Glutaraldehyde Fixation of Ribosomes. Its Use in the Analysis of Ribosome Dissociation†

Alap R. Subramanian

ABSTRACT: Dissociation of free ribosomes by hydrostatic pressure, which occurs during ultracentrifugation, can be prevented by fixation with glutaraldehyde. Such fixed ribosomes are also no longer dissociated by low concentrations of Mg^{2+}. The fixation reaction is satisfactory in buffers commonly used in studying ribosome function, is fast, and is effective over a wide range of ribosome or glutaraldehyde concentrations. An apparent loss of ribosomes by aggregation observed under certain conditions is prevented by the addition of a protective agent, bovine serum albumin, during fixation. With this technique it is shown that the physical separation of the subunits of ribosomes by dissociation factor takes place during the incubation rather than during the subsequent centrifugation.

It is now generally recognized that free ribosomes, which are not complexed with mRNA and peptidyl-tRNA, dissociate into the two subunits far more readily than ribosomes engaged in translation (complexed ribosomes), which are stabilized by the attached ligands. Thus, free ribosomes of *Escherichia coli*, but not complexed ribosomes, dissociate when centrifuged in gradients with low Mg^{2+} (Ron *et al.*, 1968), or with NaCl in place of the usual KCl (Beller and Davis, 1971). Similarly, the ribosome dissociation factor (DF)[1] (Subramanian *et al.*, 1968) which has been identified with initiation factor IF3 (Sabol *et al.*, 1970; Subramanian and Davis, 1970; Dubnoff and Maitra, 1971), dissociates only free ribosomes (Subramanian *et al.*, 1969). Recently it was shown that the hydrostatic pressure generated during ultracentrifugation also promotes dissociation of free ribosomes of sea urchin eggs (Infante and Krauss, 1971; Infante and Baierlein, 1971) and of *E. coli* (Spirin, 1971). Under the usual conditions of ultracentrifugation this pressure effect does not cause the production of subunit peaks but appears in a decrease in the *s* value of the 70S ribosomal peak.

It would be desirable to have a means of preventing such pressure-promoted dissociation, since it can cause artefacts in studies involving analysis of the distribution of ribosomal particles by ultracentrifugation. Formaldehyde, used in early electron microscopic studies on ribosomes (Huxley and Zubay, 1960), has been shown to prevent the splitting off of ribosomal proteins in CsCl gradients (Spirin *et al.*, 1966), and to fix (for analytical ultracentrifugation) the distribution between ribosomes and subunits (Spirin *et al.*, 1971). However, this method has the serious disadvantage of causing aggregation in the presence of Tris or NH_4^+ (Spirin *et al.*, 1966), which are commonly used in incubation mixtures for ribosomes. We have found that glutaraldehyde does not share this disadvantage and is quite suitable for fixation of ribosomes in the usual incubation mixtures for ultracentrifugal analysis.

Glutaraldehyde is known, since the studies of Sabatini *et al.* (1963), as an excellent fixative of cellular ultrastructures; in recent years it has also been used in studying the differential affinity of bacterial membrane-associated enzymes (Ellar *et al.*, 1971), for preservation of ribosomes for CsCl gradient centrifugation (Ceccarini *et al.*, 1971) or for electron microscopic study (Nonomura *et al.*, 1971), and for subunit-exchange studies with heavy-isotope-labeled polysomes (Subramanian and Davis, 1971a). The present paper shows that glutaraldehyde fixes ribosomal particles rapidly and over a wide range of aldehyde or ribosome concentrations: the fixed particles no longer dissociate or associate with changes in Mg^{2+} concentration or hydrostatic pressure. The reproducibility of the method is improved by carrying out the fixation in the presence of a protein such as bovine serum albumin. Some uses of this method will be discussed.

Materials and Methods

Ribosomes were prepared from *E. coli* MRE600 cells, grown with aeration at 37° in L broth (10 g of bactotryptone, 5 g of NaCl, and 2 g of yeast extract per l.) and harvested at mid-log phase, by the standard alumina-grinding procedure (Tissieres *et al.*, 1959). The ribosomes were washed with 1 M NH_4Cl and after sedimentation the pellet was dissolved in $TKM_{10}D$ buffer (10 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, and 1 mM dithiothreitol) with 20% v/v glycerol to a concentration to 500 A_{260} units/ml (*i.e.*, 33 mg/ml (Hill *et al.*, 1969)). Radioisotope-labeled complexed ribosomes were made by bovine pancreatic ribonuclease fragmentation (1 μg/ml, 0°, 5 min) of endogenous polysomes prepared by the procedure of Tai *et al.* (1972);[2] details have been presented elsewhere (Subramanian and Davis, 1971a).

Dissociation factor was obtained by washing ribosomes with 1 M NH_4Cl and collecting the fraction precipitating between 30 and 66% saturation of $(NH_4)_2SO_4$ as described by Subramanian *et al.* (1968). It was dissolved in TKM_0D buffer and was dialyzed against the same buffer. Protein concentration of this solution was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Glutaraldehyde was purchased from Fisher Scientific Co. (50% biological grade). This slightly acidic solution was diluted to 25% (v/v) for adjustment of its pH to 7.4–7.8 (using pH paper) with 1 M Tris; more dilute solutions in the

† From Bacterial Physiology Unit, Harvard Medical School, Boston, Massachusetts 02115. *Received March 20, 1972.* This work was supported by grants from U. S. Public Health Service and American Cancer Society to Dr. B. D. Davis.

[1] Abbreviations used are: DF, dissociation factor; TKM_xD, 10 mM Tris-HCl buffer (pH 7.6) with 50 mM KCl, *x* mM magnesium acetate, and 1 mM dithiothreitol.

[2] Manuscript in preparation.

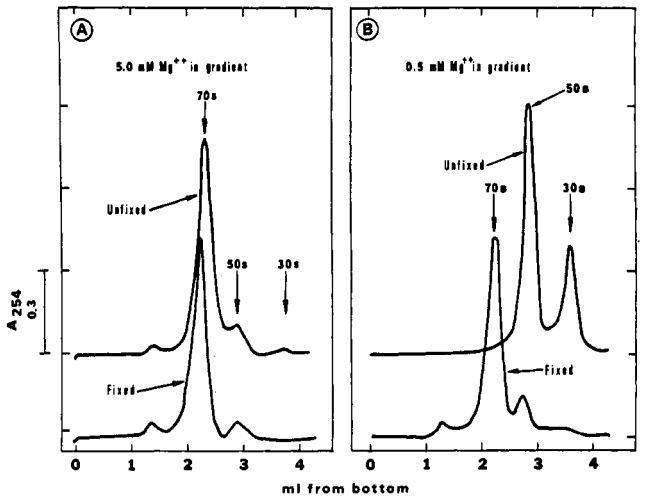

FIGURE 1: Absence of dissociation of glutaraldehyde-fixed ribosomes in low Mg^{2+} at high hydrostatic pressure. Ribosome solutions (0.1 ml; 100 μg) in TKM_5D were mixed at 0° with 0.2 ml of TKM_5 buffer with or without 2.5% glutaraldehyde. After 10 min 0.1 ml of each mixture was layered over sucrose gradients in TKM_5 (panel A) or in $TKM_{0.5}$ buffers (panel B); analyses as in Methods.

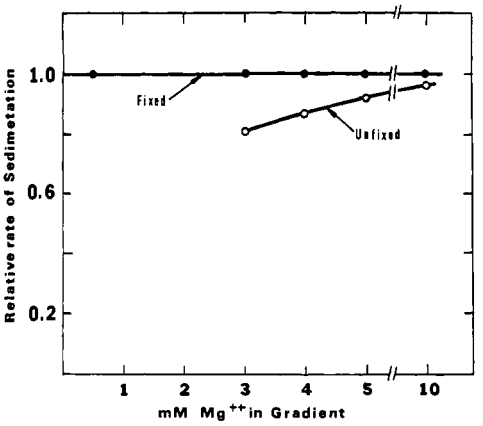

FIGURE 2: Constant sedimentation rate of glutaraldehyde-fixed ribosomes in gradients with different Mg^{2+} concentration. Ribosome solutions in TKM_5D, fixed with 1.7% glutaraldehyde or unfixed, were layered (0.1 ml; 33 μg) over 10–30% sucrose gradients (10 mM Tris-HCl (pH 7.6)–100 mM KCl) with magnesium acetate as specified. Analyses as in Methods. The distance traveled by the peak of fixed ribosomes in 10 mM Mg^{2+} was taken as unity. A twofold higher K^+ than usual was used in gradients in order to accentuate the hydrostatic pressure effect.

buffers used in the incubation of ribosomes were made just prior to use. Generally, one volume of the ribosome solution in TKM_5D buffer was mixed with two volumes of 2.5% glutaraldehyde in TKM_5 at 0° and the mixture was layered over ice-cold gradients.

On standing, the slightly alkaline glutaraldehyde solution often yields a white precipitate, which is probably the polymer product of base-catalyzed aldol condensation. It is therefore best to prepare the pH-adjusted dilute solutions just prior to use.

Gradient analysis. For most analyses the ribosome solutions after fixation (0.1–0.3 ml) were layered over 4.8-ml, linear, 10–30% sucrose gradients in TKM_5 or $TKM_{0.5}$ buffer and were spun at 4° for 70 min at 45,000 rpm in a Spinco SW 50.1 rotor. The gradients were then analyzed in an Isco gradient analyzer.

Bovine serum albumin (fraction V powder) was purchased from Pentex Biochemicals, Kankakee, Ill.; and bovine pancreatic ribonuclease from Worthington Biochemicals, Freehold, N. J. Chemicals used were analytical grade.

Results

Integrity of Glutaraldehyde-Fixed Ribosomes at Low Mg^{2+} and at High Hydrostatic Pressure. To test whether fixation would prevent dissociation, 1 M NH_4Cl-washed ribosomes[3] were compared before and after exposure to 1.7% glutaraldehyde at 0° for 10 min. Unfixed ribosomes presented the usual patterns: in a 5 mM Mg^{2+} gradient there was a predominant 70S peak and small subunit peaks, while in a 0.5 mM Mg^{2+} gradient the ribosomes were completely dissociated (Figure 1). In contrast, glutaraldehyde-fixed ribosomes presented exactly the same pattern in low Mg^{2+} as in high Mg^{2+} gradients; the 70S particles were no longer subject to dissociation by the combined effect of low Mg^{2+} and high hydrostatic pressure. In further experiments the fixed 70S particles were found to remain undissociated even in gradients without any Mg^{2+}.

It is now recognized that free ribosomes appear to sediment somewhat more slowly than complexed ribosomes under a variety of conditions, and that this effect is explained by partial dissociation by the high hydrostatic pressure in the centrifugal field (Infante and Baierlein, 1971; Spirin, 1971; Subramanian and Davis, 1971a). Since fixation prevents dissociation, it should also abolish this slowing effect. As Figure 2 shows, the sedimentation rate of fixed ribosomes was unaffected by a 20-fold variation in Mg^{2+} concentration, whereas the unfixed ribosomes yielded a slower peak at the lower Mg^{2+} concentrations. The constancy of the s value of glutaraldehyde-fixed ribosomes over such a range of Mg^{2+} concentration suggests that glutaraldehyde fixation abolishes the influence of Mg^{2+} on dissociation and possibly also on conformation.

Does Fixation Change s Value at All? Because unfixed free ribosomes dissociate in the centrifugal field they cannot evidently be used to answer this question; but complexed ribosomes ought to be suitable, since they do not dissociate under these conditions. Figure 3 shows the cosedimentation patterns of differentially labeled fixed and unfixed complexed ribosomes, after centrifugation for 4 hr in an exponential gradient: the fixed ribosomes did peak slightly ahead of the unfixed. From a plot of the distance traveled by the unfixed 70S ribosomes and subunits against their standard *s* values (which is linear in this type of gradient) the sedimentation constant of the fixed ribosomes was found to be 73 S. Fixation therefore makes ribosomes sediment slightly faster, perhaps owing to the mass of the added groups, or more likely because fixation makes ribosomes more compact.

Requirements for Completion of Fixation. Resistance to dissociation in low Mg^{2+} gradients provided a means of checking the apparent completion of the fixation reaction. The reaction was found to be fast even at 0°. Fixation was essentially complete when ribosome solutions were mixed with 1.7% glutaraldehyde and immediately analyzed (Figure 4A). This rapid fixation was observed over a wide range of glutaralde-

[3] Ribosomes washed with 1 M NH_4Cl behave like free ribosomes in their susceptibility to dissociation by lowered Mg^{2+} or by the dissociation factor (Subramanian and Davis, 1970); in addition they contain, like free ribosomes, little tRNA (Tai and Davis, 1972).

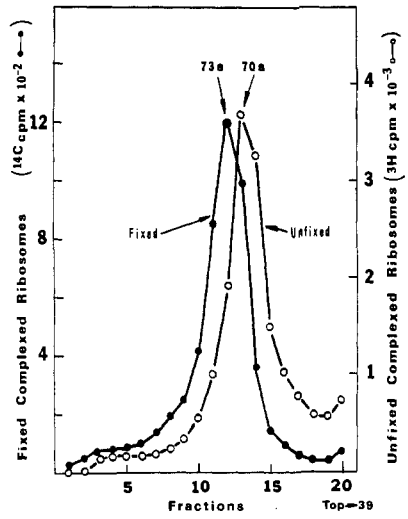

FIGURE 3: Effect of glutaraldehyde fixation on the sedimentation rate of complexed ribosomes. ^{14}C-Labeled complexed ribosomes were fixed with 1.7% glutaraldehyde, dialyzed to remove unreacted fixative, and mixed with unfixed ^{3}H-labeled complexed ribosomes. The mixture was layered over 11.8 ml of an exponential gradient (12.5–18% sucrose in TKM_{10}) and centrifuged at 40,000 rpm (SW41 Ti rotor) at 4° for 4 hr. For radioactivity measurement 9-drop fractions were collected, mixed with 10 ml of Bray's mixture, and counted in a Nuclear-Chicago scintillation counter, using low cross-over settings.

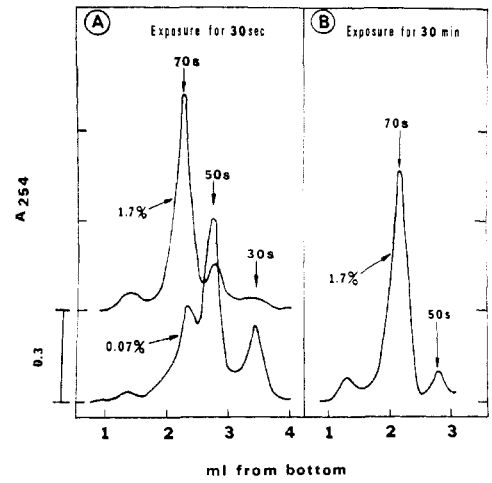

FIGURE 4: Rapidity of fixation and its dependence on concentration of glutaraldehyde. Ribosome solutions (0.1 ml, 33 μg) in TKM_5D buffer were treated with 0.2 ml of 2.5 or 0.1% glutaraldehyde in TKM_5 for the indicated periods of time. The entire reaction mixtures were then layered over sucrose gradients in $TKM_{0.5}$ and analyzed (about 10 min elapse before the samples attain full speed).

hyde concentrations. Between 5 and 0.5% (*i.e.*, 0.5–0.05 M), the pattern and the recovery of fixed ribosomes was the same. However, with 0.3% glutaraldehyde fixation was only 80% complete and with 0.07% it was clearly incomplete on such brief exposure (Figure 4A).

Although fixation is rapid, the ribosome–glutaraldehyde mixture could be kept at 0° for longer periods, at least up to 30 min, without any deleterious effect (Figure 4B). There was a slight loss of ribosomes (5–8%) when analyzed 1 hr after mixing.

Effect of Some Other Variables on Fixation. Some variation in pH during fixation did not appear to have any effect. Thus glutaraldehyde with no pH adjustment, which yielded a pH close to 6.0 in the weakly buffered incubation mixture, gave patterns similar to those obtained under the standard conditions (pH 7.6).

Variation of Mg^{2+} in the reaction mixture between 5 and 10 mM, or replacement of KCl by the same concentration of NH_4Cl, did not show any effect either on the recovery of the ribosomal particles in the gradient or on their distribution as ribosomes and subunits. However, when ribosomes were exposed to low concentrations of Mg^{2+} (at 0°) before glutaraldehyde fixation increased levels of subunits appeared. Thus fixation at 1 mM Mg^{2+} presented a pattern with 50% subunits, and at 0.1 mM Mg^{2+} the value was 100%. Since free ribosomes dissociate spontaneously at these low concentrations of Mg^{2+}, glutaraldehyde evidently fixes the subunits just as well as the ribosomes.

Fixations were routinely carried out at 0°; at 37° only about 60% of the particles were recovered in the gradient. The relative recovery of the three species (70, 50, and 30 S), however, remained unchanged.

Aggregation by Glutaraldehyde and Its Prevention by a Protective Protein. A wide variation in ribosome concentration did not appear to have any effect on the fixation reaction. The concentrations usually employed were 100–300 μg/ml, but the reaction was as rapid, and recovery as complete, at 1.5 mg/ml. At lower concentrations of ribosomes, however, even brief fixation seemed to cause a definite loss of material from the visible part of the sedimentation profile. This loss was especially evident in experiments (not reported) with radioactively labeled ribosomes, where quantitation by summation of counts is relatively simple. It was also noticed that incubation of ribosomes (at the usual concentrations) prior to fixation leads to loss of recovery (R. J. Beller, private communication). Thus ribosomes incubated at 37° for 15 min and then chilled and fixed gave only 70–80% recovery of the 70S peak as compared to controls (unincubated and fixed, or incubated but unfixed). The reason for this peculiar effect is unknown, but we found that such loss from aggregation did not occur if the incubation mixture contained *E. coli* supernatant proteins. Moreover, the presence of the supernatant proteins eliminated the loss at low concentrations of ribosomes as well.

The protective effect of the supernatant proteins could also be provided by bovine serum albumin. Ribosomes incubated in the presence of the albumin (0.3 mg/ml) gave 100% recovery upon subsequent fixation (Figure 5). It was found sufficient to add the albumin before fixation (rather than before incubation) to provide this protection. There was an additional beneficial effect from the albumin. The glutaraldehyde–ribosome mixture at 0° could be kept longer (at least 2 hr) before analysis without noticeable loss of ribosomes. Similar good recovery was obtained in the presence of the albumin with low concentrations of ribosomes.

Activity of Fixed Ribosomes. Enzymes in tissue slices often retain their activity when fixed by glutaraldehyde, as has been histochemically demonstrated by Sabatini *et al.* (1963). When ribosomes were exposed to glutaraldehyde for less than 1 min and then freed of unreacted glutaraldehyde in a Sephadex G-50 column they were found to be completely inactive in polymerizing phenylalanine in the presence of poly(U) (Nirenberg and Matthaei, 1961).

An Application of the Glutaraldehyde Fixation Technique: the Nature of Dissociation Factor Induced Dissociation. In the usual assays for DF (Subramanian *et al.*, 1968; Subra-

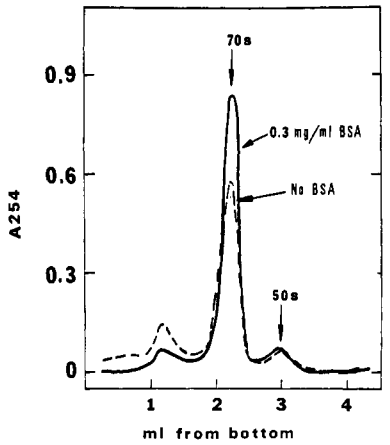

FIGURE 5: Aggregation of incubated ribosomes by glutaraldehyde and elimination of this loss by bovine serum albumin. Duplicate ribosome solutions (0.1 ml, 33 μg) in TKM_5D buffer were incubated at 37° for 15 min with 0.3 mg/ml of bovine serum albumin or without. After chilling the solutions were mixed with 0.2 ml of 2.5% glutaraldehyde in TKM_5 and the entire reaction mixtures were then layered over sucrose gradients in TKM_5 and analyzed. Analysis in $TKM_{0.5}$ gradients gave similar patterns.

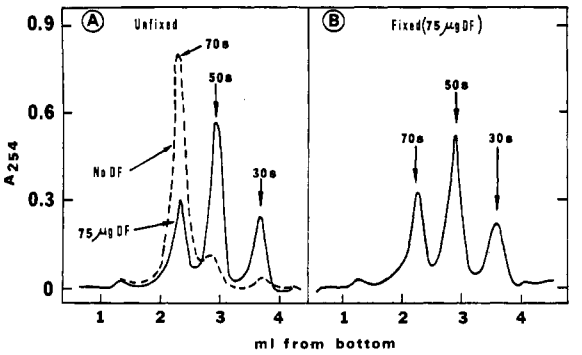

FIGURE 6: Evidence for dissociation of ribosomes by DF during incubation. Duplicate ribosome solutions (0.1 ml; 33 μg) in TKM_8D buffer were incubated at 37° for 10 min with 75 μg of dissociation factor. After chilling one mixture was fixed by the addition of 0.2 ml of 2.5% glutaraldehyde in TKM_8, and to the control 0.2 ml of TKM_8 buffer was added; both were then layered over TKM_5 gradients and analyzed.

manian and Davis, 1970) a solution of free (or 1 M NH_4Cl-washed) ribosomes is incubated between 30 and 37° with DF and then sedimented through a sucrose gradient by ultracentrifugation. The resulting pattern of ribosomes and subunits is then assumed to represent their distribution at the end of incubation. However, since dissociation can occur during centrifugation, it is conceivable that during incubation the DF produces an unstable 70S·DF complex which then dissociates in the gradient. This possibility can be tested by fixing the incubation mixture before ultracentrifugal analysis. Fixation ought to preserve such an intermediate 70S·DF complex from dissociation. But as Figure 6 shows, the two analyses, with and without fixation, gave nearly identical patterns. Evidently, DF causes physical separation of subunits in an incubation mixture, before centrifugation.

Discussion

The discovery of dissociation of free ribosomes by the high hydrostatic pressure generated during ultracentrifugation (Infante and Baierlein, 1971) makes it quite useful to have a means of preventing this alteration in samples under analysis—only then can meaningful results be obtained from studies which depend on the integrity of ribosomes. The results presented here show that glutaraldehyde is an excellent reagent for this purpose. It works fast, can be used in the presence of the ions commonly used in work with ribosomes, is tolerant to limited variations in pH and Mg^{2+} concentration, and is effective over a wide range of ribosome concentrations. The fixed ribosomes are no longer dissociated by hydrostatic pressure or low Mg^{2+}, and their *s* value remains unaffected by wide variations in the Mg^{2+} concentration of the sedimenting gradient. We did not attempt to compare other aldehydes, since Sabatini *et al.* (1963) have reported that among a series of mono- and dialdehydes glutaraldehyde gave the best preserved cellular fine structure.

Glutaraldehyde caused an apparent loss of ribosomes at low ribosome concentration, or with ribosomes incubated at 37° before fixation. However, this effect can be prevented by the addition of a protective agent, bovine serum albumin. Tris would be expected, like other primary amines, to react with glutaraldehyde and therefore, is generally avoided by electron microscopists in their fixation studies (Sabatini *et al.*, 1963). In the present procedure this reaction could not significantly lower the concentration of available fixative because of the high ratio of glutaraldehyde to Tris employed; but the removal of Tris could appreciably lower the pH. The results presented in this paper show that glutaraldehyde fixation of ribosomes is tolerant to any such change that may occur. However, in studies where pH has to be maintained constant during fixation, it would be advisable to use buffers nonreactive with glutaraldehyde.

Since glutaraldehyde is a bifunctional reagent it could conceivably link together two or more ribosomes. The sedimentation patterns of fixed ribosomal preparations always showed a dimer peak; but this may not have originated from cross-linking since unfixed preparations also contained a similar peak (Figure 1). As shown by Capecchi (1964),[4] ribosomal preparations contain a small amount of dimers, even though the use of KCl in the extracting buffer minimizes the artificially high levels of dimers observed earlier (Tissieres *et al.*, 1959). However, ribosomes incubated at 37° showed dimer formation from cross-linking but the albumin was found to suppress this reaction (Figure 5). The presence of the albumin minimized also the extent of cross-linking resulting from the prolonged exposure of ribosomes to glutaraldehyde.

It is interesting that reaction of ribosomes with glutaraldehyde eliminates the dissociating effect of low Mg^{2+}. The aldehydic group reacts primarily with amino groups, but since these are present in both the RNA and protein components, it would be difficult to localize the cause of this stickiness. A specific role for Mg^{2+} bridges between the nucleotide phosphates of the subparticles, though considered likely at one time, is no longer taken seriously, since it has been found that subunits treated mildly with formaldehyde no longer reassociate (Watson, 1964). The stickiness of glutaraldehyde-fixed ribosomes might be due to cross-linking between the subparticles by this bifunctional reagent.

This technique has already proved useful in demonstrating (Subramanian and Davis, 1971a) that in lysates free ribosomes exchange their subunits rapidly on incubation, and in showing the occurrence of an artefact in earlier exchange

[4] Quoted by J. D. Watson.

studies with heavy-isotope-labeled polysomes (Kaempfer, 1968). The present paper uses this technique to show that DF causes the physical separation of the two ribosomal subparticles (Figure 6) upon reaction with free ribosomes. This finding rules out the suggestion that DF acts by merely loosening the binding between subunits so that these may dissociate during centrifugation (Grunberg-Manago *et al.*, 1971), but it does not exclude the formation of a 70S·DF complex prior to dissociation (Talens *et al.*, 1970). Studies of Sabol and Ochoa (1971) with radioactive DF (IF3) show, however, that such a complex, if it exists, is not detected in conventional sucrose gradient analyses.

The fixation technique should be useful in studies on ligand binding to ribosomes where the binding is analyzed by ultracentrifugation. If the binding depends on intact ribosomes, dissociation during centrifugation may free the bound ligand; but fixation before centrifugation should prevent this loss. Recently fixation has been used in studying circular dichroism of ribosomes (Kabasheva *et al.*, 1971). The technique might also find application in studies involving exchange of labeled subunits with ribosomes (Falvey and Staehelin, 1970; Subramanian and Davis, 1971b), for the observed distribution of label is affected by the dissociation of unfixed ribosomes during centrifugation (unpublished observations).

Acknowledgment

I am thankful to Dr. B. D. Davis for criticism of the manuscript and to Mrs. Jennifer McGeoch for excellent technical assistance.

Added in Proof

A recent study (Korn *et al.*, 1972), using spectroscopic and nmr analyses, concludes that in aqueous solution free glutaraldehyde exists in equilibrium with at least two other molecular forms, a hydrated hemiacetal and its polymer. The reacting species is the free aldehyde.

References

Beller, R. J., and Davis, B. D. (1971), *J. Mol. Biol. 55*, 477.

Ceccarini, C., Campo, M. S., and Andronico, F. (1970), *J. Mol. Biol. 54*, 33.

Ellar, D. K., Muñoz, E., and Salton, M. R. J. (1971), *Biochim. Biophys. Acta 225*, 140.

Falvey, A. K., and Staehelin, T. (1970), *J. Mol. Biol. 53*, 21.

Grunberg-Manago, M., Rabinowitz, J. C., Dondon, J., Lelong, J. C., and Gros, F. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 19*, 193.

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

Huxley, H. E., and Zubay, G. (1960), *J. Mol. Biol. 2*, 10.

Infante, A. A., and Baierlein, R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1780.

Infante, A. A., and Krauss, M. (1971), *Biochim. Biophys. Acta 246*, 81.

Kabasheva, G. N., Sandakhchiev, L. S., and Sevastyanov, A. P. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 14*, 161.

Kaempfer, R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 106.

Korn, A. H., Feairheller, S. H., and Filachione, E. M. (1972), *J. Mol. Biol. 65*, 525.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Nirenberg, M. W., and Matthaei, J. H. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1588.

Nonomura, Y., Blobel, G., and Sabatini, D. (1971), *J. Mol. Biol. 60*, 303.

Ron, E. Z., Kohler, R. E., and Davis, B. D. (1968), *J. Mol. Biol. 36*, 83.

Sabatini, D. D., Bensch, K., and Barrnett, R. J. (1963), *J. Cell Biol. 17*, 19.

Sabol, S., and Ochoa, S. (1971), *Nature (London), New Biol. 234*, 233.

Sabol, S., Sillero, M. A. G., Iwasaki, K., and Ochoa, S. (1970), *Nature (London) 228*, 1269.

Spirin, A. S. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 14*, 349.

Spirin, A. S., Belitsina, N. U., and Lerman, M. I. (1966), *J. Mol. Biol. 14*, 611.

Spirin, A. S., Sabo, B., and Kovalenko, U. A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 15*, 197.

Subramanian, A. R., and Davis, B. D. (1970), *Nature (London) 228*, 1273.

Subramanian, A. R., and Davis, B. D. (1971a), *Proc. Nat. Acad. Sci. U. S. 68*, 2453.

Subramanian, A. R., and Davis, B. D. (1971b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1311.

Subramanian, A. R., Davis, B. D., and Beller, R. J. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 223.

Subramanian, A. R., Ron, E. Z., and Davis, B. D. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 761.

Tai, P.-C., and Davis, B. D. (1972), *J. Mol. Biol.* (in press).

Talens, J., Kalousek, F., and Bosch, L. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12*, 4.

Tissieres, A., Watson, J. D., Schlessinger, D., and Hollingworth, B. R. (1959), *J. Mol. Biol. 1*, 221.

Watson, J. D. (1964), *Bull. Soc. Chim. Biol. 46*, 1399.