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Ribosomes: Analysis by Cesium Sulfate Gradient Centrifugation

Abstract. *Ribosomes from the cytoplasm of a mammalian tissue, HeLa cells, were partially purified and then concentrated into a zone by prolonged centrifugation in Cs_2SO_4 solution (density, 1.43 grams per milliliter). Ribosomal preparations treated with bentonite prior to density-gradient centrifugation showed single peaks after centrifugation. Material recovered from the peaks represented aggregates of the original 74S particle.*

Sedimentation analyses of ribosomes have been performed with sucrose gradient centrifugation, which separates particles according to mass. Although analyses of ribosomes by equilibrium density-gradient centrifugation are desirable, since this technique provides resolution based on buoyant density, few studies have been reported. This situation is due to the instability of most ribosomal preparations at the concentration of CsCl needed to provide the proper buoyant density. However, ribosomes from *Escherichia coli* are concentrated into zones (that is, form bands) during density-gradient centrifugation in CsCl when sufficient magnesium ion is present (*1*, *2*). Attempts in this laboratory to produce similar results with ribosomes from HeLa cells in CsCl with various concentrations of Mg ions have been unsuccessful. That Cs_2SO_4 might be used as a band-forming medium for ribosomes was suggested by the fact that RNA itself remains sufficiently hydrated to form bands in a solution of this salt (*3*), and hence the ribosomal RNA–protein linkages might remain protected from salt dissociation because of retention of a water shell.

HeLa cells were grown in Eagle's medium for spinner culture with 5 percent horse serum, collected by centrifugation, and treated at 4°C as follows. Cells were washed first in unbuffered saline; then in 0.35*M* sucrose with 4 m*M* magnesium acetate and 25 m*M* KCl, *p*H 7.6; and finally in 0.35*M* sucrose with 1 m*M* magnesium acetate, 1 m*M* spermine, and 5 m*M* KCl, *p*H 7.6. The cells were then suspended in four volumes of 0.1 percent Tween-80 solution containing 0.25 m*M* magnesium acetate and 0.25 m*M* spermine at *p*H 8.3 and homogenized with a Teflon pestle at 5000 rev/min for 5 minutes. Nuclei were removed by centrifugation, and the homogenate was adjusted to 0.5*M* sucrose and treated with 1/10 volume of 5 percent sodium deoxycholate for 30 minutes. The homogenate was then mixed with a concentrated solution to bring it to 2 m*M* magnesium acetate, 80m*M* NaCl, 6m*M* $NaHCO_3$ and 4m*M* tris-HCl (*p*H 8.3, buffer A) and centrifuged at 5000 rev/min for 2 minutes. Sucrose (1.3 g per 10 ml of homogenate) was added, and the material was centrifuged (Servall SS-1) at 12,000 rev/min for 10 minutes. The supernatant was removed and homogenized at 25,000 rev/min for 270 minutes. The pellet was suspended in 10 ml of buffer A, and large cell fragments were removed by centrifugation at 25,000 rev/min for 20 minutes. The supernatant was concentrated by dialysis against Aquacide (Calbiochem). This concentrated solution contained about equal amounts of protein and RNA, and it was our partially purified ribosomal preparation. Further purification was accomplished by Sephadex-G200 filtration in buffer A and by sucrose-gradient centrifugation. Cesium sulfate (99 percent minimum purity) was purchased from Kawecki Chemical Co. and purified (*4*). Ribosome solutions were rapidly mixed with concentrated Cs_2SO_4 solution (*p*H 8.6, containing Mg ion and tris-HCl) and poured into 5-ml centrifuge tubes. The solution volume of 2.5 ml was overlaid with paraffin oil. After centrifugation in a Spinco model L, the tube was punctured and two-drop fractions were collected. After addition of 90 μg of carrier (RNA and albumin) to a sample, the mixture was precipitated with cold 8 percent trichloroacetic acid; the precipitate was collected on a Millipore filter and washed twice with 5 ml of cold distilled water and dried at 80°C. The radioactivity was then measured in a Tri-Carb scintillation spectrometer.

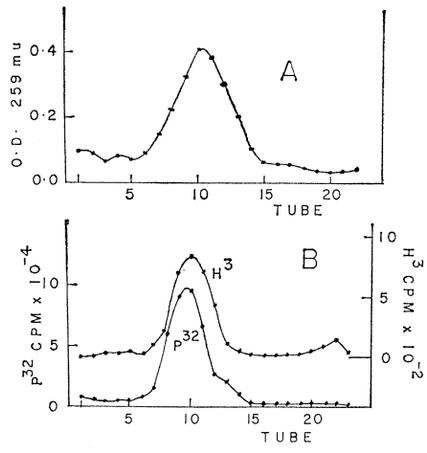

Fig. 1. Distribution of ultraviolet-absorbing and radioactive material in ribosomes. (*A*) Partially purified ribosomes (2.5 ml) were treated with bentonite; Cs_2SO_4, density 1.43 g/ml; 10 m*M* Mg ion; 39,000 rev/min, 34 hours. (*B*) Bentonite-treated ribosomes (2.5 ml) were labeled with phosphate-P^{32} and valine-H^3; Cs_2SO_4, density 1.43 g/ml; 10 m*M* Mg; 39,000 rev/min, 34 hours. Solution density increases from right to left. *CPM*, counts per minute; *O.D*, optical density.

Bentonite, used for ribosomal purification, was prepared according to Fraenkel-Conrat, Singer, and Tsugita (*5*). Bentonite treatment of ribosomal preparations from bovine pancreas removes adsorbed enzymes, including ribonuclease (*6*), and produces stable particles. Partially purified HeLa

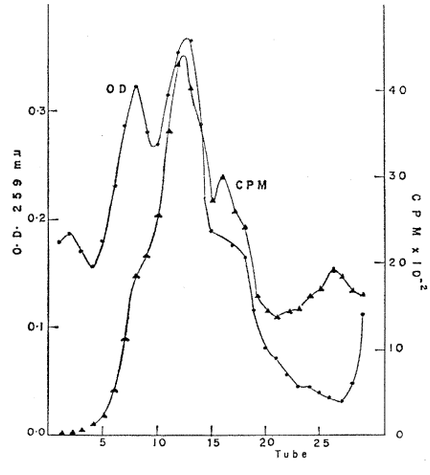

Fig. 2. Distribution of radioactivity in ribosomes not treated with bentonite. A 2.5-ml mixture of radioactive ribosomes labeled with lysine-H^3 and nonlabeled ribosomes was centrifuged in Cs_2SO_4 (density, 1.43 g/ml) containing 10 m*M* Mg ion; 38,000 rev/min for 96 hours.

cell ribosomes were suspended in 1 mM Na_2HPO_4, 0.5 mM Mg ion, pH 8.0 (buffer B) and shaken with an equal weight of bentonite in the same buffer at 0°C for 30 minutes. Bentonite was removed by centrifugation (Servall SS-1) at 12,000 rev/min for 15 minutes. The ultraviolet absorption spectrum of the supernatant, as well as colorimetric tests (*7*, *8*), indicated that the particles contained 53 to 58 percent of RNA and 47 to 42 percent of protein. Radioactive ribosomes were prepared from suspension cultures of 3×10^5 HeLa cells per milliliter grown for 16 hours in medium containing phosphate-P^{32} and valine-H^3.

Bentonite-treated ribosomes form a broad band in Cs_2SO_4 solution at 4°C after centrifugation for 30 or more hours at 35,000 to 39,000 rev/min. The Cs_2SO_4 solution, at a density of 1.43 g/ml, usually contained 10 mM Mg ion. A single peak was found for ultraviolet absorption (Fig. 1*A*), as well as for radioactivity attributable to phosphate-P^{32} and valine-H^3 (Fig. 1*B*), in the acid-insoluble fraction. Bentonite-treated samples centrifuged in Cs_2SO_4, with sodium ethylenediaminetetraacetate, pH 8.1, of sufficient concentration to remove any unbound Mg ion, give distributions similar to those found in the presence of 10 mM Mg ion. Presumably, bentonite treatment removes the necessity for the competitive-type protection which the Mg ion gives the ribosome.

The particles recovered after centrifugation tend to form aggregates which are readily sedimented at 10,000 rev/min as shown by ultraviolet absorption photographs (Spinco model E analytical centrifuge). The aggregation may be due to the conditions of resuspension, which, so far, have been a rapid fivefold dilution with ¼-strength cold Cs_2SO_4 solution and prolonged dialysis against buffer A or buffer B. Several times, 0.2 percent Tween-80 was used in the original dilution, but this did not cause any significant disaggregation to the original 74*S* particle.

Partially purified ribosomes, not treated with bentonite, form one or two broad bands in Cs_2SO_4 solution (density, 1.43 g/ml at 4°C) after 30 or more hours of centrifugation. Samples removed from the bands formed in the presence of 5 or 10 mM Mg ion and diluted into buffer A indicated a ratio of RNA to protein which is equal to or higher than that found for partially purified ribosomes which have been further purified either by bentonite treatment or by several cycles of sucrose-gradient centrifugation and Sephadex filtration. Centrifugation with higher Mg-ion concentrations (20 or 25 mM) causes trapping of some nonribosomal protein in the band, which gives samples having RNA-protein ratios similar to those of the original, partially purified ribosomes. Most of the estimates of the RNA-protein content of different samples were obtained by taking the ratio of the ultraviolet absorbancies at 259 and 235 mμ. This technique, as originally reported by Petermann and Pavlovec (*9*) for rat-tumor ribosomes, proved to be reliable and checked with results obtained with colorimetric tests (*7*). Partially purified HeLa cell ribosomes have an absorbancy ratio between 1.48 and 1.58. Bentonite-treated particles or those recovered after sucrose gradient centrifugation and Sephadex filtration have a ratio between 1.60 and 1.66. Bands recovered after Cs_2SO_4 centrifugation of bentonite-treated particles have a ratio between 1.65 and 1.75.

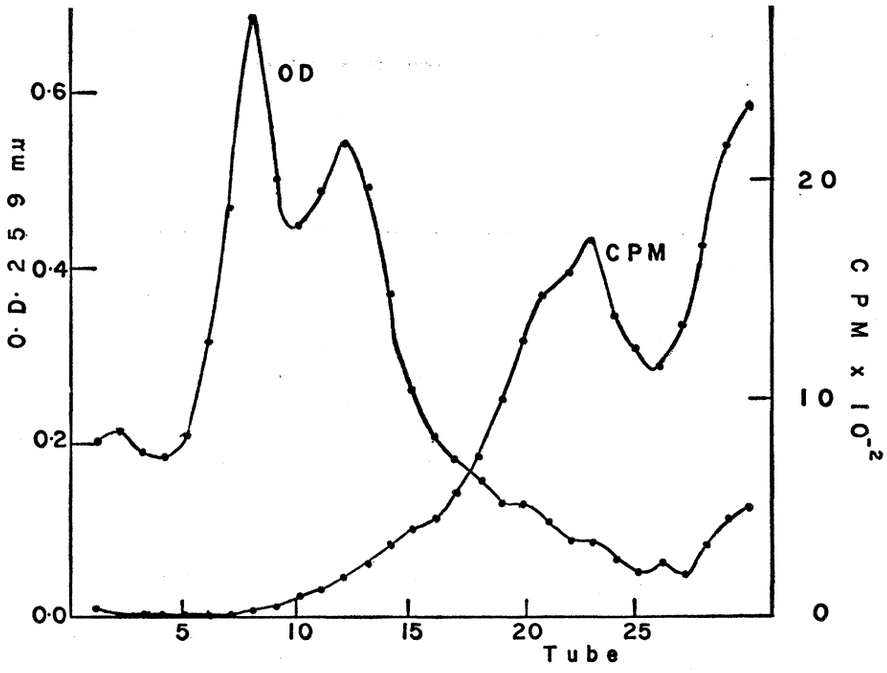

Fig. 3. Distribution of a mixture (2.5 ml) of nonlabeled ribosomes, not treated with bentonite, and soluble protein, labeled with lysine-H^3, after centrifugation in Cs_2SO_4, density 1.43 g/ml, containing 10 mM Mg ion, 38,000 rev/min, 96 hours.

In order to study the association of RNA and ribosomal protein, HeLa cells were grown in lysine-H^3 (10 μmole, 210 mc/mmole) for 16 hours. The cells were then transferred to a new growth medium containing lysine (800 μmole) for 4 more hours. Lysine-H^3 was chosen as a label since it is known that ribosomal proteins contain a high proportion of basic amino acids (*6*). The partially purified ribosomes and soluble protein fractions were isolated and dialyzed; in addition the ribosomes were filtered through Sephadex. In one experiment the protein from a crude ribosomal preparation had 1121 count min^{-1} μg^{-1}, and the soluble protein fraction, isolated at pH 8.3, had 718 count min^{-1} μg^{-1}. Figure 2 shows the distribution after centrifugation of the labeled ribosomes mixed with enough unlabeled ribosomes to produce an ultraviolet absorption pattern. The main peak of radioactivity coincides with the absorption peak in the lower-density position. The absorption peak to the left, which does not have a companion radioactivity peak, suggests that some structural protein has been removed from the ribosome, and hence some partially purified samples might not be completely stable when mixed with high concentrations of Cs_2SO_4. Radioactivity of the material near the top of the tube probably represents a mixture of protein lost from the ribosome and soluble protein. The loss of protein from *E. coli* ribosomes after CsCl density-gradient centrifugation has been reported (*2*). It is possible that the Cs_2SO_4 has modified the original ribosomal

structure so that the less dense band now represents material which more readily associates with nonribosomal protein. This association would take place during the early stages of centrifugation before the soluble protein could move to the less dense region of the gradient. The pattern of Fig. 3 for a mixture of soluble protein labeled with lysine-H^3 and nonlabeled ribosomes, indicates that this less dense band does not adsorb nonribosomal protein.

Repeated tests of the effects on partially purified ribosomes of Cs_2SO_4 centrifugation with both the Spinco SW-39 rotor and the Spinco model E analytical centrifuge, with both ultraviolet and Schlieren optics for analyses, indicate that the results are variable with respect to the formation of one or two bands. Most of the time there are two peaks but the relative amount of ultraviolet-absorbing material in one band compared with that in the other varies. Bentonite treatment of these partially purified ribosomes results in Cs_2SO_4 centrifugation patterns in which almost all the ultraviolet-absorbing material appears in one broad band.

The above technique allows further purification of partially purified ribosomes, but it is not designed to give high resolution and purification of ribosomes from very crude cell extracts. It will permit analytical studies to be made on the alteration of the structure of the ribosome (and its various complexes) brought about by treatment prior to Cs_2SO_4 centrifugation.

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16 August 1965

Reversible, Light-Screening Pigment of Elasmobranch Eyes: Chemical Identity with Melanin

Abstract. *There lies directly beneath the tapetum lucidum, in the eyes of many elasmobranch fishes, a layer of darkly pigmented choroid cells which, in bright light, extend individual strands that aggregate to form a dark, compound curtain which shields the reflecting tapetal cells. This process is reversed in dim light or in darkness; the tapetum is exposed and visual sensitivity presumably increased. The black choroid pigment has been isolated, analyzed, and shown to possess the properties of melanin.*

Elegant studies by Nicol (*1*, *2*), Denton and Nicol (*3*), earlier work by others cited by them, and a lucid review by Gilbert (*4*) focus attention upon the choroidal tapetum lucidum underlying the retina in elasmobranchs. In many sharks and in some rays, the tapetum is occlusible by a dark, compound curtain. In bright light, a layer of black, migratory cells beneath the tapetal layer extend individual, dark, finger-like processes (or dark microgranules through permanent, clear-walled channels) over the plate-like, guanine-laden reflecting cells of the tapetum lucidum. On return to darkness or to very dim light, the animal's eyes regain their eye-shine as the choroid pigmentary migration is reversed, leaving unshielded the bright, specular tapetum.

The dark, stationary choroid pigment in certain other animals, cattle, for example, is reportedly melanin (*5*, p. 226). The pigment involved in the tapetum-screening eyes of elasmobranchs has also been assumed to be melanin. However, there exist other dark pigments, such as chromolipids (*5*, pp. 69, 191 ff.), ommochromes, and sclerotins (*6*), that superficially can be confused with melanin. Since we were unaware of any analytical studies bearing on this material, we undertook diagnostic chemical examinations of elasmobranch choroid pigment.

Melanin, being an end-product of enzymic oxidation of tyrosine, is not a stoichiometric molecule, but a complex, oxidized, relatively inert polymer. It cannot, therefore, be identified with finality by any single test, but is characterized by a positive response to each of a number of chemical tests. Accordingly, the procedures and observations outlined below were applied to one or more of the species-products as designated.

We used 32 fresh eyes from the thornback ray (*Platyrhinoidis triserata*), four fresh eyes from the horned shark (*Heterodontus francisci*), and two large, formalin-preserved eyes from the great blue shark (*Prionace glauca*). The cornea, lens, iris, and intraocular fluid were removed from each eye; the orb was then cut fully open, the exposed retina lifted away with fine forceps, and the dark choroid readily separated from the underlying sclera. The choroids from *P. glauca* were leached for a few days in distilled water to remove formalin.

A *Heterodontus* specimen showed dark tapetal occlusion in an eye which had been exposed to bright illumination, while the animal's other eye, which had been masked, kept its bright tapetum unshielded. *Platyrhinoidis*, whose choroid cells are less extensively mobile, notably in the ventral half, manifested, after exposure to bright light, a darkening of the dorsal half, with partial shading of its tapetum, both dorsally and ventrally, by melanophoric extensions. Another specimen, maintained for several hours in darkness, displayed bright tapeta from which the dorsal melanistic shading had been withdrawn.

Although a living specimen of the great blue shark (*Prionace*) was not available for similar experimentation, we share Dr. Gilbert's view (personal communication) that this predacious, pelagic, oceanic species almost certainly is capable of shading the tapetum in bright light.

For complete recovery of melanin, we applied the assay method evolved by Sumner and Doudoroff (*7*), based largely on the original methods of Gortner (*5*, p. 225). The ethanol-dehydrated material, dried and extracted of lipids with ethanol-ether in a Soxhlet apparatus, was digested in 0.2 percent NaOH under reflux. The filtrate from this digestion produced, on mild acidification with dilute HCl, a dark, powdery precipitate. After decantation or centrifugation, the dark residue was redissolved several times in cold, dilute