Ringer, D., and Chladek, S. (1974), *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33,* 1336.
Schroer, R. A., and Moldave, K. (1973), *Arch. Biochem. Biophys. 154,* 422-430.
Siler, J., and Moldave, K. (1969), *Biochim. Biophys. Acta 195,* 130-137.
Skogerson, L., and Moldave, K. (1967), *Biochem. Biophys. Res. Commun. 27,* 568-572.
Skogerson, L., and Moldave, K. (1968), *Arch. Biochem. Biophys. 125,* 497-505.
Tate, W. P., and Caskey, C. T. (1973), *J. Biol. Chem. 248,* 7970-7972.
Thompson, H. A., and Moldave, K. (1974), *Biochemistry 13,* 1348-1353.
Vazquez, D., Battaner, E., Neth, R., Heller, G., and Monro, R. E. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 369-375.
Wei, C.-M., Hansen, B. S., Vaughan, M. H., Jr., and McLaughlin, C. S. (1974) *Proc. Nat. Acad. Sci. U. S. 71,* 713-717.

Evidence for Multiple Forms and Partial Resolution of Rabbit Reticulocyte α- and β-Globin Messenger RNA by Gel Isoelectric Focusing†

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ABSTRACT: Isoelectric focusing in polyacrylamide gels has been used to fractionate and characterize RNAs from rabbit reticulocytes with major emphasis on globin mRNA. Reticulocyte 18S and 28S RNAs banded essentially as single components, well separated from each other and from the multiple forms of tRNA. By contrast, mRNA was resolved into a number of major and minor components. These bands were shown to contain intact globin mRNA by translation in a messenger-dependent cell-free protein synthesizing system. One major band was enriched slightly in α-globin mRNA and a second major band was enriched considerably in β-globin mRNA. Reticulocyte supernatant mRNA, containing predominantly α-globin messenger, demonstrated only one major component which banded at the same position as the α-enriched band from total mRNA. Little of this material behaved as β mRNA either by its focusing profile or by its translation products. Globin mRNA fractions with high and low 3′ poly(A) contents also demonstrated differences in focusing distribution profiles. Although the basis for separating RNA by this technique has not been established, our results suggest that isoelectric focusing may offer a new approach to fractionation and characterization of specific mRNA species.

In the past few years, there has been considerable interest in the isolation and purification of eukaryotic mRNAs. Messenger fractions active in the synthesis of specific proteins have been prepared by a variety of methods (Moldave and Grossman, 1974). Such preparations have been valuable for direct translation studies and for synthesis of labeled complementary DNA (Ross *et al.*, 1972a; Verma *et al.*, 1972; Kacian *et al.*, 1972) to examine gene expression at both transcriptional and post-transcriptional levels (Ross *et al.*, 1972b; Housman *et al.*, 1973; Terada *et al.*, 1972; Macnaughton *et al.*, 1974).

In efforts to further purify and characterize specific mRNAs, we have explored the potential usefulness of gel isoelectric focusing. We chose the rabbit reticulocyte system as a model, since it has been well characterized and can serve as an excellent source for both mRNA and other cell-free translation components. The reticulocyte is particularly attractive for studying the properties of mRNA since (1) the bulk of its mRNA codes for α- and β-globin chains, (2) the synthesis of α- and β-globin polypeptides can be quantitated readily by well-established procedures, and (3) the focusing behavior of these two distinct but closely related mRNA species can be followed simultaneously. Our results suggest that gel isoelectric focusing may, indeed, be useful for separating discrete mRNAs from each other as well as from other RNA components. They also indicate that α- and β-globin mRNA may exist in multiple molecular forms. Preliminary accounts of these studies have been reported previously (Shafritz *et al.*, 1973b; Shafritz and Drysdale, 1974).

Experimental Procedure

Materials

All chemicals were of analytical or reagent grade and solutions were freshly prepared. Enzyme grade ammonium sulfate, ribonuclease-free sucrose, unlabeled L-amino acids, and [^{3}H]poly(U),[1] K^+ salt (specific activity 7.76 Ci/mol of phosphorus), were purchased from Schwarz/Mann, Oran-

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[1] Abbreviations used are: Temed, N,N,N′,N′-tetramethylenediamine; poly(U), poly(uridylic acid); P-enolpyruvate, phosphoenolpyruvate.

geburg, N.Y. Rabbit liver tRNA (stripped) was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, GTP, ATP, P-enolpyruvate, pyruvate kinase (rabbit muscle, 1500 I.U./ml), and dithiothreitol from Calbiochemical, San Diego, Calif., and preswollen microgranular carboxymethylcellose, Whatman CM-52 (1.0 mequiv/g) from Reeve Angel Co., New York, N.Y. Oligo(dT)-cellulose (Type T_2) was purchased from Collaborate Research, Inc., Waltham, Mass. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylenediamine (Temed) were obtained from Eastman Kodak Co., Rochester, N.Y. L-[4,5-^{3}H]Leucine (specific activity 38 Ci/mmol), L-[2,3-^{3}H]valine (specific activity 27 Ci/mmol), and [5-^{3}H]orotic acid (specific activity 21 Ci/mmol) were purchased from Amersham-Searle Corp., Arlington Heights, Ill.

Methods

Preparations of Ribosomes and Enzyme Fractions. Rabbit reticulocyte lysate, ribosomes, 105,000*g* supernatant protein, and a 0.5 M KCl ribosomal wash fraction were prepared as previously reported (Shafritz and Anderson, 1970). A concentrated crude initiation factor preparation, derived from the 0.5 M KCl ribosomal wash fraction, was obtained as previously reported (Shafritz *et al.*, 1973a), except that DEAE-cellulose treatment was used prior to ammonium sulfate precipitation to remove residual RNA from the protein fraction (Gilbert and Anderson, 1970). Free polysomes from rabbit liver were prepared as previously reported (Shafritz *et al.*, 1973a) and both liver and reticulocyte ribosomes rendered exogenous mRNA dependent by mild digestion with pancreatic RNase A (Crystal *et al.*, 1972; Shafritz *et al.*, 1973a).

Preparation of RNA Fractions. Reticulocyte lysate was used as starting material for preparation of all RNA components. For some experiments, RNA was labeled *in vivo* by injection of 2 mCi of high specific activity [^{3}H]orotic acid into the ear vein of the rabbit on the 6th day and 7th day of the phenylhydrazine treatment schedule. The rabbits were bled on the 8th day by intracardiac puncture and total RNA was extracted from lysate by the phenol–*m*-cresol–8-hydroxyquinoline method as previously reported (Shafritz *et al.*, 1973a,b). To prepare an RNA fraction enriched in 3'-poly(A) sequences (*i.e.*, mRNA), the crude RNA was adjusted to 50–75 A_{260} units/ml in 10 mM Tris-HCl (pH 7.4)–0.5 M KCl and applied to a small column containing 1 mg of oligo(dT)-cellulose as reported by Aviv and Leder (1972). Material not adsorbing to oligo(dT)-cellulose at 0.5 M KCl was used for subsequent preparation of 4S, 18S, and 28S RNA. Fractions eluting from oligo(dT)-cellulose at 0.1 M KCl and 0 M KCl were used for preparation of "9S" globin mRNA. For some experiments, the 0.1 M KCl elution step was omitted. The 4S, 18S, and 28S RNA as well as the "9S" mRNA fractions were then prepared by sucrose gradient centrifugation of the above materials, using 5–20% isokinetic sucrose gradients (Noll, 1969).

Gel Electrofocusing. Ampholine® (carrier ampholytes) LKB 1809-116, pH 4–6, Batch 1, manufactured 12/72, and LKB 81-51, pH 3–5, Batch 19, manufactured 2/69, were obtained from LKB Produkter Ltd., Sweden. Isoelectric focusing in polyacrylamide gels was performed by the procedure of Drysdale and Righetti (1972) with certain modifications. Initially, 3.6% acrylamidebisacrylamide gels were utilized, but in later experiments higher porosity gels containing 3.0% acrylamidebisacrylamide were substituted to further minimize molecular sieving effects. Twice recrystallized acrylamide and methylenebisacrylamide in a ratio of 25:1 were used for all studies (Loening, 1967). A mixture of ampholytes (2 parts pH 4–6 to 1 part pH 3–5) at a final concentration in the gels of 2% (w/v) were used routinely; 10% glycerol (v/v) was included to stabilize the pH gradient and to improve gel consistency for subsequent fractionation. All gel solutions were degassed prior to use and polymerization was catalyzed by the addition of Temed and ammonium persulfate. Gels were cast in 3 mm × 100 mm syringe barrel plexiglass tubes and electrofocusing was conducted at 0° in an apparatus from Medical Research Apparatus, Inc., Boston, Mass. After an initial electrolysis period of 30 min at 0.5 mA/gel, samples of RNA 0.1–1.5 A_{260} units were top-layered in 20 mM Tris-HCl (pH 8.0) containing 10% glycerol. For some experiments, RNA was incorporated directly into the gel during the polymerization step (Drysdale and Righetti, 1972). The current was maintained at 0.5 mA/gel until the voltage reached 400 V, where it was then held constant for the duration of the experiment. In other experiments, 400 V were applied directly with a pulse power supply (Metaloglass, Inc., Boston, Mass.) to give an average power of 0.1 W/gel over a 12-hr period. Under either of these conditions, the pH gradient formed after 6 hr and remained stable for additional focusing periods up to 18 hr. Although stable banding patterns were evident after 10 hr, electrolysis was continued to 18 hr to ensure equilibrium focusing. After electrofocusing, gels were expressed in 20-μl aliquots through a No. 20 "stub nosed" syringe needle into 40 μl of concentrated protein synthesis buffer (see below), using a Hamilton repeating dispenser (Bagshaw *et al.*, 1973). The tubes were shaken for 3 hr at 0° to extract RNA.

[^{3}H]Poly(U) Hybridization of Reticulocyte mRNA. For some experiments, small aliquots of the eluted gel fractions after focusing were removed for determination of poly(A) content by hybridization to high specific activity [^{3}H]poly(U), according to the method of Jeffery and Brawerman (1974). Incubations in a total volume of 500 μl were performed at 30° for 15 min and contained 10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mM $MgCl_2$, 2500–3000 cpm [^{3}H]poly(U) (specific activity 7.76 Ci/mol of phosphorus) and the appropriate aliquot from the eluted gel fractions or control mRNA. After this first incubation was completed, pancreatic RNase A was added and incubation continued for an additional 30 min. The reaction mixtures were then chilled in ice and 1.0 A_{260} unit carrier rabbit liver tRNA was added, followed by 2 ml of cold 10% Cl_3CCOOH. The reactions were kept on ice for 10 min and filtered onto nitrocellulose filters, and the filters were washed four times with 4 ml of cold 0.1% Cl_3CCOOH and counted by liquid scintillation spectroscopy. Blanks were generally less than 100 cpm.

Protein Synthesis Assay. Incorporation of L-[^{3}H]leucine or L-[^{3}H]leucine-L-[^{3}H]valine into protein under the direction of exogeneous reticulocyte mRNA was performed as previously reported (Shafritz *et al.*, 1973a,b) with minor modifications. In these studies, protein synthesis was performed directly in tubes containing the gel fragments in elution buffer. Incubations contained 40 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 80 mM KCl, 1 mM ATP–0.2 mM GTP–3 mM P-enolpyruvate (all adjusted to pH 7.0 with KOH), 1 mM dithiothreitol, 0.2 IU pyruvate kinase, 0.075 A_{260} unit of mRNA dependent liver or reticulocyte ribosomes, 0.3 mg of 105,000*g* supernatant protein, 0.15 mg of concentrated crude initiation factors, 0.06 A_{260} unit of

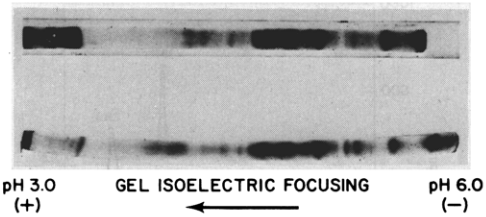

FIGURE 1. Banding pattern of reticulocyte RNAs separated by isoelectric focusing in polyacrylamide gels; 1.0 A_{260} units of unfractionated reticulocyte lysate RNA (upper gel) or 0.4 A_{260} unit of reticulocyte crude "9S" RNA (lower gel) were focused for 24 hr in 3.6% polyacrylamide gels over a pH range 3–6. Samples were top-layered in 20 mM Tris-HCl (pH 8.0)–10% glycerol and focusing performed from the cathode (−) to the anode (+). Gels were stained with 0.1% Toluidine Blue in 2% acetic acid, destained with 1% acetic acid, and photographed. See Methods for additional experimental details.

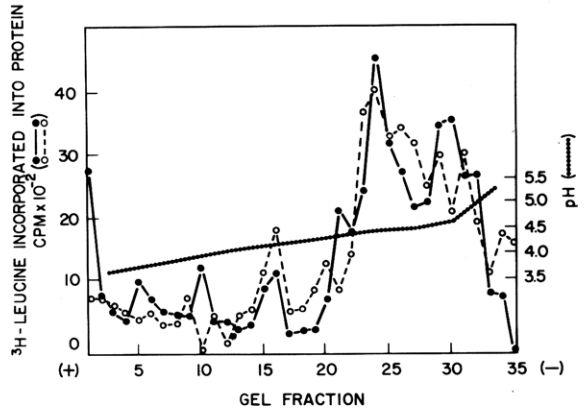

FIGURE 2: Effect of annealing on the pattern of messenger activity with focused reticulocyte RNA fractions. 2.0 A_{260} units of unfractionated total reticulocyte lysate RNA were focused in replicate 3.6% polyacrylamide gels for 24 hr over a pH range 3–6. Gels were crushed into 35 sections and eluted with protein synthesis buffer for 3–4 hr at 0°, and the first set (●–●) was tested directly for stimulation of protein synthesis in the messenger-dependent reticulocyte system (see Methods). The second set (O - - O) was heated to 65° for 10 min, cooled slowly over 1 hr, and tested for stimulation of protein synthesis as above. The pH gradient of a replicate gel (. . .) was determined by dividing it into 10 equal portions, eluting the ampholytes with 0.5 ml of H_2O and recording pH with a Beckman glass microelectrode. A blank of 1650 cpm, representing protein synthesis activity in the absence of added RNA, was subtracted from each value.

deacylated rabbit liver tRNA, 0.08 mM amino acid mixture (19 amino acids minus leucine or 18 amino acids minus leucine-valine), [^{3}H]leucine, 6 Ci/mmol, or [^{3}H]leucine-[^{3}H]valine, 6 Ci/mmol, and gel fragments containing focused RNA or control gel fragments plus exogenous reticulocyte mRNA. After incubation at 30° for 40 min, the reaction mixture was diluted to 0.5 ml with cold buffer A (10 mM Tris-HCl (pH 7.4)–100 mM KCl–1 mM dithiothreitol–0.1 mM EDTA) and gel fragments were pelleted by centrifugation at 15,000g for 2 min in an Eppendorf microcentrifuge; 100-μl aliquots of the supernatant fractions were used for determination of [^{3}H]amino acid incorporation into hot Cl_3CCOOH insoluble polypeptides (Shafritz and Anderson, 1970).

Characterization of Cell-Free Reaction Products. The translation products from the cell-free system, using either liver or reticulocyte ribosomes, were characterized by carboxymethylcellulose chromatography (Shafritz *et al.*, 1973a,b). For these studies ^{3}H-labeled products from gel fractions comprising peaks of protein synthesis (messenger activity), were pooled, as indicated in Figure 7, and processed simultaneously. The soluble fraction was separated from residual gel fragments by centrifugation in a Brinkman 3200 centrifuge at 15,000g for 2 min; 5.0 mg of carrier globin was added to each pooled sample, the globin was reisolated as an acid–acetone powder, and α and β chains were separated by carboxymethylcellulose chromatography, according to the method of Dintzis (1961).

Results

Initial Electrofocusing Studies. Total lysate RNA and crude "9S" RNA (obtained by sucrose gradient centrifugation but not purified by oligo(dT)-cellulose chromatography) were focused at 0° in 3.6% polyacrylamide gels over a pH range 3–6. Figure 1 shows the RNA banding pattern of these gels stained with Toluidine Blue. The upper gel represents total lysate RNA and the lower gel crude "9S" RNA. Both preparations demonstrated a large number of discrete bands distributed throughout the gel. Various bands in the more acidic regions represented tRNA components (Drysdale and Righetti, 1972) and were almost absent from the "9S" RNA. Differences in relative intensity of bands in other regions of the gels were also apparent. Since mRNA represents only 1% of total reticulocyte RNA (Lingrel *et al.*, 1971), it was not possible to identify mRNA bands directly in these patterns. Therefore, the position of functionally active mRNA was determined by stimulation of protein synthesis in the reticulocyte messenger-dependent cell-free system (see Methods). Focused gels were divided into 30–35 fractions and RNA was eluted in protein synthesis buffer. The pattern of messenger activity was rather complex, with at least seven peaks distributed throughout the gel (Figure 2, ●–●). Most of this activity was located between pH 4.5 and pH 5.5. Similar profiles were obtained when RNA was incorporated directly into the gel during the polymerization process. After focusing, recovery of translatable mRNA activity was approximately 50%.

To determine whether some of the focusing peaks might represent partially denatured mRNA molecules, the effect of annealing on the pattern of protein synthesis stimulation was determined (Figure 2, O - - O). A replicate gel was fractionated, heated to 65° for 10 min in the presence of Mg^{2+}, and then cooled slowly (Lindahl *et al.*, 1966). Only minor differences in the protein synthesis profile were observed after heating–reannealing, and these differences were probably within the limits of experimental variation. Certainly, any effects were considerably less than those observed with bacterial tRNA, in which there were large increases in amino acid accepting activity of certain focused tRNA fractions after reannealing (Drysdale and Righetti, 1972).

To investigate further the basis for multiple bands of messenger activity, we examined the mRNA pattern as a function of poly(A) content. Since 3′-poly(A) segments have been observed as unique and perhaps important components of most mammalian mRNAs (Darnell *et al.*, 1973), peaks of poly(A) should represent mRNA or 3′ mRNA degradation products. From the eluted gel fractions in protein synthesis buffer, there was approximately 70–75% recovery of messenger poly(A) input. With total lysate RNA there were multiple bands containing poly(A), most of which corresponded to peaks of protein synthesis stimulation (Figure 3). However, for these peaks, there was wide variation in protein synthesis activity *vs.* poly(A) content, with no progressive relationship between these two parame-

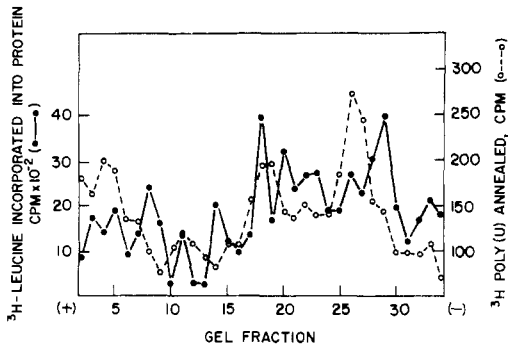

FIGURE 3: Distribution of messenger activity *vs.* poly(A) content of isoelectric focused reticulocyte RNA; 2.0 A_{260} units of unfractionated reticulocyte lysate RNA was focused as in Figure 2. After focusing, the gel was divided into 33 sections, protein synthesis buffer added, and the gel left at 0° for 4 hr with occasional shaking. A 15-μl aliquot of the aqueous layer was removed from each fraction and poly(A) content determined by annealing with [^{3}H]poly(U) (specific activity 7.76 Ci/mol of phosphorus) (O - - O) by the method of Jeffery and Brawerman (1974). The remaining portion was tested for messenger activity in cell-free protein synthesis (●-●), as indicated in Figure 2.

ters. In fact, several bands of messenger activity appeared to contain little, if any, poly(A) material.

Isoelectric Focusing Profiles of Partially Purified Labeled Reticulocyte RNAs. Reticulocyte RNAs, labeled to a specific activity of 8000–10,000 cpm/A_{260} unit, were obtained *in vivo* by injection of [^{3}H]orotic acid. Poly(A) rich material was first separated from other RNA components by oligo dT cellulose chromatography. Figure 4 shows the separation of 4S, 18S, and 28S [^{3}H]RNA (from material not adsorbed to oligo(dT)-cellulose) by centrifugation in a 5–20% isokinetic sucrose gradient. Peaks of 4S, 18S, and ≥28S were pooled, concentrated by ultrafiltration, and used for subsequent studies. A "9S" mRNA fraction (from the adsorbed poly(A) rich material) was prepared in a duplicate gradient. In addition to the "9S" peak, this RNA contained ^{3}H-labeled components at 21 S and ≥28 S. Similar peaks were observed in these regions by stimulation of protein synthesis and by poly(A) content. However, all of this material sedimented as a single broad "9S" band under denaturing conditions in a 99% Me_2SO gradient (Mendecki and Shafritz, unpublished observations).

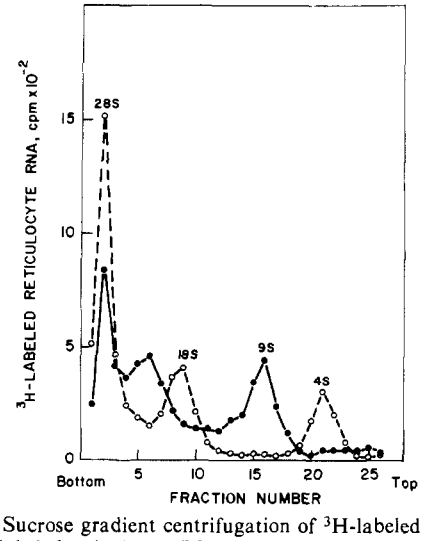

FIGURE 4: Sucrose gradient centrifugation of ^{3}H-labeled reticulocyte RNAs. ^{3}H-labeled reticulocyte RNAs, prepared *in vivo* by injection of [^{3}H]orotic acid, were separated into two fractions, one adsorbed to oligo(dT)-cellulose (●-●) and one nonadsorbed to oligo(dT)-cellulose (O - - O). These fractions were centrifuged in identical 12-ml 5–20% isokinetic sucrose gradients for 14 hr at 2°, using a Beckman SW 41 rotor at 37,000 rpm; 0.5-ml fractions were collected from the bottom of each gradient and radioactivity was determined by liquid scintillation on 100-μl aliquots of each fraction.

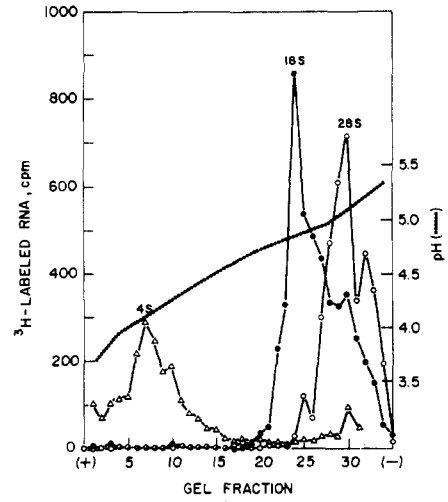

FIGURE 5: Gel isoelectric focusing of ^{3}H-labeled reticulocyte 4S, 18S, and 28S RNAs. Pooled [^{3}H]RNA fractions from Figure 4, 4S (Δ-Δ), 18S (●-●), and 28S (O-O), were focused for 18 hr in 3.0% polyacrylamide gels over a pH range 3–6. Gels were fractionated directly into counting vials, digested with 0.5 ml of NCS tissue solubilizer and counted by liquid scintillation in 5.0 ml of toluene-1,4-bis(2-(5-phenyl oxazolyl)benzene-2,5-diphenyloxazole, to which 0.1 ml of 4 M NH_4OH was added (Ward *et al.*, 1970). By this technique, there was virtually 100% recovery of input [^{3}H]RNA, except for tRNA in which the first band migrated completely thru the gel (see text).

Distribution profiles for purified focused [^{3}H]RNAs *vs.* protein synthesis activity were then obtained in a series of replicate 3% polyacrylamide gels (Figures 5–7). Figure 5 shows the focusing profiles for 4S, 18S, and 28S RNA. The first band of tRNA, generally located at approximately pH 3.6, eluted from the gel and represented 25% of the input [^{3}H]RNA. Other major bands of tRNA focused at pH 4.0 and pH 4.2, a shoulder was observed at pH 3.9, and a small amount of material remained at the top of the gel. These results were similar to those obtained previously with bacterial tRNAs (Drysdale and Righetti, 1972). 18S RNA gave one predominant band at pH 4.8 with a secondary component in the region of 28S RNA. 28S RNA gave a predominant band at pH 5.1 with a secondary band near the top of the gel. This may represent aggregated material or a higher molecular weight RNA precursors. There was also a small amount of material in the 18S region.

Focusing profiles of purified "9S" [^{3}H]mRNA were distinct from rRNA or tRNA (Figure 6). Because these mRNAs were more highly purified, there was a considerable reduction in the complexity of the banding pattern compared to crude RNA (*cf.* Figures 1 and 2). There were two major bands at pH 4.8 and pH 5.2 and several intermediate or minor bands, one of which was consistently located at pH 4.7. The mRNA fraction eluting from oligo(dT)-cellulose at 0.1 M KCl (●-●) showed an additional band at pH 4.3 and a marked enrichment in the pH 5.2 band. These findings were consistent with previous results in Figure 3, which demonstrated peaks of protein synthesis low in poly(A) content in the pH 4 and pH 5 regions.

Unlabeled globin mRNA fractions, obtained from both reticulocyte lysate (O - - O) and reticulocyte supernatant (●-●), were focused in replicate gels (Figure 7). Because mRNA input could be increased with a simultaneous reduc-

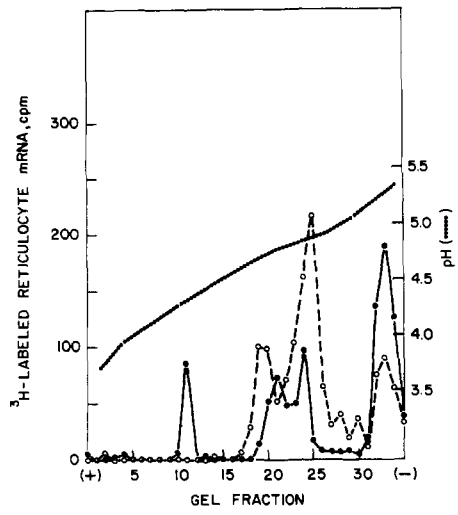

FIGURE 6: Gel isoelectric focusing of ^{3}H-labeled reticulocyte "9S" messenger RNA. For this experiment two preparations of reticulocyte mRNA were used. Both preparations were purified by a combination of oligo(dT)-cellulose chromatography and sucrose gradient centrifugation. The first preparation was eluted from oligo(dT)-cellulose at 0.1 M KCl–10 mM Tris-HCl (pH 7.4) (●–●) and the second preparation was eluted subsequently from oligo(dT)-cellulose with 10 mM Tris-HCl (pH 7.4) in the absence of KCl (O - - O). The conditions of focusing and analysis of samples were as given in Figure 5. Virtually 100% of input [^{3}H]mRNA was recovered in the gel fractions.

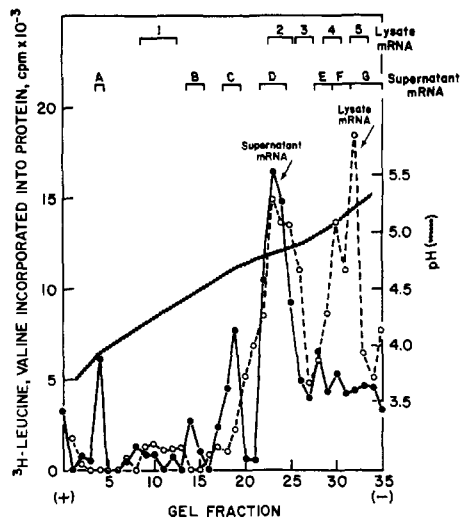

FIGURE 7: Stimulation of protein synthesis by reticulocyte globin mRNA separated by gel isoelectric focusing. Globin mRNA was isolated from both unfractionated reticulocyte lysate RNA (O - - O) and reticulocyte supernatant RNA (●–●), omitting the 0.1 M KCl elution step on oligo(dT)-cellulose chromatography. Approximately 0.25–0.30 A_{260} unit of the partially purified mRNA fractions was applied to replicate 3.0% polyacrylamide gels and focusing was performed simultaneously with the gels in Figures 5 and 6. The gels were divided into 35–36 sections and tested for messenger activity in the cell-free system, using liver mRNA-dependent ribosomes. For the peak fractions, messenger activity was approximately ⅓ saturating as compared to control globin mRNA under the same conditions, and approximately 50% of input messenger was recovered in the focused gel fractions. Other details for the assay procedure are given in Methods. In this experiment, both [^{3}H]leucine (6 Ci/mmol) and [^{3}H]valine (6 Ci/mmol) were used as labeled amino acid substrates. Material in the various peaks of protein synthesis was pooled as noted above and used for subsequent analysis of α- and β-globin chain synthesis.

tion of total RNA application, levels of protein synthesis and gel resolution were improved. To reduce potential background hemoglobin synthesis, liver ribosomes were utilized for translation of these mRNA gel fractions. The unlabeled mRNA fractions demonstrated two major bands of protein synthesis stimulation, corresponding to the positions of the major bands for [^{3}H]mRNA (*i.e.*, pH 4.8 and pH 5.2). In addition, there was an intermediate band and a number of very minor components. Compared to crude total lysate RNA, the amount of these secondary components was markedly reduced. With supernatant mRNA there was a large reduction in the pH 5.2 peak and greater activity in a minor band at pH 4.7. This also corresponded to the position of a minor ^{3}H-labeled mRNA band.

Identification of α- and β-Globin Polypeptides. Synthesis of α- and β-globin chains under the direction of focused gel fractions or control mRNA was measured by carboxymethylcellulose chromatography. ^{3}H-labeled cell free products were pooled for the various peaks, as indicated in Figure 7, and a given amount of carrier globin added. In each case 80% or more of the input ^{3}H-labeled protein was recovered with carrier globin chains and this material demonstrated one large peak on SDS gel electrophoresis of molecular weight approximately 18,000. For control globin mRNA, there was a constant relationship between the elution pattern of unlabeled carrier α and β chains and ^{3}H-labeled cell-free products (Figure 8). Small differences in cpm *vs.* A_{280} for α chain relative to β chain may reflect differences in extinction coefficients for these polypeptides. The α/β ratio for control globin mRNA, determined by summing total cpm under the α and β regions, was 0.82.

For various peaks of protein synthesis from lysate and supernatant mRNA, there were significant variations in labeled α and β chains (Figure 9). With minor adjustments for differences in elution positions of α and β chains from chromatogram to chromatogram, Figure 9 demonstrates slight enrichment for α chain in peak 2 (α/β = 1.03) and marked enrichment for β chain in peak 5 (α/β = 0.39). For

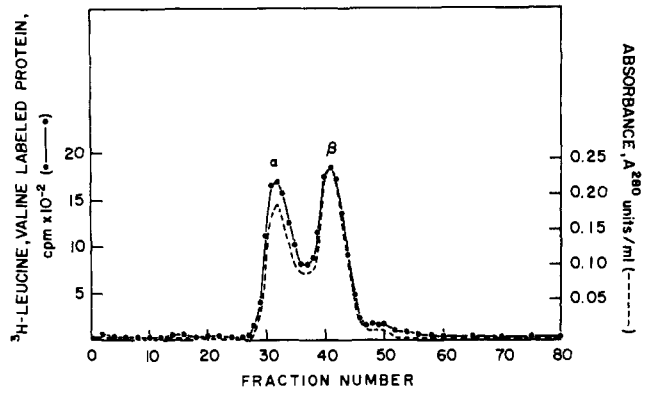

FIGURE 8: Carboxymethylcellulose chromatography of the cell-free products under direction of control reticulocyte lysate mRNA. For this study control lysate mRNA was assayed for stimulation of protein synthesis during the same series of experiments given in Figure 7. mRNA was added to near saturating levels, but not to excess; 5 mg of carrier globin was added to the cell-free product and the solution dialyzed for 48 hr against three changes of distilled H_2O to which several drops of toluene were added. An acid–acetone precipitate was prepared at −20° and stored dry at −20°. A column of 1 × 8 cm of microgranular carboxymethylcellulose (Whatman CM-52), equilibrated with 0.2 M formic acid–0.02 M pyridine, was used for separation of α- and β-globin chains. The acid–acetone powder was dissolved in 1 ml of starting column solution and a linear gradient from 0.2 M formic acid–0.02 M pyridine (70 ml) to 2.0 M formic acid–0.2 M pyridine (70 ml) was used to elute α and β chains at a flow rate of 15–20 ml/hr. Fractions of 1.5 ml were collected and 0.5-ml aliquots counted in Bray's solutions (Bray, 1960). The position of unlabeled carrier α- and β-globin chains was determined by ultraviolet absorbance at 280 mμ, using a Zeiss PMQII spectrophotometer. The α/β ratio for control globin mRNA was 0.82.

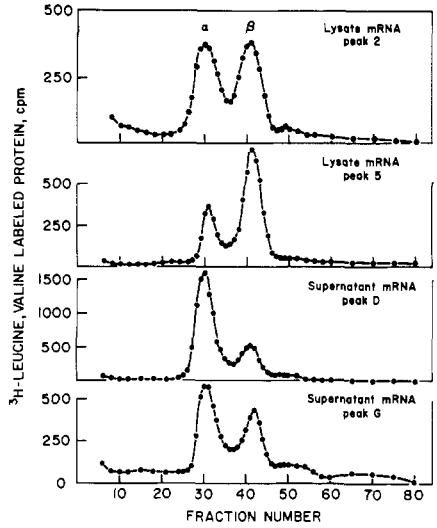

FIGURE 9: Carboxymethylcellulose chromatography of cell-free products obtained with the major peaks of focused lysate and supernatant mRNA. Peaks of protein synthesis activity were pooled, as noted in Figure 7, acid–acetone powders prepared simultaneously, and CM-cellulose chromatography performed as noted in Figure 8. In each case, elution profiles of carrier globin α and β chains were monitored by absorbance at 280 mμ and were essentially identical with that given in Figure 8.

supernatant mRNA, there was an even higher percentage of α messenger translation in peak D and again a relative enrichment for β messenger in peak G. To ensure that these results were not due to variations in recovery of α and β chains during acid–acetone precipitation, A_{280} elution patterns were monitored separately for each chromatogram and were essentially identical. Table I gives the α/β ratios for all the protein synthesis peaks designated in Figure 7. For lysate mRNA, α/β ratios ranged from 0.39 to 1.03 and for supernatant mRNA from 0.83 to 2.75. No correction was made for partial overlap of α and β chains in the chromatograms. Therefore the values listed actually represent minimum estimates for differences in α/β ratios. This is especially true for samples with large enrichment of either α or β chain, as for example peaks 5 and D. These results indicate that although there is a wide distribution of both α and β chain messengers throughout the gel, there is enrichment for α mRNA in the pH 4.7–4.8 region and enrichment for β mRNA in the pH 5.1–5.2 region.

TABLE I: α/β Ratios of Products with Reticulocyte mRNA Fractions Separated by Isoelectric Focusing.

	Lysate mRNA		Supernatant mRNA	
pI	Fraction[a]	α/β	Fraction[a]	α/β
3.9			A	0.83
4.3	1	0.87		
4.4			B	1.32
4.7			C	2.72
4.8	2	1.03	D	2.75
4.9	3	0.69		
5.0			E	1.86
5.1	4	0.71	F	1.36
5.2	5	0.39	G	1.24

[a] For determination of α/β ratios, pooled fractions are those indicated in Figure 7.

Discussion

The separation, quantitation, and characterization of discrete messenger RNA species are important steps for our understanding of rate-limiting aspects of protein synthesis in mammalian cells. Methods for isolating and purifying mRNA have included sucrose gradient centrifugation (Lingrel *et al.*, 1971), differential phenol extraction and collection on nitrocellulose filters (Lee *et al.*, 1971; Rosenfeld *et al.*, 1972), adsorption to oligo(dT)-cellulose (Aviv and Leder, 1972), gel electrophoresis (Williamson *et al.*, 1971; Lockard and Lingrel, 1972; Kazazian, 1972), gel filtration (Means *et al.*, 1974), and immunoprecipitation (Palacios *et al.*, 1972; Palmiter *et al.*, 1972; Stevens and Williamson, 1973). Within given limitations, each of these procedures takes advantage of either general properties of mRNA, *e.g.*, the 3′-poly(A) segment, or innate properties of specific mRNAs, *e.g.*, size, molecular weight, or immunologic reactivity of the proteins for which they code. Any procedure which increases the purification of specific mRNAs or provides additional information about the properties of specific mRNAs, might be an important adjunct to these methods.

In efforts to devise a general procedure for separating mRNA molecules according to differences in other physicochemical properties, we have begun investigating the potential use of gel electrofocusing. This method is highly sensitive for separating molecules on the basis of net surface charge. All components migrate to their respective isoelectric positions in a pH gradient created by a mixture of ampholyte molecules with graded isoelectric points placed in an electric field. Isoelectric focusing has been used extensively for separating and purifying proteins as well as for identifying and separating subspecies of proteins considered homogeneous by other criteria (Catsimpoolas, 1971). Drysdale and Righetti (1972) recently extended the use of this procedure to bacterial tRNAs. Various highly purified isoaccepting tRNA species gave rise to complex but rather similar banding patterns. Although there were some denaturation effects which were partially reversible and interaction of tRNA with the ampholytes could not be excluded, much of the evidence suggested that multiple forms of the given tRNAs species represented different conformations, as found by other methods (Lindahl *et al.*, 1966; Sarin *et al.*, 1966; Gartland and Sueoka, 1966; Römer *et al.*, 1970; Schulman, 1971; Egan *et al.*, 1973).

In the present study, we have explored the behavior of various reticulocyte RNAs by gel isoelectric focusing and have demonstrated distinct banding patterns for ^{3}H-labeled 4S, 18S, and 28S RNA. The profiles for mRNA, as determined by comparison of ^{3}H-labeling patterns with stimulation of protein synthesis in a messenger-dependent system, gave more complex results. There was, however, good correlation between these two methods. The complexity was reduced considerably when more purified mRNA fractions were employed. Under the latter conditions, there were two major bands of mRNA at pH 4.8 and pH 5.2, one intermediate peak, and several minor components. There was a slight enrichment of α-globin mRNA in the pH region 4.7–4.8 and considerable enrichment of β-globin mRNA (a minimum of 72% of total α and β translation) in the pH region 5.1–5.2. It should be noted for these major peaks that protein synthesis activity was stimulated to comparable levels and was in the linear portion of the messenger curve at approximately $\frac{1}{3}$ saturation. Under these conditions, translation of α- and β-globin messengers is approximately equal

(Nienhuis *et al.*; 1973). Therefore, since other experimental variables remained constant, it is unlikely that differences in total mRNA input were responsible for differences observed in α/β ratios for the major peaks.

Comparable separations of α- and β-globin mRNAs have been achieved by taking advantage of special biological properties of rabbit reticulocytes. Up to 10% of rabbit reticulocyte mRNA is present in the 105,000*g* supernatant fraction as ribonucleoprotein particles and 80–85% of this mRNA codes for α-globin chains (Jacobs-Lorena and Baglioni, 1972; Bonanou-Tzedaki *et al.*, 1972; Gianni *et al.*, 1972). Enrichment for β-globin mRNA in heavy polysomes has also been produced by treatment of reticulocytes with L-*O*-methylthreonine (Rabinowitz *et al.*, 1969; Temple and Housman, 1972). Direct fractionation of "9S" reticulocyte RNA into α- and β-globin messengers by general methods has proved more difficult. Using gel electrophoresis, two distinct bands as well as one diffuse band with secondary shoulders have been observed with unfractionated "9S" globin mRNA (Williamson *et al.*, 1971; Lockard and Lingrel, 1972; Kazazian, 1972). Subsequent studies revealed that the discrete bands could be removed from mRNA by oligo(dT)-cellulose or poly(U)-Sepharose chromatography and did not represent messenger activity (Morrison *et al.*, 1972; Lanyon *et al.*, 1972; Kazazian *et al.*, 1973). Morrison *et al.* (1974) recently reported the separation of mouse α- and β-globin mRNA by formamide gel electrophoresis, and similar results have also been obtained by other investigators (Gould and Hamlyn, 1973). Under formamide denaturing conditions, α-globin mRNA migrates slightly faster than β-globin mRNA, so that the molecular dimensions of β-chain mRNA appear somewhat greater than α-chain mRNA.

The basis for separating mRNA by isoelectric focusing, however, is not entirely clear. Since there were multiple peaks for "9S" globin mRNA, and these peaks contained varying ratios of α- and β-chain mRNA by translation studies, a simple separation of globin mRNAs cannot fully explain the results. Partial degradation of mRNA also seemed unlikely, since greater than 80% of the cell-free product could be identified as α and β chain for all major and minor peaks. Attempts to determine whether any of the multiple forms represented structural conformers were inconclusive. Although there was no appreciable difference in the protein synthesis stimulation pattern after annealing, we could not exclude the possibility that different conformations exist under the acidic pH of focusing but renature during the extraction or assay procedure. Nor could we exclude the possibility that some of the multiple forms of globin mRNA represent different conformations which have little effect on messenger translatability. Ideally, one would like to examine banding patterns in denaturing gels, but our present assay for mRNA did not allow this. Conceivably, this might be accomplished by assaying specific mRNAs with labeled specific cDNAs, or by eluting RNA and separating it from the denaturants prior to assay in the cell-free system. Other types of interaction between various RNA species or specific effects of ampholytes on mRNA banding could also contribute to our findings.

In other experiments, we found marked differences in the poly(A) content of various messenger fractions. This is consistent with recent studies by other investigators indicating the lack of a 3′-poly(A) requirement for translation of mRNAs normally containing such sequences (Bard *et al.*, 1974; Williamson *et al.*, 1974). Recently Gorski *et al.* (1974) reported discrete size classes in the 3′-poly(A) segment of mouse reticulocyte globin mRNA and suggested that these size classes represent specific metabolic events. Other investigators have found that by increasing chain length, the pK_a of poly(rA) homopolymers can be increased up to 1.5 pH units (Voet and Rich, 1970). However, since a major band of messenger activity low in poly(A) content migrated to the more alkaline region in our focusing gradients, factors other than poly(A) must also contribute to the banding position of globin mRNA molecules.

In addition to differences in poly(A) content, differences in nucleotides outside the coding region could produce multiple bands on isoelectric focusing. Therefore, primary structural differences, metabolism, or degradation of mRNA outside the coding segment might be responsible for some of the observed heterogeneity. In this regard, it is interesting that the distribution of α mRNA isolated from supernatant differed from α mRNA isolated from whole lysate (*cf.* Figure 7 and Table I). The total proportion of minor components is also greater for supernatant mRNA compared to lysate mRNA (Figure 7). These results are only preliminary but provide additional evidence for a considerable complexity in globin mRNA. The nature and biological significance of this apparent microheterogeneity remains to be determined.

Acknowledgments

The authors thank Dr. K. J. Isselbacher, Mass. General Hospital, Boston, Mass. for generous support, encouragement, and use of his facilities during the early portion of these studies, Dr. G. Brawerman for making available details of the [^{3}H]poly(U) hybridization assay for poly(A) content prior to publication, and Ms. M. Tse for excellent technical assistance.

References

Aviv, H., and Leder, P. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1408.

Bagshaw, J. C., Drysdale, J. W., and Malt, R. (1973), *Ann. N.Y. Acad. Sci. 209,* 363.

Bard, E., Efron, D., Marcus, A., and Perry, R. P. (1974), *Cell 1,* 101.

Bonanou-Tzedaki, S. A., Pragnell, I. B., and Arnstein, H. R. V. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 26,* 77.

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

Catsimpoolas, N., Ed. (1971), *Ann. N.Y. Acad. Sci. 209.*

Crystal, R. G., Nienhuis, A. W., Elson, N. A., and Anderson, W. F. (1972), *J. Biol. Chem. 247,* 5357.

Darnell, J. E., Jelinek, W. R., and Molloy, G. R. (1973), *Science 181,* 1215.

Dintzis, H. M. (1961), *Proc. Nat. Acad. Sci. U. S. 47,* 247.

Drysdale, J. W., and Righetti, P. (1972), *Biochemistry 11,* 4044.

Egan, B. Z., Weiss, J. F., and Kelmers, A. D. (1973), *Biochem. Biophys. Res. Commun. 55,* 332.

Gartland, W. J., and Sueoka, N. (1966), *Proc. Nat. Acad. Sci. U. S. 55,* 948.

Gianni, A. M., Giglioni, B., Ottolenghi, S., Comi, P., and Guidotti, G. (1972), *Nature (London), New Biol. 240,* 183.

Gilbert, J. M., and Anderson, W. F. (1970), *J. Biol. Chem. 245,* 2342.

Gorski, J., Morrison, M. R., Merckel, C. G., and Lingrel, J. B. (1974), *J. Mol. Biol. 86,* 363.
Gould, H. J., and Hamlyn, P. H. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 30,* 301.
Housman, D., Forget, B. G., Skoultchi, A., and Benz, E. J., Jr. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1809.
Jacobs-Lorena, M., and Baglioni, C. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1425.
Jeffery, W. R., and Brawerman, G. (1974), *Biochemistry* (in press).
Kacian, D. L., Spiegelman, S., Terada, M., Metafora, S., Dow, L., and Marks, P. A. (1972), *Nature (London), New Biol. 235,* 167.
Kazazian, H. H., Jr. (1972), *Nature (London), New Biol. 238,* 166.
Kazazian, H. H., Jr., Moore, P. A., and Synder, P. G. (1973), *Biochem. Biophys. Res. Commun. 51,* 564.
Lanyon, W. G., Paul, J., and Williamson, R. (1972), *Eur. J. Biochem. 31,* 38.
Lee, S. Y., Mendecki, J., and Brawerman, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1331.
Lindahl, T., Adams, A., and Fresco, J. R. (1966), *Proc. Nat. Acad. Sci. U.S. 55,* 941.
Lingrel, J. B., Lockard, R. E., Jones, R. F., Burr, H. E., and Holder, J. W. (1971), *Ser. Haematol. 4,* 37.
Lockard, R. E., and Lingrel, J. B. (1972), *J. Biol. Chem. 247,* 4174.
Loening, U. E. (1967), *Biochem. J. 102,* 251.
Macnaughton, M., Freeman, K. B., and Bishop, J. O. (1974), *Cell 1,* 117.
Means, A. R., Rosen, J. M., Woo, S., Holder, J. W., Chan, L., Harris, S. E., and O'Malley, B. W. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1541.
Moldave, K., and Grossman, L., Ed. (1974), *Methods Enzymol. 30*F.
Morrison, M. R., Brinkley, S. A., Gorski, J., and Lingrel, J. B. (1974), *J. Biol. Chem. 249,* 5290.
Morrison, M. R., Gorski, J., and Lingrel, J. B. (1972), *Biochem. Biophys. Res. Commun. 49,* 775.
Nienhuis, A. W., Canfield, P. H., and Anderson, W. F. (1973), *J. Clin. Invest. 52,* 1735.
Noll, H. (1969), *in* Techniques in Protein Biosynthesis, Vol. II, Campbell, P. N., and Sargent, J. R., Ed., New York, N.Y., Academic Press, Chapter 3, p 101.
Palacios, R., Palmiter, R. D., and Schimki, R. T. (1972), *J. Biol. Chem. 247,* 3216.
Palmiter, R. D., Palacios, R., and Schimki, R. T. (1972), *J. Biol. Chem. 247,* 3296.
Rabinowitz, M., Freedman, M. L., Fisher, J. M., and Maxwell, C. R. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 567.
Römer, R., Riesner, D., and Maas, G. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 10,* 352.
Rosenfeld, G. C., Comstock, J. P., Means, A. R., and O'Malley, B. W. (1972), *Biochem. Biophys. Res. Commun. 47,* 387.
Ross, J., Aviv, H., Scolnick, E., and Leder, P. (1972a), *Proc. Nat. Acad. Sci. U. S. 69,* 264.
Ross, J., Ikawa, Y., and Leder, P. (1972b), *Proc. Nat. Acad. Sci. U. S. 69,* 3620.
Sarin, P. S., Zamecnik, P. C., Berquist, P. L., and Scott, J. F. (1966), *Proc. Nat. Acad. Sci. U. S. 55,* 579.
Schulman, L. (1971), *J. Mol. Biol. 58,* 117.
Shafritz, D. A., and Anderson, W. F. (1970), *J. Biol. Chem. 245,* 5553.
Shafritz, D. A., and Drysdale, J. W. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1542.
Shafritz, D. A., Drysdale, J. W., and Isselbacher, K. J. (1973a), *J. Biol. Chem. 248,* 3220.
Shafritz, D. A., Isselbacher, K. J., and Drysdale, J. W. (1973b), *Proc. Int. Cong. Biochem., 9th,* 27.
Stevens, R. H., and Williamson, A. R. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1127.
Temple, G. F., and Housman, D. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1574.
Terada, M., Cantor, L., Metafora, S., Rifkind, A., Bank, A., and Marks, P. A. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3575.
Verma, I. M., Temple, G. F., Fan, H., and Baltimore, D. (1972), *Nature (London), New Biol. 235,* 163.
Voet, D., and Rich, A. (1970), *Progr. Nucl. Acid. Res. Mol. Biol. 10,* 183.
Ward, S., Wilson, D. L., and Gilliam, J. J. (1970), *Anal. Biochem. 38,* 90.
Williamson, R., Morrison, M., Lanyon, G., Eason, R., and Paul, J. (1971), *Biochemistry 10,* 3014.
Williamson, R., Crossley, J., and Humphries, S. (1974), *Biochemistry 13,* 703.