54.6; dT-dT, 59.6; pdC, 60.9; dCp, 64.5; pdT, 66.5; dTp, 68.3; pA, 76.3; pdA, 77.3; dAp, 79.8; Ap (2′,3′), 86.1, 96.3; pdG, 88.8; dGp, 91.2; pAp (2′,3′), 110, 114.8. The amount of each degradation product was determined spectrophotometrically.

References

Bennett, G. N., Mackey, J. K., Wiebers, J. L., and Gilham, P. T. (1973), *Biochemistry 12,* 3956.

Bon, S., Godefroy, T., and Grunberg-Manago, M. (1970), *Eur. J. Biochem. 16,* 363.

Chou, J. Y., and Singer, M. F. (1971), *J. Biol. Chem. 246,* 7486.

Feix, G. (1972), *Biochem. Biophys. Res. Commun. 46,* 2141.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38,* 470.

Heppel, L. A. (1966), *Procedures Nucleic Acid. Res.,* 31.

Ho, N. W. Y., and Gilham, P. T. (1973), *Biochim. Biophys. Acta 308,* 53.

Hobbs, J., Sternbach, H., and Eckstein, F. (1972a), *Biochem. Biophys. Res. Commun. 46,* 1509.

Hobbs, J., Sternbach, H., Sprinzl, M., and Eckstein, F. (1972b), *Biochemistry 11,* 4336.

Janik, B., Kotick, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., and Wilson, D. P. (1972), *Biochem. Biophys. Res. Commun. 46,* 1153.

Kaufmann, G., and Littauer, U. Z. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4,* 79.

Khurshid, M., Khan, A., and Rottman, F. M. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28,* 25.

Mackey, J. K., and Gilham, P. T. (1971), *Nature (London) 233,* 551.

McCutchan, T. F., and Gilham, P. T. (1973), *Biochemistry 12,* 4840.

Rottman, F., and Heinlein, K. (1968), *Biochemistry 7,* 2634.

Singer, M. F. (1966), *Procedures Nucl. Acid Res.,* 245.

Sulkowski, E., and Laskowski, M. (1971), *Biochim. Biophys. Acta 240,* 443.

Tazawa, I., Tazawa, S., Alderfer, J. L., and Ts'o P.O.P. (1972), *Biochemistry 11,* 4931.

Toal, J. N., Rushizky, G. W., Pratt, A. W., and Sober, H. A. (1968), *Anal. Biochem. 23,* 60.

Torrence, P. F., Bobst, A. M., Waters, J. A., and Witkop, B. (1973), *Biochemistry 12,* 3962.

Torrence, P. F., Waters, J. A., and Witkop, B. (1972), *J. Amer. Chem. Soc. 94,* 3638.

Zmudzka, B., Janion, C., and Shugar, D. (1969), *Biochem. Biophys. Res. Commun. 37,* 895.

Zmudzka, B., and Shugar, D. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8,* 52.

Ultracentrifugal Characterization of the Mitochondrial Ribosome and Subribosomal Particles of Bovine Liver: Molecular Size and Composition†

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ABSTRACT: We have measured the molecular weights of the bovine liver mitochondrial ribosome and subribosomal particles by high-speed equilibrium centrifugation. The formaldehyde-fixed ribosome, large subunit, and small subunit had sedimentation coefficients ($s_{20,w}$) of 56.3, 44.9, and 30.1 S, and had buoyant densities in CsCl of 1.42, 1.43, and 1.43 g/cm^3, respectively. These buoyant densities correspond to RNA contents of 31 and 33%. Partial specific volumes, $\bar{v}$, of 0.674 and 0.671 ml/g were calculated for these compositions and used to compute molecular weights of 2.83×10^6, 1.65×10^6, and 1.10×10^6 for the three species. Despite its low sedimentation rate, the 55 S ribosome is thus about the size of the bacterial ribosome.

Mitochondrial ribosomes are considered to be of the prokaryotic variety primarily on the bases of antibiotic susceptibility, sedimentation coefficient, and sizes of their RNAs. Generally it has been assumed that these ribosomes are about the size of typical bacterial ribosomes with the notable exception, of course, of the 55S ribosomes that occur in mammalian mitochondria. These ribosomes, thought to be considerably smaller than other prokaryotic ribosomes because of their unusually low sedimentation coefficient and very small RNA molecules, have been called "mini-ribosomes" in recent years (see review of Borst and Grivell, 1971). We were interested, therefore, in measuring the molecular weight (MW) of a representative 55S ribosome from mammalian mitochondria to define better the relation of these ribosomes to others, and also to form the basis for studies of their protein content (O'Brien *et al.*, 1974).

We have measured the molecular weight of the bovine liver mitoribosome and its subribosomal particles by high-speed equilibrium centrifugation, one of the few absolute

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methods for determining molecular weight. Despite the technical simplicity of the method, an additional datum, the partial specific volume, $\bar{v}$, is required. For material in short supply, direct pycnometric measurement of $\bar{v}$ is not feasible. Therefore, we have calculated $\bar{v}$ for these particles from their composition. The present study shows that although their sedimentation coefficient and composition differ considerably from those of other ribosomes, 55S bovine mitoribosomes are about the size of bacterial ribosomes.

Experimental Section

Procedures for the isolation and analysis of mitochondrial ribosomes and subribosomal particles from bovine liver have been described (O'Brien, 1971; O'Brien *et al.*, 1974) and further details are given in the legend of Figure 1. The ultracentrifugal techniques have been described (Hamilton, 1974). All measurements were made at about 5° with ultraviolet absorption-photoelectric scanner optics on solutions of less than 1 A_{260} unit/ml and can be considered as measurements at infinite dilution. The MW and buoyant density in CsCl of essentially homogeneous preparations of 55S ribosomes and derived large and small subribosomal particles were measured in FKTM[1] (0.3% HCHO, 50 mM KCl, 20 mM triethanolamine-HCl (pH 7.5)) containing 5 mM $MgCl_2$ (ribosomes) or 1 mM $MgCl_2$ (subribosomal particles). For the molecular weight measurements, replicate samples were centrifuged to equilibrium at two speeds, 3000 and 4000 rpm, for the ribosome, and 4400 and 5600 rpm for the subunits. The absorbancy distributions at 265 nm were plotted as ln c *vs.* r^2 (radius) and the slope of the resultant straight line computed. FKTM has a density of 1.005 g/cm^3 and a relative viscosity of 1.024.

Results and Discussion

The mitoribosomes and subribosomal particles used in this study were purified by repeated centrifugation in sucrose gradients (Figure 1). Table I presents the results of various ultracentrifugal measurements on the 55S mitoribosome and its subunits. In addition to sedimentation coefficients and buoyant densities in CsCl, data obtained by the high speed method of equilibrium centrifugation and the molecular weights calculated from these data are given. The values for $M(1 - \bar{v}\rho)$ in Table I represent all the quantities directly accessible from the ultracentrifugal data, *i.e.*, the right hand side of the expression

$$M(1 - \bar{v}\rho) = (2RT/\omega^2)\mathrm{d}\ \ln\ c/\mathrm{d}\ r^2$$

To calculate M from this expression we also require $\bar{v}$, the partial specific volume, and ρ, the solution density. The solution density was taken to be equal to the solvent density. The partial specific volume of the ribosomal particle was calculated from its RNA and protein composition using the following relationship

$$\bar{v} = \frac{\%\ \mathrm{RNA}}{100}(\bar{v}_{\mathrm{RNA}}) + \frac{\%\ \mathrm{Prot}}{100}(\bar{v}_{\mathrm{Prot}})$$

For $\bar{v}_{\mathrm{RNA}}$, we have used the value of 0.53 ml/g, measured pycnometrically for rat liver cytoribosomal RNA (Petermann and Pavlovec, 1966).[2] For $\bar{v}_{\mathrm{Prot}}$, a value of 0.74 ml/g was calculated from the amino acid composition of the proteins of rat liver cytoribosomes (Hamilton, 1971) or *E. coli* ribosomes (Craven *et al.*, 1969).

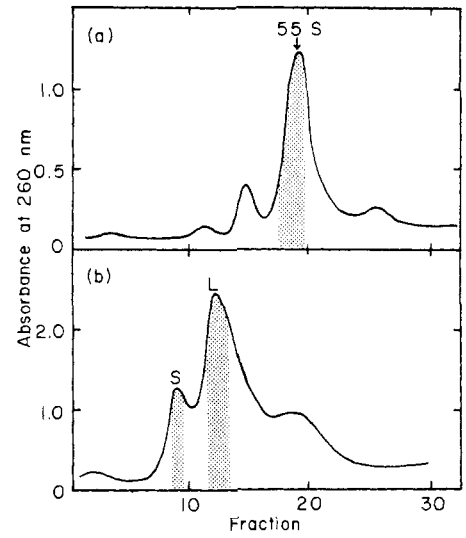

FIGURE 1: Sucrose gradient analysis of bovine (a) mitoribosomes and (b) subribosomal particles. The ribosomes were prepared from mitochondria by treatment with 0.5% sodium deoxycholate and 2% Triton X-100 in buffer T (20 mM $MgCl_2$–100 mM KCl–20 mM triethanolamine-HCl (pH 7.5)–5 mM β-mercaptoethanol) containing heparin, 50 μg/ml, and yeast oligonucleotides, 2 mg/ml, and were incubated for 5 min at 37° in buffer T containing puromycin (1 mM) to discharge nascent polypeptides (O'Brien, manuscript in preparation). (a) The 55S ribosomes, recovered from sucrose gradients in buffer T, were fixed by the addition of 37% HCHO to a final concentration of 5%. After 1 hr, the ribosomes were concentrated by vacuum dialysis against FKTM and recentrifuged in a sucrose gradient containing FKTM to obtain essentially homogeneous preparations of 55S ribosomes. Centrifugation in 10–30% sucrose gradients in the Beckman SW 27 rotor was for 5 hr at 27,000 rpm. Fractions containing 55S ribosomes were pooled as indicated, and dialyzed against FKTM in preparation for analytical ultracentrifugation. (b) Sucrose gradient analysis of subribosomal particles prepared by dialysis of unfixed 55S ribosomes against buffer O (5 mM $MgCl_2$–300 mM KCl–20 mM triethanolamine-HCl (pH 7.5)–5 mM β-mercaptoethanol). Centrifugation of the sucrose gradient in buffer O was for 14 hr at 19,000 rpm in the SW 27.1 rotor. Fractions containing small or large subribosomal particles were pooled as indicated, and dialyzed against FKTM containing 1 mM $MgCl_2$ for analytical ultracentrifugation.

We have estimated the RNA and protein content of the ribosomal particles from their buoyant densities in CsCl. For the 55S ribosome, the buoyant density in CsCl was 1.42 g/cm^3 in FKTM at 4°, while the corresponding figure for either subribosomal particle was 1.43 g/cm^3. These calculations of RNA and protein content are made by assuming that the buoyant density of ribonucleoprotein particles in CsCl reflects their chemical composition. Experience has shown that the contributions of hydration and ion binding tend to offset each other, allowing the estimation of particle RNA and protein content according to the following relationship

$$\frac{1}{\theta_{\mathrm{RNP}}} = \frac{\%\ \mathrm{RNA}}{100}\frac{1}{\theta_{\mathrm{RNA}}} + \frac{\%\ \mathrm{Prot}}{100}\frac{1}{\theta_{\mathrm{Prot}}}$$

where θ is the buoyant density of the ribonucleoprotein par-

[1] Abbreviations used are: buffer FKTM, 0.3% HCHO, 50 mM KCl, and 20 mM triethanolamine-HCl (pH 7.5) containing 5 mM $MgCl_2$ (ribosomes) or 1 mM $MgCl_2$ (subribosomal particles); buffer T, 20 mM $MgCl_2$–100 mM KCl–20 mM triethanolamine-HCl (pH 7.5)–5 mM β-mercaptoethanol; buffer O, 5 mM $MgCl_2$–300 mM KCl–20 mM triethanolamine-HCl (pH 7.5)–5 mM β-mercaptoethanol.

[2] This value is close to the average of the most disparate published values, 0.495 ml/g for bacteriophage MS2 RNA (Slegers *et al.*, 1973) and 0.557 ml/g for *E coli* 16 S rRNA (Ortega and Hill, 1973). Recalculating $\bar{v}$ with either of the extreme values gives molecular weights about 9% lower or higher.

TABLE I: Physical Data on the Bovine Liver Mitochondrial Ribosome and Subribosomal Particles.

Particle	Experimental Data: $s_{20,w}$	Buoyant Density in CsCl, θ (g/cm^3)	$M(1 - \bar{v}\rho)^a \times 10^{-6}$	Calculated Quantites: $\bar{v}^b$ (ml/g)	Mol Wt^c ($\times 10^{-6}$)	% RNA^b from θ	Mol Wt^d of RNA ($\times 10^{-6}$)	Published Data: Mol Wt of RNA ($\times 10^{-6}$) HeLa^e	*Xenopus*^{f} *laevis*	*Xenopus*^{g} *laevis*
Ribosome	56.3	1.42	0.910 (0.017,9)	0.674	2.83	31				
Large subunit	44.9	1.43	0.535 (0.008,7)	0.671	1.65	33	0.54	0.54	0.58	0.53
Small subunit	30.1	1.43	0.360 (0.007,7)	0.671	1.10	33	0.36	0.35	0.32	0.30
Sum of large and small subunits					2.75		0.90	0.89	0.90	0.83

[a] $M(1 - \bar{v}\rho) = (2RT/\omega^2)(\mathrm{d} \ln c/\mathrm{d}r^2)$ calculated from the concentration distribution at equilibrium in the analytical ultracentrifuge. The numbers in parentheses are the standard error of the mean and the number of values. [b] See text for calculation of these quantities. [c] Molecular weight calculated from $M(1 - \bar{v}\rho)$ and $\bar{v}$ with $\rho = 1.005$ g/cm^3. [d] Molecular weight $\times$ % RNA/100. See text. [e] Robberson *et al.* (1971). [f] Leister and Dawid (1974). [g] Dawid and Chase (1972).

ticle, RNA, or protein (Hamilton, 1971). The value commonly used for θ_{RNA} in CsCl is 1.89 g/cm^3 (Bruner and Vinograd, 1965). For particles of high protein content such as nuclear RNPs (Faiferman *et al.*, 1970), some viruses (Hamilton, 1971), and mammalian mitoribosomes (O'Brien *et al.*, 1974), θ_{Prot} should be determined experimentally in CsCl; the value for bovine mitoribosomal protein is 1.28 g/cm^3: (T. W. O'Brien, manuscript in preparation). With these values, the 55S ribosome, with θ_{RNP} = 1.42 g/cm^3, contains 69% protein, while the subribosomal particles, with θ_{RNP} = 1.43 g/cm^3, contain 67% protein. Using these values for protein content, the best estimates that can presently be made for $\bar{v}$ are 0.674 ml/g for the ribosome and 0.671 ml/g for the subribosomal particles. The values of M given in Table I were calculated using these estimates for $\bar{v}$. Thus, by the method of high speed equilibrium centrifugation, a MW of 2.83×10^6 has been calculated for the 55S mitoribosome, and MW's of 1.65×10^6 and 1.10×10^6 for the large and small mitochondrial subribosomal particles respectively.

While the assumptions made in estimating $\bar{v}$ seem empirically valid, it is instructive to compare these MW values with ones that can be calculated on a different basis, using the MW of their RNA components. The best estimates presently available for the MW of bovine mitoribosomal RNAs probably are the values obtained by electron microscopic length measurements of human mitoribosomal RNA (Robberson *et al.*, 1971). These values, 0.54×10^6 and 0.34×10^6 for the MW's of the large and small RNA components, can be used with the RNA content of the particles to calculate particle MW according to the relationship

$$M_{RNP} = (100/\%\ \mathrm{RNA})M_{RNA}$$

The values so obtained are 2.87×10^6 for the 55S ribosome and 1.65×10^6 and 1.06×10^6 for the large and small subribosomal particles. The close agreement of these figures with those obtained by equilibrium centrifugation provides confidence in the assumptions made in determining $\bar{v}$ and % RNA from the particle buoyant density in CsCl.

Finally, we have used the molecular weights and RNA contents of the subribosomal particles to calculate the size of their RNAs. As Table I shows, these values agree very well with literature values for the mitochondrial rRNAs determined by electron microscopy and polyacrylamide gel electrophoresis.

The most interesting aspect of this study concerns the size of 55S mitoribosomes. It was recognized only recently that, despite their low sedimentation coefficient, they are actually about the size of bacterial ribosomes (Hamilton and O'Brien, 1973; Sacchi *et al.*, 1973; DeVries and van der Koogh-Schuuring, 1973; Leister and Dawid, 1974). Thus, although they contain only about half the amount of RNA present in *E. coli* ribosomes, they are only slightly larger than these model prokaryotic ribosomes (Hill *et al.*, 1969) both in MW (Table I) and physical size (DeVries and van der Koogh-Schuuring, 1973). It would seem that their RNA deficiency is compensated by a corresponding increase in protein content. They are the most protein-rich ribosomes known (O'Brien *et al.*, 1974).

In an electron microscopic study of 55S mitoribosomes from rat liver, Aaij *et al.* (1972) noted that the mitoribosome was smaller and less asymmetric than the cytoribosome. Our data on the size of the subribosomal particles (Table I) confirm their observation. The frictional coefficient of the mitoribosome calculated from s, M, and $\bar{v}$, which includes hydration as well as asymmetry contributions, is 1.57, while that for the rat liver cytoribosome is 1.70 (Haga *et al.*, 1970).

The sum of the MW of the subribosomal particles is about 80,000 less than the MW of the ribosome. This difference probably reflects the loss of some proteins during the preparation of the subribosomal particles in the high salt buffer O. Indeed, if one compares the total protein mass of the subribosomal particles, 1.84×10^6 daltons, estimated from their per cent protein and MW, they appear to contain about 110,000 daltons less protein than is calculated to be present on the same basis in the 55S ribosomes. This mass is equivalent to three or four proteins of the average size found in bovine mitoribosomes (O'Brien *et al.*, 1974), and is a reasonable estimate for the amount likely to be washed

from the ribosome under these conditions.

Finally, in addition to their high protein content, it should be emphasized that the unusually low sedimentation coefficient of these ribosomes remains a major physical-chemical property distinguishing them from other ribosomes, whether of the eukaryotic or prokaryotic variety, including the mitoribosomes of primitive eukaryotes (Kuntzel and Noll, 1967; Chi and Suyama, 1970). In most earlier studies, the sedimentation coefficient of mammalian mitoribosomes was estimated relative to the sedimentation rate of *E. coli* or extramitochondrial ribosomes in sucrose density gradients. In view of the fact that the physical properties of 55S ribosomes differ considerably from those of either of these sedimentation standards, it is interesting that the $s_{20,w}$ of nominal 55S bovine mitoribosomes, 56.3 S, is very close to the relative sedimentation coefficient, 55.4 S, determined earlier for these ribosomes in sucrose gradients (O'Brien, 1971). Thus, while it is no longer appropriate to refer to 55S ribosomes as being "the smallest," they aptly deserve the distinction of being "the slowest."

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References

Aaij, C., Nanninga, N., and Borst, P. (1972), *Biochim. Biophys. Acta 277,* 140.

Borst, P., and Grivell, L. A. (1971), *FEBS Lett. 13,* 73.

Bruner, R., and Vinograd, J. (1965), *Biochim. Biophys. Acta 108,* 18.

Chi, J. C. H., and Suyama, Y. (1970), *J. Mol. Biol. 53,* 531.

Craven, G. R., Voynow, P., Hardy, S. J. S., and Kurland, C. (1969), *Biochemistry 8,* 2906.

Dawid, I. B., and Chase, J. W. (1972), *J. Mol. Biol. 63,* 217.

DeVries, H., and Van der Koogh-Schuurring, R. (1973), *Biochem. Biophys. Res. Commun. 54,* 308.

Faiferman, I., Hamilton, M. G., and Pogo, A. O. (1970), *Biochim. Biophys. Acta 204,* 550.

Haga, J. Y., Hamilton, M. G., and Petermann, M. L. (1970), *J. Cell. Biol. 47,* 211.

Hamilton, M. G. (1971), *Methods Enzymol. 20C,* 512.

Hamilton, M. G. (1974), *Methods Enzymol. 30F,* 387.

Hamilton, M. G., and O'Brien, T. W. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 583 Abs.

Hill, W. E., Rosetti, G. P., and Van Holde, K. E. (1969), *J. Mol. Biol. 44,* 263.

Kuntzel, H., and Noll, H. (1967), *Nature (London) 215,* 1340.

Leister, D. E., and Dawid, I. B. (1974), *J. Biol. Chem. 249,* 5108.

O'Brien, T. W. (1971), *J. Biol. Chem. 246,* 3409.

O'Brien, T. W., Denslow, N. D., and Martin, G. R. (1974), *in* The Biogenesis of Mitochondria, Kroon, A. M., and Saccone, C., Ed., New York, N.Y., Academic Press, p 347.

Ortega, J. P., and Hill, W. E. (1973), *Biochemistry 12,* 3241.

Petermann, M. L., and Pavlovec, A. (1966), *Biochim. Biophys. Acta 114,* 264.

Robberson, D., Aloni, Y., and Attardi, G. (1971), *J. Mol. Biol. 60,* 473.

Sacchi, A., Cerbone, F., Cammarano, P., and Ferrini, U. (1973), *Biochim. Biophys. Acta 308,* 390.

Slegers, H., Clauwaert, J., and Fiers, W. (1973), *Biopolymers 12,* 2033.

An Investigation of the Triplet State of Flavines and Flavoproteins by Optical Detection of Magnetic Resonance†

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ABSTRACT: The magnetic sublevels of the lowest excited triplet state of riboflavine, deazaflavine, FMN, FAD, and alloxazine, as well as two flavoproteins have been investigated by optical detection of magnetic resonance. Lifetimes, relative steady-state populations, relative radiative rate constants, as well as zero-field splitting parameters are reported for riboflavine and deazaflavine. This double resonance technique has been used to observe and characterize phosphorescence from flavodoxin and yeast hemoglobin and is shown to be a powerful technique for investigating the triplet electronic structure of weakly luminescent biological systems.

The triplet state of riboflavine makes a major contribution to the intra- and intermolecular photochemistry of flavine (Song and Metzler, 1967; Penzer and Radda, 1967; De Kok *et al.*, 1971). Triplet riboflavine also serves as a model system for mechanistic studies of flavoenzyme-catalyzed reactions. Moreover, excited flavine is implicated in a wide range of biological processes including photodynamic action as well as phototropism wherein it may function as the photoreceptor (Foote *et al.*, 1968; Song and Moore, 1968; Thomas, 1965). For these reasons the riboflavine triplet state has been extensively investigated both experimentally,

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