Properties of Mouse Globin Messenger Ribonucleic Acid and Its Preparation in Milligram Quantities*

R. Williamson,† M. Morrison, G. Lanyon, R. Eason, and J. Paul

ABSTRACT: There is good evidence that the RNA component found in reticulocyte polysomes and sedimenting at 9 S is the mRNA for globin. Using a combination of zonal ultracentrifugation and preparative polyacrylamide gel electrophoresis, milligram amounts of this mRNA have been isolated from mouse reticulocytes. The mRNA splits into two major components on analytical gel electrophoresis under conditions where the mRNAs for the α- and β-globin chains would be expected to separate. These two components label with ^{32}P to an equal extent *in vivo*. The molecular weight of the mRNA is 170,000 ± 8000; this indicates a molecule having about 65 nucleotides more than would be required to code for globin. The behavior of another RNA component, of sedimentation coefficient approximately 12 S, has also been investigated. This component does not appear to be either a degradation product of 28S or 18S rRNA arising from ribonuclease action during preparation, or mRNA for globin. The melting profiles of 9S and 12S RNAs are compared to those of 5S and 18S RNAs; the 9S mRNA appears to have considerable secondary stucture.

Mammalian reticulocytes, which synthesize mostly hemoglobin and have easily isolated polysomes, are a favored system for the study of protein synthesis (Chantrenne *et al.*, 1967). When total polysomal RNA isolated from rabbit, rat, or mouse reticulocytes is subjected to electrophoresis on polyacrylamide gels, a component is found approximately of the molecular weight expected for globin mRNA; an RNA of similar size can be isolated on sucrose gradients. This RNA can also be isolated from a characteristic mRNA–protein complex obtained after treatment of reticulocyte polysomes with chelating agents (Marbaix and Burny, 1964a; Huez *et al.*, 1967). The amount of this RNA can be correlated with polysome size (Evans and Lingrel, 1969a) and with the capacity of the reticulocyte for protein synthesis (Lamfrom and Knopf, 1964). It disappears after treatment of polysomes with low levels of ribonuclease (Marbaix and Burny, 1964a,-b) and after sonication of polysomes to monosomes (Williamson *et al.*, 1969). Its labeling kinetics differ *in vivo* from rRNA (Evans and Lingrel, 1969b), and it gives a unique "fingerprint" after degradation with specific nucleases (Labrie, 1969). Added 9S RNA causes a specific synthesis of its corresponding globin by programming ribosomes in heterologous protein-synthesizing systems (Schapira *et al.*, 1968; Laycock and Hunt, 1969; Lockard and Lingrel, 1969). Lingrel and his colleagues (1971) have verified that mouse reticulocyte 9S RNA acts as the mRNA for globin in mouse–guinea pig and mouse–duck mixed systems. They have similarly demonstrated that rabbit reticulocyte 9S RNA is the mRNA for rabbit globin in a mixed rabbit–guinea pig system.

We have undertaken the purification of large amounts of mouse globin mRNA with a view to studying its properties and its hybridization to DNA (Williamson *et al.*, 1970). We have attempted to analyze the heterogeneity of the mRNA, and have also purified and studied the properties and labeling kinetics of a second RNA component sedimenting between the 18S and 5S rRNA species, at approximately 12 S.

Materials and Methods

Reticulocytes were prepared from whole blood of Porton strain mice which had been injected intraperitoneally 4 and 7 days previously, with 0.1 ml of a 2.5% solution of neutralized phenylhydrazine hydrochloride. The cells were washed with Hanks' balanced salt solution (Paul, 1965) and the buffy coat removed. The cells were used fresh or stored at −70° until required. The reticulocyte count was usually between 50 and 80%.

Preparation of Polysomes and RNA. The elimination of ribonuclease activity from all solutions used in the preparation of polysomes and RNA was essential to obtain consistent results. Neither Macaloid (a gift from Texas Lead and Barytes Co.) nor Bentonite proved as effective as diethyl pyrocarbonate (Baycovin, a gift from Bayer Chemicals Ltd., Glasgow), which was used to sterilize all glassware and solutions prior to use, rather than as a deproteinizing agent (Fedorcsak *et al.*, 1969). This is consistent with the interpretation that RNase problems are primarily due to RNase which is added to the reticulocyte fractions during preparation, rather than to nucleases from the tissue itself, as reticulocyte polysomes are markedly free of RNase activity when isolated carefully (Williamson and Mathias, 1963).

The reticulocytes were lysed by the addition of two volumes of 0.001 M $MgCl_2$. The cell membranes and mitochondria, together with any few remaining white blood cells, were sedimented at 20,000*g* for 10 min and discarded. If total supernatant RNA was to be prepared, 4-aminosalicylic acid was added to a final concentration of 6% to the supernatant at this stage, followed by an equal volume of phenol–*m*-cresol–8-hydroxyquinoline (Kirby, 1965). After two phenol deproteinization steps, the aqueous phase was precipitated twice from ethanol and the final pellet taken up in 0.05 M KCl–0.0015 M $MgCl_2$–0.001 M Tris (pH 7.5) (TKM buffer) or 0.03 M NaH_2PO_4–0.036 M Tris–0.001 M EDTA (pH 7.8).

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† To whom correspondence should be addressed.

Alternatively, polysomes were sedimented from the post-mitochondrial supernatant, at 140,000*g* for 60 min. The polysome pellet was suspended in a 6% solution in water of 4-aminosalicylic acid and extracted with phenol–*m*-cresol–8-hydroxyquinoline as described above. Polysomes were sometimes treated with one-half volume of 0.1 M EDTA to give subunits plus a messenger ribonucleoprotein component as described by Chantrenne *et al.* (1967). It was found that a final EDTA concentration as low as 0.007 M would give dissociation into ribosomal subunits plus messenger ribonucleoprotein, and that, at below this concentration, dissociation into ribosomal subunits still occurs but no 14S mRNP component is obtained, as for dissociation with low concentrations of pyrophosphate (Holder and Lingrel, 1970) and after incubation in the presence of puromycin (Pragnell and Arnstein, 1970).

Labeling of RNA in Vivo. Either [^{32}P]phosphate (PBS-1, Radiochemical Centre, Amersham) or [^{3}H]uridine (TRA-178, Radiochemical Centre, Amersham) was injected intravenously into mice 22 hr before killing.

Zonal Ultracentrifugation. Samples were centrifuged using either the B-XIV titanium rotor or the B-XV aluminium rotor (M.S.E. Ltd., Crawley, Sussex). The conditions were B-XIV rotor, 50-ml underlay, 560-ml sucrose gradient (linear), either 5–20 or 10–30%, 10-ml sample containing up to 30 mg of RNA or 70 mg of polysomes and 110-ml overlay; B-XV rotor, 100-ml underlay, 1240 ml of sucrose gradient as for B-XIV rotor, 10-ml sample containing up to 75 mg of RNA or 200 mg of polysomes and 300-ml overlay. Various centrifugation times were used, depending on whether all components or only the mRNA were to be isolated; in general, for mRNA isolation from total polysomal RNA, a run of 4 hr at 46,000 rpm at 3° for the B-XIV rotor was used. Latterly, longer run times were used to isolate purified 9S mRNA directly. After centrifugation, the contents of the rotor were pumped through a variable path-length flow cell (Perkin-Elmer, Ltd., London) and the optical density was monitored at 260 mμ using a Unicam SP-800 spectrophotometer with a Servoscribe external chart recorder.

Preparative Gel Electrophoresis. Conditions were as previously reported (Lanyon *et al.*, 1968) except that a buffer of lower ionic strength (Tris (0.01 M)–sodium acetate (0.005 M)–EDTA (0.5 mM)) was sometimes used to shorten the time required for migration across the more concentrated gel used for improved resolution. Gels of 3% were found superior to the 2.4% gels previously described for resolution of 9S and 12S RNA.

Analytical Gel Electrophoresis. Conditions were as described by Loening (1967). The bands were visualized without staining using a Joyce-Loebl ultraviolet scanner with a Smith Servoscribe chart recorder. RNA could be eluted from gels using 0.4% SDS–0.6 M LiCl, and then pelletted from this solution by centrifugation for 24 hr at 140,000*g* (U. E. Loening, personal communication). Alternatively, the bands were stained with 0.5% toluidine blue for 1 hr, and the excess stain removed by soaking the gels overnight in water.

Counting Procedures. Solutions containing ^{32}P were usually counted without scintillator with the Beckman DPM-100 (Cerenkov counts, tritium channel, 30% efficiency). Solutions of ^{3}H- or ^{14}C-containing material were counted after mixing with a toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–Triton X-100 scintillant (efficiency approximately 55% for ^{14}C, 25% for ^{3}H).

Gels were cut into 1-mm thick slices with a Mickle gel slicer (Gomshall, Surrey, England). Slices containing ^{32}P were counted as above without scintillator or in toluene-based scintillator; those containing ^{3}H were first heated to dryness in a scintillation vial containing 10% piperidine (Loening, 1967) and then swollen in 1 ml of distilled water followed by 10 ml of toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–Triton X-100 scintillator and counted.

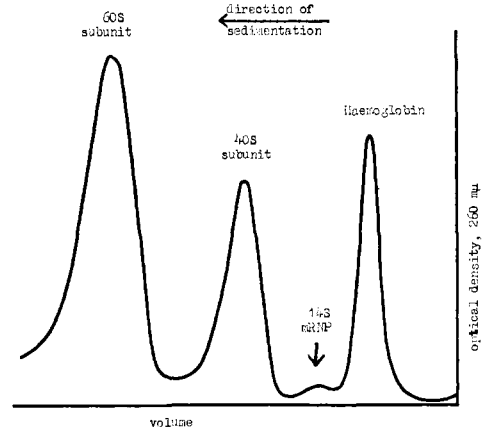

FIGURE 1: Zonal ultracentrifuge run of EDTA-treated mouse reticulocyte polysomes (40-mg total in 11 ml of 0.05 M KCl–0.001 M tris (pH 7.4)–0.1 M EDTA (pH 8.0) (2:1, v/v) layered on 10–30% sucrose gradient, 0.05 M KCl–0.001 M Tris (pH 7.4). M. S. E. B-XIV Ti rotor, 46,000 rpm, 3 hr, 7°.

Melting Curves. Samples of purified 9S and 12S RNA, as well as samples of isolated 18S and 5S rRNA, analyzed for purity by polyacrylamide gel electrophoresis shortly before use, were melted and reannealed in 0.015 M NaCl–0.0015 M sodium citrate (pH 7.0). The melting and reannealing was performed using a thermostatically controlled heating block in an SP-800 Unicam spectrophotometer attached through its amplifier to a Servoscribe chart recorder.

Base Composition. The base composition of the mRNA was determined with a Varian aerograph Model ICS-1000 ion-exchange liquid chromatograph (Varian Associates, Ltd., Walton-on-Thames, Surrey).

Analytical Ultracentrifugation. Sedimentation coefficients were determined in the presence of formaldehyde by the method of Boedtker (1968). mRNA (25 μg/ml), in 1.1 M formaldehyde–0.09 M Na_2HPO_4–0.1 M NaH_2PO_4, was heated at 63° for 15, 30, or 60 min, cooled rapidly in ice, and analyzed immediately. Sedimentation rates were measured at 20° in a Spinco Model E ultracentrifuge equipped with ultraviolet scanner–multiplex accessories.

Results

RNA Isolation. It was found that the addition of 0.1% diethyl pyrocarbonate to buffers and sucrose solutions before use was sufficient to completely eliminate ribonuclease activity. Currently we obtain only components of sedimentation coefficients approximately 9 and 12 S sedimenting between the 18S and 5S rRNAs, with a trace of a component of approximate sedimentation coefficient 7 S. In early experiments, prior to the treatment of buffers by diethyl pyrocarbonate, other RNA components were found to sediment between 18S and 5S rRNAs, giving a pattern on gel electrophoresis similar to that obtained by Labrie (1969).

Approximately 20 ml of packed reticulocytes was obtained from 100 mice; this in turn gave 60 mg of total postmicro-

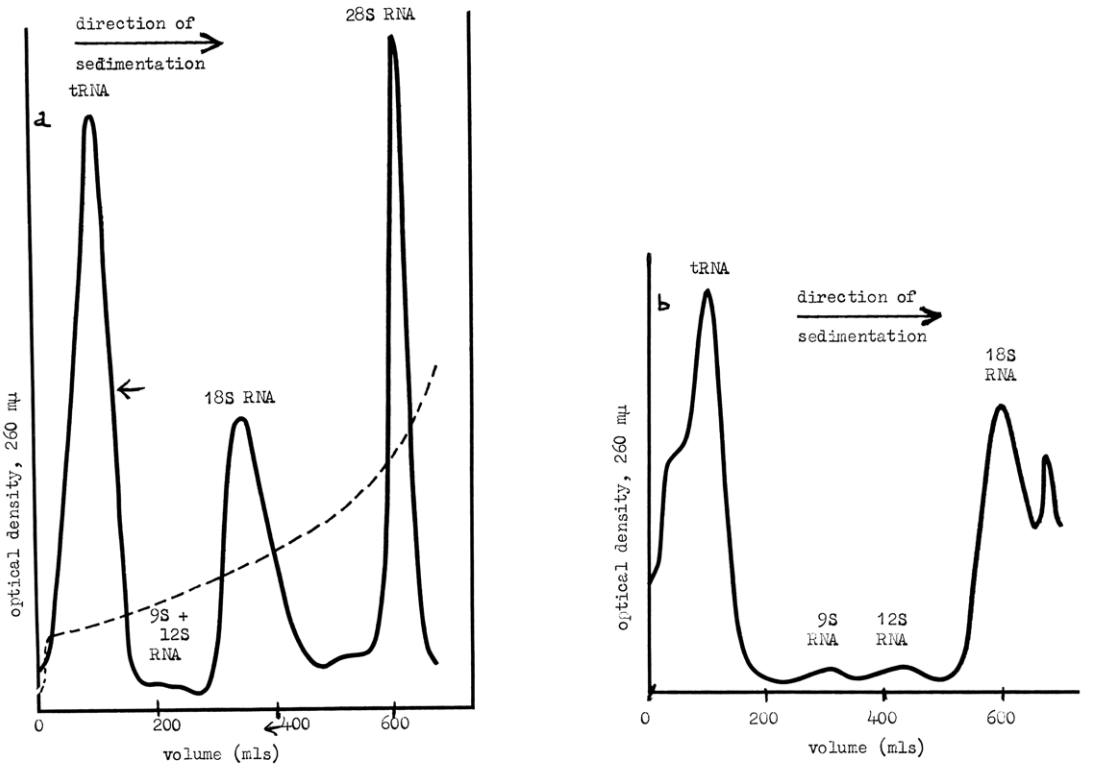

FIGURE 2: Zonal ultracentrifuge run on total mouse reticulocyte RNA (30 mg of RNA in 0.001 M Tris–0.05 M KCl–0.0015 M EDTA, pH 7.4), 5–20% sucrose gradient in 0.001 M Tris–0.05 M KCl–0.0015 M EDTA (pH 7.4). M. S. E. B-XIV Ti rotor, 45,000 rpm, (a) for 4 hr, 7°. (b) For 7 hr, 7°.

somal RNA which yielded 0.5 mg of mRNA. This yield of mRNA was obtained whether by fractionation of postmicrosomal RNA or of EDTA-treated polysomes. A maximum of 60 mg of RNA or 120 mg of polysomes was layered in a single zonal run; on short runs resolution was improved if approximately half this amount was used.

The 9S RNA represents approximately 1.3% of the total cytoplasmic RNA and is present at from two to three times the concentration of 12S RNA. The ratio of 9S to 12S RNA remains unchanged before and after zonal centrifugation (taking a cut encompassing both species), indicating that the 12 S does not arise due to breakdown of larger species once the RNA is isolated. To determine whether the 12S RNA occurs in greater amounts in monosomes (which would include "spent" ribosomes no longer active in protein synthesis) than in active polysomes, the RNA isolated from pentaribosomes was compared to that isolated from mono- and diribosomes from the same preparation, and it was found that the proportion of 12S RNA was increased by 20% relative to 18S RNA in the preparation from the smaller polysomes.

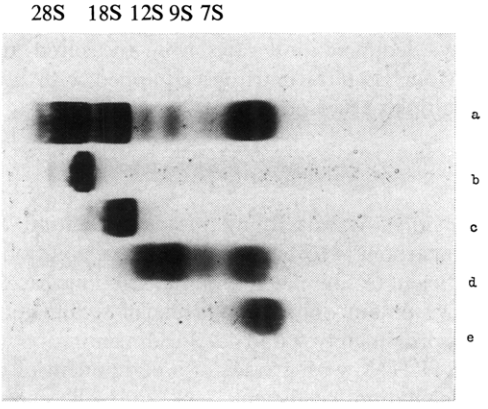

FIGURE 3: 2.6% polyacrylamide gel electrophoresis of total mouse cytoplasmic RNA and cuts obtained after zonal fractionation of the RNA in the B-XIV zonal rotor at 45,000 rpm for 4 hr at temperature setting 5. Electrophoresis was for 1 hr at 17 V/cm, 6 mA/gel. The buffer temperature was 4°. (a) Total mouse cytoplasmic RNA, (b) 28S RNA cut, (c) 18S RNA cut, (d) crude 9S cut, and (e) 5S and 4S cut.

Zonal Centrifugation. Excellent resolution was obtained with both B-XIV and B-XV zonal rotors, whether centrifuging EDTA-treated polysomes (Figure 1) or total postmitochondrial RNA (Figure 2). In the latter procedure using a B-XIV zonal rotor (now the standard method), approximately 0.35 mg of combined 9S and 12S RNA, or 0.25 mg of 9S RNA, was recovered from a zonal run starting with 30 mg of total cytoplasmic RNA. The 9S and 12S components clearly separate during the zonal run on RNA (Figure 2b). Figure 3 shows polyacrylamide gel patterns obtained from RNA cuts from a short zonal run. Similar results are obtained for EDTA-treated polysomes. The 14S mRNP[1] component gives only 9S RNA after phenol treatment. There is a trace of 12S RNA associated with the RNA from the small subunit. We have found that a zonal run of 13 hr on postmitochondrial RNA gives pure 9S components; Figure 4 shows the increasing purity of the 9S component as the zonal run time is increased.

Preparative Polyacrylamide Gel Electrophoresis. When the 9S RNA prepared from short zonal runs was found to be contaminated with 12S or 5S RNA by analytical gel analysis, several batches were pooled and a single Polyprep run was carried out under conditions where the eluted RNA gives well-

[1] Abbreviation used is: mRNA, messenger ribonucleoprotein.

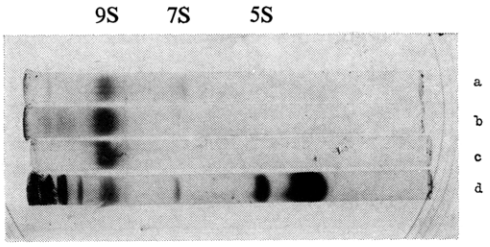

FIGURE 4: 2.4% polyacrylamide gel electrophoresis of (a) 9S RNA cut from 8-hr zonal run on postmicrosomal RNA, (b) 9S RNA cut from 4-hr zonal run, (c) 9S RNA cut from 12-hr zonal run, and (d) total mouse reticulocyte cytoplasmic RNA. Electrophoresis was for 45 min at a voltage of 17 V/cm, 6 mA/gel. The buffer temperature was 4°.

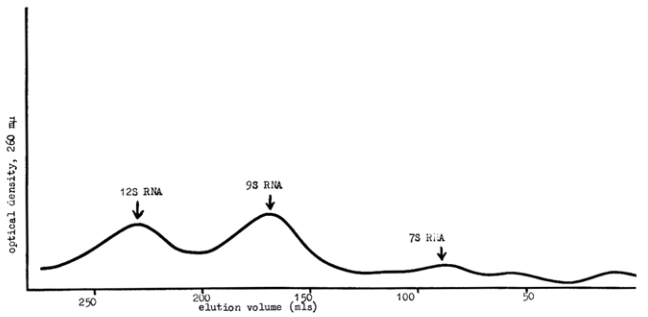

FIGURE 5: Preparative polyacrylamide gel run on 1.5 mg of pooled impure 9S RNA cuts from several zonal runs. Polyacrylamide gel (100 ml of 3%) in 0.04 M Tris–0.02 M sodium acetate–0.002 M EDTA (pH 7.8). Electrophoresis was carried out at 40 V (50 mA) for 1 hr, then at 120 V (200 mA).

defined peaks (Figure 5). The purity of the final 9S RNA product was always confirmed using analytical 2.6% polyacrylamide gel electrophoresis.

Analytical Gel Electrophoresis. When run on 2.6% analytical polyacrylamide gels, both the 9S and 12S components migrate as single bands (Figure 3). The 9S component from different runs always migrates to the same position. When run on 6% polyacrylamide gels, the 9S component splits into two major components (Figure 6), present in approximately equal amounts, as well as three faint minor components, which run slightly faster. The region of the 9S component shows diffuse staining which is absent for 18S, 12S, and 5S RNAs. The 12S component also splits into two bands, but in this case the species migrating more slowly is present in smaller amounts than the faster.

It is possible to estimate the molecular weight of an RNA species from its migration on polyacrylamide gel electrophoresis (Loening, 1967, 1969; Lewicki and Sinskey, 1970). When the distance of migration of 28S, 18S, and 5S rRNAs are plotted against the log of their molecular weight, the points are found to lie on a straight line for gels of acrylamide concentration less than 3%. We have used this technique to measure the molecular weights of 9S and 12S RNA, using 2.6% polyacrylamide gels, and find values of 225,700 ± 13,700 for 9 S and 373,400 ± 19,100 for 12 S (ten different RNA samples in each case). The standard molecular weights used were those determined by Hunt for rabbit reticulocyte RNA: 28 S, 1.53×10^6; 18 S, 0.65×10^6; and 5 S, 4×10^4 (Hunt, 1970; Forget and Weissman, 1969). The variation obtained is of the order of the error in precision of measurement (Lewicki and Sinskey, 1970). Using a similar technique, Labrie (1969) obtained an estimated molecular weight of 190,000 for what we call 9 S (10 S in his paper). However, there is as of yet little agreement in the literature on the correct value for the molecular weights of 28S and 18S rRNAs from mammalian species, and an accurate evaluation of molecular weights for other species on polyacrylamide gels must await more accurate determinations of these values by other methods.

As has been mentioned above, the 9S RNA is consistently diffuse even on 2.6% gels as compared to rRNAs or 12S RNA, and the molecular weight spread for the half-width of the band is approximately 20,000 to either side of the mean value given above. In 6% gels, the 9 S splits into two major components, and these differ by approximately 0.15 S in sedimentation coefficient, corresponding to a difference in molecular weight of approximately 5000. However, as this estimate is made by extrapolating from the positions and molecular weights of 5S and 7S RNA, rather than interpolating (as in the case of 2.6% gels) from 18S and 5S RNA, it is necessarily less precise.

Analytical Ultracentrifugation. The sedimentation coefficient, $s_{20,w}$, for the untreated mRNA determined by boundary sedimentation in 0.09 M Na_2HPO_4–0.01 M NaH_2PO_4 is 8.9 ± 0.1 S (average of four runs). Treatment of the mRNA with formaldehyde for 60 min at 63° caused no degradation of the RNA. The sedimentation coefficient after formaldehyde treatment is 6.2 ± 0.02. Using the relationship that $s_{20,w} = 0.05\ M^{0.40}$ (Boedtker, 1968), a molecular weight of 170,000 ± 8000 was obtained (standard deviation from seven runs).

Radioactive Labeling Pattern in Vivo. Marbaix and Burny (1964b), Evans and Lingrel (1969b), and Labrie (1969) have commented on the fact that the 9S component incorporates radioactive isotope at a different rate from the rRNA components in rabbit. We have examined this in mouse and extended these results to examine the incorporation into the two major components of 9 S separately, and to compare the labeling of 9 S to 12 S. Figure 7a shows the labeling pattern of RNA separated on 6% polyacrylamide gels after injection of isotope 22 hr before bleeding; as can be seen, the two 9S peaks are labeled to an identical specific activity. The 12S component (Figure 7b,c) is not only labeled to a much lower extent than the 9S RNA, but also somewhat less than 28S RNA. The relative specific activities are approximately 9 S:12 S = 5:1 and 28 S:12 S = 1.5:1.

Melting and Reannealing Profiles. The melting and reannealing profiles for 5S, 9S, 12S, and 18S RNA are shown in Figure 8. The 9S, 12S, and 18S RNAs were prepared as de-

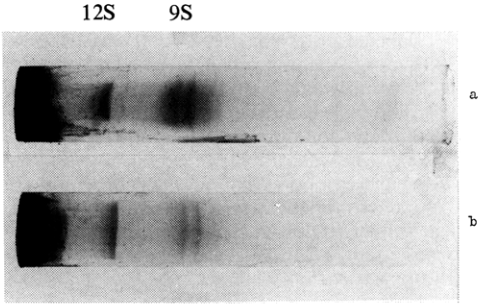

FIGURE 6: Two different 6% polyacrylamide gel electrophoresis runs on mouse reticulocyte polysomal RNA (400 μg). Gels were 1 cm diameter and 5 cm long. Electrophoresis was for 30 min at 10 V/cm, followed by 3 hr and 20 min at 20 V/cm, 13 mA/gel. The buffer temperature was 6°; migration is toward the anode, from left to right.

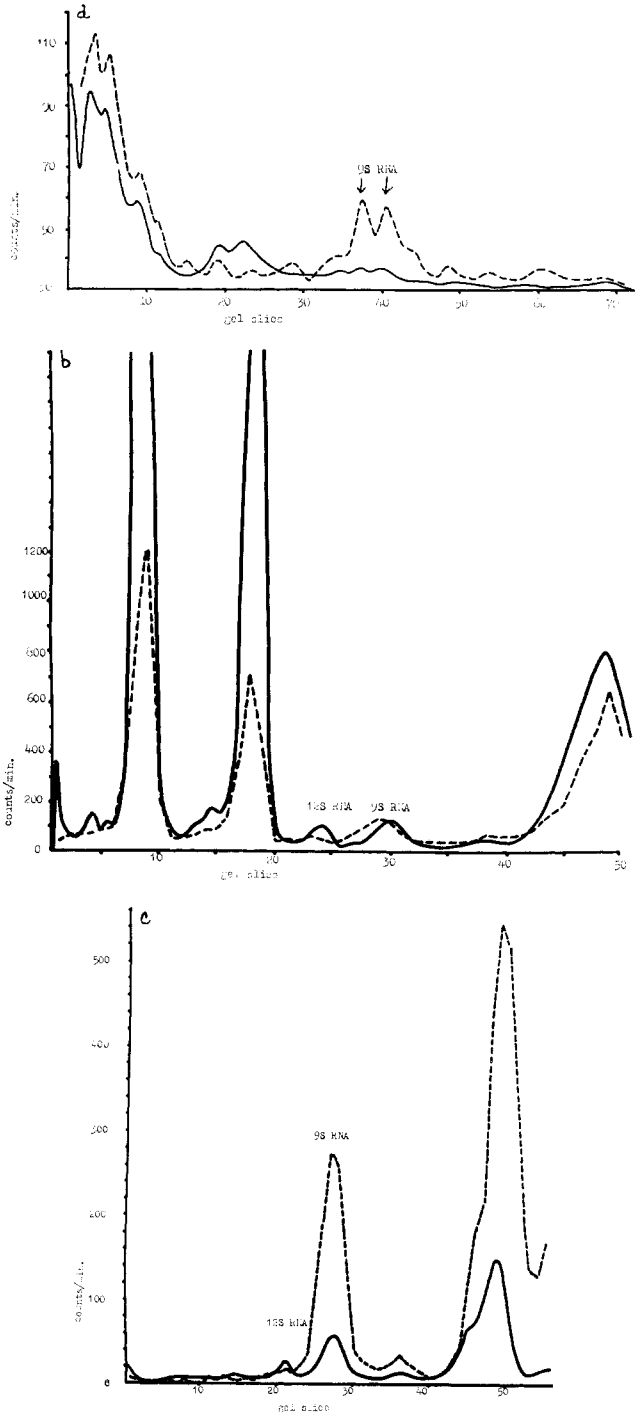

FIGURE 7: (a) 6% polyacrylamide gel electrophoresis of mouse reticulocyte ^{32}P-labeled polysomal RNA (50 μg). Electrophoretic conditions: 30 min at 6 V/cm, 3 mA/gel; 4.5 hr at 12 V/cm, 5 mA/gel. Buffer temperature was 4°. (b) 2.6% polyacrylamide gel electrophoresis of ^{32}P-labeled mouse reticulocyte polysomal RNA (50 μg). Electrophoretic conditions: 25 min at 4 V/cm, 1.5 mA/gel; 65 min at 10 V/cm, 4 mA/gel. Buffer temperature was 4°. (c) 2.6% polyacrylamide gel electrophoresis of ^{32}P-labeled mouse reticulocyte polysomal RNA cut from between the 18S and 4S RNA peaks of a sucrose gradient similar to that in Figure 2b. Electrophoretic conditions: 25 min at 4 V/cm, 1.5 mA/gel; 65 min at 10 V/cm, 4 mA/gel. Buffer temperature was 4°.

scribed above using the zonal rotor; 5S RNA was prepared by the method of Reynier *et al.* (1967). The purity of the samples was confirmed by analytical polyacrylamide gel electrophoresis. In all cases the RNA showed considerable hyperchromicity from 18% for 9S RNA to 23% for ribosomal components. The melting profiles, although considerably broader than for DNA, show evidence of secondary structure. Most of the secondary structure is reestablished upon cooling, apart from the 12S RNA, which shows no reannealing on cooling.

From the hyperchromicity it is possible to estimate the amount of base pairing in an RNA molecule (Boedtker, 1967; Cantor, 1968). Using the estimates given by Boedtker (1967), it is calculated that 9S RNA contains approximately 50% base-paired sequences, as compared to approximately 65% for 5S and 18S RNA. Our results for the ribosomal components, which were included as an internal control on our technique, are in agreement with published values (Cantor, 1968; Hartman and Thomas, 1970).

Base Composition. The base composition of the mouse globin mRNA is given in Table I, with the composition of rabbit globin mRNA (G. Marbaix, 1971, personal communication) given for comparison.

TABLE I: Base Composition of Globin mRNA (9S RNA) for Mouse and Rabbit.

	A (%)	G (%)	C (%)	U (%)
Mouse	27.4	24.1	26.6	21.9
Rabbit	28.8	25.5	26.8	19.0

Discussion

We have demonstrated that the isolation of mouse globin mRNA can be carried out reproducibly on a milligram scale, and have found that under our conditions reproducible yields and purity were obtained either using RNA isolated directly from the postmitochondrial supernatant or from the 14S mRNP obtained after EDTA treatment, under conditions stringently excluding ribonucleases. The two methods gave similar yields and purities of mRNA.

Mouse reticulocytes have proved to be as suitable as rabbit reticulocytes as starting material. In both cases one obtains two major RNA components, called here 9 and 12 S, between the 5S and 18S rRNAs. Significant amounts of other RNA components were only rarely seen in the messenger region in the mouse system.

Nonspecific fragments of RNA arising from ribonuclease action have been reported to occur between the 18S and 5S rRNA components (Cox and Kanagalingam, 1967; Tencheva and Hadjiolov, 1969). However, as reported by these authors, the first and major product of ribonuclease action on rRNA after isolation is a component sedimenting at approximately 15–16 S, and appearing very close to the 18S component on polyacrylamide gel electrophoresis at 2.5% concentration. We therefore regard it as very unlikely that the 9S or 12S components represent breakdown products of rRNA caused by the action of ribonuclease after the disruption of the cells. Competitive hybridization is, of course, one way to exclude the possibility that the minor RNA components are breakdown products of rRNA, and preliminary results indicate that there is only a very small amount of competition between 9S RNA and combined 18S and 28S rRNA (Williamson *et al.*, 1970).

The existence of a second RNA component, sedimenting at

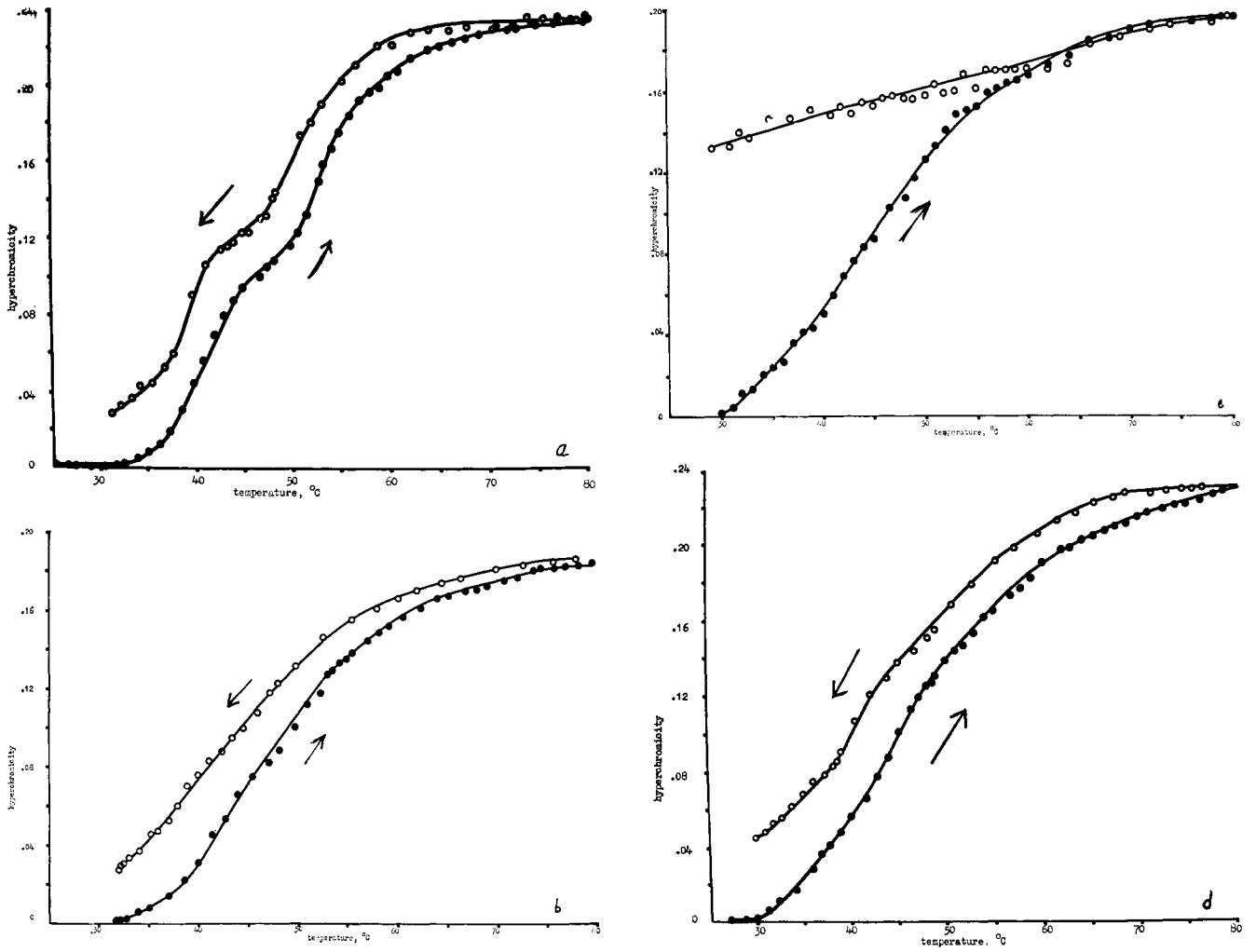

FIGURE 8: Melting and reannealing curves for purified 5S (a), 9S (b), 12S (c), and 18S (d) RNAs in 0.015 M NaCl–0.0015 M sodium citrate. Hyperchromicity is expressed as the ratio of the optical density at the indicated temperature to the optical density at 30°.

12 S, has been previously mentioned for rabbit reticulocytes by several groups (Laycock and Hunt, 1969; Labrie, 1969; Williamson *et al.*, 1969). This RNA component does not stimulate amino acid incorporation into globin-like peptides in an *E. coli* system (Laycock and Hunt, 1969), it does not disappear when polysomes are gently degraded to monosomes (Williamson *et al.*, 1969), and its oligonucleotide fingerprint after T-1 ribonuclease digestion differs from that of 9S RNA (Labrie, 1969). We have confirmed that the 12S RNA in mouse as in rabbit has a labeling pattern *in vivo* distinct from both 9S and rRNAs.

It is evident that further investigation of this component is necessary. A component of mol wt 373,000 at a total amount of less than 1% could not be present at a level of one molecule per ribosome. The 12S component may be an intermediate in rRNA breakdown *in vivo*; the data suggest that 12S RNA contains hidden chain breaks which do not cause the structure of the molecule to be disrupted until after melting. The labeling pattern is also consistent with this explanation.

The nature of the protein component associated with the mRNA in the RNP peak sedimenting at approximately 14S is still unclear. There are two major protein components in the 14S complex, which do not appear to be part of the normal spectrum of ribosomal structural proteins, but one of which migrates similarly (but not identically) to the protein associated with mRNA in the nucleus (Olsnes, 1970; Sarasin, 1969; E. M. Lukanidin, G. P. Georgiev, and R. Williamson, submitted for publication, 1971; Lebleu *et al.*, 1971). The mRNP component occurs as a homogeneous peak in cells such as rabbit and mouse reticulocytes, but as a diffuse and ill-characterized band sedimenting from approximately 10 S up to 40 S in liver. Heywood (1970) and Mathews (1970) have reported messenger-specific factors associated with reticulocyte polysomes, and some of the proteins which remain associated with mRNA on EDTA-mediated polysome dissociation may fulfil this function.

The molecular weight of the mRNA can be calculated either from its sedimentation coefficient giving a value of 170,000 ± 8000, or from its position on the polyacrylamide gel electrophorograms, giving a value of 225,000 ± 14,000. The discrepancy between these two values is too great to be due to experimental error alone, and it is probable that mRNA shows anomalous migration in polyacrylamide gel electrophoresis as compared with rRNA. Polyacrylamide gel electrophoresis migration is very dependent upon secondary structure, as can be shown by comparing migration of single- and double-stranded DNA (Williamson, 1970). The molecular weight calculated by Boedtker's method (1968) from the sedimentation behavior is independent of secondary structure and therefore far more likely to be accurate. It is still too large to be

solely "message" for the globin chain. The difference between the experimentally determined molecular weight of 170,000 and the molecular weight calculated from the base composition and the average coding polynucleotide length (148,000) corresponds to approximately 65 nucleotides. We would suggest four possible functions for these sequences: (i) they represent a rather complex starting and/or termination signal. (ii) They are concerned not with control of protein synthesis, but with control of mRNA synthesis, perhaps being among the RNA sequences corresponding to DNA binding sites for repressors of the sort postulated by Georgiev (1969) or Britten and Davidson (1969). This would imply that a portion of the regulatory DNA sequence was transcribed and processed with the messenger sequence. (iii) They are base-paired, self-complementary regions at each end of the molecule, similar to that found in certain DNA phages, permitting the mRNA to loop round and form a circle. Most reticulocyte polysomes are found in this circular conformation (Mathias *et al.*, 1964) and were this the case, it might explain the longevity of hemoglobin mRNA. (iv) They may be a sequence of extra nucleotides involved in ticketing the lifetime of the mRNA (Sussman, 1970, Lim and Canellakis, 1970). Preliminary DNA–RNA hybridization results (Williamson *et al.*, 1970) indicate that the purified mRNA hybridizes to a high percentage of the DNA. Whether this is due to hybridization of the nonmessenger sequences rather than the sequences specifying the globin chain is being investigated. Results of Kedes and Birnstiel (1971) also indicate high DNA–RNA hybridization values for the putative mRNA for histone.

The melting data indicate a surprisingly high amount of secondary structure for the mRNA; it is not at first sight evident that a coding sequence should show any base pairing at all. It has been demonstrated that both the coding and noncoding portions of R17 and Qβ viral RNAs have considerable double-stranded character, and in the case of the coding portion, three loops of length 20 base pairs have been isolated and sequenced (Steitz, 1969; Adams and Cory, 1970; F. Sanger, personal communication, 1971). Therefore it is quite possible that both the coding and the noncoding portions of a mRNA will contain double-stranded regions. It is not known whether this is to protect the molecule from nuclease action, is concerned with viral packaging, or whether such regions would provide possible recognition sites for messenger-specific factors involved either in the transport of the messenger from the nucleus to the cytoplasm (Spirin, 1969) or in recognition by messenger-specific protein factors on the polysomes (Heywood, 1970) or in the postribosomal supernatant (Mathews, 1970).

The radioactive labeling pattern and molecular weight difference between the two 9S components obtained on 6% gel electrophoresis are consistent with their being the mRNAs for the α- and β-globin chains. The great majority of these studies were carried out using reticulocytes from Porton mice having only a single major hemoglobin component on gel electrophoresis, and thus presumably having only two globin moieties (Cole *et al.*, 1968; Hunter, 1968; Craig and Russell, 1963). However, mouse hemoglobin can contain multiple polymerized components and the effect of phenylhydrazine in inducing resumption of foetal hemoglobin synthesis in mouse systems is unknown; therefore it is possible that other globin species are present in small amounts.

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References

Adams, J. M., and Cory, S. (1970), *Nature (London) 227*, 570.
Boedtker, H. (1967), *Biochemistry 6*, 2718.
Boedtker, H. (1968), *J. Mol. Biol. 35*, 61.
Britten, R. J., and Davidson, E. H. (1969), *Science 165*, 349.
Cantor, C. R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 478.
Chantrenne, H., Burny, A., and Marbaix, G. (1967), *Progr. Nucl. Acid Res. Mol. Biol. 7*, 173.
Cole, R. J., Hunter, J., and Paul, J. (1968), *Brit. J. Haematol. 14*, 477.
Cox, R. A., and Kanagalingam, K. (1967), *Biochem. J. 103*, 431.
Craig, M. L., and Russell, E. S. (1963), *Science 142*, 398.
Evans, M. J., and Lingrel, J. B. (1969a), *Biochemistry 8*, 829.
Evans, M. J., and Lingrel, J. B. (1969b), *Biochemistry 8*, 3000.
Fedorcsak, I., Natarajan, A. T., and Ehrenberg, L. (1969), *Eur. J. Biochem. 10*, 450.
Forget, B. G., and Weissman, S. M. (1969), *J. Biol. Chem. 244*, 3148.
Georgiev, G. P. (1969), *Annu. Rev. Genet. 3*, 155.
Hartman, K. A., and Thomas, G. J., Jr. (1970), *Science 170*, 740.
Heywood, S. M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1782.
Holder, J. W., and Lingrel, J. B. (1970), *Biochim. Biophys. Acta 204*, 210.
Huez, G., Burny, A., Marbaix, G., and Schram, E. (1967), *Eur. J. Biochem. 1*, 179.
Hunt, J. (1970), *Nature (London) 226*, 950.
Hunter, J. (1968), Ph.D. Thesis, University of Glasgow, Scotland.
Kedes, L. H., and Birnstiel, M. L. (1971), *Nature New Biol. 230*, 165.
Kirby, K. S. (1965), *Biochem. J. 96*, 266.
Labrie, F. (1969), *Nature (London) 221*, 1217.
Lamfrom, M., and Knopf, P. M. (1964), *J. Mol. Biol. 9*, 558.
Lanyon, G., Williamson, R., and Paul, J. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 279.
Laycock, D. G., and Hunt, J. A. (1969), *Nature (London) 221*, 1118.
Lebleu, B., Marbaix, G., Huez, G., Temmerman, J., Burny, A., and Chantrenne, H. (1971), *Eur. J. Biochem. 19*, 264.
Lewicki, P. P., and Sinskey, A. J. (1970), *Anal. Biochem. 33*, 273.
Lim, L., and Canellakis, E. S. (1970), *Nature (London) 227*, 710.
Lingrel, J. B. (1971), *in* Methods in Protein Biosynthesis, Vol. 2, Laskin, A. E., and Last, J. A., Ed., New York, N. Y., Marcel Dekker (in press).
Lockard, R. E., and Lingrel, J. B. (1969), *Biochem. Biophys. Res. Commun. 37*, 204.
Loening, U. E. (1967), *Biochem. J. 102*, 251.
Loening, U. E. (1969), *Biochem. J. 113*, 131.
Marbaix, G., and Burny, A. (1964a), *Arch. Intern. Physiol. Biochem. 72*, 689.
Marbaix, G., and Burny, A. (1964b), *Biochem. Biophys. Res. Commun. 16*, 522.
Marbaix, G., Burny, A., Huez, G., and Chantrenne, H. (1966), *Biochim. Biophys. Acta 114*, 404.
Mathews, M. B. (1970), *Nature (London) 228*, 661.

Mathias, A. P., Williamson, R., Huxley, H. E., and Page, S. (1964), *J. Mol. Biol. 9*, 154.
Olsnes, S. (1970), *Eur. J. Biochem. 15*, 464.
Paul, J. (1965), *in* Cell and Tissue Culture, 3rd ed, London, E. & S. Livingstone, p 83.
Pragnell, I. B., and Arnstein, H. R. V. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9*, 331.
Reynier, M., Aubert, M., and Monier, R. (1967), *Bull. Soc. Chim. Biol. 49*, 1205.
Sarasin, A. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 327.
Schapira, G., Dreyfus, J. C., and Maleknia, N. (1968), *Biochem. Biophys. Res. Commun. 32*, 558.
Spirin, A. S. (1969), *Eur. J. Biochem. 10*, 20.
Steitz, J. A. (1969), *Nature (London) 224*, 957.
Sussman, M. (1970), *Nature (London) 225*, 1245.
Tencheva, Z. S., and Hadjiolov, A. A. (1969), *J. Neurochem. 16*, 769.
Williamson, R. (1970), *J. Mol. Biol. 51*, 157.
Williamson, R., Lanyon, G., and Paul, J. (1969), *Nature (London) 223*, 628.
Williamson, R., and Mathias, A. P. (1963), *Biochem. J. 89*, 13P.
Williamson, R., Morrison, M., and Paul, J. (1970), *Biochem. Biophys. Res. Commun. 40*, 740.

A Cytoplasmic Protein from *Neurospora crassa* Resembling Membrane Proteins*

Charles F. Shannon and John M. Hill†

ABSTRACT: A protein component, in association with nonprotein components, has been recovered from the cytoplasm of *Neurospora crassa* and has been partially characterized. The protein component was separated from the nonprotein components by gel filtration. This protein component represented over 50% of the total soluble protein during early logarithmic phase growth of the organism. The isolated protein component appeared to be a single protein species. It electrofocused as a single band with an isoelectric point of pH 5.3. The isolated protein migrated as a single band during analytical electrophoresis at pH 7.0 in polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Multiple bands were observed in the same gel system following analytical electrophoresis of the protein when it was associated with the nonprotein components. However, preparative electrophoretic separation of these bands and subsequent amino acid analyses indicated that these bands were very similar in amino acid composition, thus providing strong evidence that only a single protein was present. Gel filtration also indicated a single protein species. Molecular weight analyses by gel electrophoresis, sedimentation equilibrium centrifugation, gel filtration, and amino acid composition were consistent with a monomer molecular weight of 15,000 with a stable aggregated species of molecular weight 45,000. The amino acid composition of the protein indicated that the protein was extremely hydrophobic. This would explain the aggregation phenomena encountered in molecular weight analyses. The amino acid composition, solubility properties, and electrofocusing pattern shows this protein is very similar to proteins isolated from cellular and organelle membranes.

A protein component was obtained from the cytoplasm of *Neurospora crassa* by Kuehn *et al.* (1969) using a mild isolation procedure. This protein component appeared to be similar to protein components isolated from cellular and organelle membranes. However, the protein components of membranes have proved to be so numerous and complex as to preclude a unified concept of membrane structure. Schnaitman (1969) has described inner mitochondrial membranes containing 23 proteins and endoplasmic reticulum with 15 proteins. Neville (1967), as well as Kiehn and Holland (1968, 1970a,b), found a complex array of proteins in mammalian cell membranes. The plasma membranes of several strains of Mycoplasma contain over 20 proteins (Rottem and Razin, 1967). The protein complexity of erythrocyte membranes has been described by several laboratories (Schneiderman, 1965; Lenard and Singer, 1966; Carraway and Kobylka, 1970). It is possible that many of these components observed in cell and organelle membranes were not different proteins, but were oligomers of the same protein.

Only relatively specialized membranes have shown an apparent simplicity in protein structure, and these appear to be the exception rather than the rule. The gas vacuole membranes from blue-green algae are assembled with a single protein of molecular weight of 14,000 and without the lipids usually found in biological membranes (Jones and Jost, 1970). Yu and Masoro (1970) claim that sarcotubular membranes from rat muscle cells are composed principally of a single protein of molecular weight of 6000–10,000.

The apparent complexity of membrane structure has been magnified by the controversy concerning homogeneity and molecular weights of protein fractions isolated from membranes of various cells and organelles. Criddle *et al.* (1962) and Richardson *et al.* (1964) solubilized organelle membranes from bovine cells using detergents and surface activating agents, and fractionated the protein components. A major protein fraction was obtained which was labeled "structural

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† To whom to address correspondence.