridge, D., *J. Gen. Microbiol.*, **19**, 497 (1958).

[7] Siegel, M. R., and H. D. Sisler, *Biochim. Biophys. Acta*, **87**, 70 (1964).

[8] Grenson, M., personal communication.

[9] De Deken, R. H., *Biochim. Biophys. Acta*, **78**, 606 (1963).

[10] Halvorson, H., *Biochim. Biophys. Acta*, **27**, 255 (1958).

[11] Gordon, J., and H. G. Boman, *J. Mol. Biol.*, **9**, 638 (1964).

[12] Halvorson, H., W. Fry, and D. Schwemmin, *J. Gen. Physiol.*, **38**, 549 (1954–1955).

[13] Boman, H. G., and S. Hjertén, *Arch. Biochem. Biophys.*, Supplement 1, 276 (1962).

[14] Markham, R., in *Modern Methods of Plant Analysis*, ed. K. Peach and M. V. Tracey (Berlin: Springer-Verlag, 1955), vol. 4, p. 278.

[15] Holden, J. T., in *Amino Acid Pools*, ed. J. T. Holden (Amsterdam: Elsevier Publishing Co., 1962), p. 78.

[16] Aronson, A. I., and S. Spiegelman, *Biochim. Biophys. Acta*, **53**, 84 (1961).

[17] Stent, G. S., and S. Brenner, these PROCEEDINGS, **47**, 2005 (1961).

[18] Kurland, C. G., and O. Maaløe, *J. Mol. Biol.*, **4**, 193 (1962).

[19] Alföldi, L., G. S. Stent, M. Hoogs, and R. Hill, *Z. Vererbungslehre*, **94**, 285 (1963).

[20] Eidlic, L., and F. C. Neidhardt, *J. Bacteriol.*, **89**, 706 (1965).