MITOCHONDRIAL AND CYTOPLASMIC RIBOSOMES AND THEIR ACTIVITY IN BLOOD AND CULTURE FORM *TRYPANOSOMA BRUCEI*

JAY HANAS, G. LINDEN, and K. STUART

From the Department of Biology, University of South Florida, Tampa, Florida 33620

ABSTRACT

Ribosomes of *Trypanosoma brucei*, a parasitic, flagellated protozoan (order Kinetoplastida), were identified on sucrose density gradients by their radioactively labeled nascent peptides. Ultraviolet absorption revealed only cytoplasmic ribosomes which served as internal sedimentation markers.

Synthesis on cytoplasmic ribosomes was completely inhibited by cycloheximide. In the presence of this antibiotic, nascent peptides were associated with ribosomes of lower sedimentation coefficient than the cytoplasmic ribosomes. Chloramphenicol blocked synthesis on these ribosomes which are probably the mitochondrial ribosomes.

These ribosomes differed from the cytoplasmic ribosomes in several ways. Their sedimentation coefficient was about 72S rather than 84S. The stability of the 72S ribosomes was less sensitive to pancreatic ribonuclease and low Mg^{++} concentrations, dissociating below 0.1 mM Mg^{++}. The 72S ribosomes were more sensitive to elevated KCl concentrations, dissociating above 0.25 M.

Protein synthetic activity associated with the 72S class of ribosomes was found in trypanosomes grown in rats. Under these conditions no cytochromes or fully active Krebs cycle is present in these cells and respiration is insensitive to cyanide.

Mitochondrial ribosomes have been observed in a wide spectrum of organisms ranging from fungi to mammals. These ribosomes differ from their cytoplasmic counterparts in antibiotic sensitivity, monomer stability at various ion concentrations, sedimentation coefficient, and the molecular weights of their RNA species (see reference no. 3 for review). The properties of mitochondrial ribosomes from different organisms differ greatly but two distinct classes seem to emerge. The first class has a sedimentation coefficient in the 70–80S range and contains RNA species of approximately 16S and 23S. This class of mitochondrial ribosomes is found in fungi and protozoa (2, 3, 6, 7, 17). The second class, frequently referred to as "miniribosomes," has a sedimentation coefficient in the 50–60S range and RNA species which are approximately 12S and 16S. "Miniribosomes" have been found in higher animals from shark to man (11–13, 19). It is noteworthy that the protein synthetic activity of mitochondrial ribosomes, regardless of source, is insensitive to cycloheximide (an inhibitor of cytoplasmic ribosomes) but is sensitive to inhibitors of bacterial protein synthesis

(3). *Crithidia*, a flagellated, parasitic protozoan (order Kinetoplastida), has been reported to contain 60S mitochondrial ribosomes, suggesting an exception to these two classes of mitochondrial ribosome (9).

We have examined the ribosomes of another kinetoplastid flagellate, *Trypanosoma brucei*, to characterize its mitochondrial ribosomes and determine if they were exceptional as well. In addition, these cells when grown in the vertebrate host (blood forms) lack cytochromes and a fully active Krebs cycle and are insensitive to cyanide (20). This situation is analogous to that of glucose-repressed or anaerobically grown yeast which have similar alterations of the respiratory system (16). Yeast grown under these conditions is capable of mitochondrial protein synthesis (15). We wished to determine if trypanosomes grown in rats were capable of mitochondrial protein synthesis.

MATERIALS AND METHODS

Growth and Isolation of Cells

T. brucei, London School of Hygiene reference no. 14/2/164 (stabilate established 1969), was used in this study (see reference 18). The cells were cultured aerobically in a liquid medium containing 3.33 g NaCl, 5.40 g Na_2HPO_4, 0.60 g NaH_2PO_4, 0.2 g KCl, 3.60 g nutrient broth (Difco Laboratories, Detroit, Mich.), 5 ml glycerol, 900 ml blood lysate (prepared by adding distilled water (9:1 vol/vol) to outdated human red blood cells and collecting the supernate after a 30-min, 13,500-rpm centrifugation in a Sorvall GSA rotor [DuPont Instruments, Sorvall Operations, Newtown, Conn.]) and 100 ml calf or beef serum. The pH was adjusted to 7.2–7.4 with 1 N NaOH. This medium was filtered through a glass fiber filter (Millipore GF, Millipore Corp., Bedford, Mass.) and filter sterilized (0.02 Selas candle filter, Selas Flotronics, Spring House, Pa.).[1] The cells were grown in 250-ml flasks containing 50 ml medium at 28°C and harvested at a cell density of about 2×10^7 cells/ml. The harvested cells were washed three times with ice-cold Locke's solution (9.0 g NaCl, 0.20 g $CaCl_2$, 0.30 g KCl, 0.20 g $NaHCO_3$) containing 0.5% glycerol (vol/vol) and 5% calf serum (vol/vol). The cells were kept at 4°C until use.

Blood form trypanosomes from a stabilate were maintained in rats for 9 mo by weekly intraperitoneal inoculation. The trypanosomes were separated from rat blood cells using sterile DEAE-cellulose (8), washed three times, and then were resuspended in Locke's solution containing 1% glucose and 5% calf serum (Locke's-serum).

[1] Adapted from A. Balber. Personal communication.

To determine the effects of various concentrations of cycloheximide, aliquots of cells in Locke's-serum with glucose or glycerol and containing different concentrations of cycloheximide were preincubated at 25°C for 10 min and replaced in ice. After the cells cooled, [^{3}H]leucine was added to 1 μCi/ml and the cells were incubated at 28°C in a shaking water bath for 25 min. After incubation, 1 ml 1% sodium dodecyl sulfate was added to each tube and then protein was precipitated by the addition of 2 ml 10% TCA. The tubes were heated at 90°C for 15 min and the resultant precipitates were collected on glass fiber filters, washed with 5% TCA, dried according to Davies and Cocking (5), and placed in 10 ml scintillation counting fluid (4 g 2,5-bis-2-(5-*t*-butyl-benzoxazolyl)-thiophene/liter toluene). Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer model 3375 (Packard Instrument Co., Downers Grove, Ill.).

For sucrose density gradient analysis, cells were suspended to a final density of about 1×10^9 cells/ml in the appropriate Locke's solution. Antibiotics, when used, were present in the suspension solution at the following final concentrations: cycloheximide, 20 mM for blood forms and 50 mM for culture forms, chloramphenicol 20 mM, puromycin 1 mM. The cells were incubated in 1 ml containing 25 μCi [^{3}H]leucine at 28°C with shaking for 5 min, after which time 3 ml ice-cold Locke's solution containing 1 mg unlabeled leucine was added. The cells were then pelleted and homogenized in 1 ml 2% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) 10 mM Tris, 50–500 mM KCl, 0–10 mM $MgCl_2$, pH 7.4 (TKM buffer) in an all glass homogenizer. RNase treatment was performed according to Rich et al. (14). In some experiments, 10 mM phosphate buffer (pH 7.4) was substituted for Tris buffer. The homogenates were centrifuged at 5,000 rpm for 5 min in a Sorvall SE-12 rotor and the supernates were layered over 28 ml linear 15–30% sucrose gradients prepared in the homogenization buffer without the Triton X-100. The gradients were centrifuged for 16 h at 17,500 rpm in a Spinco SW-25.1 rotor or 15,500 rpm in a Spinco SW-27.1 rotor (Beckman Instruments, Inc., Spinco-Div., Palo Alto, Calif.) The gradients were divided into 1-ml fractions using a Cornwall syringe (Becton, Dickinson & Co., Rutherford, N. J.) and after the A_{260} was determined 1 drop bovine serum albumin (10 mg/ml) was added to each fraction and 1 ml 10% TCA was added. The radioactivity in the precipitates was measured as described earlier.

Rat liver cytoplasmic ribosomal monomers were prepared according to Blobel and Potter (1) for use in calibrating the sucrose density gradients.

Materials

Cycloheximide and pancreatic RNase 1-A were purchased from Sigma Chemical Co., St. Louis, Mo. Radioactive [4,5-^{3}H]leucine (55 Ci/mmol, 0.5 mCi/ml) was a product of Schwartz Mann Div., Becton, Dickin-

son & Co., Orangeburg, N. Y., and chloramphenicol was supplied by Parke, Davis & Co., Detroit, Mich.

RESULTS

In order to establish the cycloheximide concentrations which would completely inhibit cytoplasmic protein synthesis, the effect on total protein synthesis by various concentrations of this antibiotic was measured. An approximately fivefold higher level of cycloheximide-resistant protein synthesis was found for cultured cells compared to cells grown in rats (Fig. 1). In addition, a higher concentration of antibiotic was necessary to reach a level above which a further increase would not result in further inhibition. Cycloheximide-resistant synthesis was about 5% in culture forms while it was about 1% in the cells grown in rats.

Characterization of the Ribosomes Associated with Cycloheximide-Resistant Amino Acid Incorporation

Sucrose density gradient analysis of cells grown in culture and labeled in the absence of inhibitors revealed a peak of absorbance near the bottom of the gradient (Fig. 2 A). Coincident with this peak was a peak of radioactivity. This peak of radioactivity was eliminated by puromycin treatment, indicating that it was due to nascent peptides (Fig. 2 B). Chloramphenicol treatment, although resulting in a decrease in the amount of radioactivity associated with the absorbance peak, did not eliminate this peak of radioactivity (Fig. 2 C). The reduction is probably due to the effects of chloramphenicol on respiration or amino acid uptake, as total synthesis is reduced as well. This peak (Fig. 2 A) of absorbance represents the trypanosome cytoplasmic ribosomes and the peak of radioactivity represents the associated nascent peptides. Upon co-sedimentation, with rat liver cytoplasmic monosomes, the peak of radioactivity is found to have a slightly greater sedimentation coefficient (approximately 84S, taking rat liver monosomes to be 80S [Fig. 2 D]). A second experiment gave identical results. Without exception, rat liver and trypanosome ribosomes examined on separate sucrose gradients differed by a minimum of 4S.

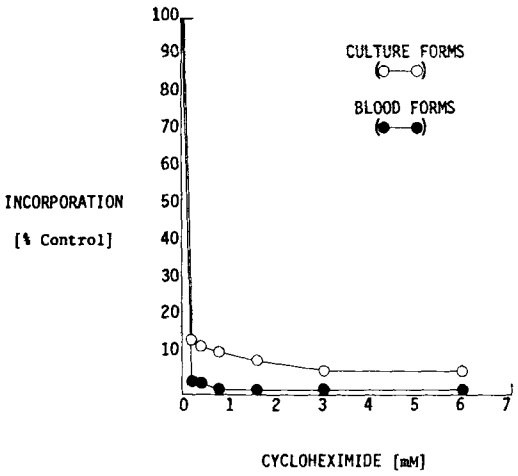

FIGURE 1 Effects of various concentrations of cycloheximide on total protein synthesis in blood and culture form cells. Trypanosomes were preincubated in cycloheximide for 10 min, then incubated in the presence of [^{3}H]leucine for 25 min at 28°C, and the amount of hot TCA-insoluble radioactivity was determined

Cycloheximide treatment resulted in almost complete loss of the radioactivity associated with the peak of absorbance, indicating essentially complete inhibition of synthesis on the cytoplasmic ribosomes (Fig. 3 A). In the presence of cycloheximide a peak of radioactivity is observed which has a sedimentation coefficient lower than that of the cytoplasmic ribosomes. The sedimentation coefficient of the material in this band was calculated to be 71.7S ± 1.8S, taking the cytoplasmic ribosome to be 84S. This peak of radioactivity was eliminated by puromycin treatment (Fig. 3 B), indicating that it also was probably due to nascent peptides on active ribosomes. Chloramphenicol inhibition of the cycloheximide-resistant production of radioactive nascent peptides by the 72S ribosomes was essentially complete (Fig. 3 C). This is in contrast to the partial (51%) inhibition of the cytoplasmic ribosomes by chloramphenicol (Fig. 2 C), a level of inhibition which would not have eliminated the 72S peak of radioactivity.

Comparison with the Cytoplasmic Ribosomes

The 72S ribosomes whose protein synthetic activity was insensitive to cycloheximide but sensitive to chloramphenicol differed from the cytoplasmic ribosomes in various ways. Homogenization and sedimentation in 0.1 mM Mg^{++} or with no Mg^{++} added resulted in complete dissociation of the cytoplasmic ribosomes but did not affect the 72S peak (Fig. 4 A). However, dialysis against buffer containing no magnesium or the addition of 1 mM EDTA (data not shown) resulted in the loss of absorbance due to the cytoplasmic ribosomes

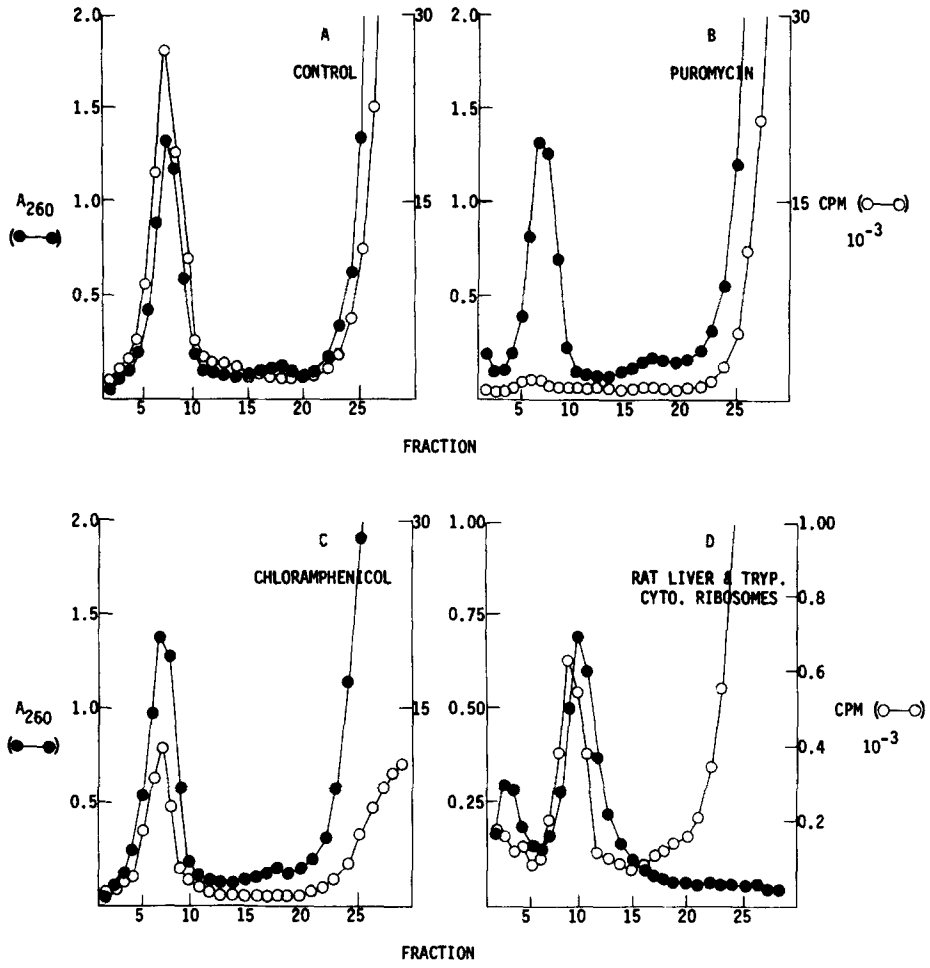

FIGURE 2 Sucrose density gradient analysis of cells labeled in the absence of cycloheximide. All sucrose gradients are presented with the top to the right. Culture form trypanosomes after suspension in Locke's-serum, glycerol, were incubated with [^{3}H]leucine for 5 min at 28°C in the absence of inhibitors (A), with puromycin (1 mM) present for the final 30 s of incubation (B), and after 10 min preincubation with chloramphenicol (20 mM) (C). Rat liver monosomes co-sedimented with samples from cells labeled in the absence of inhibitors (D). In Fig. 2 D the trypanosome sample alone would have shown an absorbance peak of about 0.025.

and the 72S peak of radioactivity, indicating that both types of ribosomes were degraded under these conditions. RNase treatment (1 μg/ml, 4°C) resulted in substantial degradation of the cytoplasmic ribosomes but did not affect the 72S peak of radioactivity (Fig. 4 B).

Although the 72S ribosomes were less sensitive to low magnesium concentrations than the cytoplasmic ribosomes, their stability was more sensitive to elevated KCl concentrations. A concentration of 0.5 M KCl resulted in dissociation of some of the cytoplasmic ribosomes as evidenced by the appearance of 64S and 48S peaks of absorbance under these conditions (Fig. 4 C). These peaks probably represent the subunits of the cytoplasmic ribosomes. This treatment also resulted in a loss of the peak of 72S cycloheximide-resistant radioactivity and the appearance of a peak of cycloheximide-resistant radioactivity with a sedimentation coefficient of approximately 52S. This appears to represent a shift in this peak, as a concentration of 0.25 M KCl produced no dissociation of the cytoplasmic ribosomes but resulted in the appearance of a broad peak of cycloheximide-resistant

radioactivity with a sedimentation coefficient ranging from 52S to 72S. The exclusion of Mg^{++} from homogenization and gradient buffers in the presence of 0.5 M and 0.25 M KCl resulted in degradation of cytoplasmic ribosomes but did not affect the profile of cycloheximide-resistant incorporation greatly. The radioactivity profile was reduced at 0.5 M KCl but the peak position differed by only one fraction (Fig. 5 A) and was essentially unchanged at 0.25 M KCl (Fig. 5 B).

Cycloheximide-Resistant Amino Acid Incorporation in Cells Grown in Rats

As noted earlier, a lower but detectable cycloheximide-resistant synthesis was observed in trypanosomes grown in rats. In order to determine if this activity was associated with the 72S ribosomes as in the culture grown cells, trypanosomes labeled in the presence of cycloheximide were analyzed on sucrose density gradients. Figure 6 A shows a sucrose gradient profile of rat-grown cells homogenized and sedimented in 250 mM KCl containing TKM buffer. A small amount of radioactivity is seen in the region of the gradient as would be expected if the 72S particle were active in synthesis and examined under these conditions (see Fig. 4 D). It had been noticed that homogenization and sedimentation in 0.5 M KCl containing TKM buffer resulted not only in a shifting of the 72S peak of radioactivity in-

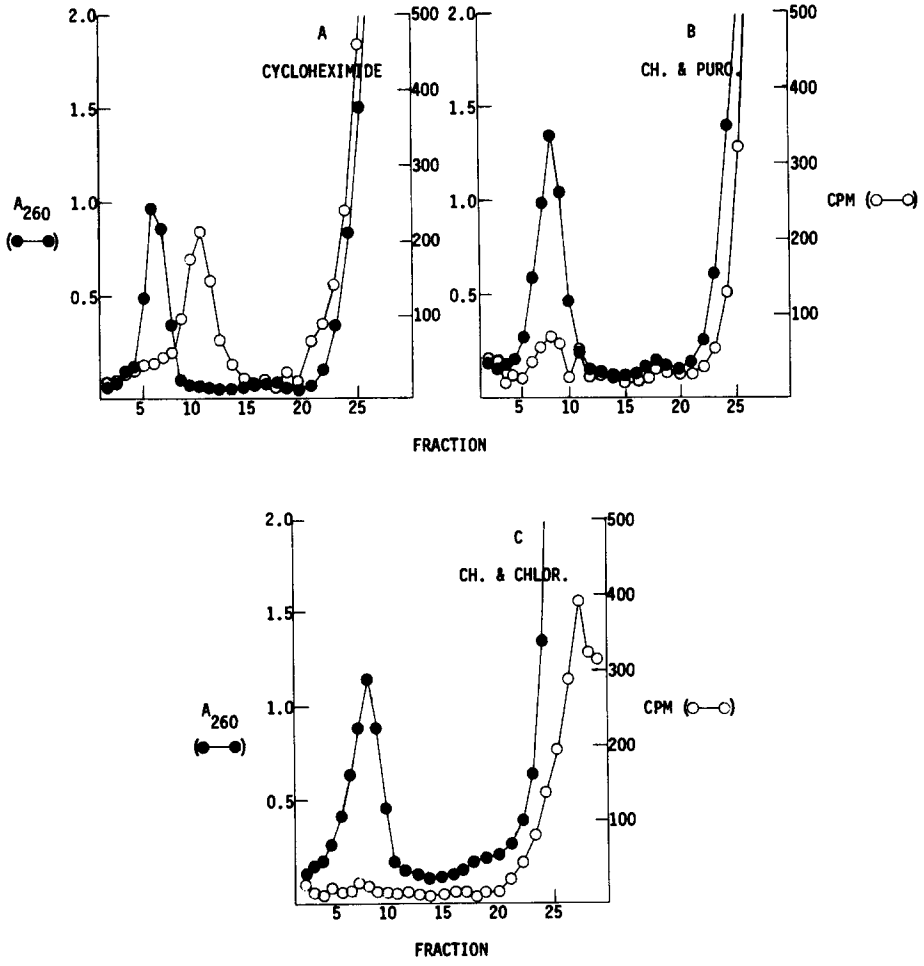

FIGURE 3 Sucrose density gradient analysis of cells labeled in the presence of cycloheximide. Culture form trypanosomes were preincubated with 50 mM cycloheximide for 10 min at 25°C, then incubated with [^{3}H]leucine for 5 min at 28°C (A). Cells so treated were also exposed to puromycin for the last 30 s of incubation (B), or chloramphenicol was also present during incubation (C).

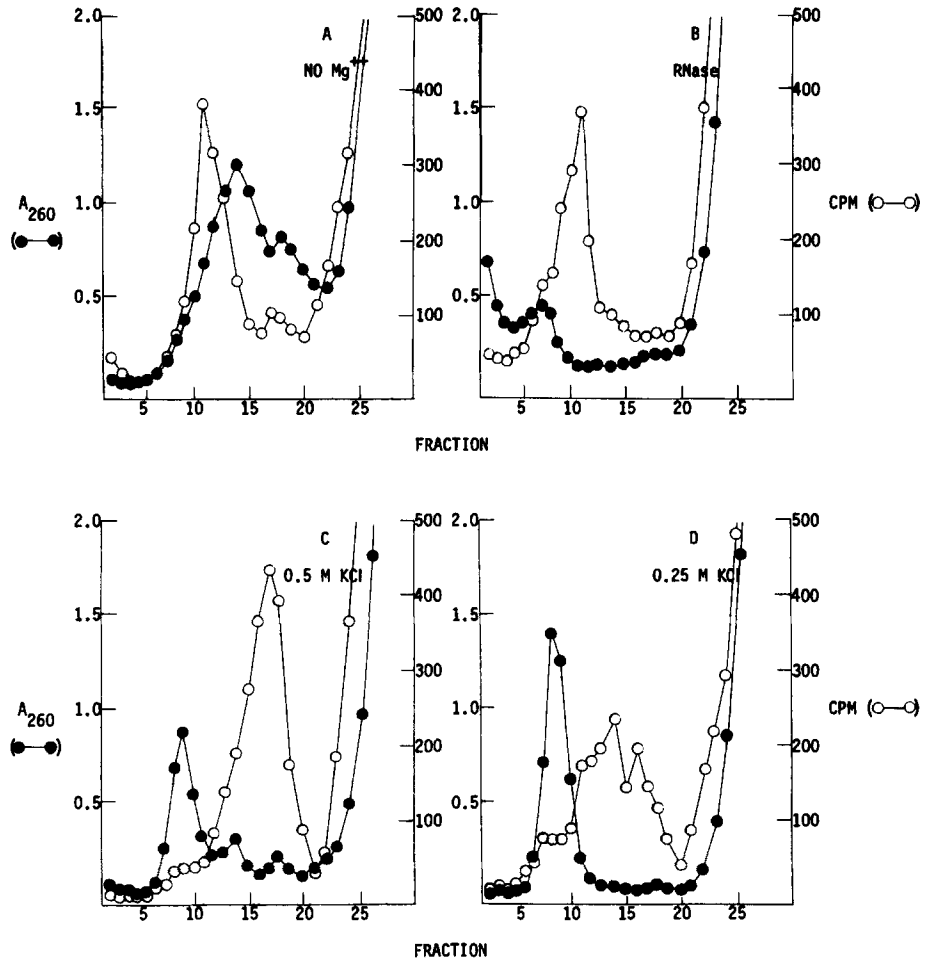

FIGURE 4 Comparisons of ribosomes labeled in the presence of cycloheximide with cytoplasmic ribosomes. Culture form trypanosomes were incubated as described in Fig. 3 A, but the lysis and sucrose gradient buffers contained no Mg^{++} (A), 0.50 M KCl (C), 0.25 M KCl (D), or the homogenate was treated with pancreatic RNase (1 μg/ml) for 20 min at 4°C before being layered on the standard gradient (B).

corporated in the presence of cycloheximide to the 52S region of the gradient, but also in the extraction of more total ribosomal material from the cells which could be seen on the gradients. Figure 6 B shows a sucrose density gradient of rat-grown cells homogenized and sedimented in 0.5 M KCl containing TKM buffer. In such gradients a distinct peak of radioactivity incorporated in the presence of cycloheximide is seen in the 52S region of the gradient. This peak corresponds in position exactly with that obtained for culture cells treated in a similar fashion (see Fig. 4 C).

DISCUSSION

Although mitochondrial ribosomes from different organisms differ greatly in their properties, they may be classified into two groups on the basis primarily of their sedimentation coefficients. The first class, which is 70–80S, is found in fungi and protozoa, while the second class (50–60S) is found in higher organisms. Regardless of source, all mitochondrial ribosomes have one property in common: their protein synthetic activity is blocked by inhibitors of bacterial protein synthesis (e.g., chloramphenicol or erythromycin) but not by

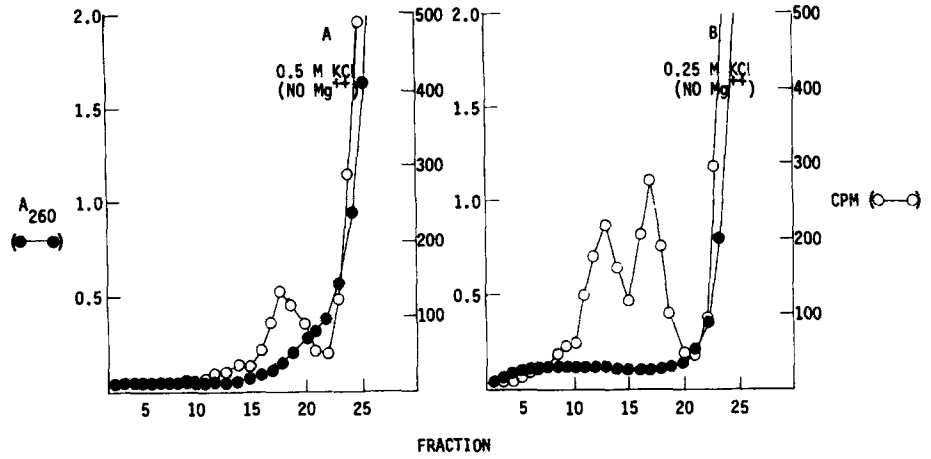

FIGURE 5 Sucrose density gradient analysis in the absence of additional Mg^{++} of trypanosomes labeled in the presence of cycloheximide. Culture form trypanosomes were treated as described for Fig. 4 C and D, except that Mg^{++} was omitted from the homogenization and sucrose gradient buffers.

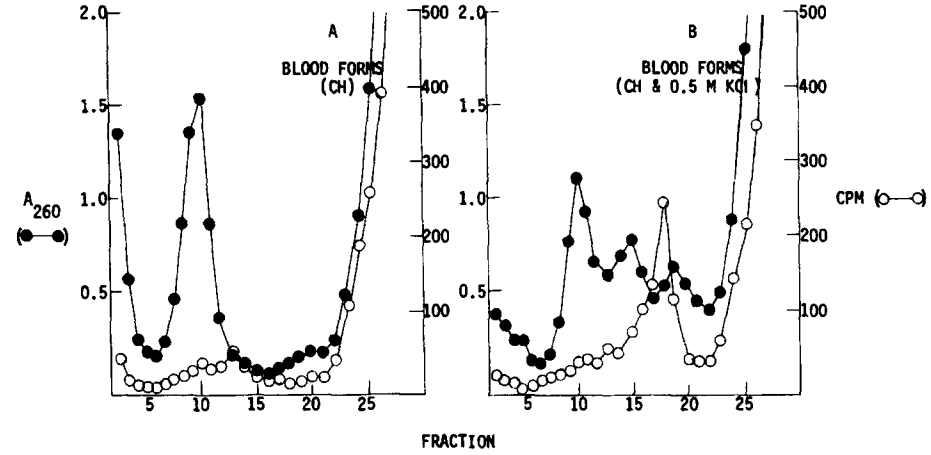

FIGURE 6 Sucrose density gradient analysis of blood form trypanosomes labeled in the presence of cycloheximide. Blood form trypanosomes were isolated from rat blood using sterile DEAE-cellulose columns, then suspended in Locke's-serum, glucose. These cells were preincubated for 10 min at 25°C in 20 mM cycloheximide, then incubated with [^{3}H]leucine at 28°C for 5 min and treated as described in Fig. 4 C and D.

inhibitors of cytoplasmic ribosomal activity such as cyloheximide (1). In this paper we describe ribosomes in *T. brucei* whose protein synthetic activity is insensitive to cycloheximide but is inhibited by chloramphenicol. These ribosomes are assumed to be mitochondrial ribosomes although mitochondrial localization was not demonstrated.

Cytoplasmic ribosomes of *T. brucei* have a monomer sedimentation coefficient of approximately 84S and subunit sedimentation coefficients of about 64S and 48S relative to rat liver ribosomes and subunits taken as 80S, 60S, and 45S. In addition to the differences in antibiotic sensitivities, the cycloheximide-resistant ribosomes differ from the cytoplasmic ribosomes in other ways. They have a lower sedimentation coefficient of about 72S. The 72S ribosomes were also unaffected by RNase treatment which caused degradation of cytoplasmic ribosomes (14). Perhaps the RNA of the 72S ribosomes is less available or less sensitive to enzyme action, or the 72S ribosomes contain a RNase inhibitor. Similar RNase resist-

ance was observed by Neupert et al. (10) using *Neurospora* mitochondrial ribosomes.

Several lines of evidence lead us to suggest that the 72S peak represents a ribosomal monomer. RNase treatment did not alter the sedimentation coefficient of this peak while degrading cytoplasmic ribosomes. This reduces the possibility that the 72S peak is due to polysomes. This peak does not seem to represent dimers. Lowering the Mg^{++} concentration which would be expected to convert dimers into monomers did not result in the production of a peak with a lower sedimentation coefficient. The 72S ribosomes were particularly resistant to dissociation by low concentrations of Mg^{++}. This resistance to dissociation at low concentrations of Mg^{++} is not found for most mitochondrial ribosomes although this anomalous behavior was reported for the mitochondrial ribosomes of another protozoan, *Tetrahymena* (17). It also appears unlikely that the 72S sedimentation coefficient represents an increased sedimentation resulting from attachment to membrane. Although treatment with 0.5 M KCl resulted in a shift of the 72S peak to the 52S region of the gradient, the position of this peak was not further affected by very low magnesium concentrations as would be expected for a typical mitochondrial monomer (Fig. 5 B). Were the 52S peak a monosome released from membrane it would have to be resistant to dissociation under conditions of 0.5 M KCl and very low Mg^{++}. We think that this is unlikely. The height of this 52S peak was reduced, but a peak representing a large subunit was not observed, probably indicating that some of the 52S large ribosomal subunits were degraded under these conditions. The proposition that adherence to membrane might explain the stability of *Tetrahymean* mitochondrial ribosomes in low Mg^{++} and might produce an artificially high sedimentation coefficient (3) appears to have been eliminated by work of Stevens et al. (17). They showed that the peculiar properties of *Tetrahymena* mitochondrial ribosomes can be accounted for by the inherent properties of the ribosomes. The possibility that the 72S ribosomes are contaminating bacterial ribosomes is excluded since cells were grown in sterile culture and the properties of the 72S ribosomes differ from those of bacterial ribosomes, especially by insensitivity to low Mg^{++}.

The 72S ribosomes of *T. brucei* fall into the 70–80S range of the mitochondrial ribosomes of other protozoa and fungi (2, 3, 6, 7, 17). The evidence presented here is in contrast to the report that another kinetoplastid, *Crithidia*, contains 60S mitochondrial ribosomes which dissociate into 45S and 32S subunits at 3 mM Mg^{++} (9). Although total protein synthesis by such a preparation was inhibited by chloramphenicol and erythromycin but not by cycloheximide, protein synthetic activity and antibiotic sensitivity of the 60S particles themselves were not demonstrated. We are unable to reconcile these differences in these closely related but physiologically dissimilar organisms.

We also report in this study that amino acid incorporation activity associated with the 72S ribosome occurs in *T. brucei* grown in the rat. These trypanosomes growing under such conditions have cyanide-insensitive respiration. They lack cytochromes and a fully active Krebs cycle (20). This situation appears analogous to that of glucose-repressed and anaerobically grown yeast where, despite the lack of a mitochondrial respiratory system, mitochondrial protein synthesis occurs (15). It should be pointed out that the strain of *T. brucei* used was pleomorphic. It exists as a morphologically heterogeneous population. Short stumpy forms contain some Krebs enzymes which slender forms lack, but all blood forms lack cytochromes and their respiration is cyanide insensitive (4). It is possible that only the stumpy forms are active in mitochondrial protein synthesis.

Received for publication 3 September 1974, and in revised form 16 December 1974.

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