[3] Mitchell, P., *Ann. Rev. Microbiol.*, **13**, 407 (1959); *Quart. J. Exptl. Physiol.*, **44**, 207 (1959).
[4] Munkres, K. D., N. H. Giles, and M. E. Case, *Arch. Biochem. Biophys.*, **109**, 397 (1965).
[5] Munkres, K. D., and F. M. Richards, *Arch. Biochem. Biophys.*, **109**, 457 (1965).
[6] *Ibid.*, p. 466.
[7] Munkres, K. D., *Arch. Biochem. Biophys.*, **112**, 340 (1965).
[8] Munkres, K. D., *Biochemistry*, **4**, 2180, 2186 (1965).
[9] Munkres, K. D., unpublished data.
[10] Luck, D. J. L., and E. R. Reich, these PROCEEDINGS, **52**, 931 (1964).
[11] Criddle, R. S., R. M. Bock, D. E. Green, and H. D. Tisdale, *Biochemistry*, **1**, 827 (1962).
[12] Woodward, D. O., and K. D. Munkres, these PROCEEDINGS, **55**, 872 (1966).
[13] Chen, R. F., and R. L. Bowman, *Science*, **147**, 729 (1965).
[14] Munkres, M. M., and K. D. Munkres, manuscript in preparation.
[15] Green, D. E., and D. M. Ziegler, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 113.
[16] Fernández-Morán, H., *Circulation*, **26**, 1039 (1962); Blair, P. V., T. Oda, D. E. Green, and H. Fernández-Morán, *Biochemistry*, **2**, 756 (1963).
[17] Allmann, D. W., and E. Bachmann, *Federation Proc.*, **24**, 425 (1965).
[18] Weber, G., *Advan. Protein Chem.*, **8**, 416 (1953).
[19] Mitchell, P., in *Membrane Transport and Metabolism* (New York: Academic Press, 1961), p. 22.
[20] Crick, F. H. C., and L. E. Orgel, *J. Mol. Biol.*, **8**, 161 (1964).
[21] Reithel, F. J., *Advan. Protein Chem.*, **18**, 123 (1963).
[22] Edwards, D. L., and R. S. Criddle, *Federation Proc.*, **24**, 223 (1965); Goldberger, R., A. Pumphrey, and A. Smith, *Biochim. Biophys. Acta*, **58**, 307 (1962).
[23] McLaren, A. D., P. Mitchell, and H. Passow, *Cell Interface Reactions* (New York: Scholar's Library, 1963); Goldstein, L., Y. Levin, and E. Katchalski, *Biochemistry*, **3**, 1913 (1964); Goldman, R., H. I. Silman, S. R. Caplan, O. Kedem, and E. Katchalski, *Science*, **150**, 758 (1965).
[24] Pardee, A., and A. C. Wilson, *Cancer Res.*, **23**, 1483 (1963).
[25] Horowitz, N. H., and R. L. Metzenberg, *Ann. Rev. Biochem.*, **34**, 527 (1965).
[26] Weiland, T., and G. Pfleider, *Advan. Enzymol.*, **25**, 329 (1965).
[27] Allen, S. L., *Ann. N.Y. Acad. Sci.*, **94**(3), 753 (1961).
[28] Markert, C. L., *Harvey Lectures, Ser. 59 (1963–64)*, **1965**, 187.
[29] Munkres, K. D., and D. O. Woodward, manuscript in preparation.

---

*THE INHIBITION BY CHLORAMPHENICOL OF NASCENT PROTEIN FORMATION IN E. COLI**

BY MICHAEL J. WEBER† AND J. A. DEMOSS

DEPARTMENT OF BIOLOGY, REVELLE COLLEGE, UNIVERSITY OF CALIFORNIA, SAN DIEGO (LA JOLLA)

Communicated by Bruno H. Zimm, March 21, 1966

It has been known for some time that chloramphenicol (CAP) inhibits protein synthesis,[1] but it is still not known precisely at which step it acts. The antibiotic does not prevent amino acid activation[2] or esterification to sRNA,[3] but interferes with some later step in protein synthesis.[4] Since CAP binds to ribosomes both *in vivo* and *in vitro*,[5, 6] it has been suggested that the antibiotic inhibits some ribosomal function. Ribosome participation in protein synthesis can be considered as a sequence of three events: (1) initiation, in which ribosomes become attached to messenger RNA to form polysomes;[7] (2) chain growth, in which the information of the message is sequentially read, peptide bonds are formed, and the nascent polypeptide chain increases in length; and (3) release, in which the completed protein

is detached from the ribosome. A number of investigators have attempted to establish which of these steps is inhibited by CAP in an *in vitro* system, but the results of these studies have frequently been ambiguous. Thus, Jardetzky and Julian,[8] and Wolfe and Weisberger[9] report that CAP inhibits the binding of messenger RNA to ribosomes, while Kućan and Lipmann[10] report the opposite, and other workers[11, 12] offer independent evidence that peptide-bond formation is directly inhibited. Furthermore, our own experience has been that cell-free protein synthesizing systems, prepared from the same strain of *E. coli*, can be highly variable in their sensitivity to CAP. We turned, therefore, to an examination of the effect of CAP on the assembly of ribosomes into polysomes and on the synthesis of nascent protein by these polysomes in growing cells. We conclude that CAP interferes with the growth of nascent polypeptide chains, and does not inhibit protein synthesis by preventing the binding of ribosomes to messenger RNA or the release from the ribosome of finished proteins. Das *et al.* have independently arrived at a similar conclusion using different techniques.[13]

Materials and Methods.—Bacterial culture conditions: The strain of *Escherichia coli* used was W-60, a nonreverting arginine auxotroph obtained from Dr. Henry J. Vogel. Bacteria were grown with forced aeration at 33°C in a mineral salts medium[14] plus 0.2% glucose. For most experiments, arginine was added at a concentration of 50 μg/ml. For amino acid starvation, arginine was added at a concentration of 20 μg/ml which allowed growth to a cell density of approximately 1.1×10^9 bacteria/ml. Aeration was continued in the exhausted medium for 30 min before the experimental manipulations were begun. Growth was followed with a Klett-Summerson colorimeter, using the green filter.

Preparation and analysis of extracts: A 700-ml culture of bacteria was grown to a cell density of approximately 1.1×10^9 cells/ml and 100-ml samples were taken by forced aeration either directly over crushed ice or into a tenfold excess of ice-cold standard buffer (0.05 *M* Tris, 0.01 *M* $MgCl_2$, 0.06 *M* KCl, pH 7.8). In the first case, cells were collected by centrifugation and, in the second case, on a 142-mm Millipore filter. The collected cells were resuspended in 3 ml of cold standard buffer and were broken in a chilled French pressure cell[15] using a Carver press registering an applied pressure of 4,000–5,000 lb. This is equivalent to a pressure of 5,000–6,250 psi inside the cell. Under these conditions cell breakage was approximately 30%. Higher pressures gave increased cell breakage but caused a sharp decrease in the polysome content of the extract. The extract was layered on a 28-ml 30–15% linear gradient of sucrose in standard buffer and centrifuged in the SW 25.1 Spinco rotor for 3 hr at 25,000 rpm. After centrifugation the bottom of the tube was punctured, the contents were pumped out with a Harvard syringe pump, and optical density at 260 mμ was monitored using a Gilford model 2000 recording spectrophotometer with a 2-mm light path flow-through cell. When radioactivity determinations were made, fractions were collected from the Gilford flow-through cell outlet. Incorporation of C^{14} amino acids into protein was determined by precipitating samples with an equal volume of 10% trichloroacetic acid in the presence of 0.5 mg/ml bovine serum albumin, and heating for 20 min at 90°C. Precipitates were then chilled and collected either on Whatman glass fiber filters (GF/A) or on Millipore filters, and were washed with 15 vol of 5% TCA. Filters were thoroughly dried under a heat lamp, placed in scintillation bottles with 10 ml of a toluene-based scintillation fluid,[16] and counted in a Nuclear-Chicago model 725 scintillation counter.

Labeling of nascent protein: C^{14} amino acids from one of two sources were used. The first was 15 μc of a reconstituted protein hydrolysate (99% L-amino acid) from Schwarz BioResearch, to which was added a carrier solution containing 0.22 μmole of each of the amino acids (alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine).

The second source of C^{14} amino acids was a mixture of 1 μc each of six amino acids from New England Nuclear (aspartic acid, isoleucine, lysine, phenylalanine, tyrosine, and valine) with the same carrier solution used with the protein hydrolysate, except that arginine was omitted (i.e., 0.22 μmole of each of 12 amino acids was added as carrier).

For the incorporation experiments, the 15 μc of protein hydrolysate plus carrier or 6 μc of amino acid mixture plus carrier were added to 50 ml of warm glucose-salts medium, which in turn was poured into a vigorously aerated culture (700 ml at a cell density of 1.1×10^9 bacteria/ml). Samples were then taken as described.

Polysome specific activity: The specific activity of the polysome region [(cpm/ml)/OD_{260}] is a measure of the average amount of nascent protein present per ribosome, which in turn reflects the activity of these ribosomes in protein synthesis. The specific activity of the soluble region measures the amount of newly synthesized completed protein. Specific activity was determined in either of two ways. (1) OD_{260} of a sucrose density gradient was determined with a Gilford model 2000, and approximately 1-ml fractions were taken. For each fraction, radioactivity was determined as described and plotted on the OD tracing. The tracing was divided into regions (Fig. 1), and the counts per minute in each region were summed. The area under the OD tracing for each region was determined with a planimeter and converted to OD units for a 1-cm light path. Specific activity for each region by this method is [(Σcpm/ml)/(ΣOD_{260})]. (2) OD_{260} was monitored with a Gilford model 2000, and each region (pellet, polysome, ribosome, soluble) was collected as it came off. The average OD for each region was determined in the Zeiss PM QII, and 1 ml of each region was then assayed for radioactivity as described. The specific activity by this method is [(cpm/ml)/OD_{260}].

Biochemicals: Chloramphenicol was a gift from Parke-Davis and Co. Ribonuclease was 5$\times$ crystallized, protease-free bovine pancreatic, from Sigma. Carrier amino acids were all L-isomer, A-grade from Calbiochem. All other chemicals were reagent grade and obtainable from commercial sources.

Results.—Nascent protein in exponentially growing cells: The specific activity of the polysome region of a sucrose gradient following a pulse of C^{14} amino acids (see *Methods*, Fig. 1) is a measure of the amount of newly synthesized protein associated with polysomes, and the amount of the label "chased" off polysomes in the presence of an excess of unlabeled amino acids is a measure of the fraction of this newly synthesized protein which is "nascent" protein.[15] Figure 2*A* shows the kinetics of appearance of nascent protein in a growing culture of *E. coli*. After addition of the C^{14} amino acids, the specific activity of the polysome region increased rapidly to a steady-state level.

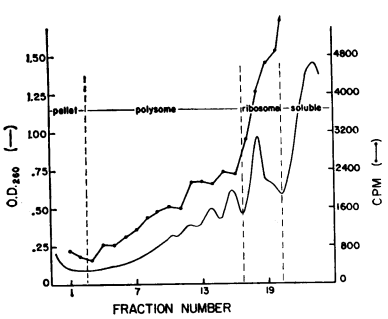

FIG. 1.—Sucrose density gradient analysis of labeled extract. C^{14} protein hydrolysate (15 μc) was added to a growing culture. After 25 sec, CAP (100 μg/ml) was added, followed immediately by a 100-fold excess of C^{12} amino acids. The sample was taken at 120 sec and treated as described in *Methods*. Essentially all of the 260-mμ absorbing material in the polysome region can be converted to ribosomes by treatment for 15 min at 4°C with 5 μg/ml RNase prior to centrifugation. The ratio of polysomes to ribosomes is the area of the polysome region (determined with a planimeter) divided by the area of the ribosome region.

The linear incorporation of labeled amino acids into soluble protein indicates that the plateau of polysome specific activity is a reflection of the steady-state level of nascent protein, and is not due to a shortage of labeled amino acids. The addition of a 100-fold excess of C^{12} amino acids to cells which had accumulated the steady-state amount of labeled nascent protein (Fig. 2*B*) resulted in an immediate drop in polysome specific activity, and the increase in specific activity of soluble proteins ceased shortly thereafter. The data demonstrate that after a 20-sec pulse with C^{14} amino acids, at least 75 per cent of the newly synthesized protein associated with polysomes is nascent, nonribosomal protein.

Does CAP Inhibit after Initiation?—If CAP inhibits protein synthesis by preventing the

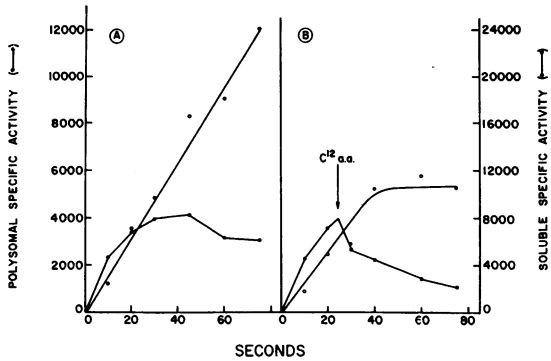

FIG. 2.—Kinetics of appearance of labeled nascent and soluble protein in exponentially growing *E. coli*. (*A*) C^{14} protein hydrolysate (15 μc) was added to a growing culture. (*B*) Same as (*A*), except that a 100-fold excess of C^{12} amino acid mixture was added at 23 sec. Sampling and analysis were as described in *Methods*. Specific activity is $[(\Sigma \text{ cpm/ml})/(\Sigma \text{ OD}_{260})]$.

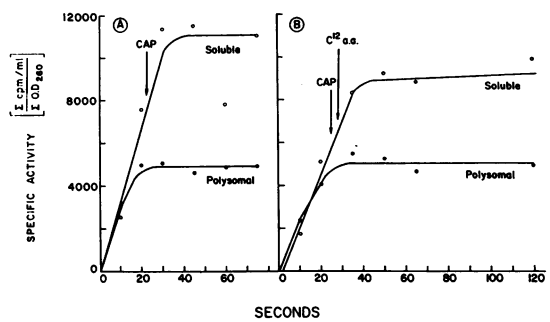

FIG. 3.—Chloramphenicol inhibition of nascent polypeptide growth. (*A*) Conditions were the same as in Fig. 2, except that at the indicated time CAP (100 μg/ml) was added to the culture. (*B*) Same as (*A*), except that a 100-fold excess of C^{12} amino acids was added immediately after the CAP.

attachment of ribosomes to messenger RNA[8, 9, 17] or by preventing any other step in initiation, then one might expect to see in CAP-treated cells the completion of already initiated polypeptide chains and a decrease in the cell's polysome content. The opposite result was obtained, however (Figs. 1 and 3). Cells in the presence of CAP maintained their full complement of polysomes, and nascent protein associated with these polysomes was not completed and released, nor could it be chased by unlabeled amino acids. The data indicate that CAP inhibits protein synthesis at some step after initiation, and thus could not act simply by preventing ribosome binding to messenger RNA.

Further evidence that CAP does not prevent the binding of ribosomes to messenger RNA *in vivo* depends on the observation of D. W. Morris in this laboratory that starving an amino acid auxotroph for its required amino acid causes a reduction in the cell's polysome content (unpublished observations). Under our conditions the ratio of polysomes to ribosomes (see Fig. 1) in an exponentially growing culture, or in a culture inhibited with CAP, was generally 1.5–1.8. A culture starved 30 min for arginine generally had a polysome-to-ribosome ratio of 0.2–0.6.

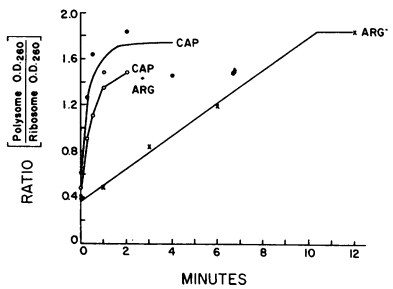

FIG. 4.—Kinetics of polysome return after amino acid starvation. Cultures were deprived of arginine for 30 min, and then the indicated additions were made (arginine at 20 μg/ml, CAP at 100 μg/ml).

Addition to a starved culture of CAP + arginine, or of CAP alone, resulted in the rapid formation of polysomes (Fig. 4). Polysome formation subsequent to readdition of only the required amino acid was substantially slower. Thus, CAP seems to stimulate the formation of polysomes. The fact that polysomes are formed in the presence of CAP demonstrates that the antibiotic does not inhibit the binding of ribosomes to messenger RNA.

Several related observations support the validity of the above conclusion. For example, it might be argued that CAP inhibits ribosome attachment to messenger RNA, but that the polysomes derived from CAP-treated cells were formed after breakage of the cells. We have eliminated this objection by determining that CAP-treated cells resuspended and broken in the presence of 100 μg/ml of CAP had a polysome-to-ribosome ratio identical to that of CAP-treated cells resuspended and broken in the absence of CAP. Alternatively, one might suggest that cells starved for an amino acid are not deficient in polysomes, but that breakage releases a nuclease from these cells that destroys polysomes in the extract. This objection was eliminated by mixing an extract from an amino acid-starved culture with an equal volume of an extract from a CAP-treated culture and incubating at 4°C for 20 min prior to centrifugation. The polysome-to-ribosome ratio of the mixed extract was the average of the two unmixed extracts, indicating that a starved culture does not have an enhanced nuclease activity. Finally, it might be argued that polysomes formed in the presence of CAP are ribosomal aggregates not formed on a messenger RNA. However, treatment with 5 μg/ml of RNase at 4°C for 15 min converted essentially all of these polysomes to ribosomes, indicating that the ribosomes were attached to a messenger RNA. We therefore conclude that the polysome-to-ribosome ratio we obtain in extracts from growing, starved, and CAP-treated cultures is a reflection of the true state of polysomal association in the cell, and that CAP does not inhibit the binding of ribosomes to messenger RNA *in vivo*.

Does CAP Inhibit Release?—We have demonstrated that CAP inhibits the growth of already initiated polypeptide chains, but have not yet distinguished between a mode of action which blocks polysome function by inhibiting the release of finished proteins and one which inhibits the growth of nascent chains in a more direct manner. If CAP inhibits only release, then the polysomes formed by adding CAP to an amino acid-starved (polysome-deficient) culture must be "normal" polysomes, i.e., formed by ribosomes attaching to the messenger RNA, synthesizing protein along its length, and then, due to the presence of the antibiotic, stopping at the end. Polysomes thus formed should be rich in nascent protein. If the growth of nascent polypeptide is directly inhibited, however, no protein synthesis should take place during the CAP-stimulated formation of polysomes, and the polysomes formed in the presence of CAP should be deficient in nascent protein. Figure 5 demonstrates that the latter is the case. A mixture of C^{14} amino acids added during the course of CAP-stimulated polysome formation was incorporated

at a rate 1 per cent of the rate in the absence of CAP, and the burst of incorporation one would expect to see if nascent protein synthesis was taking place concomitantly with polysome formation was not seen. Moreover, CAP-stimulated polysome formation in a culture recovering from amino-acid deprivation resulted in a decrease in the specific activity of the polysome region (Fig. 6). Since polysomes formed by continued protein synthesis should display a constant, or increased, specific activity, this further demonstrates that polysomes formed in the presence of CAP are deficient in nascent protein. We therefore conclude that CAP does not act by inhibiting the release from ribosomes of finished proteins, but inhibits in some other manner the growth of nascent polypeptide chains.

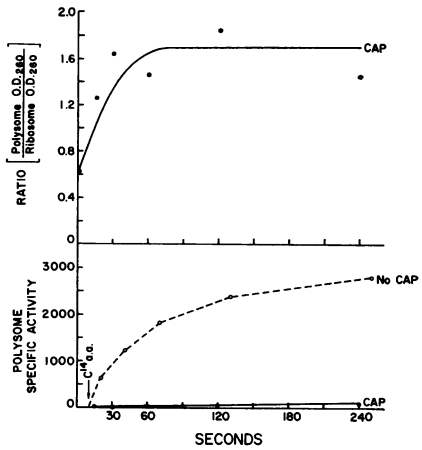

FIG. 5.—Absence of protein synthesis during the CAP-stimulated formation of polysomes. A culture was deprived of arginine for 30 min and CAP (100 μg/ml) was then added. Ten sec later the arginine-free C^{14} amino acid mixture (6 μc) was added. The formation of polysomes and the specific activities were determined as described in *Methods* (Fig. 1). The specific activity of the soluble region (not shown here) was half that of the polysome region. The *dashed line* represents the increase in polysome specific activity of a culture to which CAP had not been added. Specific activity is [(cpm/ml)/OD_{260}].

Discussion.—We have demonstrated that CAP stops the growth of nascent polypeptide chains on polysomes, that it does not prevent the binding of ribosomes to messenger RNA and that it does not inhibit protein synthesis by blocking the release from the ribosome of finished proteins. Therefore, the antibiotic would appear to inhibit some step which is common to the formation of each peptide bond of a protein. From *in vitro* studies it is known that at least three soluble components participate in the formation of peptide bonds on polysomes: charged transfer RNA, and the two transfer factors.[18] It is possible that CAP inhibits directly either their interaction with, or their activity on, polysomes. Further work *in vitro*, with systems sensitive to CAP, will be necessary to clarify this point.

If CAP acts by directly inhibiting one of the components of peptide-bond synthesis, then the polysomes formed in the presence of CAP must represent ribosomes which are attached only to the initiation sites on a messenger RNA. However, when CAP is added to an amino acid-starved culture, the size distribution of the polysomes formed seems to be the same as that found in exponentially growing cultures, even though no nascent protein has been synthesized. This might suggest that either (1) there are a

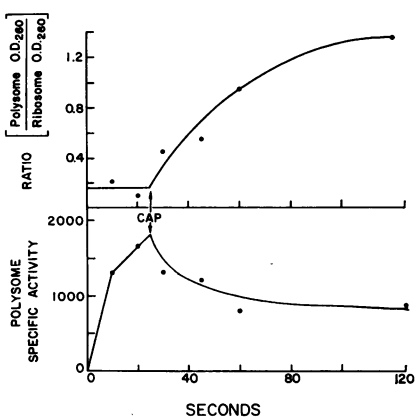

FIG. 6.—Formation of inactive polysomes during recovery from starvation. A culture was deprived of arginine for 30 min and then C^{14} protein hydrolysate (15 μc) was added with excess arginine (20 μg/ml). Twenty-five sec later, CAP (100 μg/ml) was added. Sampling and analysis were as described in *Methods*.

number of initiation sites on many messenger RNA's, or (2) several ribosomes can bind to a single initiation site. A third alternative is that CAP may alter the ribosomes in such a way that they bind at sites on the message other than the initiation sites. If this is the case, it would suggest a more indirect role for CAP in preventing nascent chain growth than an inhibition of the specific components of peptide-bond formation. Clarification of this point requires a more precise understanding of the nature of polysomes formed in the presence of CAP, and of the normal processes of initiation.

Summary.—The effect of CAP on the assembly of ribosomes into polysomes and on the functioning of these polysomes has been examined in growing cultures of *E. coli*. The following conclusions are made: (1) CAP does not cause the disappearance of polysomes or prevent the assembly of ribosomes into polysomes. It therefore does not inhibit the binding of ribosomes to messenger RNA. (2) Polysomes formed in the presence of CAP do not form nascent protein. Thus, the antibiotic does not act simply by blocking the release from the ribosome of finished proteins. (3) The growth of nascent polypeptides is inhibited by CAP, indicating that the antibiotic in some way interferes with a step common to the formation of all peptide bonds of a protein.

The authors would like to thank D. W. Morris for making some of the initial observations, and for engaging in many stimulating discussions.

* This work was supported by USPHS research grant 1-RO1-GM-13,200-01.

† USPHS predoctoral fellow.

[1] Gale, E. F., and J. P. Folkes, *Biochem. J.*, **53**, 493 (1953).

[2] DeMoss, J. A., and G. D. Novelli, *Biochim. Biophys. Acta*, **22**, 49 (1956).

[3] Lacks, S., and F. Gros, *J. Mol. Biol.*, **1**, 301 (1959).

[4] Nathans, D., and F. Lipmann, these PROCEEDINGS, **47**, 497 (1961).

[5] Vazquez, D., *Biochem. Biophys. Res. Commun.*, **12**, 409 (1963).

[6] Wolfe, A. D., and F. E. Hahn, *Biochim. Biophys. Acta*, **95**, 146 (1965).

[7] Polysomes are considered here as the active protein-synthesizing units, consisting of one or more ribosomes attached to a messenger RNA, and possibly including other components necessary for protein synthesis.

[8] Jardetzky, O., and G. R. Julian, *Nature*, **201**, 397 (1964).

[9] Wolfe, S. M., and A. S. Weisberger, these PROCEEDINGS, **53**, 991 (1965).

[10] Kućan, Z., and F. Lipmann, *J. Biol. Chem.*, **239**, 516 (1964).

[11] Julian, G. R., *J. Mol. Biol.*, **12**, 9 (1965).

[12] Traut, R. R., and R. E. Monro, *J. Mol. Biol.*, **10**, 63 (1964).

[13] Das, H., A. Goldstein, and L. Kanner, *Mol. Pharm.*, in press.

[14] Sypherd, P. S., and N. Strauss, these PROCEEDINGS, **49**, 400 (1963).

[15] Schaechter, M., *J. Mol. Biol.*, **7**, 561 (1963).

[16] Morris, D. W., and J. A. DeMoss, *J. Bacteriol.*, **90**, 1624 (1965).

[17] Rendi, R., and S. Ochoa, *J. Biol. Chem.*, **237**, 3711 (1962).

[18] Nishizuka, Y., and F. Lipmann, these PROCEEDINGS, **55**, 212 (1966).