**DIRECTED PROTEIN SYNTHESIS BY
MESSENGER RIBONUCLEOPROTEIN AND RIBOSOMES FROM
DIFFERENT MAMMALIAN SPECIES***

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There have been a number of attempts to direct protein synthesis using RNA particles containing specific genetic information. Although a variety of RNA preparations can stimulate polypeptide synthesis in bacterial cell-free systems, transfer of information has been demonstrable only with viral RNA or with synthetic polyribonucleotides.¹⁻⁵ The ability to direct protein synthesis with either viral messenger RNA or with synthetic polyribonucleotides has given rise to the concept that bacterial ribosomes are nonspecialized structures which can synthesize polypeptides dictated by the messenger they happen to contain at a given time.^{6, 7}

In mammalian systems there has been no unequivocal demonstration of directed protein synthesis with natural template RNA. Mammalian RNA preparations are capable of stimulating polypeptide synthesis by both bacterial and mammalian ribosomes, but a specific protein has not been identified in these systems.⁸⁻¹² Attempts to induce enzyme formation in tumor cells or to direct antibody synthesis with RNA have been inconclusive.^{13, 14} Hendler¹⁵ has pointed out that an essential type of evidence for the firm establishment of the messenger concept would be the demonstration that a protein-synthesizing system of one species could be induced to make a specific protein characteristic of a completely different species, by the addition of RNA characteristic of the second species. For mammalian systems, reticulocyte ribosomes provide a favorable system for such studies. The hemoglobin obtained from various species differs significantly in physical properties, amino acid composition, and antigenicity. In cell-free systems, reticulocyte ribosomes synthesize globin, and the species specificity of the globin synthesized is associated with the ribosomes.¹⁶⁻¹⁹ Presumably, the information controlling the specificity of the globin is encoded in a messenger RNA (mRNA) and this RNA could be used to study the possibility of directing protein synthesis.

In the following report a ribonucleoprotein complex (mRNP) was obtained from deer reticulocyte ribosomes and combined with rabbit reticulocyte ribosomes. The resultant hybrid polyribosome complex was shown to synthesize polypeptide fragments characteristic of deer globin.

Methods.—Preparation of rabbit reticulocyte ribosomes: Reticulocytosis was produced in New Zealand white rabbits with phenylhydrazine, and ribosomes were prepared by differential centrifugation as previously described.²⁰ The final ribosomal preparation was suspended in 0.25 *M* sucrose, and aliquots were stored at -65°C . Protein concentrations in the final preparations ranged from 1.5 to 3.0 mg/ml.

Preparation of deer reticulocyte ribosomes: North American white-tailed deer (*Odocoileus virginianus*) were maintained in captivity at the Cleveland Zoological Gardens. Reticulocytosis was produced by incorporating powdered phenylhydrazine, 1 gm daily for 10 days, in the feed. A reticulocytosis of 50% or more was necessary to obtain adequate amounts of ribosomes. Heparinized blood (150–250 ml) was obtained by jugular puncture; ribosomes were prepared as previously described²⁰ and were stored in 0.25 *M* sucrose at -65° .

Preparation of mRNP fractions: Stored, frozen ribosomes were quickly thawed at 37°C and were allowed to stand for 30–60 min at 4°C. The ribosomal suspensions were then centrifuged for 2 hr at 105,000 *g* in a Spinco model L preparative ultracentrifuge at 4°C. The supernatant fraction (mRNP) usually contained 10–30 μ g RNA/ml with a protein-to-RNA ratio of approximately 3:1. Both deer and rabbit mRNP fractions were prepared in the same manner and were assayed for activity in cell-free reaction mixtures. The rabbit ribosomal pellet remaining after supernatant mRNP was removed was resuspended in 0.25 *M* sucrose and used in reaction mixtures with deer mRNP.

Cell-free protein synthesis: The cell-free system employed was previously described²⁰ and consisted primarily of preincubated rabbit reticulocyte ribosomes, a supernatant fraction (pH 5) prepared from rabbit reticulocytes, an energy-generating system, amino acids, and buffer. The incorporation of C¹⁴ amino acids into hot trichloroacetic acid-(TCA) precipitable material was used to measure polypeptide synthesis. When C¹⁴ L-valine or C¹⁴ L-lysine was employed in the reaction mixtures, the amino acid mixtures were made deficient in the respective L-amino acid. The optimum magnesium concentration for this reaction mixture was determined to be between 0.0025 and 0.005 *M*. All tubes were adjusted to contain exactly the same amounts of water and 0.25 *M* sucrose and were then incubated for 1 hr at 37°C. Reactions were terminated by precipitation with 5% TCA, and the precipitate was washed and counted as previously described.²⁰ The ability of mRNP to stimulate protein synthesis in this system was determined, and the specificity of the protein synthesized was assayed in the following manner.

Immunologic assay of reaction products: Hemoglobin solutions prepared from lysed, washed deer erythrocytes were dialyzed and isolated by DEAE column chromatography. Antibody to deer globin was prepared by repeated injections of deer hemoglobin mixed with complete Freund's adjuvant into the footpads of New Zealand rabbits. Antibodies to deer globin were demonstrable by agar gel diffusion 6 weeks after the initial injection. The rabbits were exsanguinated and the serum was stored at –65°C. The equivalence point was determined by precipitating various concentrations of deer hemoglobin with the antiserum, and the optimum amount of antigen (150 μ g/ml antiserum) was used as carrier for specific immune coprecipitation determinations.

Following incubation of rabbit reticulocyte ribosomes with either deer mRNP or rabbit mRNP in cell-free systems, the ribosomes were removed by centrifugation at 105,000 *g* for 2 hr. Immunologic assays were performed by a modification of the method of Kabat²¹ on both the supernatant fractions and on the ribosomal pellets resuspended in 0.25 *M* sucrose to the original volume.

Two nonspecific immune coprecipitation reactions were carried out on the samples prior to specific immune coprecipitation. The nonspecific immune coprecipitation reactions were performed with egg albumen as the antigen and rabbit anti-egg albumen as the antibody. The equivalence point with the egg albumen system was 650 μ g of antigen per ml antiserum.

Immune assays were performed as follows. The antiserum was heated to 56°C for 30 min, centrifuged at 40,000 *g* for 10 min, and the sediment discarded. Optimum amounts of carrier antigen and antiserum were mixed with the reaction mixture, incubated at 37°C for 1 hr, and allowed to stand at 4°C overnight. The precipitate was collected by centrifugation at 15,000 *g* for 30 min at 4°C, washed three times with cold phosphate-buffered saline (PBS) pH 7.2, dried, dissolved in concentrated formic acid, and counted in a liquid scintillation counter.

Sucrose density gradient studies: Sucrose density gradient studies were performed to determine the distribution of protein synthesis in relation to the polysomes in the reaction mixture. Linear 10–30% sucrose gradients containing the same ionic concentration as the material to be analyzed were employed. One ml of sample was layered over 30 ml of the gradient and centrifuged for 3 hr at 25,000 *g* in a Spinco model L ultracentrifuge using a SW 25 rotor. After centrifugation, optical densities at 254 m μ were recorded by flow through an Isco UV analyzer (Instrumentation Specialties Co., Lincoln, Nebraska) and 1-ml samples were collected for further analysis.

Fingerprint analyses: Peptide analyses of the cell-free reaction mixtures were obtained as follows. Ten to 30 ml of the reaction mixtures were incubated at 37°C for 1 hr, dialyzed against distilled water at 4°C for 4 hr, and then lyophilized. The lyophilized material was resuspended in a solution of rabbit hemoglobin, and fingerprints were obtained as previously described.²²

Materials and other analyses: C¹⁴ L-leucine (ul), specific activity 220 mc/mM; C¹⁴ L-valine (ul), specific activity 200 mc/mM; C¹⁴ L-lysine (ul), specific activity 200 mc/mM, were purchased from Tracerlab Company, Waltham, Massachusetts. The other materials and analyses were as previously described.^{20, 22}

FIG. 1.—Stimulation of rabbit reticulocyte ribosomal polypeptide synthesis by deer mRNP. Addition of deer mRNP to the cell-free system results in an increased incorporation of C^{14} L-leucine into TCA-precipitable material (induced protein synthesis). Total volume of reaction mixture was 1.0 ml; the amount of rabbit ribosomes employed was 0.32 mg of protein, and the remaining components were as described in *Methods*. The deer mRNP contained 13 μ g of RNA and 41 μ g of protein per ml. The system became saturated when 3.9 μ g of deer mRNA was added, resulting in a net gain of 3814 cpm/ml reaction mixture, equivalent to an incorporation of 17.5 μ moles of C^{14} L-leucine. The unstimulated system incorporated 222 cpm/ml reaction mixture.

Results.—Stimulation of polypeptide synthesis by added mRNP: Addition of deer mRNP to rabbit reticulocyte ribosomes in the cell-free system resulted in a marked increase in the incorporation of C^{14} L-leucine into TCA-precipitable material (Fig. 1). Sucrose density gradient studies indicate that the increase in protein synthesis was associated with the polyribosomes (Fig. 2B), and that the amount of radioactivity associated with the monosomes was the same with or without added mRNP.

No ribosomes were demonstrable in the mRNP fractions by sucrose density gradient studies and no polypeptide synthesis was obtained in cell-free systems unless ribosomes were added to reaction mixtures containing mRNP. Sucrose density gradient studies of the mRNP revealed a peak of stimulatory activity with a sedimentation coefficient of approximately 26S. The presence of both RNA and protein were essential for the stimulatory activity of the mRNP. Further attempts at purification or deproteination by column chromatography or by phenol extraction invariably resulted in loss of stimulatory activity. The stimulatory activity was also destroyed by treating the material with ribonuclease (0.01 μ g/ml). This concentration of ribonuclease did not suppress endogenous protein synthesis. In most experiments the mRNP was assayed immediately after preparation since there was a progressive decrease in stimulatory activity when stored at -65°C , with an approximate 50 per cent loss in 5 days.

Alteration of amino acid ratios in polypeptide synthesis induced by deer mRNP: When deer mRNP was added to rabbit reticulocyte ribosomes, the amino acid composition of the protein formed differed from that formed in control reaction mixtures. The amount of incorporation of C^{14} leucine, C^{14} valine, and C^{14} lysine into TCA-precipitable material was determined in cell-free systems with rabbit reticulocyte ribosomes (endogenous protein synthesis). The amount of incorporation of these same amino acids was then determined for the polypeptide synthesis induced when identical cell-free systems were stimulated by adding deer mRNP. The leucine:lysine ratio with endogenous polypeptide synthesis was 7.8 compared to 2.05 for the polypeptide synthesis induced by adding deer mRNP. The valine:lysine ratio with endogenous synthesis was 2.4 compared to 6.1 for the induced polypeptide synthesis.

Immunologic assays: The specificity of the protein or polypeptide fragments synthesized by rabbit reticulocyte ribosomes when stimulated by deer mRNP was

TABLE 1
COMPARISON OF RADIOACTIVITY PRESENT IN TOTAL SYNTHESIZED PROTEIN WITH
THAT PRESENT IN SPECIFIC IMMUNOPRECIPITATES

Components added to reaction mixture	Precipitation with TCA		Protein Synthesis Precipitation with Anti-Deer Hemoglobin Antibody	
	Cpm	$\mu\mu$ Moles C^{14} leucine	Cpm	$\mu\mu$ Moles C^{14} leucine
Rabbit ribosomes + deer mRNP	3,301	15.2	7,287 $\pm$ 192	33.6
Rabbit ribosomes + deer mRNP + puromycin	46	—	102 $\pm$ 6	—
Rabbit ribosomes	1,847	6.7	215 $\pm$ 2	0.1
Deer mRNP	260	0.12	278 $\pm$ 6	0.13
Rabbit ribosomes + rabbit mRNP	16,929	78.1	240 $\pm$ 12	0.11

Radioactivity present in aliquots precipitated with TCA is compared with that present when aliquots were precipitated by reaction with rabbit anti-deer hemoglobin antibody. Immunoprecipitations were performed on protein remaining after removal of ribosomes by ultracentrifugation. The figures given for the specific immunoprecipitation were obtained after previously reacting the material twice with nonspecific immunoprecipitations. Values are expressed per ml reaction mixture; 0.1 ml of rabbit reticulocyte ribosomes (0.3 mg protein), 0.3 ml of deer mRNP (2.4 μ g RNA, 6.9 μ g protein) or 0.3 ml of rabbit mRNP (10.2 μ g RNA, 42.2 μ g protein) were added per ml reaction mixture where indicated; 0.1 μ mole of puromycin per ml was added to one of the reaction mixtures.

studied by determining the radioactivity present when aliquots were precipitated by reaction with rabbit anti-deer globin antibody. After incubation of the reaction mixtures, an aliquot was precipitated with TCA to determine total protein synthesis; the ribosomes were removed from the remainder of the reaction mixture by ultracentrifugation, and immunologic precipitations were performed on both the supernatant fraction containing soluble protein and on resuspended ribosomal pellets.

(a) *Assay of supernatant fractions:* The results are summarized in Table 1. Total polypeptide synthesis was stimulated when either deer mRNP or rabbit mRNP was added to the cell-free system. The net increase in C^{14} leucine incorporation was much greater with rabbit mRNP. No significant polypeptide synthesis occurred when deer mRNP was added to the reaction mixture in the absence of rabbit reticulocyte ribosomes. Synthesis was inhibited by adding puromycin to the reaction mixture.

The radioactivity present in specific immunoprecipitates was determined after previously reacting the material twice with a nonspecific immunoprecipitation system, as described in *Methods*. Duplicate tests were performed with each sample, and the average results with their standard deviations are shown in Table 1. A large amount of the protein (or polypeptides) formed when deer mRNP was added to rabbit reticulocyte ribosomes was precipitable by rabbit anti-deer globin antibody. In the experiment shown, the radioactivity present in the immune precipitates exceeded that present in the aliquot precipitated with TCA. Similar results were obtained in one other experiment, but in two other experiments the radioactivity present in the immune precipitates was less than

TABLE 2
COMPARISON OF RADIOACTIVITY PRESENT
IN TOTAL SYNTHESIZED PROTEIN WITH
THAT PRESENT IN SPECIFIC
IMMUNOPRECIPITATES

	Radioactivity present in TCA precipitates (cpm)*	Radioactivity present in specific immunoprecipitates (cpm)†
Expt. 1	1,545	1,264
Expt. 2	3,363	7,062
Expt. 3	3,833	4,730
Expt. 4	1,454	553

* Cpm per ml of reaction mixture precipitated with TCA. Deer mRNP added to rabbit ribosomes in each experiment.

† Cpm per ml reaction mixture precipitated with rabbit anti-deer globin antibody. Figures expressed as cpm above control values after previously reacting the material twice with nonspecific immunoprecipitations. Controls and amounts of rabbit ribosomes and deer mRNP were the same as in Table 1.

TABLE 3
COMPARISON OF RADIOACTIVITY PRESENT IN
NONSPECIFIC AND IN SPECIFIC
IMMUNOPRECIPITATES

Components added to reaction mixture	Non- specific precipitation* (cpm/ OD ₂₈₀)	Specific immune precipitation† (cpm/ OD ₂₈₀)	Ratio (specific/ non- specific‡)
Rabbit ribosomes and deer mRNP	523	14,350	26.8
Deer mRNP	565	542	0.96
Rabbit ribosomes	501	423	0.84
Rabbit ribosomes and rabbit mRNP	632	482	0.76

* Nonspecific precipitation with egg albumen and rabbit anti-egg albumen antibody added to the cell-free reaction mixture. The figures given are for the second nonspecific immune precipitate and are expressed as radioactivity per optical density unit of the precipitate.

† Specific immune precipitation with rabbit anti-deer globin antibody. Carrier deer hemoglobin was added to the cell-free reaction mixture after it had previously been subjected to two nonspecific immune precipitations. Specific antibody to deer globin was then added, and the radioactivity is expressed per optical density unit of the immune precipitate.

‡ Ratio = (cpm per OD₂₈₀ of precipitate with specific immune serum)/(cpm per OD₂₈₀ of precipitate with nonspecific immune serum). Amounts of rabbit ribosomes, deer mRNP, and rabbit mRNP employed were the same as in Table 1.

that present in the TCA precipitate (Table 2). When the immune precipitates were further washed with hot TCA, approximately 50 per cent of the radioactivity present in the precipitates was removed. However, the differences remained statistically significant.

Considerably more stimulation of total protein synthesis was obtained when rabbit mRNP was added to the reaction mixture compared to that obtained when deer mRNP was added. However, when the reaction mixtures were precipitated with rabbit anti-deer globin antibody, only that stimulated by deer mRNP exhibited significant activity in the specific immunoprecipitates (Table 1).

The ratio of the radioactivity present in specific immunoprecipitates was determined with respect to that present

in nonspecific immunoprecipitates. When rabbit reticulocyte ribosomes were stimulated with deer mRNP, the radioactivity precipitated with rabbit anti-deer globin antibody was 26.8 times as great as that precipitated by nonspecific precipitation (Table 3). In contrast, the ratios of radioactivity in the control reaction mixtures approached unity, indicating that nonspecific trapping of radioactivity was not a significant factor in the high values obtained when polypeptide synthesis was stimulated by deer mRNP.

The specificity of the reaction was further demonstrated by adding deer mRNP to cell-free reaction mixtures after previously incubating the system for 1 hr to allow endogenous protein synthesis to be completed. The radioactivity present in TCA-precipitable material was 3,850 cpm/ml reaction mixture. The radioactivity present when the reaction mixture was precipitated with rabbit anti-deer globin antibody was only 93 cpm/ml. The radioactivity present in control reaction mixtures precipitated with rabbit anti-deer globin antibody was 114 cpm/ml. This again indicates that nonspecific trapping within the antigen-antibody complex is minimal.

(b) *Immunologic assay of ribosomal fractions:* The ribosomes which were sedimented from the reaction mixture after 1 hr of incubation were assayed for the presence of immunologically reactive material. The radioactivity present in specific immunoprecipitates was found to be less than 1 per cent of that present in the supernatant fractions. However, when assays were performed on the ribosomes after a 10-min pulse, significant amounts of immunologically specific material were found to be associated with the polyribosomes (Fig. 2C). The increase in protein synthesis attributable to the added mRNP was also found to be associated with the polyribosome fraction (Fig. 2B). There was no increase in protein synthesis

FIG. 2.—Sucrose density gradient studies of (A) ribosomal distribution, (B) cell-free protein synthesis with respect to polysomes, and (C) specificity of protein synthesis. Cell-free reaction mixture (1.0 ml) was incubated for 10 min at 37°C, chilled to 4°C, layered on 30 ml of a linear 10–30% sucrose gradient, and centrifuged for 3 hr at 25,000 *g* in a Spinco ultracentrifuge using a SW 25 rotor. Optical densities were recorded at 254 μ by flow through an Isco UV analyzer and 1.0-ml aliquots were collected after flow through the instrument for assay of TCA-precipitable radioactivity and precipitation with rabbit anti-deer globin antibody. Fractions were collected and numbered beginning with the meniscus. Control tubes consisted of rabbit reticulocyte ribosomes (0.3 mg protein) in the complete system; experimental tubes consisted of rabbit reticulocyte ribosomes (0.3 mg protein) and deer mRNP (3.1 μ g RNA, 10.9 μ g protein) in the complete system.

(A) Normal polysome and monosome distribution with both experimental and control fractions. (B) Polypeptide synthesis has been stimulated in the experimental fractions as compared with the control fractions and the increase in protein synthesis is associated with the polysomes. (C) Protein synthesized by the polysomal fraction of the experimental reaction is precipitable by rabbit anti-deer hemoglobin. The immune precipitations were obtained by pooling the sucrose gradient samples as indicated by the vertical dotted line. The protein synthesized by the polysomal fraction of the control reaction is at a background level. No specific immunologically reactive material was demonstrable with the monosomal fraction.

by the monosomes and no demonstrable immunologically specific material associated with them.

Fingerprint analyses: The peptide fingerprints of deer hemoglobin differ significantly from those of rabbit hemoglobin.²³ Peptide fingerprint analyses were obtained by concentrating the reaction mixtures and adding carrier rabbit hemoglobin. There were no demonstrable differences between control and experimental reaction mixtures when the fingerprints were compared following development with ninhydrin. The fingerprints were then cut into 1-in. squares and eluted with concentrated formic acid, and the radioactivity was determined by liquid scintillation counting. At least five separate areas of radioactivity which were not present in fingerprints obtained from control reaction mixtures were present in fingerprints obtained from the experimental reaction mixtures. The radioactivity in these areas ranged from 150 to 300 cpm above background. These areas were not definitely identified as deer peptides.

Discussion.—These data suggest that when deer mRNP is combined with rabbit reticulocyte ribosomes, a hybrid polyribosomal complex is formed which is capable of synthesizing globin fragments or subunits containing sufficient antigenic sites to be recognized by rabbit anti-deer globin antibody. It is suggested that primarily peptide fragments are synthesized because of discrepancies between the radioactivity present in TCA precipitates of the reaction product and that recovered with specific immunoprecipitation. Presumably, some of the peptide fragments

were soluble in 5 per cent TCA, but were sedimented with antigen-antibody complexes washed with saline. The results are in accord with the suggestion that mammalian ribosomes as well as bacterial ribosomes may be relatively nonspecialized structures which can synthesize polypeptides dictated by the messenger they contain.

Mg²⁺ is required for the formation of mRNA-ribosome complexes which are capable of polypeptide synthesis.²⁴ Although mRNA can readily be removed from *E. coli* ribosomes by lowering the Mg²⁺ concentration,²⁵ the mRNA of reticulocyte ribosomes is more firmly bound and is not readily dissociated.²⁶ Methods usually employed to obtain template RNA from mammalian systems may either degrade mRNA or may be ineffective in recovering the active material. The methods employed in these experiments demonstrate that an mRNA-protein complex can be separated from ribosomes in the presence of low Mg²⁺ concentration and that this complex can then recombine with ribosomes from another species in the presence of Mg²⁺ to form a hybrid polyribosome capable of polypeptide synthesis. The ability of this complex to direct polypeptide synthesis was dependent upon the presence of RNA since all activity was lost following exposure to small amounts of ribonuclease insufficient to interfere with endogenous protein synthesis. The presence of protein was also essential for the activity of the complex since deproteinization resulted in loss of stimulatory activity. Miller *et al.*²⁷ also separated a stimulatory fraction from reticulocyte ribosomes by sedimentation with 0.5 *M* KCl in the absence of Mg²⁺, but directed protein synthesis was not demonstrated. The stimulatory activity of this fraction was also destroyed by ribonuclease, phenol extraction, or deproteinization.

The exact role of the protein in the complex is unknown. It may be required to stabilize the complex, but the possibility that peptidyl-sRNA fragments containing deer globin antigenicity are present in the mRNP complex and that peptide chain synthesis may proceed from these fragments is not ruled out. However, this possibility seems remote, since such fragments in *E. coli* are bound to larger ribosomal subunits (50S) and do not dissociate in low magnesium.²⁸⁻³⁰ Similar observations have been made with rat liver ribosomes.³¹ The sedimentation coefficient of the active material employed in our experiments (approximately 26S) indicates that functioning ribosomal subunits are not present.

Other attempts have been made to alter information in reticulocyte ribosomes by using combinations of ribosomes and enzyme supernatant fractions from different species.^{17, 18, 32, 33} Lamfrom³² as well as Kruh *et al.*³³ have found that small amounts of hemoglobin were synthesized in these mixtures which could be attributed to a supernatant factor. Other investigators have not been able to confirm these observations,^{18, 19} and it is generally accepted that the specificity of hemoglobin synthesis resides in the template RNA of the ribosomes.¹⁷⁻¹⁹ There is a possibility, however, that material containing information was dissociated from the ribosomes during preparation and was present in supernatant factors which appeared to alter information in these experiments. Schaeffer *et al.*¹⁰ attempted to transfer information from reticulocyte ribosomes to *E. coli* ribosomes. Although altered amino acid ratios were obtained, no specific polypeptides were identified. Conceivably, there may be sufficient differences between mammalian ribosomes and bacterial ribosomes to preclude the directed synthesis of recognizable protein.

Summary.—A ribonucleoprotein complex (mRNP) was obtained from deer reticulocytes after sedimentation in low magnesium ion concentration. Evidence is presented that deer mRNP can combine with rabbit reticulocyte ribosomes to form a hybrid polyribosomal complex capable of synthesizing globin fragments or subunits containing sufficient antigenic sites to react with rabbit anti-deer globin antibody.

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¹ Tsugita, A., H. Fraenkel-Conrat, M. W. Nirenberg, and J. H. Matthaei, these PROCEEDINGS, **48**, 846 (1962).

² Nathans, D., G. Notani, J. H. Schwartz, and N. D. Zinder, these PROCEEDINGS, **48**, 1424 (1962).

³ Ohtaka, Y., and S. Spiegelman, *Science*, **142**, 493 (1963).

⁴ Nirenberg, M. W., and J. H. Matthaei, these PROCEEDINGS, **47**, 1588 (1961).

⁵ Nirenberg, M. W., J. H. Matthaei, and O. W. Jones, these PROCEEDINGS, **48**, 104 (1962).

⁶ Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).

⁷ Brenner, S., F. Jacob, and M. Meselson, *Nature*, **190**, 576 (1961).

⁸ Fischer, S., G. P. Bruns, B. A. Lowry, and I. M. London, these PROCEEDINGS, **49**, 219 (1963).

⁹ Braverman, G., L. Gold, and J. Eisenstadt, these PROCEEDINGS, **49**, 630 (1963).

¹⁰ Schaeffer, J., G. Favelukes, and R. Schweet, *Biochim. Biophys. Acta*, **80**, 247 (1964).

¹¹ Mach, B., and P. Vassalli, *Science*, **150**, 622 (1965).

¹² Mach, B., and P. Vassalli, these PROCEEDINGS, **54**, 975 (1965).

¹³ Imsande, J., and B. Ephrussi, *Science*, **144**, 855 (1964).

¹⁴ Friedman, H. P., and A. Stavitsky, *Science*, **149**, 1106 (1965).

¹⁵ Hendler, R. W., *Science*, **142**, 402 (1963).

¹⁶ Schweet, R., H. Lamfrom, and E. Allen, these PROCEEDINGS, **44**, 1029 (1958).

¹⁷ von Ehrenstein, G., and F. Lipmann, these PROCEEDINGS, **47**, 941 (1961).

¹⁸ Arnstein, H. R. V., R. A. Cox, and J. A. Hunt, *Nature*, **194**, 1042 (1962).

¹⁹ Bishop, J., G. Favelukes, R. Schweet, and E. Russell, *Nature*, **191**, 1365 (1961).

²⁰ Weisberger, A. S., S. Wolfe, and S. Armentrout, *J. Exptl. Med.*, **120**, 161 (1964).

²¹ Kabat, E. A., *Kabat and Mayer's Experimental Immuno-chemistry* (Springfield, Illinois: Charles C Thomas, 1961), 2d ed., chap. 2.

²² Weisberger, A. S., these PROCEEDINGS, **48**, 68 (1962).

²³ Weisberger, A. S., *Proc. Soc. Exptl. Biol. Med.*, **117**, 276 (1964).

²⁴ Wolfe, S. M., and A. S. Weisberger, these PROCEEDINGS, **53**, 991 (1965).

²⁵ Gros, F., H. Hiatt, W. Gilbert, and C. G. Kurland, *Nature*, **190**, 581 (1961).

²⁶ Gould, H. J., H. R. V. Arnstein, and R. A. Cox, *J. Mol. Biol.*, **15**, 600 (1966).

²⁷ Miller, R., E. Hausmann, R. Erickson, and E. R. Schweet, *Federation Proc.*, **24**, 283 (1965).

²⁸ Gilbert, W., *J. Mol. Biol.*, **6**, 389 (1963).

²⁹ Schlessinger, D., and F. Gros, *J. Mol. Biol.*, **7**, 350 (1963).

³⁰ Cannon, M., R. Krug, and W. Gilbert, *J. Mol. Biol.*, **7**, 360 (1963).

³¹ Wettstein, F. O., and H. Noll, *J. Mol. Biol.*, **11**, 35 (1965).

³² Lamfrom, H., *J. Mol. Biol.*, **3**, 241 (1961).

³³ Kruh, J., J. Rosa, J. C. Dreyfus, and G. Schapiro, *Biochim. Biophys. Acta*, **49**, 509 (1961).