STUDIES ON THE RIBOSOME OF *CRITHIDIA FASCICULATA*

by

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ABSTRACT

Studies on the Ribosome of *Crithidia fasciculata*

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Cosedimentation analyses of RNA extracted from the insect hemoflagellate *Crithidia fasciculata* with *E. coli* and rat liver RNAs give sedimentation values of 18s and 25s for the two large ribosomal RNAs. The values obtained are consistent with the findings of other eukaryotes. The larger of the two rRNAs, i.e. the 25s, is thermolabile; and after appropriate heat treatment the resulting segment(s) of the molecule sediments in the region of the 18s.

The kinetics of synthesis of the ribosomal RNAs in *Crithidia* was followed using uracil-2-^{14}C and methionine-methyl-^{3}H. Cells were grown to midlog stage and pulsed with radioisotope; RNA was extracted and analyzed by sucrose gradient centrifugation. By these methods a rapidly sedimenting fraction was detected. Subsequent studies showed that this fraction (nominally 35s) could be "chased" into the two large ribosomal RNAs. The patterns obtained and the presence of a common precursor to 18s and 25s rRNAs indicates a similarity to other eukaryotic systems studied. In

prokaryotes the presence of a common precursor has not been detected and the rRNAs may not be transcribed as a polycistronic unit.

Other work using radiolabeled precursors has traced the entrance of the ribosomal subunits and rRNAs into the "cytoplasm". Cells were labeled for various periods of time; the cells broken and the ribosomal patterns from 11,000 x g supernatant fractions analyzed by sucrose gradient sedimentation. The gradient patterns indicate that the 35s rpreRNA itself may be packaged in an 80s ribonucleoprotein particle. After appropriate modification of the rpreRNA the 18s makes its appearance in the smaller ribosomal subunit, prior to the presence of label in the larger subunit. The data also indicate that heterogeneous and presumably messenger RNA is attached to the smaller subunit. These results are considered in light of the role of subunits in the proposed ribosome cycle during protein synthesis.

The ribosome of Crithidia, unlike most other eukaryotic ribosomes dissociates into subunits upon removal of Mg ions from the suspension medium. As with prokaryotic ribosomes, Crithidia ribosomes readily dissociate and reassociate upon removal and addition, respectively, of exogenous Mg^{++}.

It has been proposed that Crithidia fasciculata serve as a model organism for pathogenic parasites of the family

Trypanosomatidae; specifically with reference to studies on the site(s) of action of trypanocidal agents. Preliminary investigations, primarily *in vitro*, indicate that some trypanocides may act at the level of ribosomes and nucleic acids. Our findings *in vivo* indicate a strong resistance of *Crithidia* to some of these agents and suggests that selective toxicity is based on permeability differences between the host and parasite.

During these studies on the ribosome and RNAs of *Crithidia* a polysaccharide contaminant was discovered in the aqueous phase of phenol extracts of whole cells. The polysaccharide was isolated and characterized as to monomeric composition; i.e. primarily mannose. The evidence suggests that this material may be part of the cell coat.

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INTRODUCTION

In light of the role of the ribosome in protein synthesis (for review see Lengyel and Soll, 1969 and Cold Spring Harbor Symposium on Quantitative Biology, 1969); many studies have examined the structural organization and assembly of its nucleic acid and protein components. These investigations have proceeded along two major lines: 1) the artificial disruption of the ribosome and examination of individual constituents followed by reassembly *in vitro* of functional units from their components (for review see Nomura, 1970 and Traub, 1970); and 2) the biological synthesis of the ribosomal components and the formation of intact units *in vivo*.

Most investigations on the biosynthesis of ribosomes have employed bacterial or mammalian systems. We have attempted to examine such processes in the protozoan hemoflagellate *Crithidia fasciculata*. Previous studies on rRNA and ribosome synthesis in protozoa have been limited mostly to work on the more advanced ciliate *Tetrahymena pyriformis* (Leick and Plesner, 1968 a and b; Leick, 1969; Leick *et al*, 1970; Kumar, 1970).

Crithidia is a member of the entirely parasitic family Trypanosomatidae. Members of this group appear typically

eukaryotic in several respects: internal membrane, nuclear histones (Steinert, 1965) and nucleolar structure. At the ultrastructural level, Crithidia is seen to contain a single large mitochondrion which has a specialized region, the DNA containing kinetoplast. This feature is unique to the order Kinetoplastidae. The single anterior flagellum enters a collar shaped region of the cell with the basal body of the flagellum lying near the kinetoplast. Posterior to the latter is the nucleus containing a single well-defined nucleolus (Kusel et al, 1967). The cytoplasm is relatively devoid of endoplasmic reticulum although many free ribosomes are seen.

Crithidia offers a system well suited to the types of study described herein. It is readily culturable on a defined medium (see Materials and Methods) and can be grown in bulk. Previous work in our laboratory (Kahan et al, 1968) showed that cell free extracts and isolated ribosomes of Crithidia participate in conventional protein systhesis.

Ribosomes are composed of two ribonucleoprotein subunits (Osawa, 1968). The larger ribosomal subunit consists of two ribosomal RNAs (rRNAs): a high molecular weight polyribonucleotide and a smaller molecule composed of approximately 120 nucleotides. The smaller subunit contains an RNA polynucleotide which is roughly half the size of the larger subunit. Both subunits bear characteristic proteins;

however, the number and complexity of these constituents have limited their study (Wittman, 1970).

Ribosomes can be subdivided into two groups according to a number of properties which seem to differentiate the ribosomes of prokaryotes and eukaryotes. The prokaryotic ribosome and those of mitochondria and chloroplasts have sedimentation values of 70s and are composed of 50s and 30s subunits. The eukaryotic ribosome is larger and sediments at 80s with subunits of 60s and 40s (for review see Attardi and Amaldi, 1970). Classification of ribosomes into prokaryotes and eukaryotes may also be made on the basis of the size of rRNAs (Loening, 1970); prokaryotic rRNAs are smaller than eukaryotic rRNAs. It should be noted that most references to rRNA imply the larger two of the three rRNAs. The smaller 5s RNA of the large subunit has been studied less extensively. This RNA species is the same size in both E. coli (Brownlee et al, 1968) and in human tissue culture cells (Forget and Weissman, 1969). Prokaryotic rRNAs are 16s and 23s with molecular weights of 0.56 x 10^6 daltons, respectively. In contrast, the smaller high molecular weight eukaryotic rRNA is 18s and 0.7 x 10^6 daltons. The larger rRNA of eukaryotes is more variable; in protozoa and plants it is classified as 25s and it has a molecular weight of 1.3 x 10^6 daltons; in metazoa this rRNA is assigned 28s and varies from 1.4 x 10^6 daltons in some invertebrates to 1.75 x 10^6 daltons in mammals. Although

the foregoing size classification may not be as rigid as implied, as evidenced by a number of exceptions (Loening, 1970), overall relationships are clear.

The structural differences between ribosomes of eukaryotes and prokaryotes are also expressed as differences in the following properties of the ribosome:

1) An apparent resistance of eukaryotic ribosomes to dissociate into subunits at reduced magnesium ion concentration (Peterman, 1964; Martin et al, 1969; Martin and Wool, 1969); i.e., animal ribosomes, including those of Tetrahymena pyriformis, are stable in solutions with little or no added Mg^{++} (Martin and Wool, 1969; Weller et al, 1968). Bacterial ribosomes begin to dissociate at Mg^{++} concentrations immediately below the optimum for in vitro protein synthesis (Tissieres et al, 1960) and readily reassociate and regain activity upon restoration of Mg^{++}. The significance of this property is not clear and may be exaggerated.

2) Prokaryotic and eukaryotic ribosomes are sensitive to chloramphenicol (CA) and cycloheximide (CX) respectively, as expressed by inhibition of protein synthesis. These drugs have been useful in identifying the prokaryotic nature of mitochondrial and chloroplast ribosomes (Clark-Walker and Linnane, 1967; Ellis, 1969).

3) A possible distinction between requirements for peptide chain initiation in eukaryotic and prokaryotic systems (Liew et al, 1970; Culp et al, 1970).

4) Differences in ribosome and rRNA synthesis in prokaryotes and eukaryotes.

Early studies on the ribosome centered on the rRNAs in the hope that the elucidation of the nature of this material would help to explain how genetic information is transferred into protein. Subsequent studies on bacterial protein synthesis showed that an RNA fraction, exclusive of rRNA, was involved in relaying the genetic information to the ribosomes. This so-called messenger RNA (mRNA) fraction was also characterized as being both rapidly labeled and turned over, and similar in base composition to cellular DNA (Gros et al, 1961 a and b; Brenner et al, 1961). An examination of rapidly labeled RNA in a eukaryotic system, e.g. HeLa cells in culture (Scherrer and Darnell, 1962), revealed the presence of material sedimenting at 45s. The kinetics of incorporation of label into this 45s fraction and the subsequent appearance of label into stable 18s and 28s rRNAs suggested that the 45s was a precursor to rRNA. In eukaryotic systems four lines of investigation have provided evidence for a precursor-product relationship. The lines of evidence are:

1) Pulse and Chase Experiments. The fate of label incorporated into the precursor material can be traced by culturing cells for a short period of time in a radio-labeled RNA precursor such as uracil or methionine (methyl labeled). Further incorporation of label is prevented by administration of excess unlabeled precursor or Actinomycin D -- an inhibitor of RNA polymerase (Reich and Goldberg, 1964). (Girard et al, 1964; Kumar, 1970; Landesman and Gross, 1969; Muramatsu et al, 1966; Perry, 1965; Rake and Graham, 1964; Scherrer et al, 1963; Taber and Vincent, 1969 b; Weinberg et al, 1967.)

2) Cellular Dissection. In order to ascertain the site of synthesis of the ribosomal precursor RNA (preRNA) and its subsequent fate within the cell, cells are fractionated and incorporation of label into the RNAs of the subcellular fractions, in particular nucleolus, nucleus and cytoplasm, is determined. (Greenberg and Penman, 1966; Liau et al, 1968; Muramatsu and Fujisawa, 1968; Muramatsu et al, 1966; Penman et al, 1966; Penman, 1966; Perry, 1964; Vesco and Penman, 1968.)

3) Primary Structure Analysis. Similarities in primary structure, derived from base composition and RNase digestion pattern analyses, support the arguments for a precursor-pattern relationship. (Amaldi and Attardi, 1968; Choi and Busch, 1969; Jeanteaur et al, 1968; Marks et al, 1969; Muramatsu et al, 1966; Roberts and D'Ari, 1968; Wagner et al,

1967; Wikman et al, 1969; Willems et al, 1968; Zimmerman, 1968; Zimmerman and Holler, 1967.)

4) DNA-RNA Hybridization. Competition between RNA molecules for complementary sites on the DNA indicates the same cistronic origin for the suspected precursor and ultimate product. (Birnstiel et al, 1968; Brown and Weber, 1968 a and b; Dawid et al, 1970; Perry et al, 1964; Quagliarotti et al, 1970; Reeder and Brown, 1970; Retel and Planta, 1970; Taber and Vincent, 1969 b).

Recent electron microscope observations of RNA fibers from Novikoff Hepatoma cells (Busch and Smetana, 1970) and isolation of 65s and 85s nucleolar RNAs (Hidvegi et al, 1971) have led to the proposal that the precursor may first be transcribed as multiples of 45s rpreRNA and secondarily cleaved to material sedimenting at 45s. In any event, a number of intermediates of the processing sequence have been detected. These intermediates presumably arise from selective scission of the rpreRNA and/or changes in conformation. Various schemes have been proposed to show the transition of the presumed polycistronic precursor to the rRNAs of the ribosome. (For review see Busch and Smetana, 1970 (page 264.)

Although few organisms have been studied as extensively as mammals it seems clear that similar processing of a rpreRNA occurs in all eukaryotes (Attardi and Amaldi, 1970;

Loening, 1968; Loening et al, 1969; Loening, 1970) including protists; i.e., yeasts (Retel and Planta, 1970; Taber and Vincent, 1969 a); Tetrahymena (Kumar, 1970) and a dinoflagellate (Rae, 1970). It appears that the precursor itself may have undergone change during evolution; i.e. the more advanced organisms including birds and mammals have larger precursors than lower vertebrates and the remaining eukaryotes (Perry et al, 1970). However much of the excess sequences in the rpreRNA of birds and mammals may be lost during processing (Roberts and D'Ari, 1968; Wagner et al, 1967; Weinberg and Penman, 1970; Zimmerman and Holler, 1967).

By contrast, an analogous precursor RNA to the 16s and 23s rRNA of prokaryotic ribosomes has not been found. (For review see Osawa, 1968.) However recent studies on E. coli RNA synthesis following refampicin inhibition of RNA chain initiation suggest that 16s, 23s and 5s stable rRNAs may be synthesized as a single transcriptional unit (Jacobson, 1971; Pato and von Meyenburg, 1970). There is evidence (Adesnik and Levinthal, 1969; Hecht and Woese, 1968) that each bacterial rRNA has its own precursors is converted into its own mature chain. Recent evidence (Bernhardt and Darnell, 1969; Hecht et al, 1968; Jordan et al, 1970) indicated that other RNA molecules, including 5s and tRNA, may have precursors, i.e. initial transcription products which are lengthier polynucleotides than the mature molecules. There

is evidence for such precursors in both eukaryotes and prokaryotes. Mangiarotti et al (1968) suggested that the 23s rRNA is made from the linkage of two shorter, ca. 16s, polynucleotides. Their idea was based on the finding that that the rate of synthesis of 23s rRNA is the same as that of 16s rRNA and that no incompletely transcribed material with sedimentation coefficients between 16s and 23s is present. However, the 23s is not merely a dimer or aggregated form of 16s rRNA since sequence analyses has shown the 5' end (Takanami, 1967) and 3' end (McIlreavy and Midgely, 1967) of both species are different from each other; and RNase digest analyses reveal different primary sequences for the mature 16s and 23s rRNAs (Fellner, 1969).

In eukaryotes, syntheses of rRNA is localized within a distinct morphological entity -- the nucleolus. The functional relationships between the nucleolus, nuclear RNA and cytoplasmic protein synthesis have been recognized for some time (see Brachet, 1957 and references therein). The following lines of accumulated evidence validate the conclusion that the nucleolus is the site of synthesis of rRNA and ribosomes in eukaryotes:

1) rRNA precursors are found in the nucleoles (see above "Cellular Dissection"). However other types of RNAs may be synthesized in the nucleolus (Choi and Busch, 1969).

2) Selective damage to nucleoli inhibits rRNA syntheses (Perry et al, 1961).

3) Anucleolate mutants of Xenopus laevis are unable to make rRNA (Brown and Gurdon, 1964) and the number of nucleolar organizors present in Drosophila is proportional to the amount of the rRNA complementary to the DNA (Ritossa and Spiegelman, 1965).

4) Hybridization of rRNA to nucleolar DNA indicates the nucleolus as the site of origin (Quagliarotti et al, 1970). Although it is apparent from this work that at least in some cell lines the final cleavages to 28s and 18s occurs outside of the nucleolus.

In addition to transcribing rpreRNA the nucleolus apparently modifies and packages these polynucleotides into ribosomes. It has been shown that rRNA is methylated at the level of the large precursor, probably at or near the site of transcription (Greenberg and Penman, 1966; Greenberg, 1969; Taber and Vincent, 1969 b). Curiously, an apparent single methylation, perhaps of regulatory significance, also occurs at the time of 45s cleavage in HeLa cells. Zimmerman and Holler (1967) found a dimethylaminopurine residue in mature 18s rRNA absent in the rpreRNA molecule. Other rRNA modifications occurring at the level of the large precursor was noted by the conversion of uridylic acid to pseudouridylic acid (Amaldi and Attardi, 1968).

Several ribonucleoprotein particles containing different stages of rRNA processing starting with the large precursor

molecule have been demonstrated in the nucleolus (Izawa and Kawashiwa, 1969; Liau and Perry, 1969; Warner and Soeiro, 1967). Vaughn et al (1967) have demonstrated the existence of small quantities of ribosomal subunits within the extra nucleolar region of the HeLa cell nucleus. The subunits presumably after modification from the nuclear RNP, are released into the cytoplasm with the small subunit preceeding the larger (Girard et al, 1965; Leick and Plesner, 1968a). Present evidence (Leick and Plesner, 1968b; Perry and Kelly, 1966; Perry and Kelly, 1968; Taber et al, 1969) indicates that the newly synthesized subunits entering the cytoplasm are not structurally identical to mature subunits but may undergo further modification within the cytoplasm. Results of experiments with Amoeba (Craig and Goldstein, 1969) indicate that at least in this organism ribosomal proteins are added on in the cytoplasm. In bacteria nascent rRNAs are built-up into mature subunits by a series of protein additions (Osawa, 1968; Attardi and Amaldi, 1970); however, this formation of ribosomes does not occur in a morphologically discernible nucleolus.

Ribosome synthesis is under strict control. Wild type bacteria, when starved for an essential amino acid, terminate bulk RNA, primarily rRNA, immediately (Edlin and Broda, 1968). Inhibitors of protein synthesis or mutation may relax this control and allow rRNA synthesis to continue in the absence of protein synthesis (Ryan and Borek, 1971). The deprivation

of valine or lysine from HeLa cell cultures slows down the rate of transcription of 45s rRNA and upsets the relative proportion of ribosomal subunits. Synthesis is not inhibited completely, however, because of protein turnover (Maden, 1969; Vaughn et al, 1967). HeLa cells grown in a medium lacking methionine continue to synthesize a 45s precursor; however, this material is not processed into ribosomes, presumably because it is not properly methylated (Maden and Vaughn, 1968; Vaughn et al, 1967). Yeast (Taber and Vincent, 1969a) treated with cycloheximide show accumulation of precursor and inhibition of cleavage of this material indicating a posttranscriptional control. HeLa cells treated with the same antibiotic continue rRNA synthesis and process it into ribosomes, which may indicate a pool of ribosomal proteins (Warner et al, 1966), although puromycin, another inhibitor of protein synthesis, prevents ribosome formation in the same cells. Further evidence for specific controls of rRNA synthesis is strengthened by the findings that cordycepin (3' deoxyadenosine) specifically blocks rRNA synthesis (Siev et al, 1969) and the discovery that a specific RNA polymerase confined to the nucleolus is distinct from other RNA polymerases in sea urchin embryos and rat liver (Roeder and Rutter, 1969 and 1970).

Crithidia fasciculata offers a suitable system to extend our knowledge of the processes involved in the synthesis of a eukaryotic ribosome. In addition Crithidia as a member of

the entirely parasitic family Trypanosomatidae, has been offered (Newton, 1962) as a model organism to study effects and mode of action of trypanocidal drugs, on members of the genus Trypanosoma. Members of this genus are responsible for the South American, Chagas' disease and the African sleeping sicknesses. With regard to drug studies, trypanocidal activity may be located at the level of the ribosome and protein synthesis (Chesters, 1966; Kahan et al, 1968). Newton (1966) has postulated that the trypanocide, Antrycide (quinapyramine) specifically inhibits ribosomal RNA synthesis. Likewise, studies by Wallis (1966 a and b) have focused on the interaction of ribosomal extracts of Crithidia oncopelti with a number of trypanocidal agents, including Antrycide, ethidium bromide and pentamidine.

The aim of this work was to examine and compare ribosome and rRNA synthesis in the hemoflagellate Crithidia fasciculata with such syntheses in eukaryotes and prokaryotes. It was hoped that this insect trypanosomatid would also be useful in discerning the mode of action of some trypanocidal drugs at the level of the ribosome.

MATERIALS AND METHODS

Reagents

L-methionine-methyl-^{3}H (2.8 C/mmole) was purchased from Schwarz Bioresearch (Orangeburg, N.Y.); L-methionine-methyl-^{14}C (49 mC/mmole); uracil-2-^{14}C (54.9 mC/mmole) from Amersham-Searle (Des Plaines, Ill.); and uracil-6-^{3}H (12 C/mmole) from New England Nuclear (Boston, Mass.). RNase free sucrose and Actinomycin D were products of Mann Research Labs (New York, N.Y.). Chloramphenicol (CA) and cycloheximide (CX) were purchased from Sigma (St. Louis, Mo.). Antrycide was obtained from Imperial Chemical Industries (Alderley Park, Cheshire, England). All other chemicals were reagent grade and purchased from commercial sources.

Buffers

1) Glycine, pH 9.5: 0.1 M glycine; 0.1 M NaCl; 0.01 M EDTA.

2) LiAc: 0.01 M lithium acetate, pH 5.0.

3) NT: 0.1 M NaCl; 0.01 M Tris, pH 7.6.

4) RSM: 0.25 M sucrose; 10% glycerol; 35 mM Tris, pH 7.6; 25 mM KCl; 10 mM $MgCl_2 \cdot 6H_2O$; 4 mM β-mercaptoethanol.

5) SB: 0.05 M sodium borate, pH 9.2.

6) TKM: 10 mM Tris, pH 7.6; 10 mM KCl; 10 mM $MgCl_2 \cdot 6H_2O$.

7) TK: 10 mM Tris, pH 7.6; 10 mM KCl.

8) TMN: 50 mM Tris, pH 7.6; 2 mM $MgCl_2 \cdot 6H_2O$; 0.14 M NaCl.

All buffers were prepared at room temperature.

Growth Conditions and Labeling

Crithidia fasciculata (ATCC 11745) was grown on a defined medium containing hemin modified after Bacchi et al (1969) by deletion of: uracil, thymine, orotic acid and biopterin. Cells from test tube cultures were inoculated into half-filled 1 liter DeLong flasks (Bellco Glass Co., Vineland, N.J.). Cultures were incubated at 25° with moderate shaking in a Psycrotherm incubator (New Brunswick Scientific, New Brunswick, N.J.). Log-phase cells were harvested by centrifugation at 2500 x g in Sorvall RC2-B centrifuge at 0-4° for 5 minutes. Cells were washed once in TKM buffer. The washed cell pellet was suspended in 50% glycerol (w/v) and stored at -74°. For labeling, cells were grown to midlog (2×10^7 cells/ml) and concentrated aseptically by centrifugation and resuspension in fresh culture medium. Pulse labeling of RNA was carried out by exposing cultures to uracil-2-^{14}C (0.1 uC/ml) for the indicated periods of time. For labeling with methionine, cells were grown as above to midlog in defined medium, washed once in the same medium minus methionine and resuspended in one sixth the original volume of fresh defined medium minus methionine. Cells were incubated for one hour or more, to use up endogenous methionine, prior to the addition of methionine-methyl-^{3}H (4 uC/ml). Incubation was then carried out as indicated. For pulse and chase experiments, cells were labeled for one minute with methionine as described above, an aliquot removed and a thousand fold excess of cold methionine added. In all

cases incorporation was stopped by pouring the labeled cells over two volumes of cold medium at 0°. Cells were harvested and stored at 74° as described. For these experiments the cells were analyzed during a period of two to three weeks after the labeling was performed.

To determine the incorporation of exogenously supplied radioisotope into acid precipitable material cells were grown to midlog, isotope (either uracil-2-^{14}C, 0.03 uC/ml, or methionine-methyl-^{14}C, 0.03 uC/ml) added and aliquots removed at given time points. The aliquots (generally one ml) were immediately precipitated with four ml of cold 5% trichloroacetic acid (TCA). The precipitates were measured as described below.

Cell Fractionation

For the isolation of cytoplasm, stored frozen cells were thawed and homogenized in the cold in one volume of RSM buffer with twenty strokes of a Potter-Elvehejm homogenizer. The homogenate was centrifuged for five minutes at 3000 x g to remove whole cell and precipitated hemin. The supernatant fluid was removed and centrifuged at 11,000 x g for thirty minutes. Microsomes and ribosomes were prepared according to Kahan et al (1968). Colorimetric determinations for RNA and DNA were performed according to Schneider (1957).

Isolation of RNA

RNA from whole cells was isolated as modified from the method of Click and Hackett (1966) as follows. To one volume of frozen glycerol treated cells was added ten volumes of phenol-extraction buffer mixture (2:1 v/v). Commercially obtained phenol was distilled and stored in 50 ml aliquots in dark bottles at -20°. Prior to the isolation this phenol was thawed and made 88% v/v with 10 mM EDTA. The extraction buffer contained 1.0% sodium deoxycholate (DOC) and 0.5% bentonite in glycine buffer, pH 9.5. The extraction mixture was shaken for one hour on a reciprocating shaker at 2°-4°. Similar sucrose gradient patterns were obtained when the phenol extraction step was performed at 60° for 15 minutes. The emulsion was broken by centrifugation at 10,000 x g for 15 minutes. Hemin, which precipitates slightly from the medium upon autoclaving and adheres tenaciously to harvested cells was extracted into the phenol layer, and thus did not interfere with the isolation of RNA from the top aqueous phase. The latter was removed and extracted two to three times with ether. The remaining ether was blown off with a gentle stream of nitrogen. The RNA was precipitated from the aqueous phase with two volumes of ethanol, and stored in this form at -20°. Before use the RNA was centrifuged at 11,000 x g and dissolved in pH 9.5 glycine buffer at a concentration of approximately 1 mg/ml, assuming an $E^{1}_{1\%}{}^{cm}$ of 200. Escherichia coli strain K12 (Worthington, Freehold, N.J.) and Long-Evans

rat liver were extracted under identical conditions.

RNA was isolated from sucrose gradient fractions (after Leick and Plesner, 1968a) as follows: contents of tubes to be extracted were added to three ml of TMN buffer and one ml of cold 11,000 x g supernatant fluid as carrier. To this was added 0.5 ml of 25% sodium dodecyl sulfate (SDS) and an equal volume of phenol saturated with TMN buffer. The mixture was shaken for four minutes in the cold. The emulsion broken by centrifugation; the aqueous was removed and the bottom phenol layer was reextracted with 1.0 ml of TMN buffer. The combined aqueous phases were precipitated by addition of two volumes of 96% ethanol containing 0.2 moles of sodium acetate per liter; and was stored at -20°. Before use, the RNA was collected by centrifugation and dissolved in TMN buffer.

Sucrose Gradient Analyses

RNA extracted from whole cells was sedimented in 2 to 20% linear sucrose gradients made up in the pH 9.5 glycine buffer. All gradients were prepared approximately 8 - 12 hours before use by means of a Buchler single outflow device. The 11,000 x g supernatant fraction was placed on 15 to 30% linear sucrose gradients made in TKM buffer and centrifuged as indicated. Ribosomes and microsomes were suspended in TKM buffer prior to centrifugation. RNA released from total 11,000 x g supernatant fluid was placed

on 5 to 30% sucrose gradients made up in NT buffer containing 0.5% SDS. RNA isolated from gradient fractions was sedimented in 2 to 20% sucrose gradients made up in TMN buffer. In all cases sedimentation was carried out in a Spinco Model L-2 ultracentrifuge at 24,000 rev/min for lengths of times as indicated in the legends to the figures. Centrifugations were run at 2^{o} except in the cases of SDS gradients which were run at 20^{o}. The SW 25.1 rotor and accompanying band forming caps (Beckman Instruments; Mountainside, N.J.) were used during the course of this work. In all cases approximately 10 $A_{260\ nm}$ units were placed on top of the gradient.

Gradients were analyzed spectrophotometrically at 254 nm by means of an ISCO (Lincoln, Neb.) density gradient fractionator and model UA-2 ultraviolet analyzer. One ml fractions were collected and precipitated with four ml of cold 5% TCA. These precipitates as well as those from incorporation studies (see above Growth Conditions and Labeling) were poured onto Millipore (Cambridge, Mass.) HAWP 25 mm filters (0.45u pore size) and washed with an additional 50 ml of cold 5% TCA. After drying and addition of 8 ml of toluene based scintillation fluid (Spectrafluor; Des Plaines, Ill.) the samples were counted in a Beckman LS-200 scintillation counter.

Carbohydrate Analysis

The ethanol precipitated material from the aqueous phase of phenol extracted whole cells was dissolved in LiAc buffer and fractionated on DEAE cellulose (Whatman DE 32 microgranular, fines removed, 1.0 meq/g) either on columns or in bulk. Aliquots were monitored for carbohydrate by the anthrone reaction (Ashwell, 1967) at 620 nm and RNA by absorbance at 260 nm by a Beckman DU spectrophotometer. In the case of isotopically labeled cells 100 ul samples were added to Bray's scintillation fluid (Bray, 1960) and radioactivity determined in a Beckman LS-200 scintillation counter. Fractions containing anthrone-positive material were pooled and lyophilized. The lyophilisate was taken up in a minimum volume of water, the polysaccharide was precipitated with cold absolute ethanol, collected by centrifugation and the precipitate hydrolyzed in 1.0 ml of 0.6N H_2SO_4 for one to two hours at 100°. The hydrolysate after neutralization with saturated $Ba(OH)_2$, was chromatographed in the following systems: butanol:acetic acid:water (4:1:5); aqueous phenol; and butanol:pyridine:water (6:4:3) on thin layer sheets (Brinkman "MN 300 cel") and on paper (Whatman #1) in a descending system. Chromatograms were sprayed with p-anisidine phosphate (0.5 g p-anisidine HCl in 50 ml ethanol and 2 ml H_3PO_4) and developed by heating at 100° for five minutes. Electrophoresis of the hydrolyzed sample was carried out in SB buffer, 15 minutes at 100 v on Millipore "Phoroslides"; the slides were developed with

p-anisidine as described for paper chromatography. Glucose oxidase and galactose oxidase assays were performed on the hydrolysate using "galactostat" and glucostat clinical reagent kits (Worthington Biochemical Corp., Freehold, N.J.). The cysteine H_2SO_4 colorimetric assay for mannose and other hexoses was performed on both hydrolyzed and unhydrolyzed material according to Ashwell (1957). Absorption spectra were made on a Cary 14 recording spectrophotometer.

As a check on the isolation procedure for carbohydrate, cells were homogenized in 0.5N perchloric acid (PCA). The acid soluble fraction was removed and carbohydrate precipitated from it by the addition of 1.2 volumes of cold absolute ethanol. The precipitate was dissolved in water and treated as described above. In addition, carbohydrate was isolated by the alkaline hydrolyses method employed by von Brand et al (1959).

RESULTS

Sedimentation Properties of *Crithidia* RNA

Cosedimentation of isolated *Crithidia* RNA with *E. coli* and rat liver RNAs is shown in Fig. 1. The relative position of the *Crithidia* RNA yields nominal values of 18s and 25s for the two large and presumably rRNA peaks. The presence of a shoulder on the lighter side of the *Crithidia* 18s peak (see Fig. 1B) represents breakdown product(s) of the larger rRNA. This is confirmed by heating the RNA for various times at 60° and monitoring the decrease in absorbance in the 25s region with the concommitant increase in absorbance in the 15-16s region of the gradient. Fig. 2 shows complete disappearance of the 25s material after a five minute incubation at 60°. Differences in total absorbance in both profiles can be accounted for by the amount of material placed on each gradient. The lability of 25s is also observed upon vigorous agitation of the RNA solution.

Polysaccharide Characterization

During the course of these investigations on *Crithidia* RNAs a polysaccharide has been found which co-extracts with the RNA. Contamination of RNA preparations by carbohydrate is not unknown and may represent a source of error in nucleic acid determinations. For example, radiolabel from thymidine

Fig. 1. Cosedimentation of Crithidia fasciculata RNA with E. coli and rat liver RNAs. RNA from Crithidia labeled with uracil-2-^{14}C was mixed with unlabeled samples of rat liver (A) and E. coli (B) RNAs. Radioactive material contributed less than 5% of total absorbance. Samples were placed on 2-20% sucrose gradients made up in glycine pH 9.5 buffer and centrifuged for 11 hours at 24,000 rpm in the SW 25.1 rotor in Spinco model L-2 ultracentrifuge at 2^{o}. Gradients were assayed as described in "Materials and Methods". In this and all other gradients solid line = absorbance and dotted line = radioactivity. Direction of sedimentation in all gradients is from right to left.

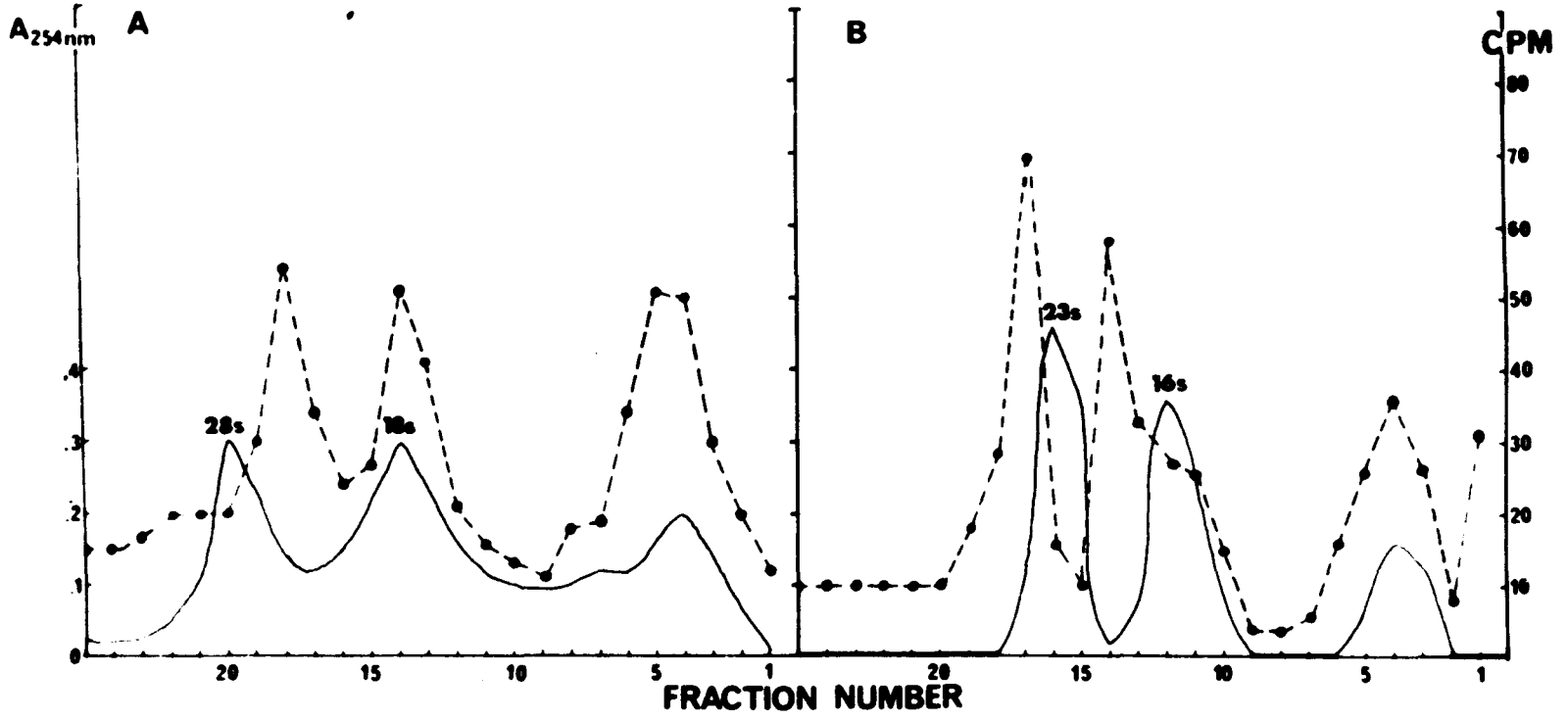
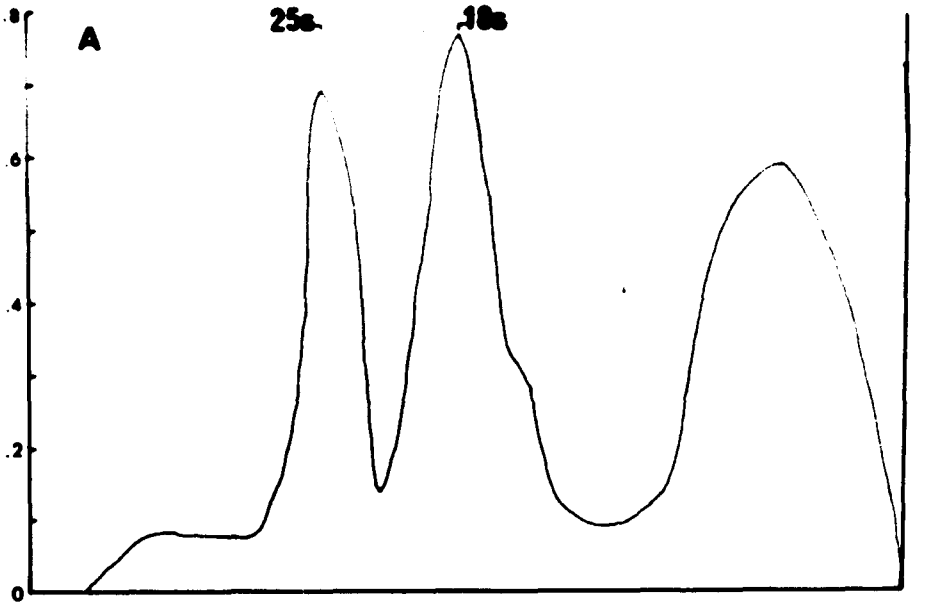
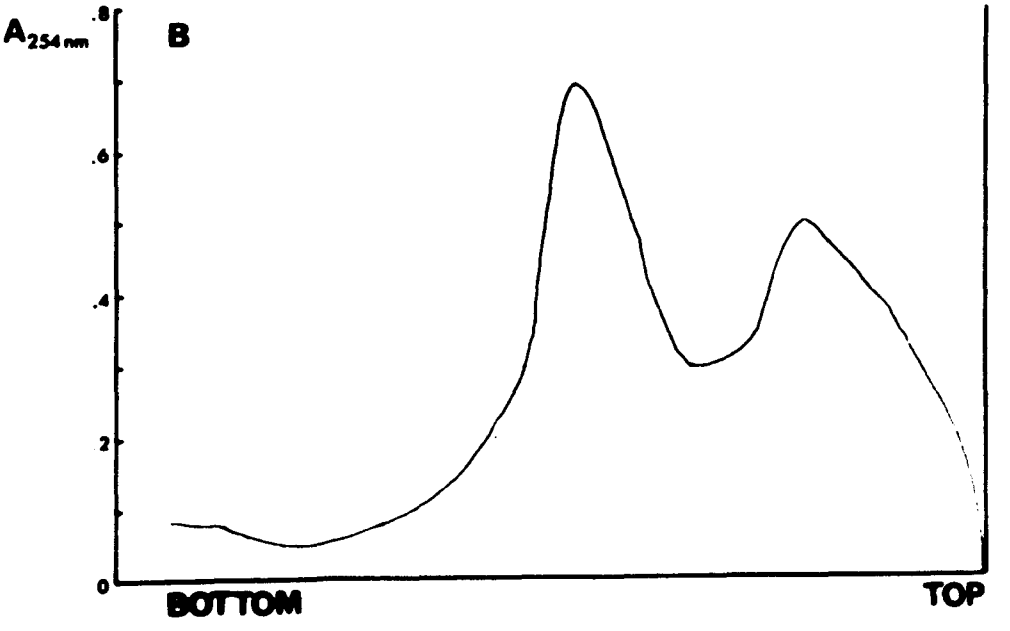

A254nm
A
28s
18s
B
23s
16s
CPM
FRACTION NUMBER

Fig. 2. Heat treatment of Crithidia RNA. A. Crithidia RNA extracted in cold and maintained at 0° prior to centrifugation. B. Crithidia RNA extracted in cold and incubated for 5 minutes at 60° prior to placement on gradient. Details of gradient centrifugation as in Fig. 1.


A
25s
18s
.8
.6
.4
.2
0


$A_{254\,nm}$
B
.8
.6
.4
.2
0
BOTTOM
TOP

and methionine appears in cell polysaccharides (Piko 1970; Lanzetta and Berech, 1971). Apart from its being a nuisance in determination of nucleic acids; i.e. colorimetric assays of nucleic acids (Schneider, 1957) make use of sugar residues; this polysaccharide may be of immunological importance. Moreover, further knowledge of the chemical composition of this trypanosomatid would be of value.

The carbohydrate, which was first detected by the anthrone reaction carried out on the aqueous phase of phenol extracts, was separated from RNA by DEAE cellulose ion exchange chromatography, as shown in Fig. 3. It is seen that this polysaccharide does not contribute to UV absorption nor is it labeled by methionine-methyl-^{14}C. Therefore we believe that this material does not interfere with RNA measurements on sucrose velocity gradients. Therefore separation of the CHO was not routinely employed during the course of these studies.

The anthrone positive material after separation from the RNA is treated as described in Materials and Methods. After hydrolysis of the polysaccharide the sugar is seen to co-chromatograph with mannose in the thin-layer and paper systems employed. The results of electrophoresis (Fig. 4) show that the only detectable monosaccharide is mannose. Para-anisidine positive areas in the "Phoroslide" and thin layer systems are fluorescent under shortwave UV light at

Fig. 3. Separation of contaminating carbohydrate from Crithidia RNA. DEAE cellulose ion exchange chromatography of ethanol precipitated material from the aqueous phase of phenol extracted Crithidia, labeled with methionine-methyl-^{14}C for 7 hours. Column was equilibrated against 0.01 M LiAc buffer pH 5.0. Sample was dissolved and applied to column in the same buffer. The carbohydrate was eluted with buffer and the RNA eluted with buffer made 1.0 M with LiCl. Twenty ml fractions were collected and 1 ml samples were assayed for: RNA by absorbance at 260 nm (---X---X---); incorporation of methyl label by precipitation with cold 5% TCA and liquid scintillation counting of the filtered precipitates (---0---0---); and carbohydrate by the anthrone reaction at 620 nm (————).

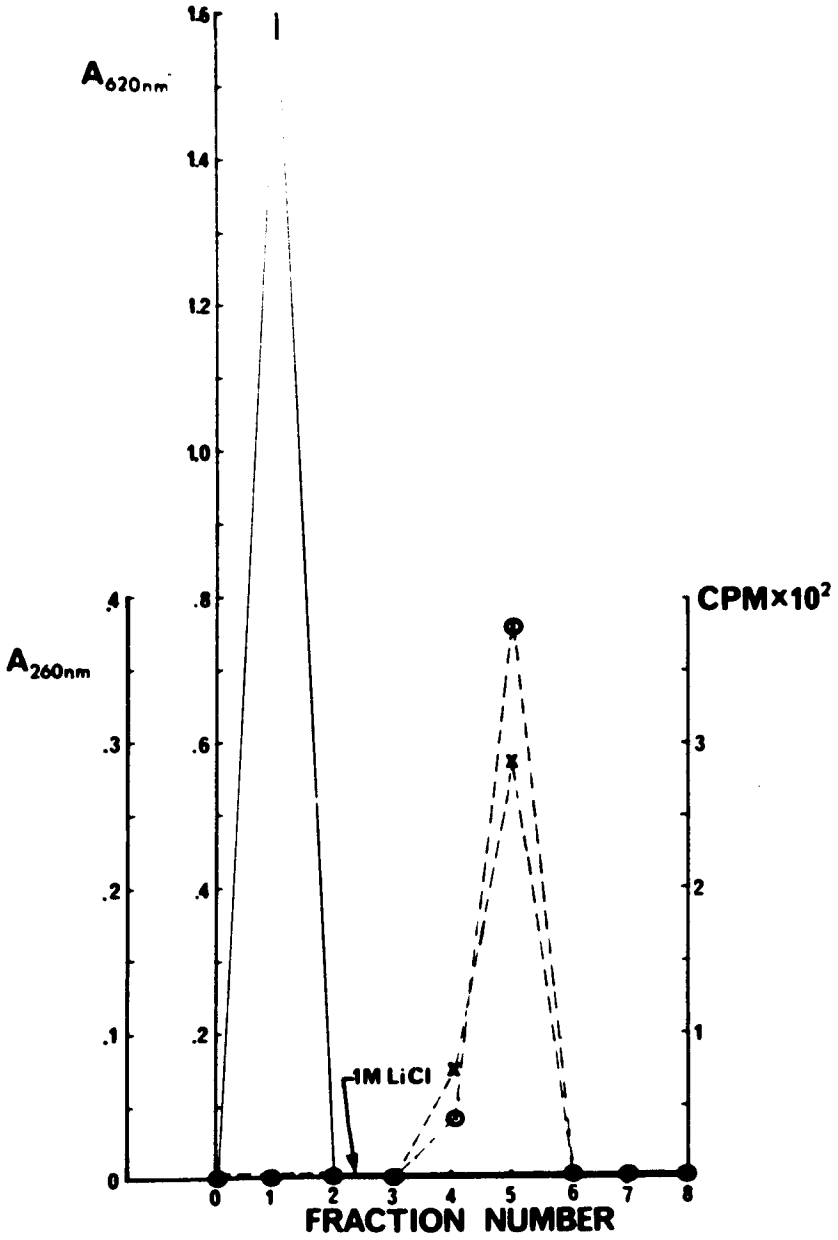
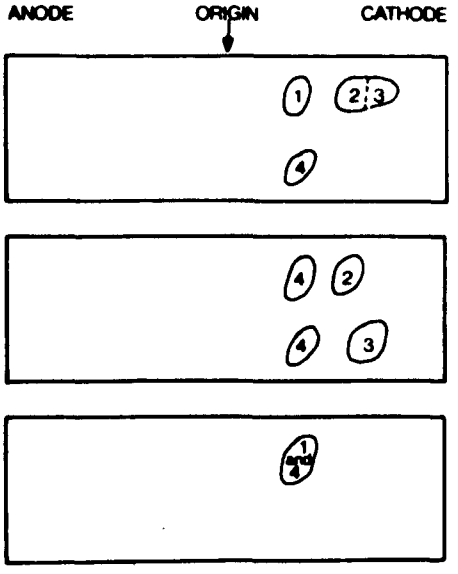

A620nm
A260nm
CPM×10²
1M LiCl
FRACTION NUMBER
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.4
.3
.2
.1
0
3
2
1
0 1 2 3 4 5 6 7 8

Fig. 4. Electrophoretic separation and identification of the monosaccharide derived from Crithidia hydrolyzed polysaccharide (1), galactose (2), glucose (3) and mannose were used as standards on Millipore "phoroslides". Approximately 3 ug of sugar was spotted at the center of the slide using the Millipore electrophoretic apparatus. The separation was carried out for ten minutes at 100 v, in SB buffer.


ANODE
ORIGIN
CATHODE
1
2
3
4
4
2
4
3
1
4

257 nm and this is extremely helpful in detecting less than 2 ug amounts of sugar. The "Phoroslide" electrophoretic system described, which has not been previously used in the determination of sugars, is an ideal system for the rapid and sensitive identification of hexoses.

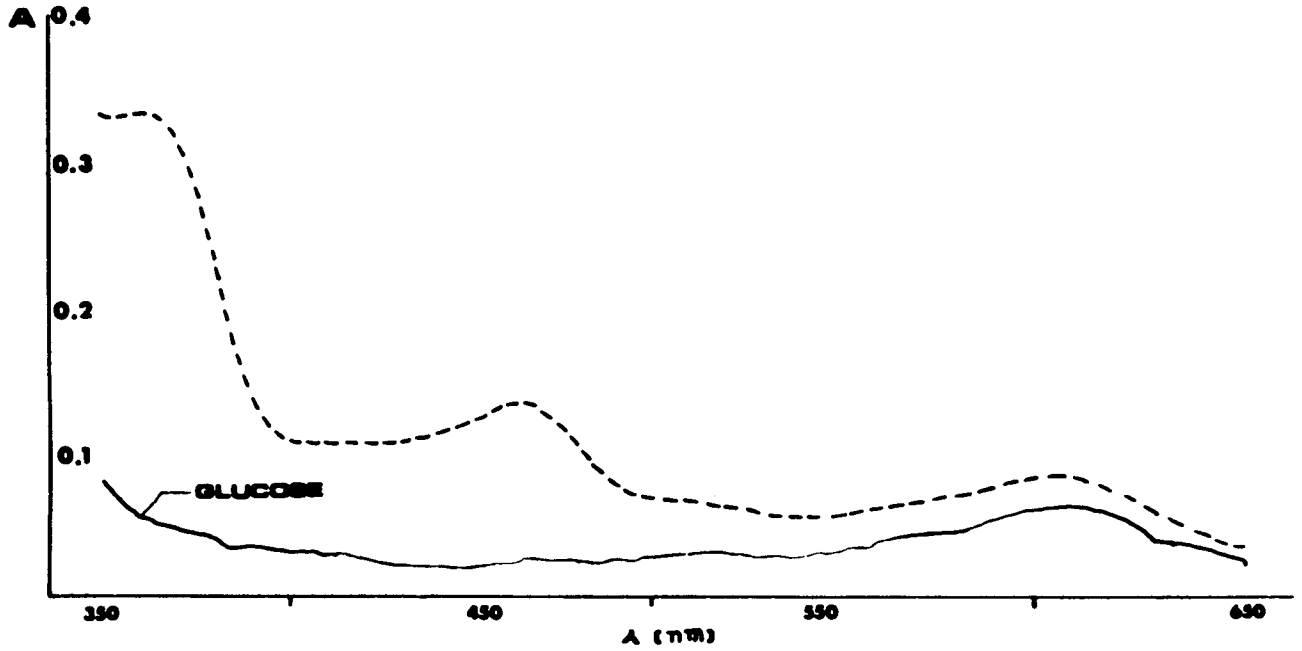
Enzymatic determination of glucose in the hydrolyzed sample is negative. Galactose determination by the galactose oxidase assay reveals what may be a minor component (less than 10% by weight of total sugar in the hydrolysate).

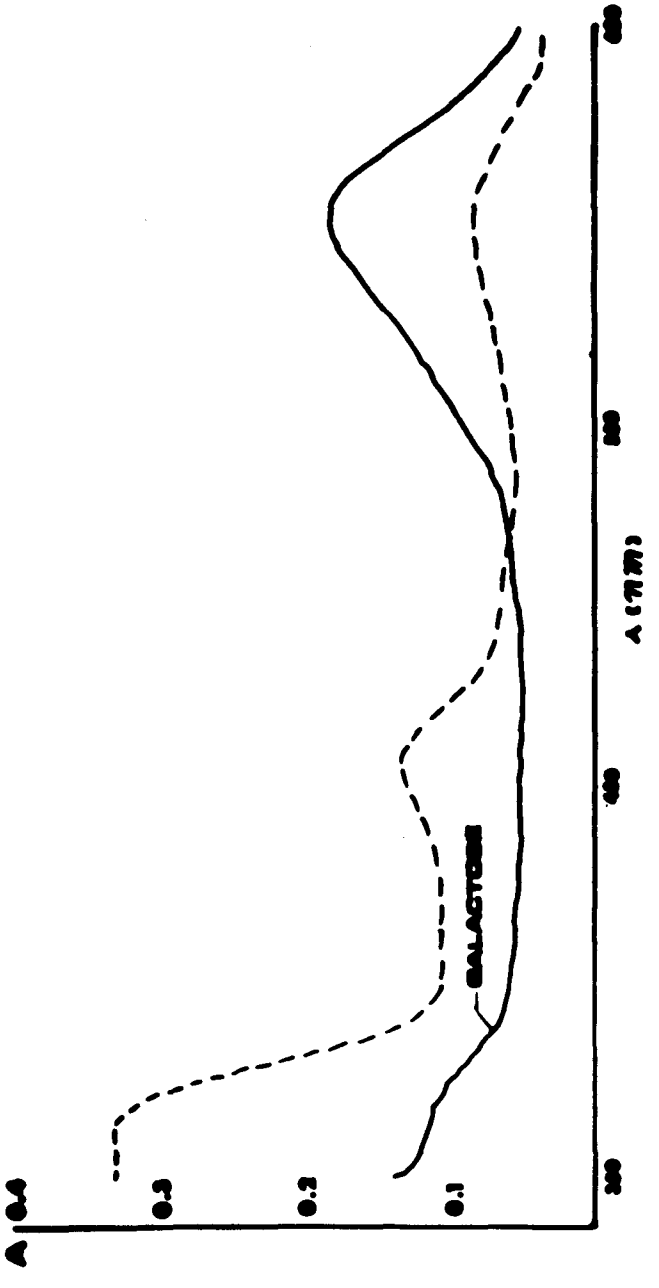
The most convincing evidence for the nature of the composition of the polysaccharide derives from the cysteine-H_2SO_4 reaction. Superimposed absorption spectra, reproduced in Fig. 5, indicate the nearly identical reactivity of Crithidia sample and standard mannose. The polysaccharide fraction comprised as much as 30% by weight of the RNA fraction; i.e., measured as the ratio of anthrone positive material to $A_{260\ nm}$ material.

Uracil-2-^{14}C Pulse Labeling of Crithidia RNA

Cells incubated with uracil-2-^{14}C for various times are used to determine the labeling pattern of Crithidia RNA (Fig. 6). The shorter pulse periods show a substantial amount of heterogeneously sedimenting material whereas an increase in incubation time reveals a peak sedimenting at about 35s. Still longer labeling shows an increase in

Fig. 5. The cysteine H_2SO_4 colorimetric assay of the Crithidia hydrolyzed polysaccharide. Absorption spectra of the Crithidia product (--------) is superimposed on absorption spectra of glucose, galactose and mannose assay standards (————).


A
0.4
0.3
0.2
0.1
GLUCOSE
350
450
550
650
λ (nm)


GALACTOSE

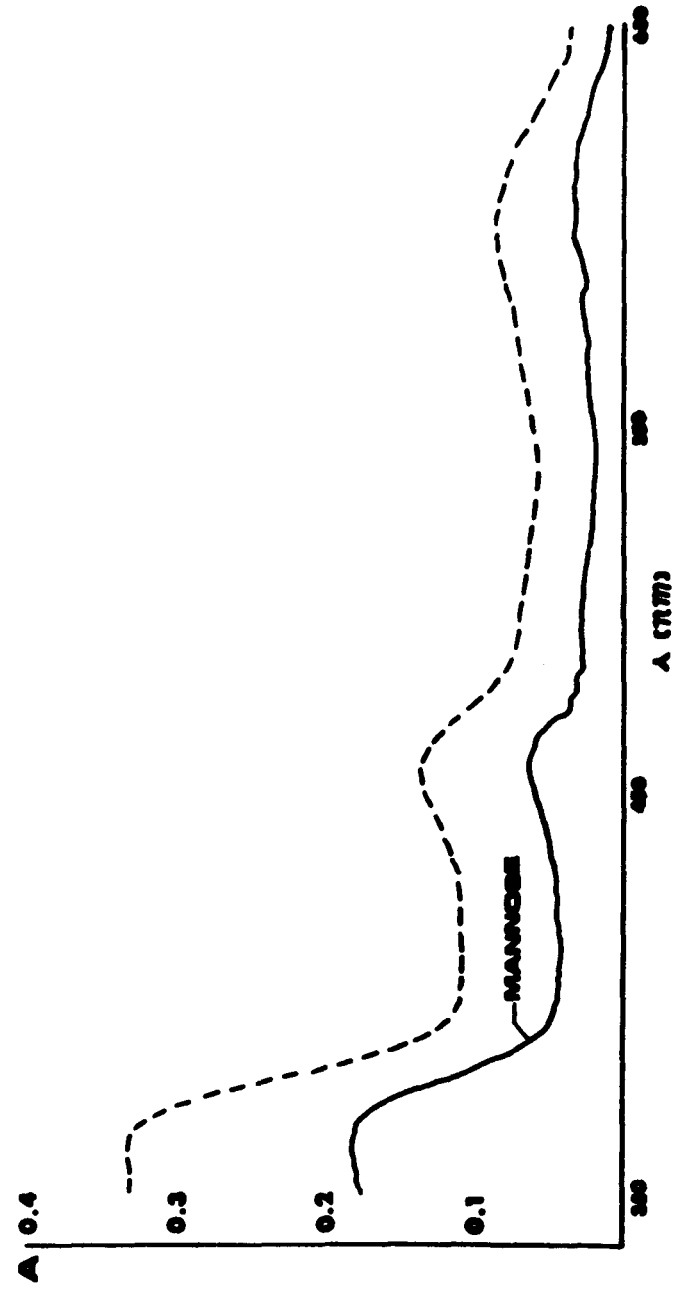
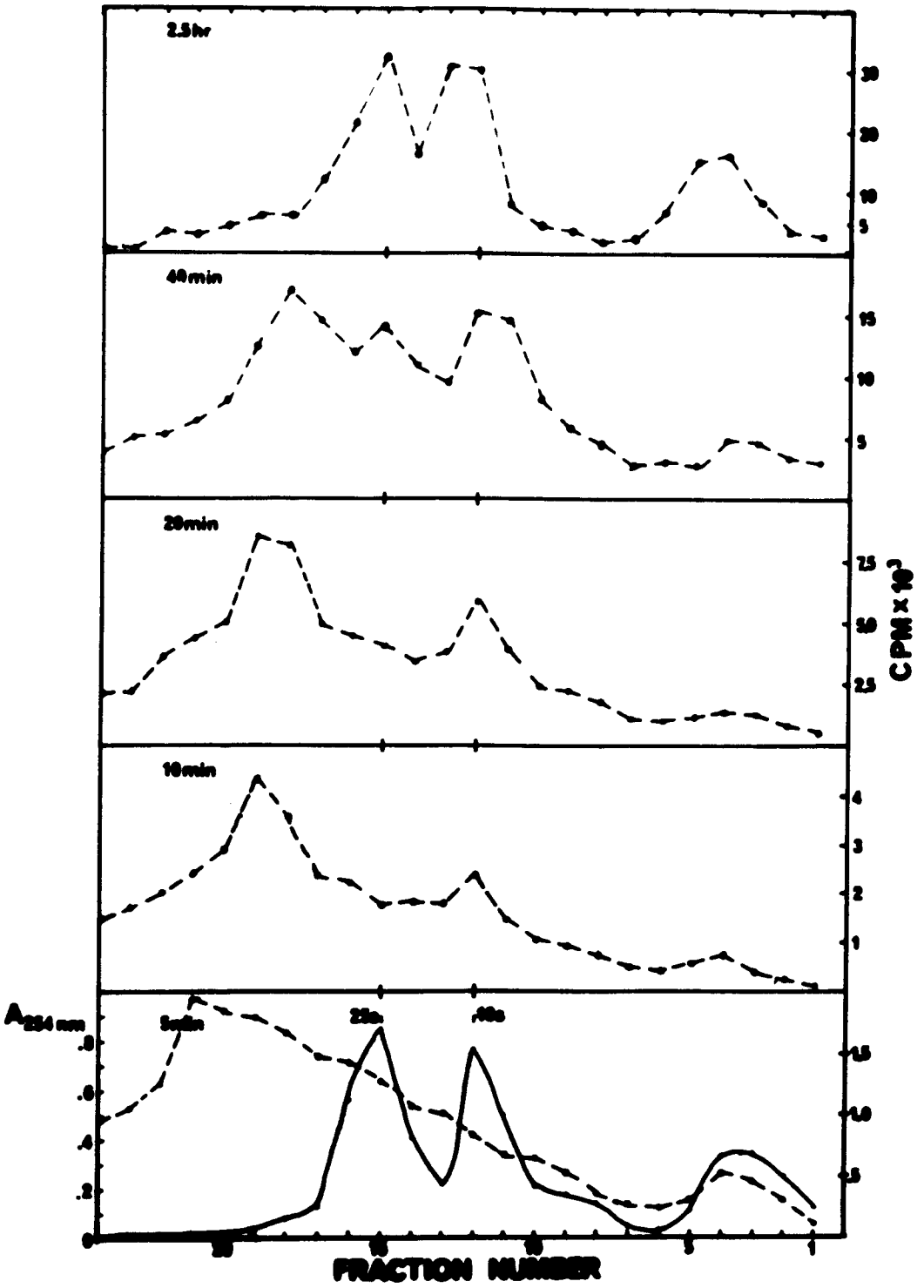

A
0.4
0.3
0.2
0.1
MANNOSE

Fig. 6. Uracil-2-^{14}C pulse labeling patterns of Crithidia RNA. Crithidia was labeled for indicated periods of time, RNA extracted and placed on sucrose gradients (details as in Fig. 1). Absorbance is shown only for 5 min pulse.


2.5hr
40min
20min
10min
5min
25m
40m
$A_{254\,nm}$
CPM x 10^3
FRACTION NUMBER

activity in the 18s region. No detectable peak in the 25s region nor shift in the more heavily sedimenting material is seen at 10 minutes of label. After 40 minutes label appears in both large rRNA fractions and thereafter a steady state is reached during which the radioactivity profile is similar to the absorbance pattern. The labeling pattern as shown in the figure, though not definitive of a precursor-product relationship agrees well with the established findings from other eukaryotic systems (Loening et al, 1969; Perry et al, 1970). That is, the rapidly labeled, heavily sedimenting material serves as precursor for the mature rRNAs.

Methionine-Methyl-^{3}H Pulse Labeling

Methionine-methyl-^{3}H is used to identify the relationship between the more rapidly sedimenting material (nominally 35s) and mature rRNA. Radio-labeled methionine is a particularly useful tracer; it is well known that bulk RNA, i.e. rRNA and tRNA, are methylated (for review see Starr and Sells, 1969) whereas mRNA, defined also as heterogeneously sedimenting RNA, is not methylated to any detectable extent (Moore, 1966). Greenberg and Penman (1966) have shown that the methylation of RNA occurs at the level of the large precursor in HeLa cells.

RNA extracted from intact cells of Crithidia incubated with methionine-methyl-^{3}H for various periods of time are

analyzed and the results shown in Fig. 7. It can be seen that after two minutes of incubation label appears primarily in the 35s region, and only subsequently does label enter into the two mature species. A delay is seen in the labeling of the 25s with respect to the 18s material, i.e. from the figure it is seen that only after 40 minutes does significant activity appear in the 25s peak. These results are consistent with our uracil-2-^{14}C labeling data.

The elimination to a large extent of heterogeneous RNA in radioactivity profiles by use of a methyl label, and the appearance of such label in the 35s region support the view of a precursor-product relationship for rRNA synthesis in Crithidia.

Pulse and Chase Experiments

A next step to help confirm the inferred precursor-product relationship is the use of pulse and chase techniques. Our initial studies have shown that uracil-2-^{14}C is not chased by the addition of cold base. Attempts to chase, i.e. inhibit immediately any further incorporation, with Actinomycin D were unsuccessful; at concentration of drug as high as 50 ug/ml and 2 x 10^7 cells/ml a 50% inhibition of uracil incorporation is achieved, but only after 60 minutes. This apparent resistance to Actinomycin endures even after pretreatment of Crithidia with EDTA. Such treatment confers sensitivity to this antibiotic in otherwise resistant E. coli (Leive, 1965). These results are seen in Fig. 8B.

Fig. 7. Methionine-methyl-^{3}H pulse labeling profiles of Crithidia RNA. Cells were labeled for indicated periods of time, RNA extracted and placed on sucrose gradients (details as in Fig. 1). Absorbance is shown only for two minute pulse and top 1/3 of gradients are not shown.

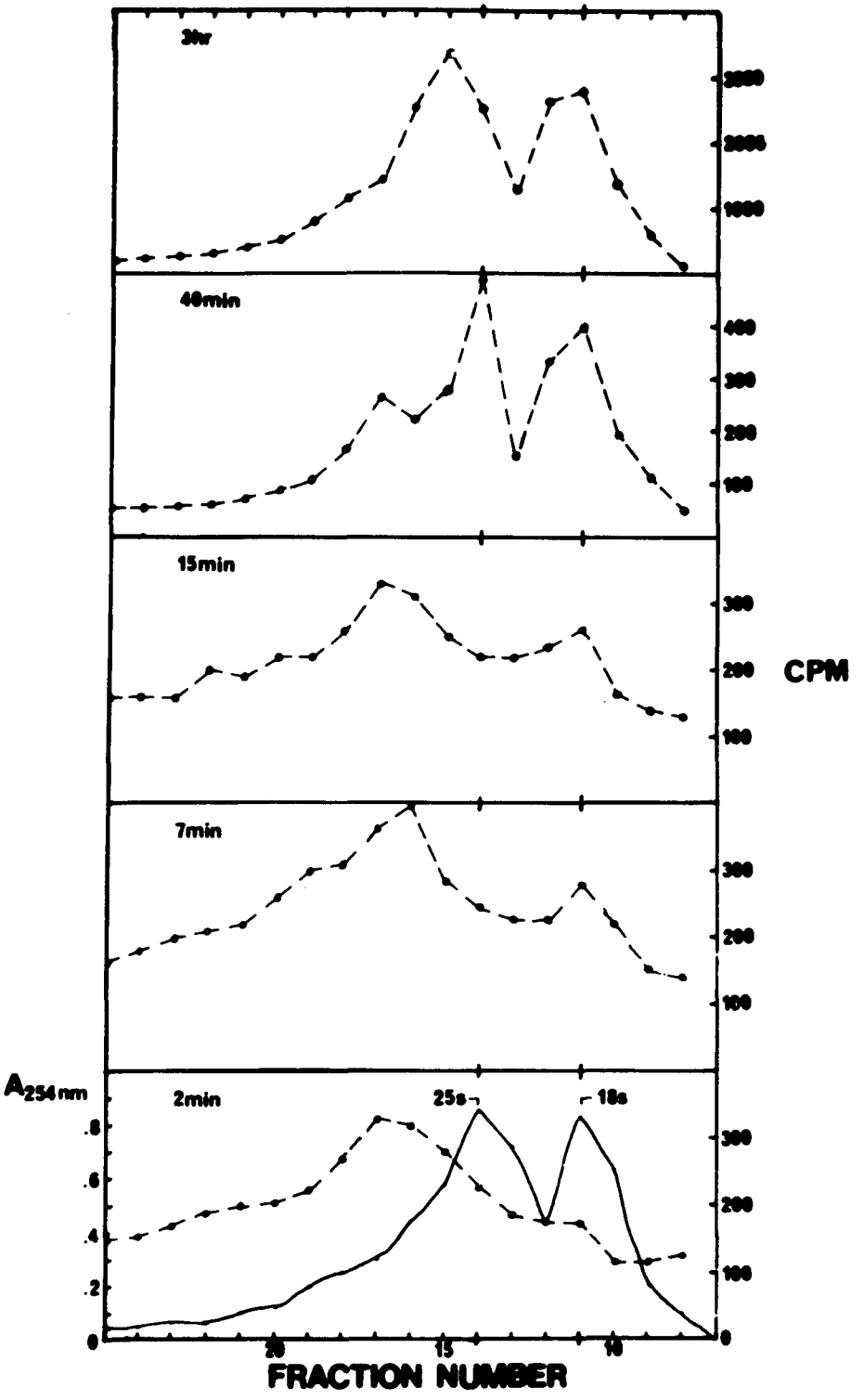
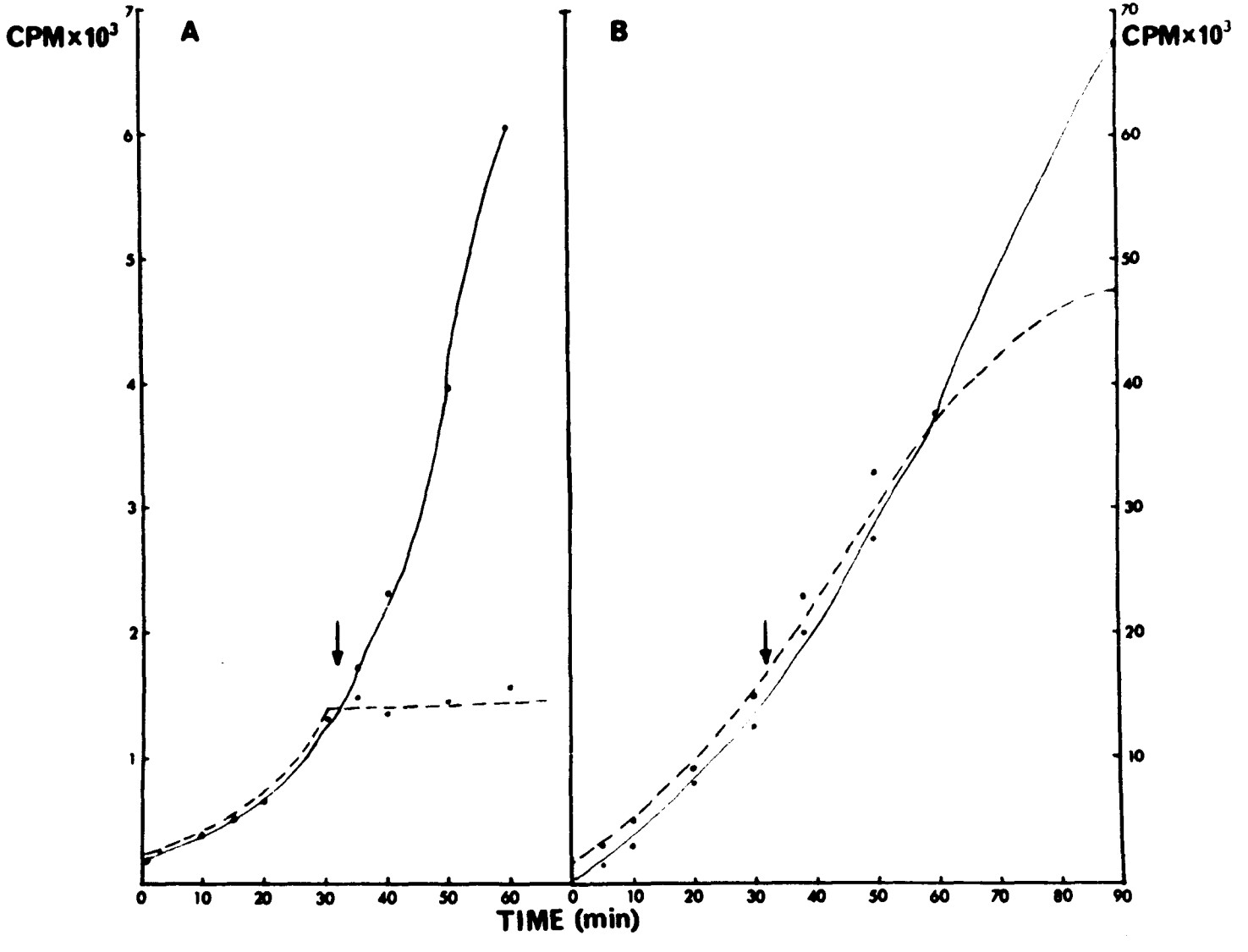

3hr
40min
15min
7min
2min
25s
18s
CPM
A_{254nm}
FRACTION NUMBER

Fig. 8. Pulse and Chase Experiments. A. At 0 time 100 ml midlog cultures of Crithidia received 2 uC of methionine-methyl-^{14}C. At 32 minutes (arrow) the experimental flask (--------) received 1000 fold excess of cold methionine and the control flask (————) an equal volume of distilled water. B. At 0 time 100 ml cultures of Crithidia received 2 uC of uracil-2-^{14}C. At 32 minutes (arrow) the experimental flask (--------) received 25 ug/ml of Actinomycin D. The control (————) received equal volume of distilled water.

Aliquots were removed at points indicated and precipitated with cold 5% TCA. Precipitates were assayed as described in Materials and Methods.


CPM×10³
A
B
CPM×10³
TIME (min)

Crithidia lends itself to pulse and chase with radiolabeled methionine (see Fig. 8A). The addition of excess cold amino acid inhibits any further incorporation of the label. After a one minute pulse of methionine-methyl-^{3}H *Crithidia* is chased with cold methionine. The profiles of the extracted RNA is seen in Fig. 9. The profiles indicate that the material sedimenting in the 35s region serves as precursor for both mature rRNAs, a conclusion made evident from the increase in specific activity in 18s and 25s RNAs with concommitant decrease in the 35s region.

Crithidia Ribosomes

The non-coordinate appearance of label into each of the two large rRNAs predicts a differential synthesis and/or appearance of ribosomal subunits into the cytoplasm. The cytoplasm, defined as the 11,000 x g supernatant fraction, appears free of DNA as shown by standard colorimetric assay (Table I). Therefore gross contamination with either nuclei or kinetoplast occurs.

Figure 10 shows the results of sucrose velocity sedimentation of the 11,000 x g supernatant. At 10 mM Mg^{++}, the optimum concentration for cell free protein synthesis (Kahan *et al*, 1968) virtually no free subunits are present as detected by UV absorption profile (Fig. 10A). However, when the Mg^{++} concentration is reduced in both the homogenization medium and gradients complete dissociation of the

Fig. 9. Crithidia pulse and chased RNA. A 200 ml midlog culture of Crithidia received 2 mC of methionine-methyl-^{3}H for one minute, an aliquot removed and 1000 fold excess cold methionine added. After one hour the remainder was removed. A. RNA extracted from one minute pulsed cells. B. RNA extracted from one minute pulsed and one hour chased cells. Conditions as in Fig. 1.

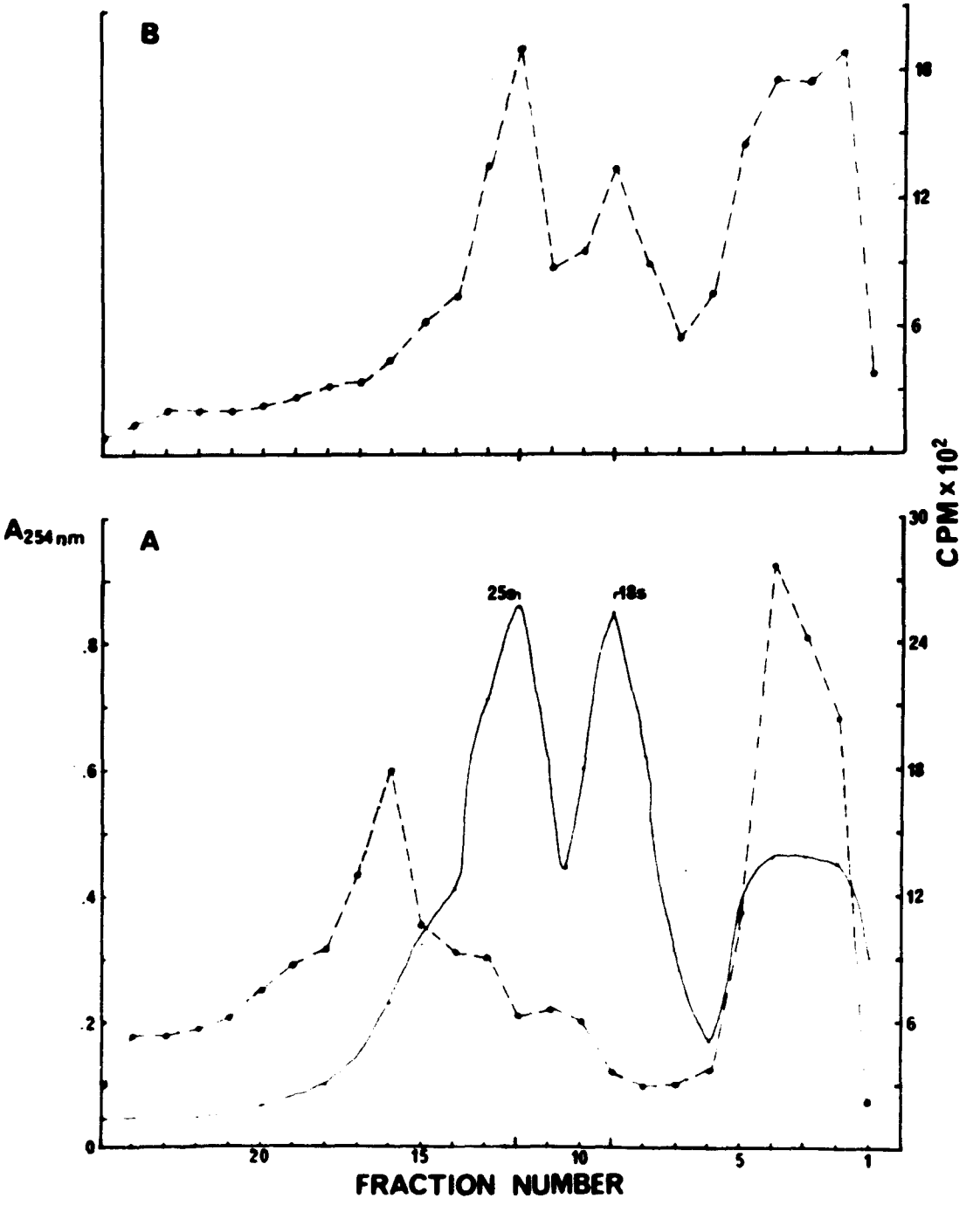
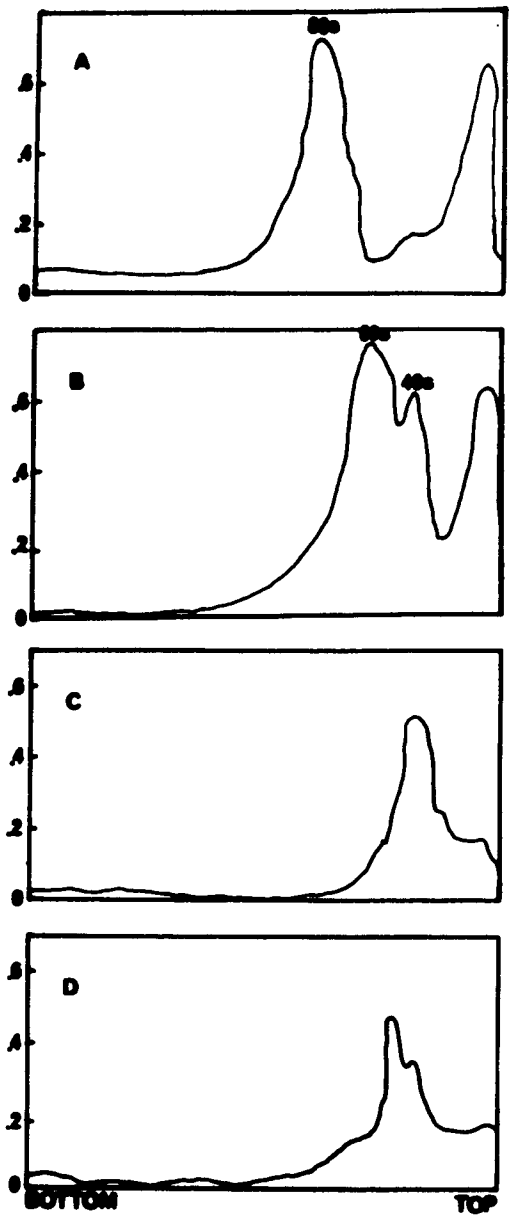

B
A
$A_{254\,nm}$
CPM x 10^2
25s
18s
FRACTION NUMBER
0
.2
.4
.6
.8
6
12
18
24
30
20
15
10
5
1

TABLE I

Tube	DNA	$A_{600\ nm}$
1	40 ug (salmon sperm std.)	0.065
2	80 ug " " "	0.11
3	120 ug " " "	0.16
4	160 ug " " "	0.21
5	0.1 ml (11,000 x g pellet)	0.055
6	0.2 ml (11,000 x g pellet)	0.12
7	1.0 ml (11,000 x g supt. fluid)	0.00

DNA was contained in two ml final volume, 4 ml of diphenylamine reagent was added and reaction mixture boiled for 15 minutes. DNA from 1.5 gm net weight of cells was extracted according to Schneider (1957). Each determination was performed in triplicate.

Fig. 10. Gradient centrifugation of 11,000 x g supernatant fluid and ribosomes. A. 11,000 x g supernatant fraction was obtained as described in Materials and Methods and placed on 15-30% sucrose gradients made up in TKM buffer. B. As for A except no Mg^{++} present in either the isolation buffer or gradients. C. Isolated ribosomes, resuspended, resuspended in TK buffer. Centrifugation as for Fig. 1 except for four hours. D. As for C except centrifuged for 6 hours.

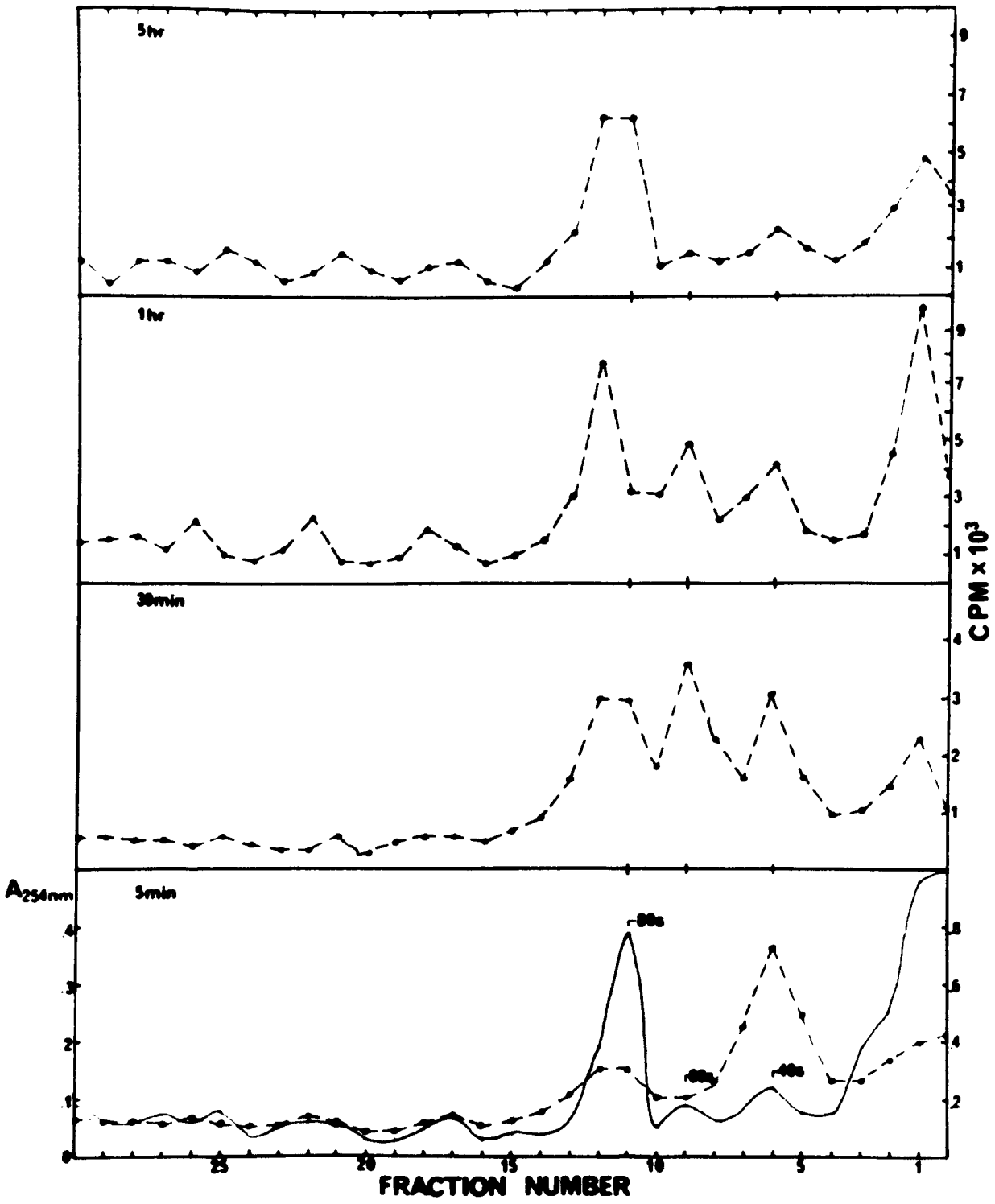

A
B
C
D
.8
.4
.2
0
BOTTOM
TOP

ribosomes into subunits occurs (Fig. 10B). This is in agreement with the results of Kahan et al (1968) and Cross (1970) on isolated Crithidia spp. microsomes and ribosomes. The use of Mg^{++} free gradients make possible the localization of the relative positions of the subunits on the gradients. Unlike the results of Cross (1970), a considerable difference in the sedementation behavior of microsomes and ribosomes is seen; i.e. ribosomes, DOC treated microsomes, sedimented much more slowly than untreated microsomes (Fig. 10C). Even after six hours of centrifugation (Fig. 10D) the ribosomal subunits from the 11,000 x g supernatant fraction have sedemented further than microsomal subunits have in four hours (Fig. 8B). These results apply to both free subunits and intact ribosomes. It appears, therefore, that DOC treatment of the microsomal fraction removes supernatant and/or ribosomal protein or loosens ribosomal conformation. However, these differences may also be attributable to the relatively low K^+ concentration in our buffer compared to that used by Cross (1970).

Uracil-2-^{14}C Pulse Labeling of Cytoplasm

Uracil-2-^{14}C is used to monitor the appearance of ribosomes into the cytoplasm (Fig. 11). After five minutes of incubation label appears primarily in the region of the small subunit. Radioactivity is also seen in the region of the monosomes and polysomes. Only subsequently does label appear in the larger subunit. In addition an increase in

Fig. 11. Pulse labeling profiles of the 11,000 x g supernatant fluid. Cells were labeled for the periods indicated with uracil-2-^{14}C. 11,000 x g supernatant fractions were isolated. The supernatant fluid was placed on gradients and centrifuged as in Fig. 10. Absorbance is shown only for the 5 minute label.

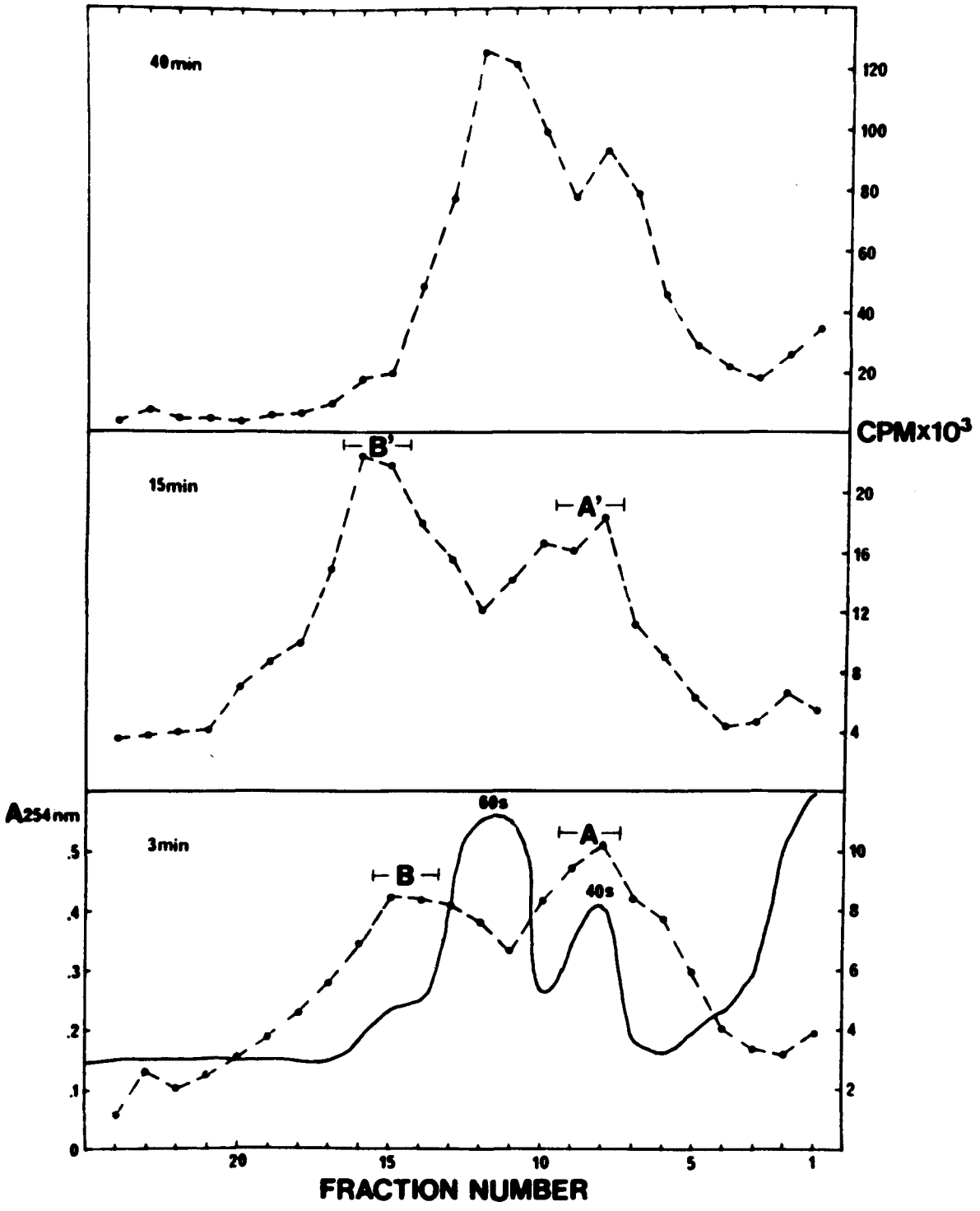

5hr
1hr
30min
5min
CPM x 10³
A254nm
80s
60s
40s
25
20
15
10
5
1
FRACTION NUMBER

labeling time results in an increased ratio of label in the single ribosome as compared to the subunits.

From the results of the uracil-2-^{14}C and methionine-methyl-^{3}H labeling experiments it is known that after short periods of incubation with label radioactivity appears in heterogeneous RNA and 18s rRNA. From this the cytoplasmic data can be interpreted as follows: the label in the small subunit, monosomes and polysomes is due to the labeled mRNA and 18s rRNA. Since mRNA is attached to the small subunit it is expected that whole ribosomes and polysomes are hybrids; i.e. small subunits are labeled whereas the large subunits are unlabeled. To test this, cells were labeled for various lengths of time and homogenized as before, except that Mg^{++} was omitted so as to display only ribosomal subunits (see above) in sucrose gradient centrifugation (Fig. 12). After three minutes of labeling with uracil-2-^{14}C radioactivity is seen in the region of the small subunit and in a rapidly sedimenting region which does not correspond to a UV absorbing peak. The label in this heavy region corresponds in sedimentation properties to single 80s region of the 5 minute pulse pattern of Fig. 11. Labeling for longer periods shows an increased specific activity in the large subunit region.

The label in the rapidly sedimenting region is unexpected. It is possible that radioactivity in the region sedimenting

Fig. 12. Pulse labeling patterns of the 11,000 x g supernatant fluid from cells broken in Mg^{++} deficient medium. Cells were labeled for the periods indicated with uracil-2-^{14}C. 11,000 x g supernatant fractions were isolated in Mg free buffer. Centrifugation was as in Fig. 1 except for 8 hours. Absorbance is shown only for the three minute label. Fractions indicated by bar lines were removed and RNA extracted (see Fig. 13).

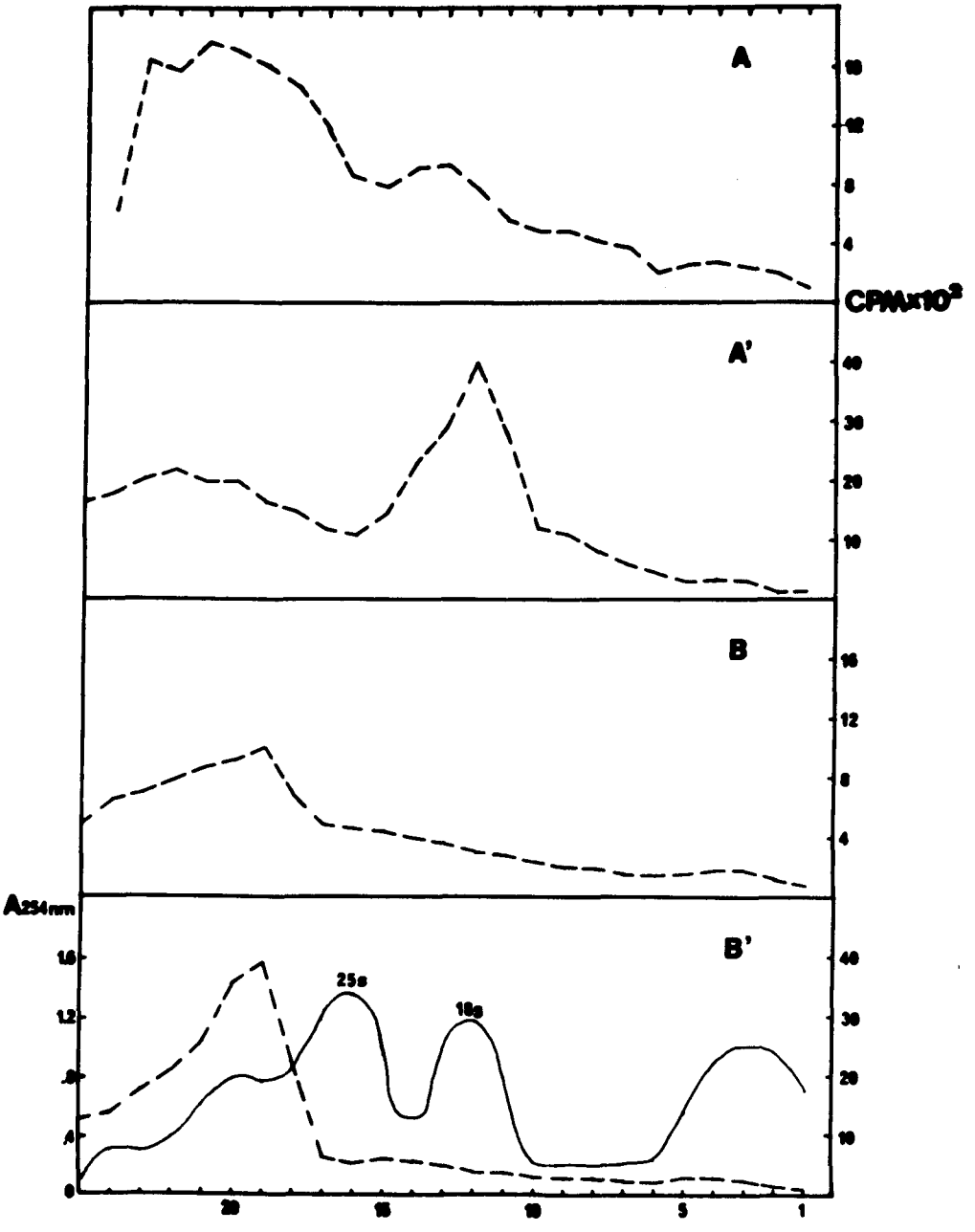

40min
120
100
80
60
40
20
CPMx10³
B'
15min
A'
20
16
12
8
4
A254nm
60s
A
3min
B
40s
.5
.4
.3
.2
.1
0
10
8
6
4
2
20
15
10
5
1
FRACTION NUMBER

faster than 60s subunits represents monosomes, i.e., for some reason these ribosomes, may be stable initiation complexes, which do not dissociate upon removal of Mg^{++}. Alternatively it is conceivable that the particle represents a precursor RNP particle. In order to discriminate between the two alternatives RNA is isolated specifically from those gradient fractions containing this peak. If this rapidly sedimenting particle represents a newly complexed ribosome, it should contain 18s rRNA plus heterogeneous, presumably m, RNA. If, on the other hand, this particle is a precursor ribonucleoprotein particle it should contain a ribosomal precursor RNA or 28s rRNA. The gradient fractions extracted are indicated by bar lines in Fig. 12. As a control, RNA is also isolated from the gradient but from those fractions containing small subunits. The RNAs are extracted from the three minute and 15 minute pulsed cells. The results are shown in Fig. 13.

From the data of Fig. 13 A and A' it is seen that RNA extracted from the small subunit sediments heterogeneously with a peak at 18s. The specific activity of this latter peak is much enhanced in the gradient of the 15 minute pulsed cells as compared to its activity in the gradient of the three minute pulse. Therefore the radioactivity of the 40s subunit is due to mRNA and 18s rRNA.

From Fig. 13 B and B', it is seen that the radioactive

Fig. 13. Identification of RNA species from particles of the 11,000 x g supernatant fluid. The fractions indicated by bar lines in Fig. 12 were removed and phenol extracted with cold carrier supernate as described in Materials and Methods. The isolated RNA was dissolved in TMN buffer and placed on 2-20% sucrose gradients made up in the same buffer. A. Fractions from the small subunit from the 11,000 x g supernate of cells labeled with uracil-2-^{14}C for three minutes. A'. As for A except cells were labeled for 15 minutes. B. Fractions from the rapidly sedimenting particle of the 11,000 x g supernate from cells labeled for three minutes. B'. As for B except cells labeled for 15 minutes. Centrifugation as in Fig. 1.


A
A'
B
B'
CPMx10²
A254nm
25s
18s

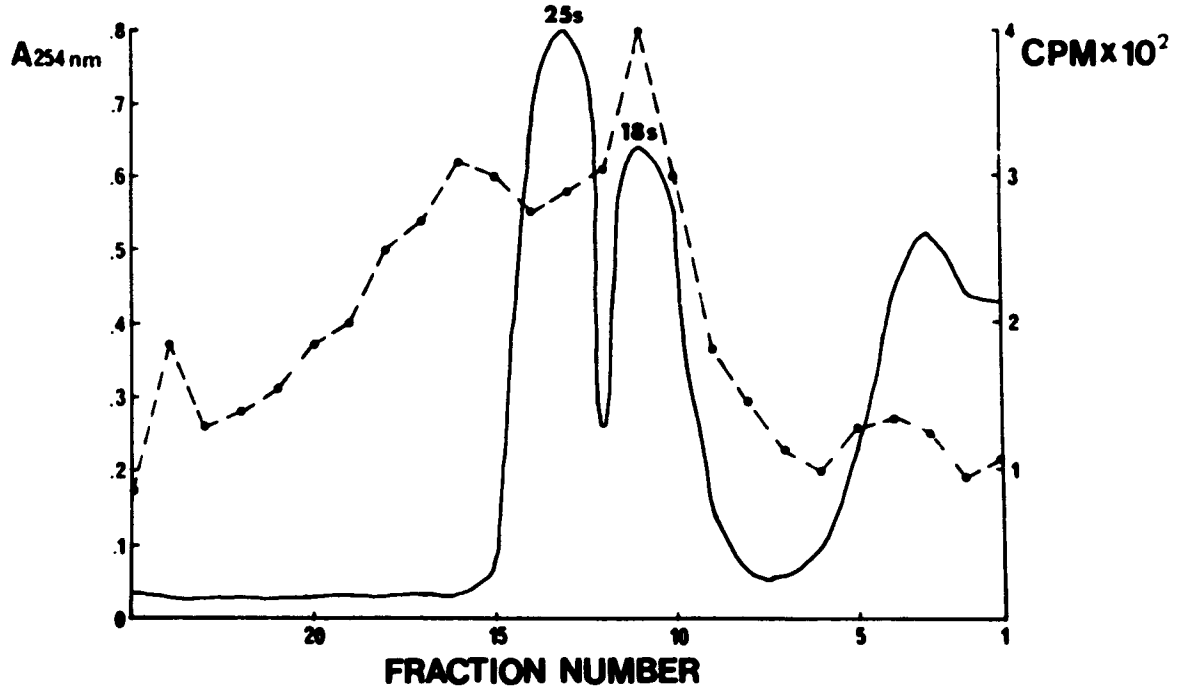
RNA from the approximately 80s particle sediments as uniform band of 35s. It therefore seems likely that this material represents a precursor, containing perhaps rpreRNA. That is, the newly synthesized precursor rpreRNA to the rRNAs may be packaged in a larger presumably ribonucleoprotein particle.

In order to determine whether the above results are artifacts of the Mg^{++} free conditions, the following experiment was performed. Cells are labeled with uracil-2-^{14}C for thirty minutes. The cells are fractionated as described and RNA is released by the SDS method (see Materials and Methods) from the 11,000 x g supernatant fraction. This RNA is centrifuged as shown in Fig. 14. From the data it is seen that not only is there a peak at 18s with a considerable shoulder at 25s, but there is a rapidly sedimenting fraction which may correspond to 35s. Therefore the presence of the putative rpreRNA can be detected in the 11,000 x g supernatant fluids of cells which have been pulsed for sufficiently short periods of time.

Effect of Inhibitors on RNA Synthesis in Crithidia

As has been pointed out in the Introduction protein synthesis inhibitors CA and CX have been used to distinguish between prokaryotes and eukaryotes. In addition by employing these inhibitors one can study the effect of an absence of protein synthesis on RNA synthesis; in particular ribosome synthesis which is presumably under rigid control. As is

Fig. 14. Cytoplasmic (11,000 x g supernate) RNA gradient centrifugation. RNA isolated by the SDS method from 11,000 x g supernate (see Materials and Methods) of cells labeled for thirty minutes with uracil-2-^{14}C. RNA was placed on 5-30% sucrose gradients made up in NT buffer made 0.5% with SDS. Gradients were centrifuged as for Fig. 1 except at 20°.


A254 nm
CPM x 10²
25s
18s
FRACTION NUMBER
.8
.7
.6
.5
.4
.3
.2
.1
0
4
3
2
1
20
15
10
5
1

shown in Fig. 15B, CX, but not CA, inhibits the incorporation of methionine-methyl-^{14}C into cold acid insoluble material. Therefore, in addition to the inhibition of protein synthesis methylated RNA species may not be made after the addition of CX. Examination of uracil-2-^{14}C incorporation following addition of CX shows (Fig. 15A) that RNA synthesis levels off very shortly after the introduction of the drug. It is possible that inhibition of protein synthesis leads to inhibition of RNA synthesis or rapid degradation of newly synthesized molecules.

The difficulty in culturing African trypanosomes has led to the use of Crithidia spp. (e.g. Kahan et al, 1968; Newton, 1966; Wallis, 1966a and b) in studies on the site and nature of activity of trypanosides. Newton (1966) has argued that Antrycide inhibits specifically the synthesis of rRNA in C. oncopelti. In the hope of further elucidating the mechanism of action of this compound, we have treated C. fasciculata with this agent. As shown in Fig. 16, at concentrations of $10^{-6}M$ there is no inhibition of RNA synthesis. At $10^{-4}M$ Antrycide a slight inhibition is noted which is probably due to the precipitation of the medium which begins at this concentration. Studies on overall growth rate indicate a similar negative effect until concentrations are reached at which components of the medium precipitate out of solution. Another important trypanocide, Berenil, also shows neither an effect on the

Fig. 15. Effect of cycloheximide (CX) on incorporation of radioactive label. A. 100 ml cultures of Crithidia received 2 uC of uracil-2-^{14}C at 0 time. At 32 minutes (arrow) experimental flask (---x---x--) received to a final concentration 100 ug/ml of CX and control flask (——·——·—) received equal volume of water. B. 100 ml cultures of Crithidia received 2 uC methionine-methyl-^{14}C at 0 time. At 32 minutes (arrow) one experimental flask (---x---x--) received 100 ug/ml of CX; another flask (---o---o--) received 100 ug/ml of CA and the control flask (——·——·—) received an equal volume of distilled water. Aliquots were removed at points indicated and precipitated with cold 5% TCA.

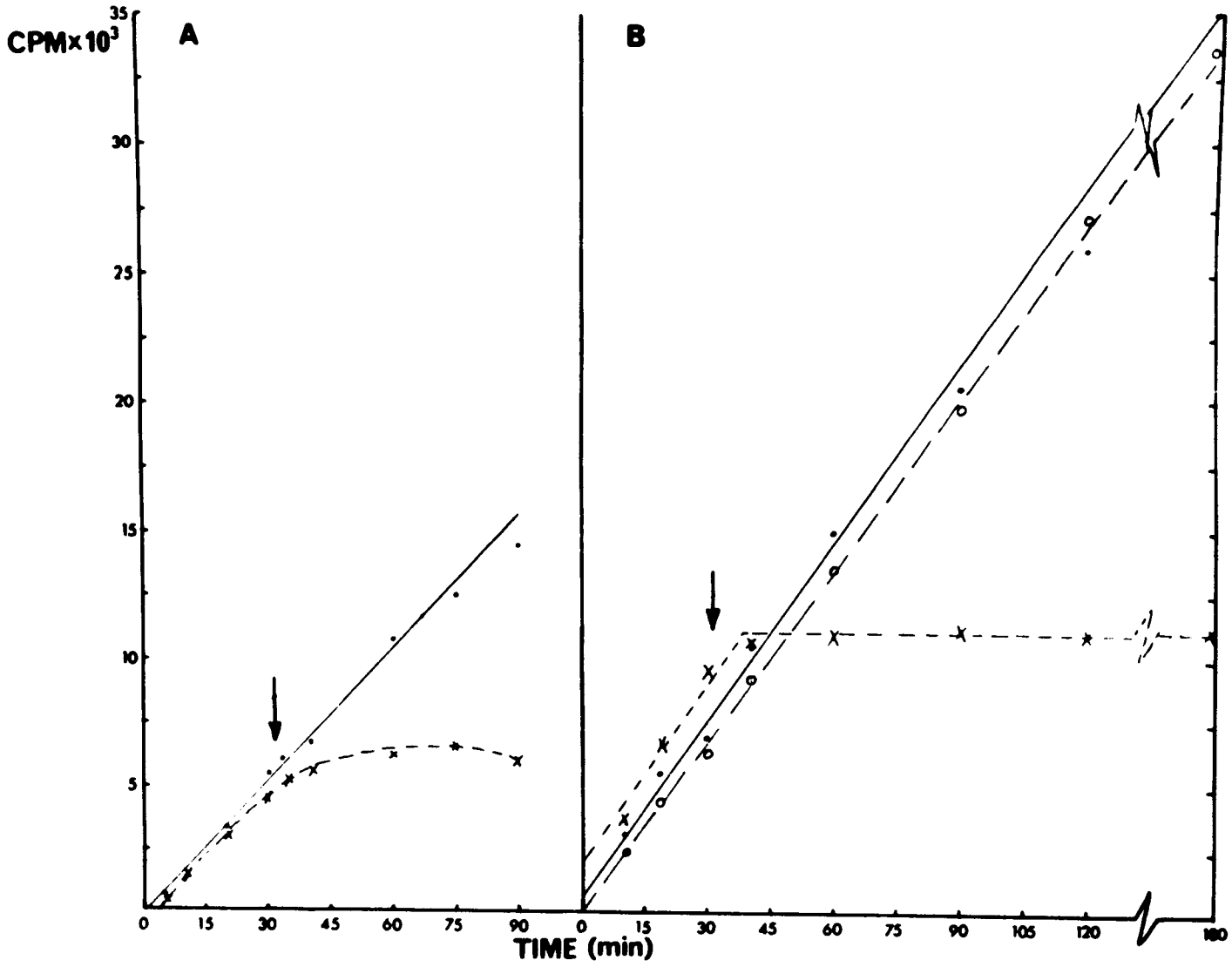
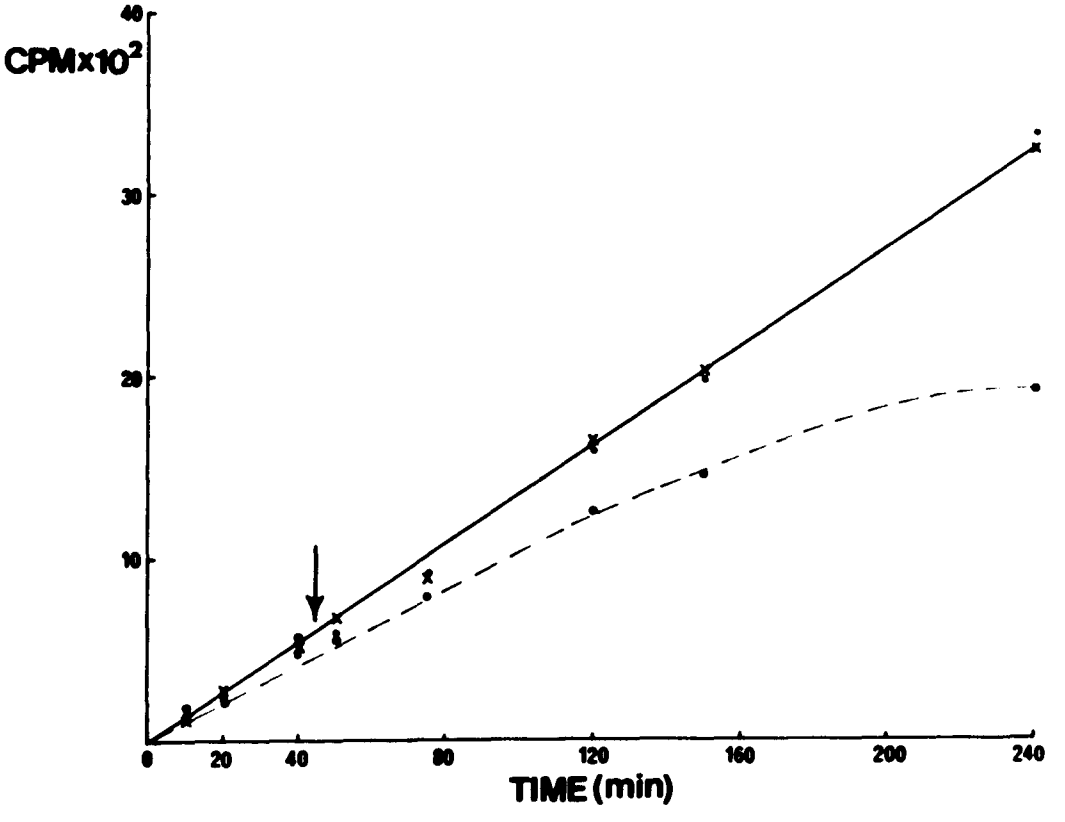

CPM×10³
35
30
25
20
15
10
5
A
B
0
15
30
45
60
75
90
0
15
30
45
60
75
90
105
120
180
TIME (min)

Fig. 16. Effect of Antrycide on the incorporation of uracil-2-^{14}C. 100 ml cultures of Crithidia received 2 uC of label at 0 time. At 45 minutes (arrow) one experimental flask (——x——x——) received one ml of $10^{-4}M$ antrycide (final concentration $10^{-6}M$); another experimental flask (---.---.--) received one ml of $10^{-2}M$ antrycide (final concentration $10^{-4}M$); control flask (——·——·—) received equal volume of water. Aliquots were removed at points indicated and precipitated with cold 5% TCA.


CPMx10²
40
30
20
10
0
20
40
80
120
160
200
240
TIME (min)

growth nor RNA synthesis of our strain of C. fasciculata (M. Zahalsky, pers. comm.). As in the case of Antrycide, Bernil is used at concentrations far above those normally employed in vivo; i.e., those concentrations exhibiting trypanocidal activity.

DISCUSSION

This study investigated the ribosome of the protist Crithidia fasciculata. We hoped thereby to add to our understanding of the biological characteristics of the Trypanosomatidae -- a clinically and economically important group of organisms.

Phenol Extraction of Crithidia

A polysaccharide, as noted above, was detected in the aqueous phase of phenol extracted cells. There are few detailed reports (for review see von Brand, 1966) on the presence and composition of polysaccharides in Trypanosomatidae. Galactose containing polymers in culture forms of Trypanosoma cruzi and Crithidia fasciculata were reported by von Brand (1959) and Cosgrove and Hanson (1962) respectively. The sole monomeric component of these carbohydrates was identified by two chromatographic systems of only limited value for separating aldohexoses. These carbohydrates were contaminated by nucleic acid fragments thought by us to be artifacts of the isolation procedure; i.e., alkaline hydrolysis of whole cells.

Identification of a mannose polysaccharide in our strain of Crithidia raises the question of its universality in

Trypanosomatidae, in fact even among different strains of Crithidia. Also, are these polysaccharides storage products or structural components? Storage carbohydrates are not unknown in parasitic organisms. Glycogen-like polymers have been reported in other parasitic flagellates, specifically Trichomonadidae (Manners and Ryley, 1955; Shorb, 1964) and many other parasites including mesozoa, helminths and arthropods (von Brand, 1966).

Ryley (1955) noted in C. oncopelti a small amount of material, estimated as glycogen, was present but not metabolized under conditions of endogenous respiration. Read (1970) notes a lack of phosphorylase in trypanosomes further suggesting an inability to utilize storage polysaccharides. Furthermore Cosgrove and Hanson (1962) reported the presence of periodic acid Schiff positive material only in pellicular regions of Crithidia suggesting that the polysaccharide isolated from this organism was supportive rather than reserve in nature. Other non-D-glucopyranose polymers have been isolated, of these immunologically active ones have been reported from T. cruzi (von Brand, 1966) and from Trichomonas foetus (Feinberg and Morgan, 1953). Recently a serologically specific polysaccharide, presumably surface coat, was identified as containing both galactose and mannose (Njogu and Zahalsky, unpublished observations). Evidence from electron microscopy (Wright and Hales, 1970) suggests that a component of the

pellicle of T. brucei is periodic acid Schiff positive and that carbohydrate is associated with protein, as determined by trypsin digestion. This surface material is non-anionic since staining with colloidal iron was negative either before or after protease action. These latter findings can be extended to Crithidia since this species is not retained on DEAE cellulose filters (pers. obs.); a technique used to separate bloodstream trypanosomes from other cellular components.

Crithidia rRNAs

As noted in Fig. 3 the presence of the carbohydrate does not interfere with the detection of RNA in the preparations, and essentially can be ignored. The smaller rRNA of Crithidia exhibits the same sedimentation properties as rat liver 18s and is larger than E. coli 16s rRNA. The larger rRNA sediments faster than the E. coli bacterial standard, but is smaller than rat liver 28s (Fig. 1). Among eukaryotes the size of the larger rRNA parallels the relative phylogenetic position of the organism (Loening, 1968). Exceptions to these findings appear, and include in protozoa Amoeba and Euglena in which the larger of the rRNAs have unusually high sedimentation coefficients (Craig and Goldstein, 1969; Loening, 1968). The significance of these exceptions is unclear.

The 25s rRNA of Crithidia is readily degraded to slower sedimenting material under conditions which may produce a

disruption of hydrogen bonding in a discontinuous polynucleotide (Fig. 2). That is, incubation of isolated Crithidia RNA at elevated temperature results in the selective degradation of the 25s rRNA; other species are resistant to such treatment. Similar findings in Acanthamoeba (Stevens and Pachler, 1970); silkmoth pupae (Applebaum et al, 1966); Drosophila (Greenberg, 1969) and Tetrahymena (Bostoch et al, 1971) suggest a state of association of two RNA molecules held together by noncovalent bonding perhaps induced by an extremely specific nuclease. As pointed out by Applebaum et al (1966) and our personal observation the degradation is inhibited by the presence of Mg^{++}. The identification of specific degradation of rRNA has not been limited to the larger molecule. Nemer and Infante (1967) observed a specific conversion, induced by heating, of 18s rRNA onto 13s material of RNA extracted from the eggs of sea urchins, Strongylocentrotus purpuratus. Nor is such degradation limited to eukaryotes as evidenced by the erroneous belief based on such degradation data that bacterial 23s rRNA was in fact a dimer of 16s molecules (Midgley, 1965).

Ribosomal RNA Synthesis in Crithidia

Crithidia synthesizes its ribosomes in a manner typical of eukaryotes, i.e. an initial large precursor (rpreRNA) is rapidly synthesized. This rapidly labeled precursor material can be chased into the two high molecular weight rRNAs of the ribosome. From its position in the sucrose gradient the

rpreRNA was assigned a sedimentation coefficient of 35s. This value is consistent with an apparent dichotomy in the size of eukaryotic precursor molecules (Loening et al, 1969; Perry et al, 1970); i.e., the precursor or transcriptional unit for rRNA is considerably larger in birds and mammals than in the remaining eukaryotes.

In Crithidia, as in other eukaryotes, methylation of rRNA occurs at the level of the large precursor. After one minute of incubation with methionine-methyl-^{3}H radioactivity is distributed in the region of the suspected precursor (Fig. 7). This distribution is skewed to the lighter side suggesting that partially transcribed molecules are methylated as they are being polymerized (Greenberg and Penman, 1966). Significant radioactivity sediments faster than the rpreRNA. Such sedimentation behavior may be attributable to artifactual aggregation due to the relatively large amount of material placed on these gradients. All the gradient patterns of RNA labeled with methionine-methyl-^{3}H show heterogeneously sedimenting material. Although there is a purine requirement in Crithidia it has been suggested that under conditions of labeling the methyl label may enter into the purine ring during de novo synthesis. In other cell systems such incorporation is inhibited by addition of adenosine and guanosine to the labeling medium (Weinberg et al, 1967). The necessity for the large quantity is the low specific activity of methyl labeled RNA -- an indication of a low level of methylation in this species.

Under normal conditions the number of ribosomes in a cell generally double prior to a subsequent division. It is not surprising, therefore, that the rate of processing of rpreRNA into mature RNA species is proportional to the growth rate. Pulse labeling studies (Fig. 5 and 6) on Crithidia having a generation time of 6 hours show that 18s rRNA is detected earlier than the similar processing step in HeLa cells, where the generation time approximates 24 hours (Penman, 1966). Rapid processing of precursor material is also seen in other protists; e.g., yeasts (Taber and Vincent, 1969b), Tetrahymena (Kumar, 1970) and Dictyostelium discoideum (Iwabuchi et al, 1971).

In eukaryotic systems studied to date intermediates in the processing of rpreRNA have been identified (Weinberg and Penman, 1970 for example). In particular, another precursor polynucleotide makes its appearance coincident with the 18s rRNA. This second precursor polynucleotide apparently is the remainder of the first detected precursor after the 18s has been cleaved off; thus this material represents a specific precursor to the larger rRNA. We have failed to detect in the Crithidia system such a specific precursor to the 25s rRNA, although some eighteen minutes elapse before significant label sediments in the 25s region after the appearance of the label in the 18s peak. We may offer the following hypotheses to help explain this observation: 1) In Crithidia the 18s and 25s rRNAs may be synthesized as one

polynucleotide, from which, subsequent to cleavage of the 18s rRNA, the remaining fragment exhibits sedimentation properties identical to the initial precursor molecule. 2) Alternately, each species may be synthesized separately as a large and distinct precursor molecule. Accordingly, the 18s would be made and the remnant of its specific precursor degraded to acid soluble constituents. The existence of two immediate precursors has been suggested for rRNA synthesis in carrots (Leaver and Key, 1969; Loening, 1970). From other studies involving DNA-RNA hybridization competition studies and RNase digestion pattern analyses (see for example, Quagliarotti et al, 1970; Reeder and Brown, 1970) it seems unlikely that two distinct precursors, one for each mature rRNA, would be made, at least not in those organisms studied.

An additional hypothesis to account for the origin of the 25s conceives of an initial processing step in which the larger rpreRNA is cleaved at several sites; subsequently the 25s is put together from non-covalently bound segments of the precursor molecule. This view is consistent with the presence of a postulated nick in the 25s molecule. However, short pulses with methionine-methyl-^{3}H reveal acid insoluble material only in the 18s region and not in the shoulder trailing the 18s peak (Fig. 6). If the 25s is ultimately put together from smaller segments one would expect to find components sedimenting nearly identically to the segments

obtained from heat treated 25s; none are found.

Should future results on Crithidia and other eukaryotes reveal the precessing of 18s and 25s from one initially transcribed, polycistronic molecule, it would perhaps reveal a selective advantage to a eukaryotic cell for coordination of rRNA synthesis and hence ribosome subunit synthesis. Although such coordination can be achieved in eukaryotes in other ways; as exemplified by control of 5s synthesis (Brown and Weber, 1968). Prokaryotes, similarly, may coordinate the synthesis of 16s and 23s rRNAs without the aid of a common precursor. The presence of rRNA precursors in such presumably primitive eukaryotes as a dinoflagellate (Rae. 1970), an organism lacking histones, and Crithidia may make this a fundamental distinction between prokaryotes and eukaryotes. A recent study (Brown and Haselkorn, 1971) has demonstrated a ribosomal precursor RNA and eukaryotic-like maturation pattern in Euglena, a photosynthetic flagellate.

Entry of Ribosomes and Subunits into Crithidia Cytoplasm

In order to determine how and where ribosome synthesis proceeds in Crithidia one objective was to isolate intact nuclei and other subcellular fractions. The separation of cell homogenates into purified fractions failed, apparently due to a similarity in size and density of the nucleus and combined kinetoplast-mitochondrion complex. We, therefore, elected to study the kinetics of entry of the ribosomes, subunits and rRNAs into the cytoplasm (defined as the DNA

negative, 11,000 x g supernatant fluid). Pulse labeling studies in Crithidia showed that label appears in the smaller subunit, ribosomes and polysomes after a five minute pulse with uracil-2-^{14}C (Fig. 11). This result, which shows an absence of label in the larger subunit, suggested to us that label in polysomes and single ribosomes derives from the smaller subunit; i.e., 18s rRNA plus mRNA. That is according to the ribosome cycle, as demonstrated in both eukaryotic and prokaryotic systems (Guthrie and Nomura, 1968; Kabat and Rich, 1969; Kaempfer, 1969; Sobol et al, 1970), the newly emerged smaller subunit may initiate a new round of translation by coupling with mRNA and a preexisting cytoplasmic 60s subunit. To test this the 11,000 x g supernatant fraction was isolated under Mg^{++} free conditions which would allow for the display of only ribosomal subunits in sucrose gradients (Fig. 12). We had expected to find, in the gradient patterns of cells pulsed for short periods of time, label associated only with the small subunit; however, there was a peak of radioactivity sedimenting faster than 60s subunits at about 80s. There existed at least two possibilities to explain the nature of this particle. On the one hand, this material may actually be whole ribosomes, which do not dissociate in the absence of Mg^{++}. Such intact ribosomes may represent stable complexes which are involved in initiating a new round of translation. Alternatively the 80s material may be a precursor particle. Investigations (Taber et al, 1970; Leick and Plesner, 1968a) from a number of systems indicated the presence of mature

rRNA species within subunit precursors in the cytoplasm. In vertebrate cells (Warner and Soeiro, 1967; Rogers, 1968) precursor ribonucleoprotein particle, containing ribosomal precursor RNA species, have been demonstrated in the nucleus. To help clarify the nature of the rapidly labeled material of the Crithidia 11,000 x g supernatant fraction, the gradient fractions containing it were isolated and RNA extracted from them (Fig. 13). In addition RNA was extracted from fractionated 40s subunits. The results show that no mature 18s and 25s rRNA was found in the 80s particle. The sedimentation behavior of the extracted RNA is very similar to that of rpreRNA. It would appear that the rpreRNA is contained within a particle which sediments at 80s. This would also explain the presence of label in whole ribosomes in the five minute profile shown in Fig. 11. The presence of such a particle may indicate the contamination of the 11,000 x g supernatant fraction. As indicated in the Introduction the site of synthesis and initial processing of ribosomes is clearly the nucleolus. If this 80s material is not cytoplasmic its presence in the 11,000 x g supernatant fluid may arise from damage to the nucleus which selectively frees the precursor whereas DNA is not released (as detectable by the diphenylamine reaction).

The labeling pattern of RNA extracted from the small subunit shows clearly the presence of mature 18s rRNA and heterogeneously sedimenting, presumably messenger, RNA. This

is consistent with the role of the small subunit in protein synthesis. This would not necessarily indicate that newly emerged small subunits are programmed within RNA, since the subunits may be derived from polysomes during the Mg^{++} free conditions. In fact in HeLa cells Gerard *et al* (1965) have shown that newly emerged small subunits contain only 18s rRNA.

The *Crithidia* Ribosome

The *Crithidia* ribosome readily dissociates into its subunits upon depletion of Mg^{++} (Fig. 10). These observations are in agreement with the results of Kahan *et al* (1968) on *C. fasciculata* and *C. onocopelti* (Cross, 1970). The dependency on Mg^{++} and ready reversibility of the subunits to reassociate is usually associated with prokaryotic systems but is also seen in another flagellated eukaryote, i.e. *Chlamydomohas* (Sager and Hamilton, 1967). The ribosomes of higher cells dissociate and reassociate if treated with high concentrations of K^{+} (Martin and Wool, 1969). The physiological significance of Mg^{++} induced dissociation as a step in a ribosome cycle involving subunits seems questionable because it has been proposed that: 1) both eukaryotic and prokaryotic ribosomes dissociate prior to a new round of translation (Kaempfer *et al*, 1968; Kaempfer, 1969); and 2) specific protein factors are held to be responsible for dissociation in *E. coli* (Subramanian and Davis, 1970) and yeast (Petre, 1970).

We have observed a change in sedimentation behavior after the purification of ribosomes from the microsomal fraction (Fig. 10). Olsnes (1970) has reported the removal of protein from ribosomes by DOC treatment in the presence of EDTA. Although EDTA is not present we feel that such DOC treatment, used in preparation of Crithidia ribosomes, capable of carrying out cell free protein synthesis, accounts for the change in sedimentation behavior.

Activity of Inhibitors and Trypanocides on Crithidia

The protein synthesis inhibitors, CX and CA have been widely used to distinguish between "80s" and "70s" ribosomes. Our experiments (Fig. 15) show that Crithidia is refractory to CA, whereas CX produces an immediate cessation of further incorporation of methionine. CX has also been used to study control of RNA synthesis, in particular rRNA, after protein synthesis has shut down. From Fig. 15 it is seen that addition of CX inhibits further incorporation of methionine-methyl-^{14}C and uracil-2-^{14}C into cold TCA insoluble precipitable material. These data suggest that inhibition of protein synthesis may inhibit further syntheses of stable RNA or if synthesis continues then there must be rapid degradation. Yeast cells (Taber and Vincent, 1969a) treated with CX show buildup of precursor material (rpreRNA) without a subsequent cleavage. Starvation of HeLa cells for methionine results in a similar inhibition of cleavage of the 45s rpreRNA, though synthesis of 45s itself may not be

affected (Maden, 1968; Vaughn *et al*, 1967). Preliminary results have indicated that *Crithidia* starved for methionine continue to synthesize and process the rpreRNA normally.

Newton (1962) has suggested that *Crithidia* can serve as a model for investigations on the mode of action of some clinically effective trypanocides on members of the genus *Trypanosoma*. We cannot share this view in light of recent studies in our laboratory. That *Crithidia* is not a useful model organism for drug studies may be seen from the following: Newton (1966) reports that Antrycide specifically inhibits the appearance of label into a ribosomal pellet of *C. oncopelti*, a finding which suggests that rRNA synthesis alone is inhibited by the drug. Were this indeed the inhibitory response, our system might make possible the identification of the site(s) of drug action. Selective inhibition by drugs of rRNA synthesis is not unknown as evidenced by studies with Actinomycin D (Perry and Kelley, 1970 and references therein) and Cordycepin (Siev *et al*, 1969). In our studies (Fig. 16) the growth of *Crithidia fasciculata* was not inhibited by antrycide (10^{-4} to 10^{-7}M) nor was the incorporation of uracil-2-^{14}C into acid insoluble material stopped or slowed down, until the concentration of drug produced a marked precipitation of the medium. Similar results have been obtained with Berenil and confirmed in a recent report by Newton (1970) where *C. fasciculata* rather than *C. oncopelti* was studied.

Antrycide and Berenil plus other trypanocides inhibit both the charging and transfer reactions equally well in in vitro protein synthesizing systems obtained from Crithidia (Kahan et al, 1968) and rat liver (Landez et al, 1969). The failure to detect in vitro effects by Antrycide and Berenil specific to Crithidia coupled with the lack of in vivo effects on Crithidia by these drugs indicate that the lack of sensitivity may be due to permeability barriers. Evidence that a differential toxicity of these trypanocides on Crithidia and Trypanosoma may be due to permeability phenomena is that isolated ribosomes of a pentamidine resistant strain of C. oncopelti (Wallis, 1966a) show as similar an affinity to pentamidine as do ribosomes from a sensitive strain. In addition, resistant C. oncopelti cells take up much less drug than do sensitive cells. Furthermore, Ethidium bromide (Riou and DeLain, 1969) and Berenil (Newton and LePage, 1967) appear to react most readily with the kinetoplast DNA. Thus, the fact that vertebrate bloodstream trypanosomes are KCN insensitive and may be made dyskinetoplastic (Hill and Anderson, 1970), presumably due to repression of the kinetoplast-mitochondrion complex, strengthens the case against studies on the action of trypanocides on Crithidia which have an active cytochrome system.

The advantages that accrue to Crithidia as a representative trypanosomatid, namely its ability to be grown in bulk and under defined conditions outside of its natural host, its

ability to be stored and its clonability (Hutner et al, 1968) offer excellent opportunity for biochemical studes of a flagellated protozoan. These advantages also make practical studies on the evolutionary position and relationships of this organism to other eukaryotes. However, these practical advantages alone plus our in vitro and in vivo studies do not support the contention that Crithidia is a valid model organism to assess the mode of action of drugs that are active against Trypanosoma. Since African blood-stream trypanosomes may be maintained in the laboratory in suitable mammalian hosts, we believe that infectious bloodstream trypanosomes should be used to study the mode of action and selective toxicity of trypanocides. The value of Crithidia appears to be in its use as yet another convenient eukaryote for studies in cell biology.

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