the expression of the gene entirely intracellular, we might expect the carrier, who is a mosaic of normal and Hunter cells, to display symptoms—as is the case for heterozygotes of some variants of glucose-6-phosphate dehydrogenase deficiency, likewise an X-linked trait (*10, 11*).

Isolation and identification of the corrective factors in the medium will clarify the normal mechanism of MPS degradation, and, eventually, open up possibilities of replacement therapy. An obvious immediate use of the technique of cell mixing is in the differentiation of the genetic mucopolysaccharidoses.

JOSEPH C. FRATANTONI
CLARA W. HALL
ELIZABETH F. NEUFELD
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

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4. Correction of the defect in the chase experiments (Fig. 1B) is only partial, while that in the accumulation experiments (Fig. 1A) is essentially complete. This is probably due to a difference in experimental procedure: in the accumulation experiments, cell mixtures had been plated together 2 days before labeling, while in the chase, observation was begun immediately after mixing. We speculate that time is required to decrease the large pool of preexisting MPS of Hurler and Hunter cells before normal kinetics can be achieved; indeed, cells plated together for 5 days before beginning the chase (2 days without $^{35}SO_4$ and 3 days in the presence of isotope) lose 80 percent of the label in 24 hours, as do normals.
5. In both cases, the product recovered in the medium migrates as inorganic sulfate on electrophoresis in 0.05*M* sodium tetraborate; however, no definitive identification has yet been performed.
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11. Danes and Bearn (9) have observed the presence of about 50 percent metachromatic cells in uncloned fibroblasts from Hunter heterozygotes. Superficially, this seems to be at variance with our results, in which normal cells correct the defect of Hunter cells mixed with them. However, it is obvious that the concentration of complementary factors must depend on conditions of growth, especially on cell density and medium volume, so that the degree of correction will vary in different experimental conditions. Metachromasia, which is not a quantitative test, should not be expected to reveal partial correction.
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Isopycnic Separation of Subcellular Components from Poliovirus-Infected and Normal HeLa Cells

Abstract. *A rapid method for determining the density of ribonucleoprotein particles and complexes has been developed. The method involves glutaraldehyde fixation of sucrose gradient fractions and immediate centrifugation for 5 hours through preformed cesium chloride gradients. There is little or no aggregation of particles, so that components which co-sediment in sucrose gradients are resolved by the cesium chloride gradient. By this method the densities of HeLa cell ribosomes, polyribosomes, and subribosomal particles have been determined. Furthermore, the poliovirus replication complex has been separated from polyribosomes and its density has been found to be unaffected by treatment with ethylenediaminetetraacetic acid.*

The separation of subcellular particles containing RNA is usually effected by rate-zonal centrifugation through gradients of sucrose. As investigation of cellular physiology has progressed, problems have arisen which cannot be solved by rate-zonal analysis because of its low resolving power and the apparent co-sedimentation of disparate elements. This is especially true in the study of virus replication. A separatory method based on isopycnic banding of formaldehyde-fixed subcellular complexes in cesium chloride has provided a second dimension of analysis and has been successfully used in a number of studies (*1, 2*). The aldehyde fixation is necessary to stabilize the complexes against the dissociative effects of CsCl (*3*). The methods which have been published, however, are lengthy and the steps of the procedure may produce selective losses.

To obviate these difficulties we have developed a rapid method of isopycnic centrifugation using glutaraldehyde fixation and preformed cesium chloride gradients. Glutaraldehyde was chosen because it is a bifunctional reagent which is known to fix tissue specimens rapidly (*4*). In this report we present the method and illustrate its utility in the investigation of certain questions (*5*).

To carry out the analysis, cytoplasmic extracts are prepared and then fractionated through 15 to 30 percent linear sucrose gradients in RSB (0.01*M* tris, 0.01*M* NaCl, 0.0015*M* $MgCl_2$ at *p*H 7.4) by standard techniques (*6*). After centrifugation, the gradients are separated into fractions while the absorbance at 260 mμ is monitored by a Gilford recording spectrophotometer. Samples of 0.8 ml from appropriate regions or individual fractions of the sucrose gradients are fixed at 4°C by the addition of 0.2 ml of 33 percent glutaraldehyde (Fisher Scientific Co., biological grade) which has been neutralized to *p*H 7.0 with 1*M* $NaHCO_3$ just prior to use. The fixed samples are immediately layered onto preformed linear gradients of CsCl of appropriate density (*7*). Sucrose need not be removed from the samples, because the density of CsCl at the top of the gradients is always greater than the density of 30 percent sucrose (1.14 g/cm^3). Comparison of samples containing sucrose with samples freed of sucrose by dialysis or centrifugation shows that the sucrose layer above the CsCl gradient does not alter the apparent densities of subcellular particles. Dialysis of materials after exposure to glutaraldehyde often leads to artifacts and should be avoided.

The linear CsCl gradients are preformed in a volume of 5 ml in 9/16 by 3¾ inch (1.4 by 9.5 cm) cellulose nitrate tubes (Spinco) with CsCl solutions of two different densities made up in RSB containing 0.8 percent BRIJ-58. Addition of the non-ionic detergent BRIJ-58 prevents loss of materials due to absorption to glass and cellulose nitrate tubes. However, the solubility of BRIJ-58 in CsCl becomes limited when the density is higher than 1.70 g/cm^3. Polyallomer tubes have been found to produce artifacts and should not be used. After layering the sample, the tube is filled with Nujol or another mineral oil. The tubes are then centrifuged for 5 hours at 4°C at a speed chosen to produce 170,000*g* at the midpoint of the gradient. These tubes can be centrifuged either in the SW41 Spinco rotor or in the SB-283 IEC rotor. After deceleration with the brake on, drops are collected from the bottom of the tubes directly into disposable glass tubes and are assayed for refractive index, absorbancy at 260 mμ, and acid-precipitable radioactivity (*6*). Densities are obtained from the refractive index measurement. Because of the sucrose and glutaraldehyde on the top of the gradient, neither density nor absorbancy in the top fractions is shown in the figures. Recovery of radioactivity in the

CsCl fractions is about 90 percent of the input.

To illustrate the method, the densities of normal HeLa ribosomes and 60*S* and 40*S* subribosomal particles were determined. Cells were prelabeled for 20 hours with H^3-uridine, washed, and then incubated for another 20 hours in label-free medium. Prior to analysis the cells were exposed to actinomycin D for 3½ hours. Figure 1A shows the clear separation in sucrose of 74*S* monoribosomes and 60*S* and 40*S* subunits. Peak fractions from the appropriate regions in sucrose were recentrifuged separately in CsCl gradients. Ribosomes formed a single band in CsCl at a density of 1.55 g/cm³ (Fig. 1D); 60*S* subunits banded at a density of 1.57 g/cm³ (Fig. 1C), and 40*S* subunits banded at a density of 1.49 g/cm³ (Fig. 1B). The results shown in Fig. 1 are in good agreement with determinations made on L-cell ribosomal subunits by the formaldehyde method (see *2*; ribosomes = 1.55, 60*S* particles = 1.57, 40*S* particles = 1.49). Similarly prelabeled poliovirus-infected HeLa cells also yielded 74*S* ribosomes and 60*S* and 40*S* ribosomal subunits with the same density as those from uninfected cells.

The density of glutaraldehyde-fixed monoribosomes has been determined 30 different times by their absorbancy at 260 mμ or by prelabeling with H^3-uridine of infected and uninfected HeLa cells. The absolute density of the fixed ribosomes varied from 1.54 to 1.56, but the separation between ribosomes and other particles is a constant value. Such differences have been noted previously (*2*).

To assure that equilibrium is reached by 5 hours of centrifugation at 170,000*g* for the subcellular complexes studied, two types of experiments were done. In one type of experiment, phenol-extracted 35*S* poliovirus RNA was fixed and layered over a preformed CsCl gradient from 1.20 g/cm³ to 1.65 g/cm³. The fixed RNA sedimented to the bottom of the CsCl gradient after 5 hours of centrifugation. Thus an RNA molecule sedimenting as slowly as 35*S* (*8*) will pass through the gradient quantitatively in 5 hours. The second experiment was to extend the time of centrifugation of fixed ribosomes to 18 hours. This did not change the observed buoyant densities of the particles. Moreover, the degree of similarity between the previously reported densities of L-cell ribosomes and their subunits (*2*) with those re-

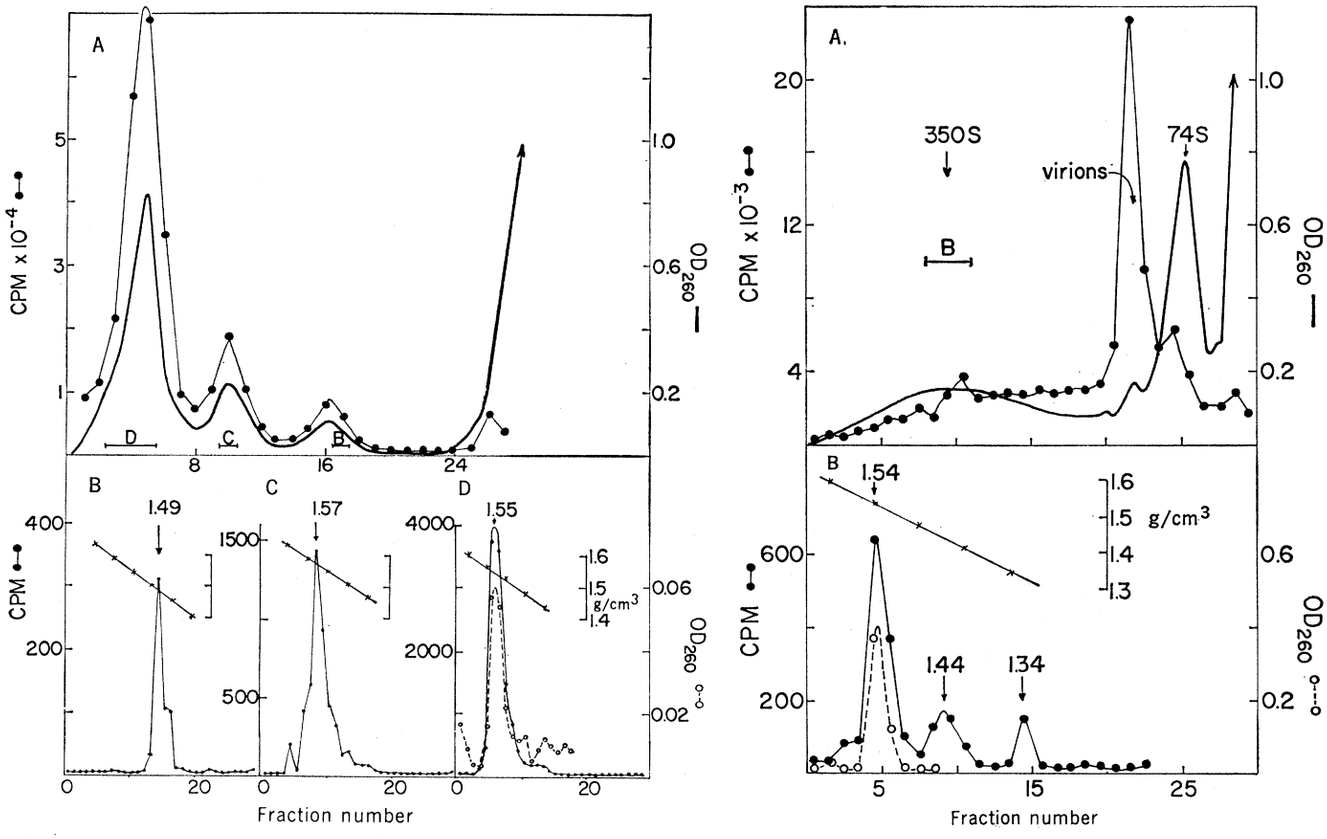

Fig. 1 (left). Density of ribosomal subunits (40*S* and 60*S*) and ribosomes (74*S*) from HeLa cells. One × 10^7 cells were exposed to 1 μc/ml of H^3-uridine in 50 ml of spinner-modified Eagle's medium (*13*) plus 7 percent horse serum for 20 hours at 37°C, washed, and resuspended in fresh minimal essential medium (MEM) for another 20 hours at 37°C. They were then concentrated 10× and incubated in MEM containing 5 μg of actinomycin D (*14*) per milliliter for 3.5 hours at 37°C. Cytoplasmic extracts were prepared, treated with 1 percent sodium deoxycholate and 1 percent BRIJ-58, and fractionated through a 15 to 30 percent sucrose-RSB gradient in the SB110 rotor of the IEC B-60 centrifuge at 54,000*g* for 18 hours at 4°C. (A) Sucrose gradient. —, OD_{260}; ●—●, acid-precipitable H^3-uridine. Horizontal bars indicate the peak fractions, of each of which 0.8 ml was fixed and recentrifuged to equilibrium. (B) 40*S* subunits banded in 33 to 55 percent CsCl. (C) 60*S* subunits banded in 33 to 55 percent CsCl. (D) 74*S* ribosomes banded in 25 to 55 percent CsCl and centrifuged for 10.5 hours. ○- - -○, OD_{260}; ●—●, acid-precipitable H^3-uridine. Fig. 2 (right). Separation of viral polyribosomes, replication complex, and poliovirus by equilibrium centrifugation. Four × 10^7 HeLa cells were infected by standard techniques (*15*). Beginning at 3 hours post-infection the cells were exposed to H^3-uridine at 1 μc/ml for 30 minutes, after which they were harvested. Cytoplasmic extracts were made, treated as in Fig. 1, and then layered over a 15 to 30 percent sucrose-RSB gradient. Centrifugation was in the IEC SB 110 rotor at 75,000*g* for 2 hours at 4°C. (A) Sucrose gradient. —, OD_{260}; ●—●, acid-precipitable H^3-uridine. The horizontal bar over the 350*S* viral polyribosome region indicates the combined fractions, of which 0.8 ml was recentrifuged in CsCl. (B) CsCl gradient (25 to 53 percent). ○- - -○, OD_{260}; ●—●, acid-precipitable H^3-uridine. CPM, counts per minute; OD, optical density.

ported here for HeLa materials further supports the conclusion that the particles reach equilibrium after 5 hours.

The utility of this method depends on its ability to separate particles of similar sedimentation rate but different density. It is therefore critical to show that the glutaraldehyde does not nonspecifically aggregate particles. To do this, advantage was taken of the observation that phenol-extracted poliovirus RNA, when added to a cytoplasmic extract, sediments in a sucrose gradient at about the same rate as ribosomes (*9*). When a fraction from the sucrose gradient containing both ribosomes and viral RNA was fixed with glutaraldehyde and centrifuged to equilibrium in CsCl, the two species separated completely. The ribosomes banded at 1.54 g/cm^3, whereas the viral RNA banded at 1.40 to 1.42 g/cm^3 (because of proteins bound to it). Thus, the two species were fixed separately by the glutaraldehyde and were not aggregated.

Cytoplasmic extracts as usually prepared (*6*) cannot be analyzed by the fixation and banding procedure prior to their fractionation on sucrose gradients because, owing to the high concentration of proteins, glutaraldehyde links proteins to particles and the resultant CsCl profiles are meaningless. The particles must be sedimented some distance through sucrose to separate them from soluble proteins.

A further demonstration of the utility of this method comes from analysis of the polyribosome region of a sucrose gradient of the cytoplasm from poliovirus-infected HeLa cells (*10*) which were labeled with H^3-uridine for 30 minutes (Fig. 2A). The fractions in the polyribosome region marked *B* gave three peaks in CsCl (Fig. 2B). The heaviest peak corresponds to the viral polyribosomes and contains all of the label after an amino acid pulse. It has the same density as normal ribosomes. The middle peak (at 1.44 g/cm^3) represents the replication complex, the site of poliovirus RNA synthesis (*11*). It has been shown previously that the replication complex sediments in the region of polyribosomes in sucrose gradients (*11*). Infected cells labeled for only 5 minutes with uridine show only this peak in the polyribosome region, and in CsCl all of the label is at 1.44 g/cm^3; treatment with ethylenediaminetetraacetic acid does not alter the position of the peak in sucrose or CsCl gradients. The third peak, at 1.34 g/cm^3, represents virions. This has been verified by analysis of purified virions and is consistent with published values for the density of unfixed poliovirus (*12*). The finding of virions sedimenting in the 350*S* region of the sucrose gradient is surprising in light of the fact that the peak of virions is at about 150*S*. This result has been found repeatedly and must be due to virions binding to other materials or to their sliding down the walls of the centrifuge tubes.

The experiment shown in Fig. 2 demonstrates that co-sedimenting species of RNA can be easily separated and quantitated by the fixation and isopycnic centrifugation technique. The rapidity of the method and the small number of necessary manipulations recommends it over the more laborious formaldehyde method (*1, 2*).

DAVID BALTIMORE
ALICE S. HUANG

Department of Biology,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139

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Immunoglobulin M Antibodies with Ten Combining Sites

Abstract. *Immunoglobulin M rabbit antibodies to a hapten are shown to have ten binding sites per molecule. The affinity for the specific hapten is approximately 100 times greater for one-half of the sites than for the other half. All sites are retained in the five 7S subunits produced by reduction and alkylation of the immunoglobulin M. Each of the 7S subunits of the IgM molecule apparently has one strong and one weak site.*

Our studies (*1*) on the combining sites of rabbit IgM-class antibody to a hapten led to the detection of five sites per molecule of IgM on the basis of its having a molecular weight of 900,000. No additional sites were detected when the IgM molecule was separated into its subunits by reduction and alkylation. The average binding constant of the sites in intact IgM or in the subunits was of the order of 1 to 5×10^5 liter/mole, as measured with the homologous hapten, *p*-iodobenzenearsonate. Except for Merler *et al.* (*2*), other workers have also reported five sites per molecule (see *3, 4*).

Studies on the structure of human IgM by Miller and Metzger (*5*) indicated that the molecule was composed of five subunits, each apparently composed of two light chains and two heavy chains (*6*). These authors proposed that IgM antibody possesses ten potential combining sites per intact molecule with only half being effective in binding antigen. Onoue *et al.* showed that there were ten Fab-like fragments per molecule (*7*) and suggested that half of the potential sites might not be detected because of their low affinity for antigen.

We have examined the situation with IgM antibody against a haptenic group different from that used earlier. The antiserums used were against the 1-azonaphthalene-4-sulfonate (azo-NS) group and were obtained from rabbits injected with *Salmonella typhimurium* to which azonaphthalene sulfonate groups had been coupled. This particulate type of antigen is particularly effective for the production of IgM-class antibodies to haptens (*8*).

Antibodies to azo-NS were specifically purified by adsorbing them on an immunoadsorbent prepared by coupling diazotized 1-aminonaphthalene-4-sulfonate to an insoluble polymer of rabbit serum albumin (azo-NS-poly RSA) (*9*), and then eluting the antibodies with 0.2*M* naphthalene-1-sulfonate. The free hapten and the antibodies of IgM and IgG classes were separated by gel filtration on Sephadex G-200 before the hapten was removed. The antibody of each class was further purified by a second gel filtration and then the hapten bound to antibody was removed by