Skeletal Muscle Ribosome Subunits and Peptidyl Transfer Ribonucleic Acid*

William S. Stirewalt,† James J. Castles,‡ and Ira G. Wool§

ABSTRACT: When muscle ribosomes containing radioactive nascent chains were dissociated, most of the radioactivity cosedimented with the undissociated 75S monomers; however, a shoulder of radioactivity on the trailing edge of the monomer peak was coincident with the 60S ribosome subunit. There was also radioactivity at the top of the gradient, but no discrete peak with the 40S subunit. When fractions of each ribosome component were reanalyzed, radioactivity cosedimented with the 75S monomer and the 60S subunit but not the 40S subunit. The radioactivity associated with the 60S subunit was precipitated with hot trichloroacetic acid indicating it was in peptide chains rather than phenylalanyl-tRNA or free phenylalanine; moreover, the sedimentation coefficient of the radioactive material was changed from 3.6 to 2 by treatment with pancreatic ribonuclease suggesting the peptide is attached to tRNA. Puromycin released nascent peptide chains from 75S monomers and from the 60S subunit. We conclude that when ribosomes containing nascent chains are dissociated a portion of the peptidyl-tRNA remains bound to the 60S subunit.

When muscle ribosome subunits were prepared from a mixture of monomers and polysomes, a fraction of the ribosomes (75S monomers) remained associated (Martin and Wool, 1968; Martin *et al.*, 1969). The percentage of 75S monomers was the same as the proportion of active ribosomes determined by the formation of peptidylpuromycin (Wool and Kurihara, 1967); it appeared then that the subunits were derived from ribosomes not in the process of translating cellular mRNA (Martin *et al.*, 1969). However, the fraction of ribosomes resistant to dissociation was only one-half the percentage of ribosomes sedimenting as polysomes (Stirewalt *et al.*, 1967). Thus it was still possible that some of the ribosome subunits were derived from active ribosomes.

The source of mammalian ribosome subunits may be important, for some properties of bacterial ribosome subunits depend upon the functional state of the ribosomes from which they are derived. For example, free ribosome subunits present in extracts of bacteria associate to form monomers only when initiation of protein synthesis can occur (Schlessinger *et al.*, 1967); whereas particles derived from bacterial polysomes form monomers in less stringent circumstances because of the presence of peptidyl-tRNA on 50S ribosome subunits derived from polysomes (Schlessinger *et al.*, 1967; Gilbert, 1963). Therefore, we have attempted to determine whether muscle ribosome subunits are derived in part from ribosomes actively translating mRNA. We have done this by following the distribution of nascent polypeptide chains upon dissociation of muscle ribosomes.

Materials and Methods

Materials. The materials used for the preparation of muscle ribosomes and for the *in vitro* incorporation of radioactive amino acids into protein have been described (Wool and Cavicchi, 1967). [^{3}H]Phenylalanine (5 Ci/mmole) was purchased from New England Nuclear Corp.

Preparation of Skeletal Muscle Ribosomes. Ribosomes were prepared by a modification of the method of Florini and Breuer (1966). Abdominal wall, thigh, and gastrocnemius muscles from male Sprague–Dawley rats (150–180 g) were pooled and homogenized at 0° in two volumes of buffer containing 50 mM Tris-HCl (pH 7.6), 12.5 mM $MgCl_2$, 250 mM KCl, and 0.25 M sucrose, for 30 sec at a setting of 80 in a VirTis 45 homogenizer. The homogenate was centrifuged at 13,000*g* for 15 min; the supernatant was collected and the pellet resuspended in one volume of the same buffer and recentrifuged. The combined supernatants were filtered through cheesecloth and centrifuged for 2 hr at 78,000*g*. Ribosomes were isolated from the 78,000*g* pellet in a low potassium (80 mM) medium as described before (Martin *et al.*, 1969); ribosome pellets were stored at −20°.

Before each experiment, ribosome pellets were suspended in medium A (50 mM Tris-HCl (pH 7.3), 12.5 mM $MgCl_2$, 80 mM KCl) and the suspension clarified by centrifugation for 5 min at 3000*g*.

The concentration of ribosomes in suspension was estimated from the absorbancy at 260 mμ; 1 OD_{260} unit is equivalent to 90 μg of ribosomes.

Preparation of Ribosomes Bearing Radioactive Nascent Peptides. Ribosomes (20 mg) were incubated at 37° for 30 min in 10 ml of a medium containing 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 12.5 mM $MgCl_2$, 10 mM β-mercaptoenthanol, 5 mM ATP, 0.05 mM GTP, 1 mM phosphoenolpyruvate, 100 μg of pyruvate kinase, 6 mg of muscle supernatant protein, and 10 mg of tRNA acylated with [^{3}H]phenylalanine and 19 other nonradioactive amino acids (Wool and Cavicchi, 1967). After incubation, the reaction mixture was cooled at 4° and 5-ml portions were layered on 7 ml of medium A containing 0.5 M sucrose and centrifuged at 50,000 rpm in a Spinco Ti50 rotor for 5 hr. The ribosome pellets were stored at −20°.

Preparation of Muscle Ribosome Subunits. Ribosomes were dissociated in high KCl buffer (50 mM Tris-HCl (pH 7.3),

* From the Departments of Physiology, Biochemistry and Medicine, University of Chicago, Chicago, Illinois 60637. *Received October 19, 1970.* The expenses of the research were met by grants from the National Institutes of Health (AM-04248 and AM-13807) and the John A. Hartford Foundation.

† Present address: Department of Physiology, Rutgers Medical School, New Brunswick, N. J.

‡ Recipient of a U. S. Public Health Service Research Career Development award.

§ To whom to address correspondence.

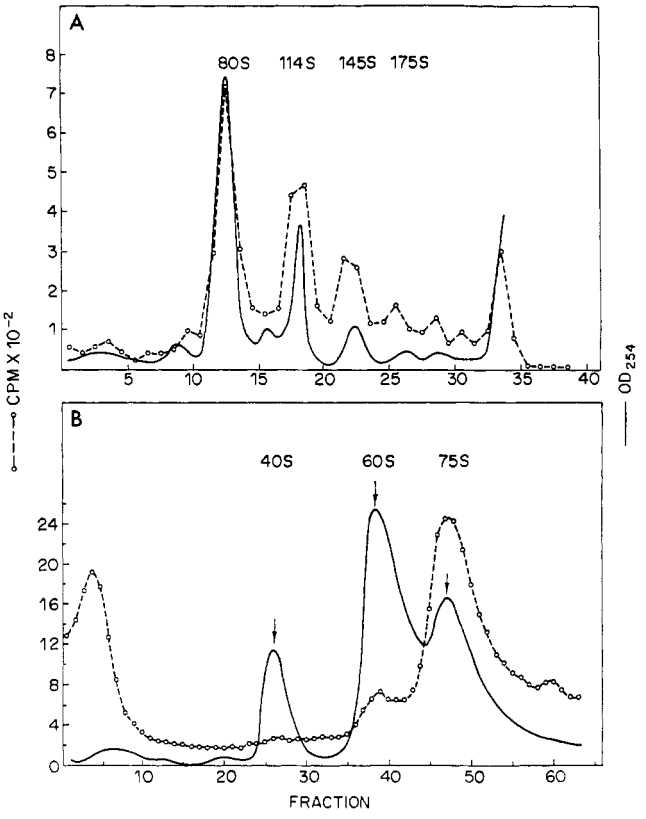

FIGURE 1: Distribution of radioactive nascent peptide chains on skeletal muscle ribosomes and ribosome subunits. Skeletal muscle ribosomes which had been incubated with [^{3}H]Phe-tRNA to label nascent peptide chains were suspended in medium A and (A) 0.6 OD_{260} unit (6900 cpm) was analyzed on a 10–30% sucrose gradient in medium A by centrifugation at 28° in a Spinco SW65 rotor at 60,000 rpm for 60 min; fractions (0.15 ml) were collected and the radioactivity was determined. The KCl concentration of a portion of the original suspension of ribosomes was adjusted to 880 mM and (B) 54 OD_{260} units (610,000 cpm) was analyzed on a 10–30% sucrose gradient in high KCl buffer by centrifugation at 28° in a Spinco SW27 rotor at 27,000 rpm for 4 hr; fractions (0.5 ml) were collected and the radioactivity of 0.05-ml samples was determined.

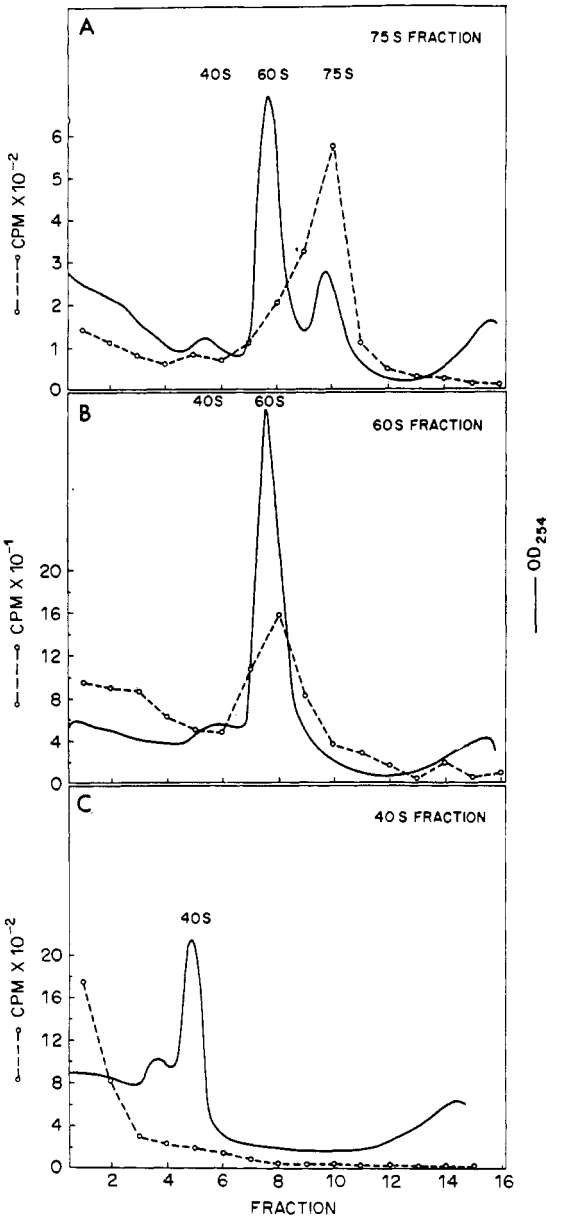

FIGURE 2: Determination of the association of nascent peptide chains with ribosomes and ribosome subunits. Ribosome fractions from the preparative sucrose gradient (indicated by the arrows in Figure 1B) were diluted with high KCl buffer to lower the sucrose concentration. A sample (0.2 ml) was layered on a 10–30% sucrose gradients in the high KCl buffer. Centrifugation was at 28° for 50 min at 60,000 rpm in a SW65 rotor: (A) 75S fraction; (B) 60S fraction; (C) 40S fraction. Samples (0.3 ml) were collected and the radioactivity was determined.

12.5 mM $MgCl_2$, and 880 mM KCl) and the subunits isolated as described before (Martin and Wool, 1968; Martin *et al.*, 1969).

Analysis of Ribosome Particles on Sucrose Gradients. Sucrose gradients were prepared and analyzed as described before (Stirewalt *et al.*, 1967). Gradients containing sodium dodecyl sulfate were prepared according to Gilbert (1963) except that they were linear instead of exponential; the gradients contained 100 mM NaCl, 5 mM Tris-HCl (pH 7.3), and 0.5% sodium dodecyl sulfate in 5–20% sucrose. Fractions from the gradients were collected in glass vials and the volume adjusted to 1 ml with water; 10 ml of Triton–toluene scintillation ffuid (Patterson and Greene, 1965) was added and the radioactivity was measured in a Packard Tri-Carb spectrometer; the counting efficiency was 18%.

Results

Distribution of Nascent Polypeptide Chains on Muscle Ribosomes and Ribosome Subunits. Skeletal muscle ribosomes containing nascent chains labeled *in vitro* with [^{3}H]phenylalanine were incubated at 37° for 5 min in a buffered medium containing either 80 mM or 880 mM KCl, then analyzed in sucrose gradients of the same ionic composition (Figure 1). In 80 mM KCl almost all the ribosomes and radioactivity sedimented with a coefficient of 80 or greater (Figure 1A). When preparations of ribosomes were incubated in 880 mM KCl they formed 40S and 60S subunits and 75S particles (Figure 1B). Approximately 55% of the radioactivity cosedimented with the undissociated 75S monomers. A shoulder of radioactivity on the trailing edge of the monomer peak was coincident with the 60S ribosome subunit. The remaining radioactivity was found near the top of the gradient; no discrete peak accompanied the 40S ribosome subunit. All of the radioactivity present in the fractions collected from

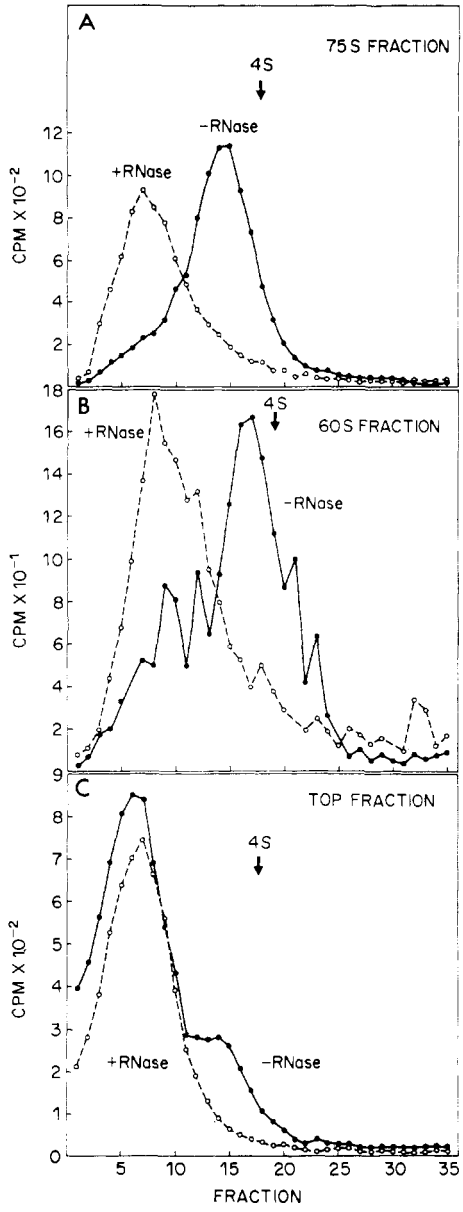

FIGURE 3: Effect of RNase treatment on sedimentation of nascent peptide chains. Pancreatic RNase (10 μg/ml) was added to one-half of (A) the 75S fraction; (B) the 60S fraction; and (C) a fraction collected from the top of a preparative gradient—the fractions were from a gradient similar to that shown in Figure 1B. The hydrolyzed and control samples were dialyzed for 6 hr against 1 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.3) at room temperature. Sodium dodecyl sulfate was added (the final concentration was 0.5%) and the samples were analyzed on sodium dodecyl sulfate gradients with 0.5 mg of tRNA as a marker. Centrifugation was at 20° in a Spinco SW27 rotor at 23,000 rpm for 36 hr. The position of the optical density peak due to tRNA is indicated by an arrow. Fractions (0.9 ml) were collected and the radioactivity was determined.

the gradient (Figure 1B) was precipitated in hot 10% trichloroacetic acid indicating that the radioactivity was in peptide chains—not in [^{3}H]Phe-tRNA or [^{3}H] phenylalanine.

To distinguish between nascent peptide chains specifically attached to ribosome particles and chains that had been detached during centrifugation, a single fraction from the absorbancy peak of each of the three ribosome components (see arrows Figure 1B) was rerun on analytical gradients (Figure 2). Radioactivity again cosedimented with the 60S subunit and the 75S monomer but not with the 40S subunit.

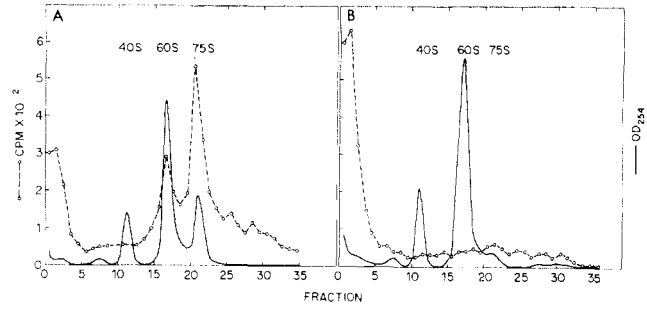

FIGURE 4: The release of nascent peptide chains from skeletal muscle ribosomes by puromycin. Skeletal muscle ribosomes with labeled nascent peptide chains were suspended in high KCl buffer as in Figure 1B and were incubated for 15 min at 37° (A) without or (B) with puromycin (10^{-5} M). Ribosomes (0.7 OD_{260} unit and 10,500 cpm) were layered on 10–30% sucrose gradients in high KCl buffer. Centrifugation was at 28° in a Spinco SW65 rotor at 60,000 rpm for 60 min. Fractions (0.3 ml) were collected and the radioactivity was determined.

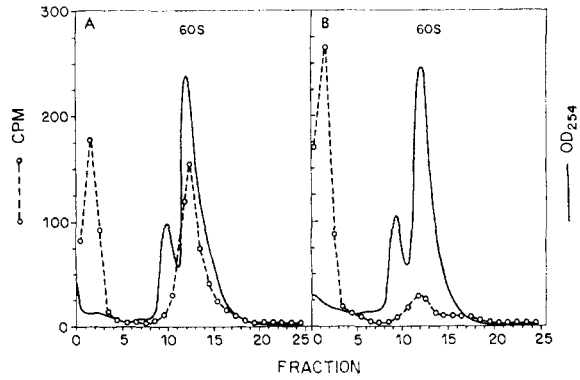

FIGURE 5: The release of nascent peptide chains from 60S subunits by puromycin. Skeletal muscle 60S subunits were isolated from a gradient similar to that shown in Figure 1B and incubated for 15 min at 37° (A) without or (B) with puromycin (10^{-5} M). The samples were layered on 10–30% sucrose gradients in high KCl buffer. Centrifugation was as in Figure 4.

Characteristics of the Nascent Polypeptide Chain Associated with Ribosomal Particles. Nascent polypeptide chains associated with 70S monomers and 50S subunits of *Escherichia coli* are attached to tRNA (Gilbert, 1963). We sought to determine if nascent peptides also remained attached to tRNA when muscle ribosomes were dissociated. Samples from the 75S and 60S fractions and from the top of a preparative gradient were incubated in the presence or absence of pancreatic RNase. After incubation, sodium dodecyl sulfate was added, and the samples were analyzed on sucrose gradients containing sodium dodecyl sulfate. In the absence of RNase, the radioactive polypeptide associated with the 75S particle had a sedimentation coefficient of approximately 3.6, slightly slower than a 4S tRNA marker (Figure 3A). (A similar difference in sedimentation rate between polyphenylalanyl-tRNA and tRNA has been reported; Traut and Monro, 1964.) After digestion with RNase, the sedimentation coefficient was 2 (Figure 3A). The change in the sedimentation coefficient from 3.6 to 2 after RNase treatment indicated that most of the nascent polypeptide associated with 75S ribosomes was attached to RNA. Similarly, 70% of the radioactivity isolated with the 60S subunit was 3.6 S (Figure 3B). This proportion corresponded closely with the per-

centage of radioactivity that remained associated with the 60S particle on recentrifugation (Figure 2B). After treatment with RNase, the radioactive nascent peptide isolated in association with 60S subunits had a sedimentation coefficient of 2. The experiments support the view that the nascent peptides associated with ribosomal particles were attached to tRNA.

Twenty per cent of the radioactivity from the top of the preparative gradient sedimented in a way similar to peptidyl-tRNA (3.6 S); the remainder appeared to be free peptide (2 S) (Figure 3C). The results suggest that some peptidyl-tRNA was released when ribosomes were dissociated to subunits. The free peptide may derive from hydrolysis of released peptidyl-tRNA or hydrolysis of the peptidyl-tRNA associated with ribosomal particles; in the latter instance, tRNA might remain associated with the ribosomal particles. Our experiments do not permit a decision regarding the origin of free peptides.

Release of Nascent Polypeptide Chains from Ribosome Particles by Puromycin. The addition of puromycin (10^{-5} M) to a ribosome suspension in 880 mM KCl buffer released all nascent peptide chains from the 75S monomer; there was a concommitant dissociation of 75S particles to 60 and 40S subunits—no nascent chains were associated with the 60S subunit (Figure 4). Nascent chains were also released from isolated 60S subunits by puromycin (Figure 5); the observation, incidently, confirms that peptidyl transferase is a component of the 60S subunit.

Discussion

Inactive ribosome subunits with altered conformation (47 S and 32 S) are formed when mammalian ribosomes are exposed to EDTA (Tashiro and Siekevitz, 1965) or to low concentrations of magnesium (Hamada *et al.*, 1968); in each case nascent peptide was retained on the putative large (47 S) subunit. However, nascent chains on the 47S particle were not released with puromycin (Hamada *et al.*, 1968) and the separate subunits could not be reassociated to form active ribosome monomers.

When active subunits are prepared by dissociation of skeletal muscle ribosomes in 880 mM KCl some of the nascent peptide chains are released and some remain attached to 75S monomers and 60S subunits. The nascent peptide chains associated with ribosome particles are attached to tRNA. When ribosomes are incubated in 880 mM KCl with puromycin nascent chains are released and 75S monomers completely dissociate to 40S and 60S subunits. It is likely that peptidyl-tRNA is a factor in stabilizing ribosome monomers for most ribosomes with nascent chains do not dissociate, whereas monomers lacking peptidyl-tRNA dissociate completely; nonetheless not all ribosomes bearing nascent chains are resistant to dissociation. For that reason the fraction of resistant monomers cannot be equated with the percentage of particles active in protein synthesis as has been done (Martin *et al.*, 1969; Martin and Hartwell, 1970). We do not know what distinguishes the fraction of ribosomes with nascent chains that do dissociate. It occurred to us that the determining factor might be the length of the peptide chain—longer chains conferring greater resistance to dissociation. However, the mean size (determined by filtration on Sephadex G-50) of nascent chains associated with 75S ribosomes were actually somewhat less than that of peptides on the 60S subunit (results not shown).

When ribosomes treated with puromycin were dissociated, no nascent peptide remained bound to the 60S subunit. Thus peptidyl-tRNA must have been bound to the same site on the ribosome monomer and the 60S subunit. In the two site model of translocation during protein synthesis (Watson, 1964), peptidyl-tRNA shuttles back and forth between an acceptor and donor site on the ribosome (Heintz *et al.*, 1966). Only peptidyl-tRNA in the donor site will transfer peptide to puromycin. All the peptidyl-tRNA that remained associated with ribosomes in the presence of 880 mM KCl must then have been in the donor site. It may be that peptidyl-tRNA was in the donor site because the ribosomes were contaminated with aminoacyltransferase II, or perhaps in 880 mM KCl peptidyl-tRNA in the acceptor site was released. Spirin (1969) has presented evidence which suggests that upon dissociation of *E. coli* ribosomes peptidyl-tRNA in the donor site is retained on the 50S subunit whereas peptidyl-tRNA in the acceptor site is released. If high concentrations of KCl release nascent chains from the acceptor site then the remaining chains should be in the donor site and all should be discharged with puromycin. We have found that in 880 mM KCl, 75% of the nascent chains are released by 10^{-5} M puromycin, whereas in 80 mM KCl only 40% are released (W. S. Stirewalt, unpublished results). The results are not decisive but they do support our interpretation, although we recognize it is also possible that high ionic strength merely changes the reactivity of the ribosome to puromycin (Skogerson and Moldave, 1968).

The release of nascent peptide from isolated muscle 60S subunits by puromycin confirms that the peptidyl-tRNA occupied the donor site. The peptidyl-tRNA bound to the 60S subunit can also donate peptide to aminoacyl-tRNA since in low concentrations of magnesium and in the absence of aminoacyltransferase II Phe-tRNA bound to ribosomes reassociated from subunits is found covalently bound to the carboxyl terminus of nascent peptide (Castles *et al.*, 1971). Apparently, at low magnesium concentrations only ribosomes with attached peptidyl-tRNA will bind Phe-tRNA and incorporate the amino acid into a peptide. The observation underscores the importance of peptidyl-tRNA in the apparent function of ribosomes and ribosome subunits.

Acknowledgment

We thank Mrs. A Fox and Mr. W. Saito for expert technical assistance.

References

Castles, J. J., Rolleston, F. S., and Wool, I. G. (1971), *J. Biol. Chem.* (in press).

Florini, J. R., and Breuer, C. B. (1966), *Biochemistry 5*, 1870.

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

Hamada, K., Yang, P., Heintz, R., and Schweet, R. (1968), *Arch. Biochem. Biophys. 125*, 598.

Heintz, R., McAllister, H., Arlinghaus, R., and Schweet, R. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 633.

Martin, T. E., and Hartwell, L. H. (1970), *J. Biol. Chem. 245*, 1504.

Martin, T. E., Rolleston, F. S., Low, R. B., and Wool, I. G. (1969), *J. Mol. Biol. 43*, 135.

Martin, T. E., and Wool, I. G. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 569.

Patterson, H. S., and Greene, R. C. (1965), *Anal. Chem. 37*, 854.

Schlessinger, D., Mangiarotti, G., and Apirion, D. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1782.

Skogerson, L., and Moldave, K. (1968), *Arch. Biochem. Biophys. 125*, 497.
Spirin, A. S. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 197.
Stirewalt, W. S., Wool, I. G., and Cavicchi, P. C. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1885.
Tashiro, Y., and Siekevitz, P. (1965), *J. Mol. Biol. 11*, 166.
Traut, R. R., and Monro, R. E. (1964), *J. Mol. Biol. 10*, 63.
Watson, J. D. (1964), *Bull. Soc. Chim. Biol. 46*, 1399.
Wool, I. G., and Cavicchi, P. C. (1967), *Biochemistry 6*, 1231.
Wool, I. G., and Kurihara, K. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2401.

Effects of Monazomycin on Ion Transport and Oxidative Phosphorylation in Liver Mitochondria*

Sergio Estrada-O and Carlos Gómez-Lojero

ABSTRACT: The antibiotic monazomycin stimulates an energy-linked uptake of Li^+, Na^+, K^+, Rb^+, or Cs^+ in exchange for protons in liver mitochondria. Simultaneous with the induced ion movements it also stimulates ATP hydrolysis and substrate oxidation in the absence of P_i acceptor. In the presence of K^+, the ATPase activity induced by monazomycin varies exponentially with the third power of the antibiotic concentration. The rate of ATP hydrolysis is also dependent on the concentration and radius of the alkali metal cations in the order: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$. In medium free of added alkali metal cations, the antibiotic stimulates ATP hydrolysis, succinate but not glutamate or α-ketoglutarate oxidation, and a rapid efflux of K^+ from the mitochondria. Substrate oxidation induced by the antibiotic in the absence of added cations, P_i, or acetate, is inhibited by low concentrations of Li^+, K^+, Rb^+, Cs^+, or Mg^{2+} but not Na^+. This latter cation actually opposes the effect of added Mg^{2+}. The inhibited state caused by alkali metal cation is overcome by phosphate, acetate, or nigericin. Inorganic phosphate stimulates oxygen uptake according to the sequence Cs^+, Rb^+, K^+, and Li^+. The inhibited state mediated by Mg^{2+} is reversed by P_i, ATP, or EDTA. Monazomycin appears to stimulate Mg^{2+} uptake in the presence of P_i. It does not affect the respiratory control induced by rutamycin in submitochondrial sonic particles. It is suggested that cations, protons, or anions influence the cooperative aggregation of monomers of the antibiotic, to form a conducting oligomer which translocates ions acting either in parallel or in series with cation carriers existing in the mitochondrial membrane.

Monazomycin is an antibiotic produced by *Streptomyces mashiuensis* (Akasaki *et al.*, 1963), which is known to contain 16 OH^- groups, 1 galactose, 1 dissociable amino group, and no amino acids (Mitscher *et al.*, 1967).

Lardy *et al.* (1967) and Ferguson and Lardy (1968) reported that the antibiotic induces ion transport, uncouples respiration and stimulates ATP hydrolysis in liver mitochondria. Recent observations by Mueller and Rudin (1969) indicate that monazomycin translocates K^+, Na^+, or H^+ across lipid bilayers. This compound was found to be capable of transporting ions as a function of its concentration and of applied electric potentials. From these results, Mueller and Rudin (1969) suggested that voltage or chemically controlled changes may cause the monomers of monazomycin to rearrange in conducting oligomers which translocate ions across lipid membranes by acting more like a channel than like a mobile carrier.

The present results in liver mitochondria indicate that the stimulation of ion movements as well as uncoupling of oxidative phosphorylation mediated by monazomycin are also subject to cooperative transitions. The possibility is proposed that such cooperative behavior could be explained by the assembly of several monomers of the antibiotic into one conducting oligomer controlled in its formation by cations, protons, anions, and membrane potential changes.

Materials and Methods

Mitochondria were prepared from livers of male rats weighing 150 g as described by Johnson and Lardy (1967). A continuous recording of oxygen consumption, light-scattering changes, and variations in the extramitochondrial concentration of alkali metal cations and protons was carried out by means of an apparatus designed, developed, and constructed by Chance, Mayer, and Pressman (Pressman, 1965, 1967; Graven *et al.*, 1966). ATPase activity was measured by the method of Lardy and Wellman (1953) and inorganic phosphate was determined by the method of Sumner (1944). The submitochondrial sonic particles were prepared and isolated according to Graven *et al.* (1967). Monazomycin was a kind gift from Dr. Henry A. Lardy. The antibiotic LLA491, which is very similar, if not identical with monazomycin (Mueller and Rudin, 1969), was a kind gift from Dr. E. L. Patterson.

Results

At a concentration of approximately 2.3×10^{-7} M, monazomycin causes an oscillatory uptake of Li^+, Na^+, K^+, Rb^+, or Cs^+ supported by ATP hydrolysis in liver mito-

* From the Departamento de Bioquímica, Facultad de Medicina, Ciudad Universitaria, México 20, D. F., México. *Received September 10, 1970.*