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PERTURBATION OF RIBOSOME SUBUNIT INTERACTION BY GLUTARALDEHYDE FIXATION

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Ultracentrifugal analysis of ribosomal purity is complicated by the rapid reequilibration of ribosomes with their subunits, and this is further enhanced by the effects of hydrostatic pressure. Fixation of the ribosome system prior to ultracentrifugal analysis supposedly freezes the reequilibration, and thus tends to obviate these difficulties. However, no redistribution of the ribosome-subunit population must be allowed to occur during fixation. Thus, it is necessary that fixation be extremely rapid compared to the ribosome-subunit reequilibration, in order to avoid errors in analysis. It was the purpose of this investigation to make a direct experimental comparison of the rates of these two processes, fixation and ribosome-subunit reequilibration, using the stopped-flow technique with a light-scattering detector, under a variety of buffer environment readjustments. The following findings resulted from this study: (1) Fixation in Tris buffers could not be followed by light scattering because of interaction of glutaraldehyde with Tris, leading to continuous production of high molecular weight contaminants. (2) Polymerization of glutaraldehyde leads to increased ultraviolet absorption, which must not be confused with scattering changes. (3) Undialyzed ribosome solutions prepared by dissolving stock suspensions stored at high levels of magnesium and univalent electrolyte into a known standard buffer produced solutions having free Mg^{2+} levels lower than those of original buffer, complicating kinetic observations and threatening ribosome stability. (4) Addition of malonic acid as a buffer for Mg^{2+} largely eliminated this problem. (5) The kinetics of glutaraldehyde were measured quantitatively by studying the perturbation of ribosome association and dissociation kinetics produced during shifts of Mg^{2+} levels. (6) At fixative levels not producing coagulation, fixation kinetics can be competitive with those of association-dissociation. (7) Mass action effects like those of dilution can be caused by fixation, and can give rise to excess subunits, which may be mistaken for loose couples in original ribosome preparations.

1. Introduction

Some of the complications during ultracentrifuge analysis of ribosomal purity have been discussed in a reassessment of the effects of interaction microheterogeneity [1]. In order to obviate such reequilibration effects, fixation of ribosomal preparations prior to ultracentrifuge analysis has been introduced, but this leads to complications caused by undesired cross-linking [2], since a large excess of glutaraldehyde is usually employed to assure rapid fixation of ribosomes. Even the addition of neutral target material, for example, bovine plasma albumin, to absorb the excess glutaraldehyde, does not successfully prevent all undesirable cross-linking reactions in the ribosomal material [2]. For the purpose of this study, the inclusion of an additional material such as bovine plasma albumin serves only to complicate the experimental

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measurements of reaction rates and their interpretation. Thus, the stopped-flow measurements have been confined to variations of the concentration of glutaraldehyde in the various mixing buffer systems.

2. Experimental

Buffers were prepared in freshly boiled distilled water, to minimize air bubbles in stopped-flow experiments. All stopped-flow measurements were performed in a commercially available Durrum-Gibson apparatus equipped with 90° light-scattering observation. Its use in ribosome subunit interaction kinetics has been carefully described [3].

Escherichia coli MRE600 ribosomes were kindly supplied for this research by Professor A.J. Wahba. Their preparation has been previously described [4]. They were shipped and stored at dry ice temperature in a buffer made up to pH 7.8 and containing 20 mM Tris-HCl, 0.5 M NH_4Cl, 10 mM magnesium acetate, 1 mM dithiothreitol, and 50% glycerol. This stock frozen preparation was thawed and diluted directly into various buffers, just prior to experiments which used Tris buffers. If other buffers were required, an aliquot of the stock ribosome solution was passed through a short Sephadex G-25 column which had been preequilibrated with desired buffer. Glutaraldehyde was specially purified grade I, obtained as a 25% solution from Sigma Chemical Co. The stock solution was stored at −20 °C.

The first experiments involved diluting ribosomes in Tris buffer with glutaraldehyde in the same buffer. The ribosome solution to be mixed in the stopped-flow apparatus was prepared by diluting 1 vol. of stock ribosome preparation with 10 parts of a buffer (hereafter referred to as buffer H) of the following composition: 10 mM Tris-HCl, 66 mM KCl, 2.25 mM $MgCl_2$, to give a final composition containing 60 mM KCl and nominally 3 mM $MgCl_2$, at pH 7.8. The buffer to be mixed with this ribosome solution in the stopped-flow apparatus consisted of 1 vol. of storage buffer and 10 vols. of buffer H. In order to be able to interpret the effect of the addition of glutaraldehyde, a blank experiment must first be performed by mixing the ribosome solution with the same buffer without glutaraldehyde.

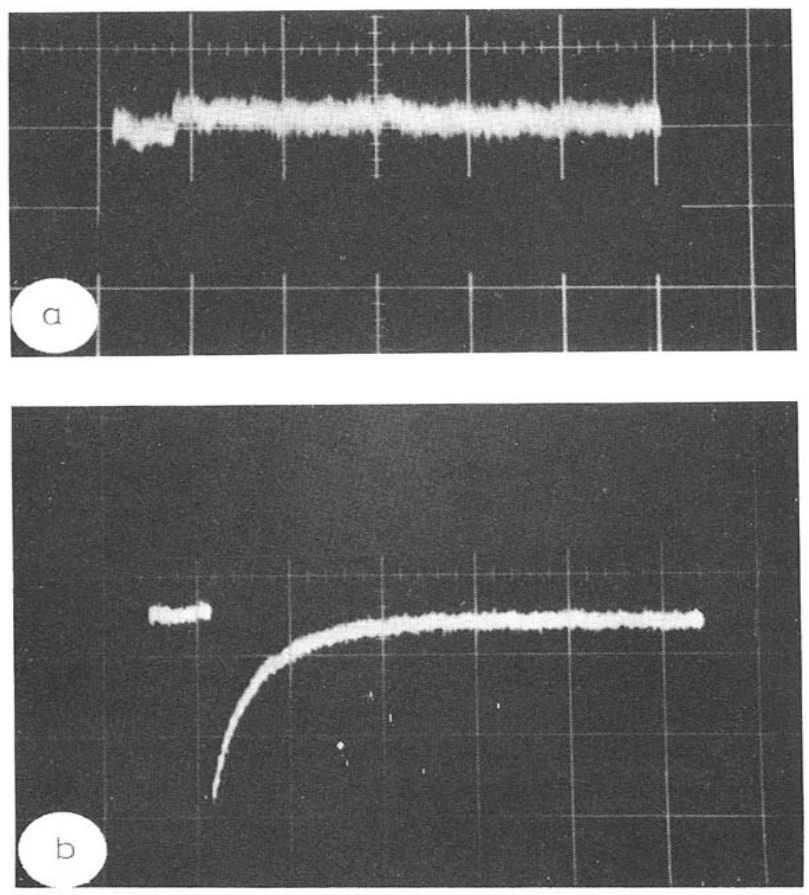

Fig. 1. (a) Stopped-flow dilution of ribosomes to a final concentration of 0.191 μM, by mixing with an equal volume of 10 mM Tris-HCl (pH 7.8) buffer, containing 60 mM KCl, 45.5 mM NH_4Cl and 3 mM Mg^{2+}. The output of the Durrum stopped-flow apparatus in the 90° scattered light mode was recorded with a Biomation Model 1015 waveform recorder and played back on a Tektronix 545 B oscilloscope. The effective sweep time was 69.4 s/division. (b) Stopped-flow dilution of ribosomes to a final concentration of 0.733 μM in the same buffer system. The temperature was 26 °C, and scattered light was monitored at 436 nm. The effective sweep time was 34.7 s/division.

In fig. 1a is shown the light-scattering response to the mixing of a solution of ribosomes in Tris buffer, as described, at a ribosome concentration of 0.191 μM, with an equal volume of the buffer adjusted to 3 mM $MgCl_2$. It appears that the effect of dilution is insufficient to be observed in this experiment. In fig. 1b is shown the result for a similar experiment in which the ribosome concentration is 0.733 μM. Instead of showing a drop of scattering as a result of dilution, there is an

increase of scattering after the initial mixing.

The explanation for this peculiar result is that the free Mg^{2+} concentration in the ribosome solution is actually lower than the 3 mM of the buffer solution with which it is being mixed. This phenomenon of reduced Mg^{2+} level in solutions prepared by diluting stock ribosome aliquots into buffers with known Mg^{2+} levels was discovered by Chaires et al. [5]. The reason for the reduction in free Mg^{2+} level was provided by A. Wishnia (personal communication). In the storage buffer, the high level of univalent electrolyte effectively displaces Mg^{2+} from the phosphate groups in the ribosomes. When this ribosome solution is diluted into a buffer containing a relatively low level of univalent electrolyte (approx. 105 mM in place of 500 mM), the ribosomes regain capacity to bind Mg^{2+}, thereby significantly reducing the level of free Mg^{2+}, the more so at higher ribosome concentrations.

This explanation is confirmed by the results shown in fig. 2, where the titration of ribosomes with magnesium in Tris buffers at pH 7.8 is followed by static light scattering in a Perkin-Elmer spectrofluorimeter. The abscissa is the total Mg^{2+} concentration, including that from the stock ribosome solution. It is noted that as the ribosome concentration is increased from 0.34 to 0.64 μM, the titration curve shifts slightly toward higher

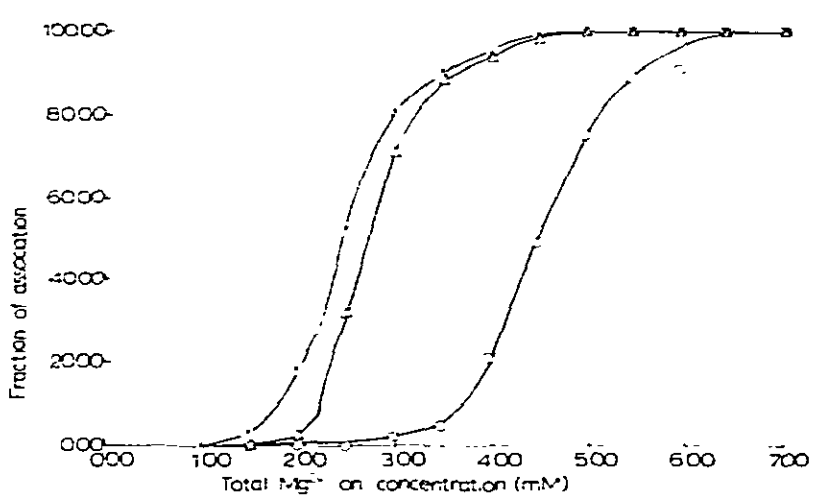

Fig. 2. Mg^{2+} titrations of ribosomal solutions prepared by dilution of concentrated stock ribosome solution. Final buffer conditions: 10 mM Tris-HCl (pH 7.8), 60 mM KCl, 50 mM NH_4Cl, and total Mg^{2+} concentrations as indicated. The temperature was 25°C. Ribosome concentrations: (×), 0.34 μM, (△) 0.64 μM, (○) 2.53 μM.

Mg^{2+} concentrations, but when the ribosome concentration is increased to 2.53 μM, there is a very large shift of the titration curve to higher Mg^{2+} concentrations. At the same level of total Mg^{2+}, the association of ribosomes is much lower at the highest ribosome concentration. This implies that the free Mg^{2+} concentration is much reduced at the highest ribosome concentration, verifying the explanation for fig. 1b.

In order to overcome this problem, it was necessary to add some component to the buffer system to buffer Mg^{2+}. A magnesium chelate in equilibrium with a small amount of free Mg^{2+} and free chelating agent constitutes a satisfactory type of buffering system. Frequently, H^+ and metal ions would compete for the same binding sites on the chelating agent, but their individual binding affinities are governed by the acid dissociation constant and the metal chelate dissociation constant. If the desired buffered levels of H^+ and Mg^{2+} are such that the magnesium chelate has appreciable buffering capacity for both ions, then the two ionization processes will be undesirably coupled. Since we desire to work at pH 7.8, it is necessary to find a good magnesium chelating agent whose buffering capacity for protons at pH 7.8 is very small. A search for such a chelating agent revealed that malonic acid has two acid dissociation constants with pK values of 2.66 and 5.32, and one magnesium chelate dissociation constant with a pK value of 1.91 [6]. Thus, at pH 7.8, both protons of malonic acid are dissociated, while the maximum buffering capacity for Mg^{2+} will be at 12.3 mM Mg^{2+} concentration. This system should have useful buffering capacity for Mg^{2+} between 1.23 and 123 mM Mg^{2+} levels.

A buffer was prepared similar to buffer H, described above, by adding to buffer H 25 mM malonic acid and 8.26 mM Mg^{2+}, which results in a buffered level of 3 mM Mg^{2+}. In fig. 3a is shown the results of a stopped-flow experiment in which a 0.186 μM ribosome solution in this buffer system was mixed with the corresponding buffer produced by diluting one part of storage buffer with 10 parts of this modified Buffer H. The expected slow decrease of light scattering, after the initial mixing period, shown in fig. 3a is in contrast to the results shown in fig. 1a, where no

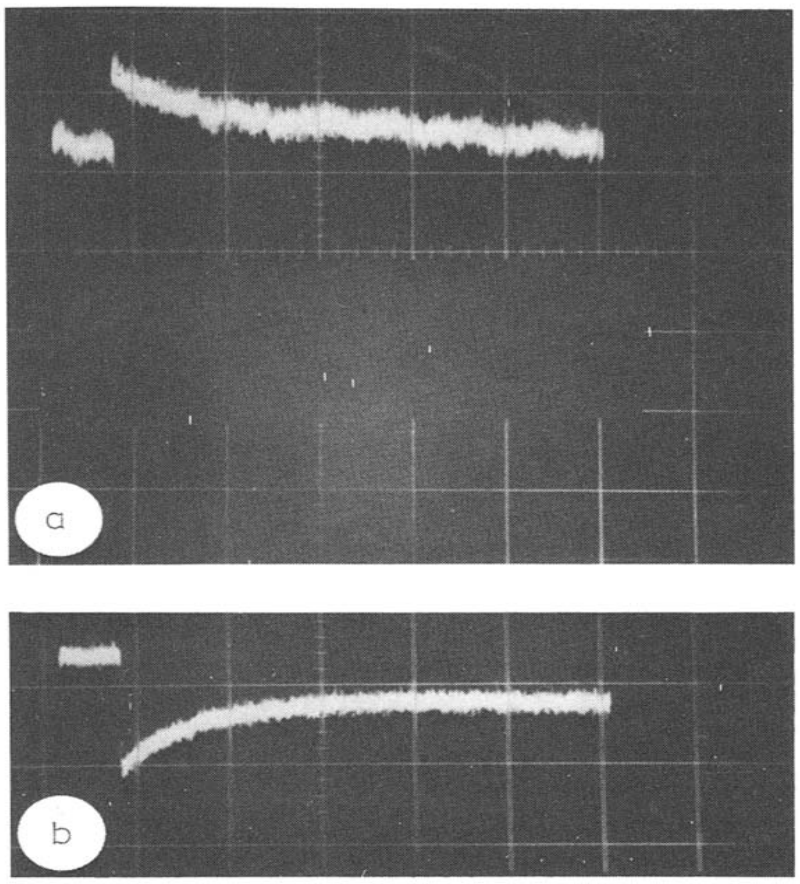

Fig. 3. (a) Stopped-flow dilution of ribosomes to a final concentration of 0.186 μM, by mixing with an equal volume of Tris-malonic buffer at pH 7.8. Final buffer conditions: 25 mM malonic acid, 10 mM Tris-KOH, 45.5 mM NH_4Cl, and 8.26 mM $MgCl_2$ (buffered at 3.0 mM free Mg^{2+}). The effective sweep time was 69.4 s/division. (b) Stopped-flow dilution of ribosomes to a final concentration of 0.844 μM in the same buffer. The effective sweep time was 34.7 s/division.

decrease was observed. This implies that in the experiment shown in fig. 1a, there was a complete compensation of dissociation due to dilution by association due to a jump in Mg^{2+} concentration. In fig. 3b is shown the results of a mixing experiment in which 0.844 μM ribosomes in the modified buffer H are mixed with the corresponding buffer. In this case, there is still a rise in light scattering after initial mixing, implying that the Mg^{2+} buffering capacity of this buffer is insufficient for such a high ribosome concentration. In the case of the pH, the H^+ concentration is almost 6 orders of magnitude smaller than the concentration of the Tris buffer. In the case of Mg^{2+}, however, an equivalent buffering capacity would require a chelate molarity of 3000. While this is obviously impossible, some improvement in Mg^{2+} buffering capacity would be obtainable in practice by raising the malonic acid concentration, above the 25 mM used here. Many of the experiments to be described below were at low ribosome concentrations such as that in fig. 3a. For experiments using high ribosome concentrations, corrections were made, taking into account the large number of magnesium-binding sites in a ribosome unit [7], to estimate more correctly the final free Mg^{2+} concentration [8].

In fig. 4 is shown the results of mixing 2.5 vols. of a solution of 0.245 μM ribosomes in 60 mM KCl, 10 mM Tris-HCl, 4.95 mM NH_4Cl, 6 mM Mg^{2+}, at pH 7.8, with 1 vol. of a buffer containing 60 mM KCl, 10 mM Tris-HCl, 0.35% glutaraldehyde, with sufficient EDTA to reduce the final Mg^{2+} concentration to 3 mM. If fixing of ribosomes and subunits were extremely rapid compared to the rate of ribosome-subunit reequilibration, we should expect no shift of light scattering after initial mixing. Instead, we see that there is a small decrease in light scattering, followed by a continual rise in light scattering. Numerous replicate experiments all showed similar effects, indicating that the phenomenon is real. It was finally recognized that the increase in scattering was the result of the interaction of the Tris component of the buffer with glutaraldehyde. This was confirmed by observation of the light scattering from the buffer containing glutaraldehyde alone. Consequently, it became impossible to assess reliably the kinetics of the glutaraldehyde-ribosome interaction in Tris buffers, even though all experiments would be completed within two horizontal divisions of fig. 4.

A phosphate buffer, hereafter referred to as buffer G, was therefore used for further studies, consisting of 60 mM KCl, 25 mM malonic acid, 5 mM sodium phosphate-KOH, at pH 7.8, with Mg^{2+} levels adjusted as desired.

In addition to the difficulty caused by the interaction of Tris with glutaraldehyde, it was found that an apparent drift in scattering still took place at long times after mixing. This was correlated with a yellowing of glutaraldehyde solutions, the result of polymerization, which gave rise to appreciable absorption of light at 436 nm. In fig. 5a is shown the results of a fixation experiment at

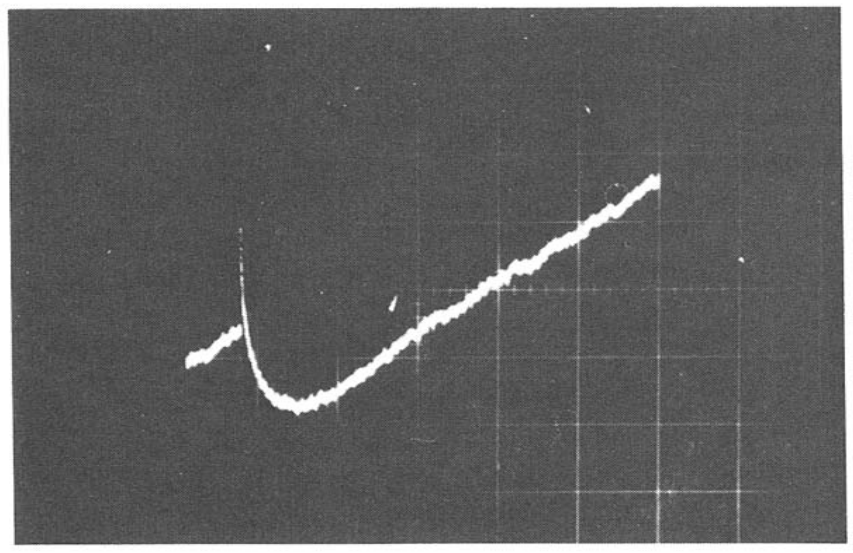

Fig. 4. Stopped-flow results of mixing 2.5 vols. of a solution of 0.245 μM ribosomes in 60 mM KCl, 10 mM Tris-HCl, 4.95 mM NH_4Cl, 6 mM Mg^{2+}, at pH 7.8, with 1 vol. of a buffer containing 60 mM KCl, 10 mM Tris-HCl, 0.35% glutaraldehyde, with sufficient EDTA to reduce the final Mg^{2+} concentration to 3 mM. The effective sweep time was 694 s/division. The temperature was 25 °C, and scattered light was monitored at 436 nm.

nominally constant Mg^{2+} concentration, monitored at 436 nm. The apparent drop in scattering was accompanied by appreciable increase in light absorption at 436 nm. Fig. 5b shows the results of a duplicate stopped-flow experiment monitored at 590 nm. The expected drop in scattering due to dilution reaches a constant value when monitored at 590 nm, because of greatly reduced sensitivity to the increase in glutaraldehyde polymerization. Consequently, all further scattering experiments were done at 590 nm. It was found necessary to substitute K^+ for NH_4^+ when using phosphate buffers, to avoid precipitation of $MgNH_4PO_4$. The NH_4^+ present in the stock ribosome solution required removal.

Since it was necessary to remove also the Tris which came from the storage buffer, aliquots of stock ribosome preparation were passed through a short Sephadex G-25 column which had been preequilibrated with the appropriate buffer G at the desired Mg^{2+} concentration. It is noted that simple dilution of ribosomes produces a relatively slow dissociation process with small amplitude, and consequent poor signal-to-noise ratio. In order

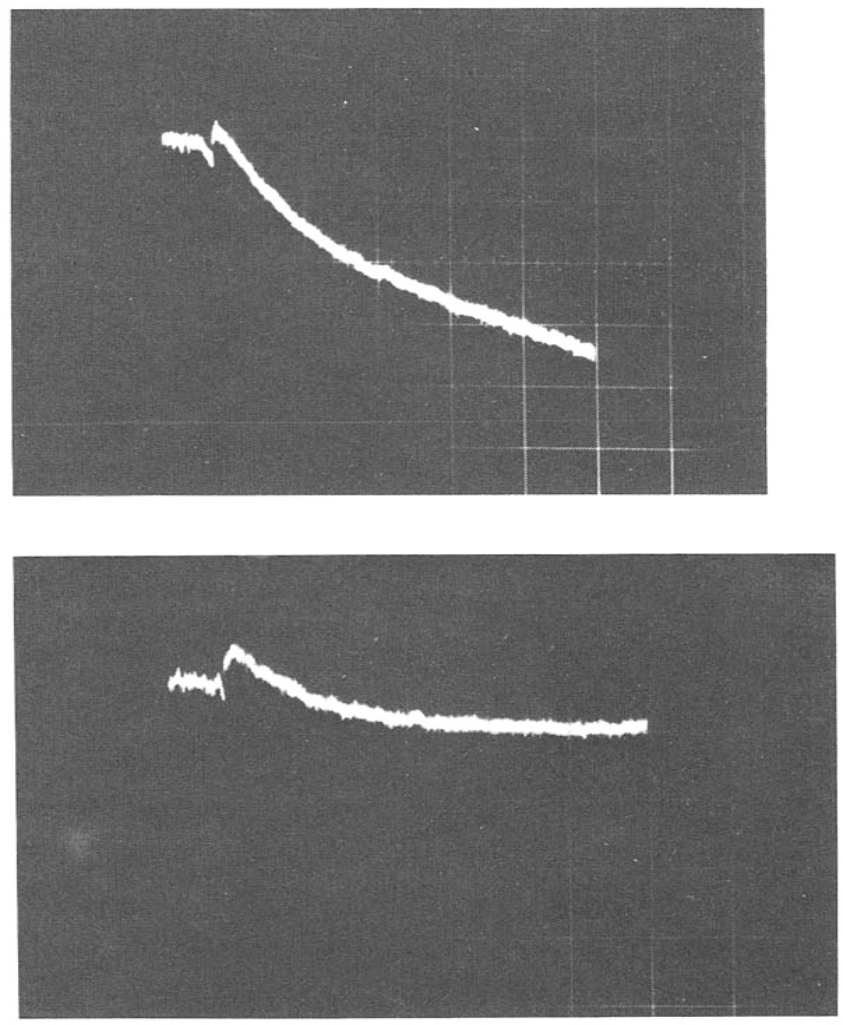

Fig. 5. (a) Stopped-flow dilution by mixing 2.5 vols. of a solution of 0.57 μM ribosomes in 25 mM malonic acid, 5 mM sodium phosphate-KOH, 10 mM NH_4Cl, 8.26 mM Mg^{2+}, at pH 7.8, with 1 vol. of the same buffer containing 0.3% glutaraldehyde. The temperature was 25 °C. Scattered light was monitored at 436 nm. The effective sweep time was 136.5 s/division. (b) Scattered light was monitored at 590 nm. The effective sweep time was 136.5 s/division.

to have a reference process for the glutaraldehyde fixation which is faster and has more amplitude, it was decided to perform further experiments with jumps of Mg^{2+} concentration. In fig. 6a–c are shown the results of Mg^{2+} jump experiments, with both decreasing and increasing Mg^{2+} level. The dissociation process in which the final solution contains only 1 mM Mg^{2+}, causing complete dissociation, is relatively quite fast, as shown in fig. 6a. Reference to fig. 6b shows that a drop of Mg^{2+} concentration from 6 to 2.95 mM results in a much slower ribosome dissociation process.

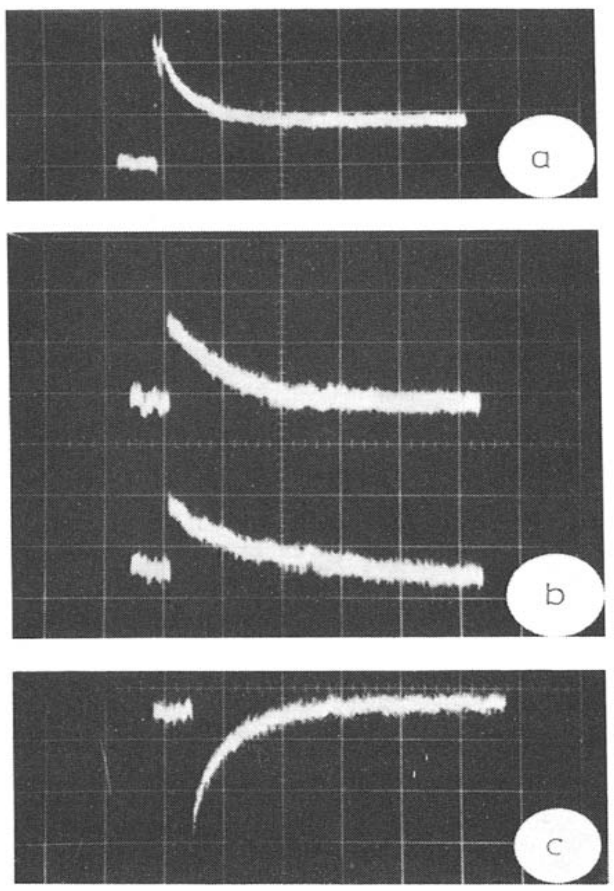

Fig. 6. (a) Stopped-flow kinetic results of mixing a solution of 0.370 μM ribosomes in buffer G at 4 mM free Mg^{2+} with equal volume of control buffer G, containing 4.47 mM EDTA, so as to reduce the final free Mg^{2+} concentration to 1 mM. Sweep time 1.37 s/division. (b) Duplicate stopped-flow kinetic results of mixing a solution of 0.340 μM ribosomes in buffer G at 6 mM free Mg^{2+} with equal volume of control buffer G, containing no Mg^{2+}, so as to reduce the final free Mg^{2+} concentration to 2.95 mM. Sweep time 140 s/division. (c) Stopped-flow kinetic results of mixing a solution of 0.996 μM ribosome in buffer G at 2.5 mM free Mg^{2+} with equal volume of control buffer G, containing higher Mg^{2+} concentration, so as to increase the final free Mg^{2+} concentration to 3.0 mM. Sweep time 70 s/division.

3. Results

The experimental data for fig. 7 and the figures to follow were treated as follows: primary two-coordinate microcomparator readings directly from stopped-flow light-scattering photographs were plotted on graph paper. With the aid of French curves, best-fit continuous curves were drawn through these data. Smoothed secondary experimental data were then interpolated from these curves. These smoothed readings from the curves

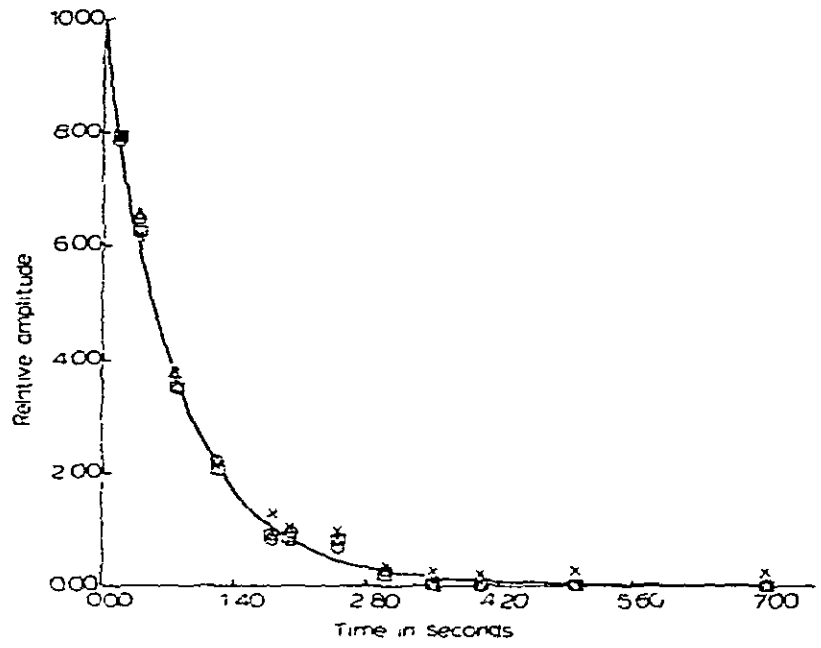

Fig. 7. Stopped-flow kinetic results of mixing solutions of 0.370 μM ribosomes in buffer G at 4 mM free Mg^{2+} with equal volume of control buffer G, containing 4.47 mM EDTA, so as to reduce the final free Mg^{2+} concentration to 1 mM. Glutaraldehyde concentrations: (○) zero, (□) 0.063%, (×) 0.313%. The subunit recombination rate constant k_1 was assumed to be 0. The average value of the ribosome dissociation rate constant k_{-1} was 1.26 s^{-1}.

were plotted as experimental points in fig. 7 and the figures to follow. They were also used to compute values for the pertinent rate constants, as described in section 4. With the aid of the derived rate constants, simulations of the kinetic processes were developed, and these simulations are shown in the figures as continuous curves.

A large number of fixation experiments were performed at various glutaraldehyde concentrations, with shifts of Mg^{2+} levels similar to those in fig. 6. In fig. 7 are shown the results of mixing solutions of 0.370 μM ribosomes in buffer G at 4 mM free Mg^{2+} with control buffer G and with two different levels of glutaraldehyde in buffer G, containing 4.47 mM EDTA, so as to reduce the final free Mg^{2+} concentration to 1 mM. It is seen that even up to a final glutaraldehyde concentration of 0.313%, all of the results are identical, showing that the Mg^{2+}-driven dissociation of ribosomes is in fact much faster than the rate of fixation by glutaraldehyde. Ultracentrifugation of the fixed solution revealed the absence of all 70 S

material, confirming that there was too little time for fixation, before all the 70 S ribosomes had dissociated.

In the next set of fixation experiments, a jump of Mg^{2+} from 3.8 to 6.0 mM was used to increase the extent of ribosome association from approx. 65% to approx. 100% at relatively low ribosome concentration. These conditions were chosen in an attempt to isolate the fixation rate of subunits from the fixation rate of 70 S ribosomes. The half-time for ribosomal association under these conditions is rather rapid, approx. 10 s, but appreciable amplitude is still visible at times greater than 40 s, as shown in fig. 8. When glutaraldehyde is added to the buffer drive syringe, a decrease in amplitude is observed. As the glutaraldehyde concentration is raised, the fixation process increases in speed, allowing less and less of the association to take place.

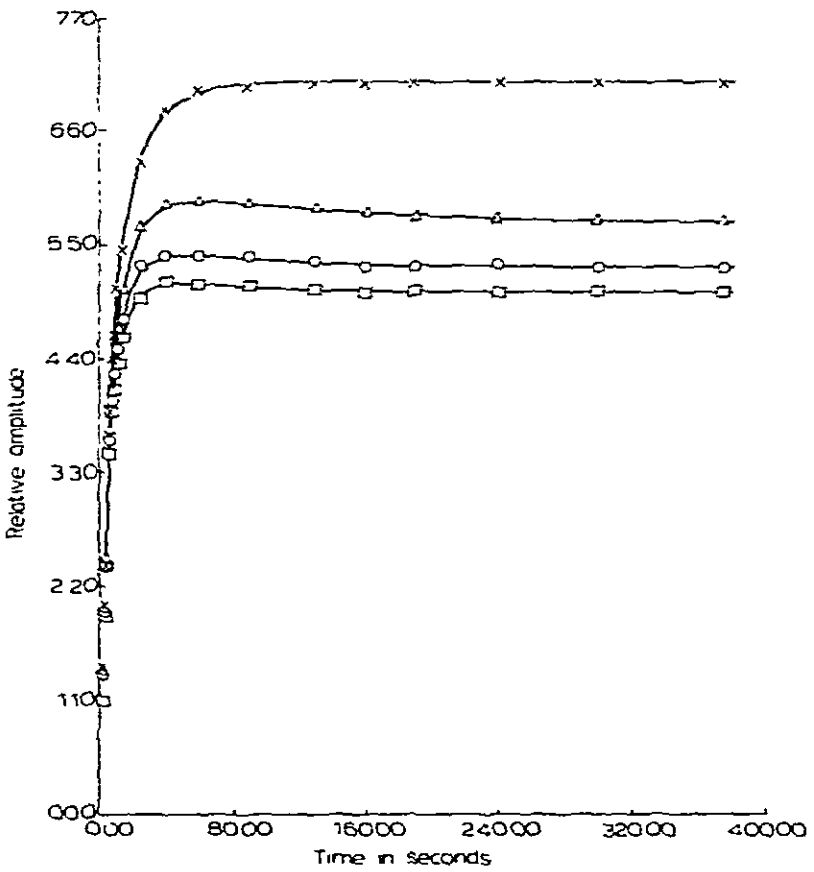

Fig. 8. Stopped-flow kinetic results of mixing solutions of 0.506 μM ribosomes in buffer G at 3.8 mM free Mg^{2+} with equal volume of control buffer G, containing higher Mg^{2+} concentration, so as to increase the final free Mg^{2+} concentration to 6 mM. Glutaraldehyde concentrations: (×) zero, (△) 0.075%, (○) 0.125%, (□) 0.156%. The average value of the subunit recombination rate constant k_1 was 1.66×10^6 M^{-1} s^{-1}. The ribosome dissociation rate constant k_{-1} was estimated to be 10^{-3} s^{-1}. The derived values of the glutaraldehyde fixation rate constant were 1.40, 2.28 and 1.76 l/mol per s for concentrations of 0.075, 0.125 and 0.156%, respectively.

An unexpected result is observed during the latter part of the fixation. After the association has reached its maximum, a slight decrease in light scattering intensity is observed, rather than a flat response. The lower the glutaraldehyde concentration used, the larger is the amplitude of this decrease. This decrease cannot be explained if one assumes no dissociation of ribosomes at 6 mM free Mg^{2+}. The assignment of a nonzero dissociation rate constant provides a pathway for the observed decrease in light scattering. The continuous curves in fig. 8 are again drawn with the aid of the kinetic constants determined as explained below.

The next series was specifically designed so that the final equilibrium position would be at approx. 50% association, where both the dissociation and reassociation rate constants are appreciable. In this way, a small difference in the fixation rate constants for the subunits will be detectable. In this series, the Mg^{2+} was dropped from 3.4 to 3.0 mM, in buffer G. This provided a sensitive test to ascertain whether the fixation rate constants of the 30 S, 50 S and 70 S species needed to be assigned different values. The results are shown in fig. 9. High concentrations of glutaraldehyde are seen to decrease the amplitude of the dissociation curve. A decrease of the glutaraldehyde concentration slows the fixation, and thus allows more of the ribosomal dissociation to be completed. At the lowest glutaraldehyde concentration used in this series, 0.05%, this reaction has a longer half-time of reaction and a greater reaction amplitude than the reaction with no glutaraldehyde. What is occurring here can be explained by the relatively slow fixation rate at low glutaraldehyde concentrations. After the reactants are mixed, the ribosomes attempt to reequilibrate according to the rate constants dictated by the new ionic conditions. The glutaraldehyde, no longer of sufficient concentration to freeze the association-dissociation reaction before significant further dissociation has taken place, slowly removes 30 S, 50 S and 70 S particles from the reactive pool in solution. This effect is akin to dilution. As the concentrations of all active

species decrease, readjustments to satisfy mass action requirements cause further dissociation. When fixation is complete, the scattered light intensity becomes independent of time.

Analogous to the previous set of experiments, a series in which the Mg^{2+} concentration was jumped from 2.5 to 3.0 mM was designed so that both the association and dissociation rate constants were appreciable. The results are shown in fig. 10. As expected, as the concentration of glutaraldehyde was increased, the reaction amplitudes relative to the control experiments without glutaraldehyde were decreased. The late-time glutaraldehyde-induced dissociation, observed in figs. 8 and 9, was also observed in this series.

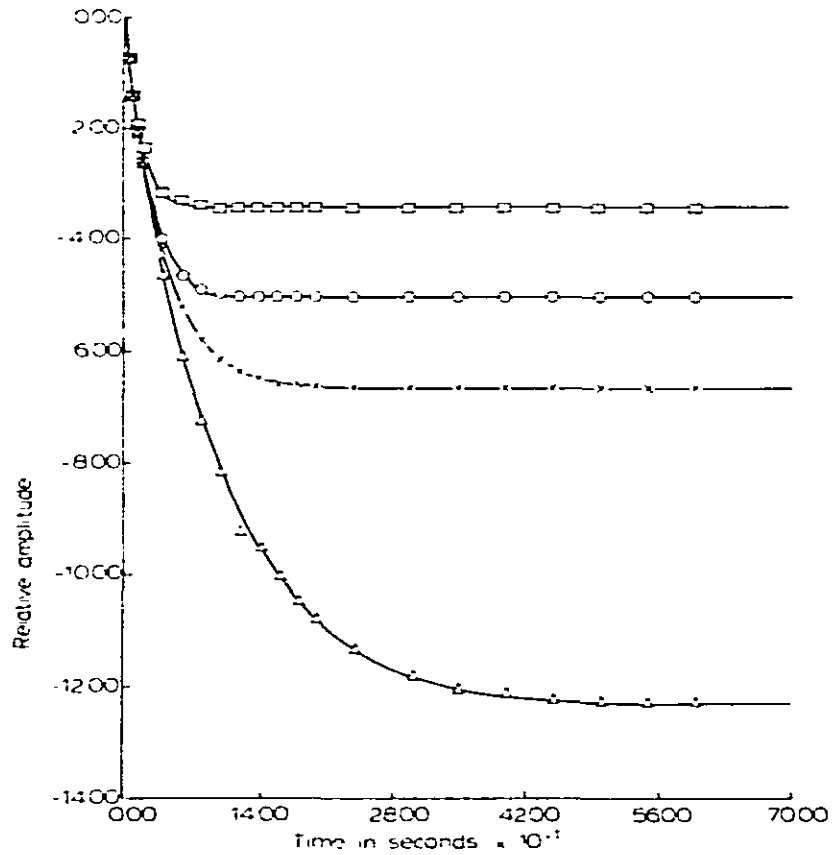

Fig. 9. Stopped-flow kinetic results of mixing solutions of 1.632 μM ribosomes in buffer G at 3.4 mM free Mg^{2+} with equal volume of control buffer G, containing lower Mg^{2+} concentration, so as to reduce the final free Mg^{2+} concentration to 3 mM. Glutaraldehyde concentrations: (×) zero, (△) 0.050%, (○) 0.250%, (□) 0.400%. The average value of the ribosome subunit recombination rate constant was 2.2×10^{4} M^{-1} s^{-1}. The ribosome dissociation rate constant was 8.5×10^{-3} s^{-1}. The derived values for the glutaraldehyde fixation rate constant were 1.66, 1.79 and 1.38 l/mol per s at glutaraldehyde concentrations of 0.05, 0.25 and 0.40%, respectively.

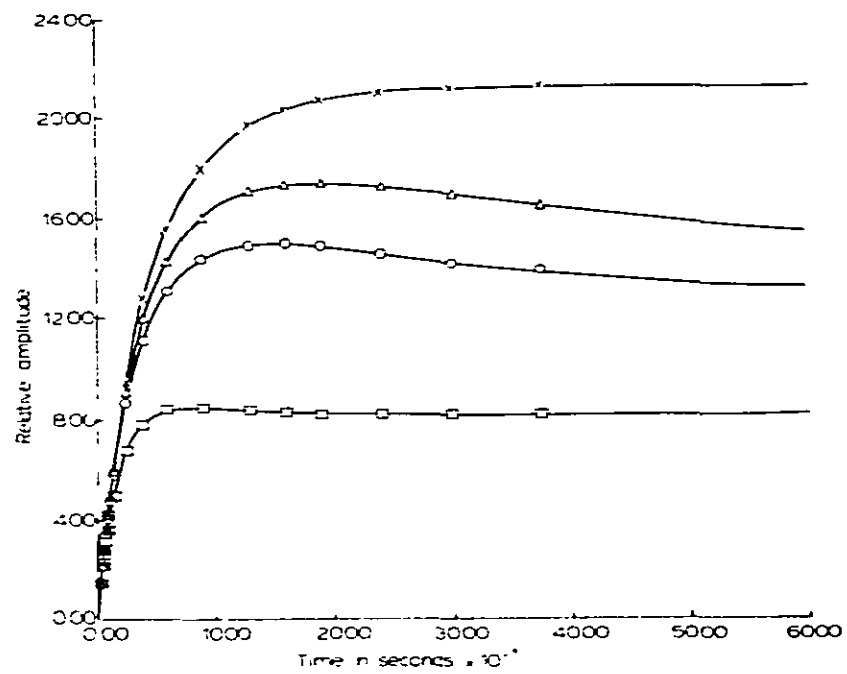

Fig. 10. Stopped-flow kinetic results of mixing solutions of 0.996 μM ribosomes in buffer G at 2.5 mM free Mg^{2+} with equal volume of control buffer G, containing higher Mg^{2+} concentration, so as to increase the final free Mg^{2+} concentration to 3 mM. Glutaraldehyde concentrations: (×) zero, (△) 0.0125%, (○) 0.025%, (□) 0.10%. The subunit recombination rate constant k_1 was 4.2×10^{4} l/mol per s. The ribosome dissociation rate constant k_{-1} was 4.1×10^{-3} s^{-1}. The derived values for the glutaraldehyde fixation rate constant were 1.79, 2.34 and 1.85 l/mol per s at glutaraldehyde concentrations of 0.0125, 0.025 and 0.10%, respectively.

4. Kinetic analysis

The simplest analytical method for obtaining rate constants for a bimolecular reaction is that of relaxation kinetics. For small perturbations, the decay to equilibrium is a simple exponential function of time. However, reaction amplitudes are necessarily small. In the present case, the glutaraldehyde fixation reaction was a perturbation superimposed upon the bimolecular ribosomal reequilibration process. When the small amplitude required for relaxation observation was perturbed in this way, as in the case of dilution experiments or stopped-flow experiments with very small changes of Mg^{2+} concentration, the changes in reaction speeds and reaction amplitudes caused by the perturbation due to glutaraldehyde fixation were too small for accurate analysis. Conse-

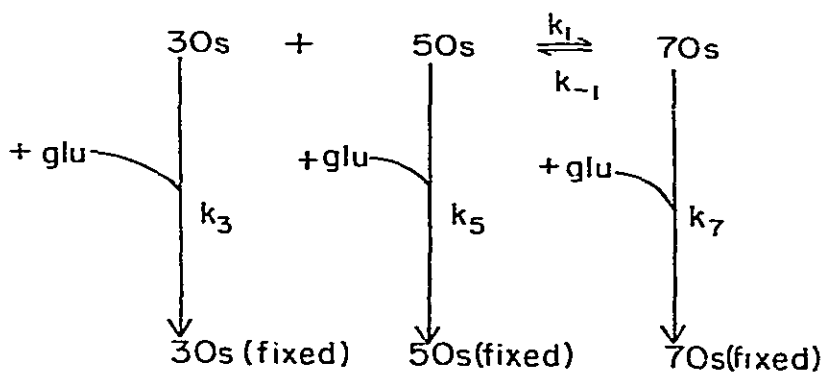

Fig. 11. Mechanism of the reaction processes, and associated rate constants.

quently, it was decided to employ large perturbations, as illustrated by the figures shown in section 3, and to solve the ensuing nonlinear kinetics.

The mechanism assumed for the coupled reactions, and the pertinent rate constants are illustrated in fig. 11.

$w(t)$, $x(t)$, $y(t)$ and $z(t)$ are taken to represent the time-dependent increments of concentration, since the time of mixing, of the species 70 S (total), 30 S (fixed), 50 S (fixed) and 70 S (fixed), respectively. The initial concentrations of unfixed 30 S, 50 S and 70 S species immediately after mixing are denoted by c_3, c_5 and c_7. The time-independent concentration of glutaraldehyde is denoted by G_0. The glutaraldehyde was present in such large excess that it was possible to assume that its concentration did not change during the course of the experiments. The time-dependent concentrations of unfixed 70 S, 30 S and 50 S species are therefore given by $c_7 + w - z$, $c_3 - w - x$, and $c_5 - w - y$, respectively.

The differential equations for the kinetics are

$$dw/dt = k_1(c_3 - w - x)(c_5 - w - y) - k_{-1}(c_7 + w - z) \quad (1)$$

$$dx/dt = k_3(c_3 - w - x)(G_0) \quad (2)$$

$$dy/dt = k_5(c_5 - w - y)(G_0) \quad (3)$$

$$dz/dt = k_7(c_7 + w - z)(G_0) \quad (4)$$

k_1 and k_{-1} denote the rate constants for subunit recombination and ribosome dissociation, respectively.

The fixation rate constants for the 30 S, 50 S and 70 S species are k_3, k_5 and k_7, respectively.

For the case where no glutaraldehyde is present, an exact solution is available by integration [3,8–11]. With the aid of this solution, the rate constants for ribosome dissociation and subunit reassociation have been determined for each fixation experiment from reference experiments under identical conditions, but without glutaraldehyde. Once these constants are known, they are used to evaluate the glutaraldehyde fixation rate constant. For the corresponding fixation experiments, a numerical integration by the Runge-Kutta method was employed [8,12,13]. The necessary computer programs in BASIC were very kindly furnished to us by Dr. Arnold Wishnia, and were appropriately adjusted to operate at the University of Connecticut IBM computer system [8].

For all of the cases described by the figures, the continuous curves were computed with the aid of the rate constants derived from this mathematical treatment of the differential equations. The experimental data were analyzed on the basis of several different assumptions in which the rate constants for fixation by glutaraldehyde were nonidentical [8]. In addition, a statistical analysis was made in which it was assumed that $k_3 = k_5 = k_7$. This indicated within a 90% confidence limit that the assumption of identical fixation rate constants was sufficient to explain the observed results. The average value of the derived fixation rate constant was observed to be $1.7 \pm 0.4\ M^{-1}\ s^{-1}$. While individual experiments were separately fitted with derived values for the fixation rate constant, no systematic variation with Mg^{2+} concentration was observed.

The individually determined average values for the rate constants k_1 and k_{-1} for ribosome subunit recombination and ribosome dissociation corresponding to the conditions for the Mg^{2+} shifts in figs. 7–10 are indicated in the legends to the figures. The individual fixation rate constants $k_3 = k_5 = k_7$ derived in these experiments are also indicated in the figure legends.

For a given set of solutions, the replicate values for k_1 and k_{-1} always agreed with these average values within 15% or better. It is conjectured that the large difference between the rate constant values in figs. 9 and 10 may be due to residual

differences in free Mg^{2+} levels. Fig. 3b shows that even in Tris buffers containing 25 mM malonate, the Mg^{2+} buffering capacity is still inadequate to maintain the nominal 3 mM Mg^{2+} level of the buffer, when the stock ribosomes are dissolved directly in the buffer and their final concentration in the stopped-flow cell is 0.844 μM. Sephadex G-25 column buffer replacement was done for experiments in phosphate buffer G. However, in the phosphate-malonate buffer experiments of fig. 9, where the final ribosome concentration is 0.816 μM, the Mg^{2+} level might possibly still be depressed somewhat further below the nominal final value of 3 mM than in the related experiments of fig. 10, where the final ribosome concentration is only 0.498 μM. The values of k_1 and k_{-1} change precipitously with falling $[Mg^{2+}]$ in this range of $[Mg^{2+}]$.

5. Discussion

Since we now have a rate constant for glutaraldehyde fixation, it is possible to visualize the competitive kinetics of fixation and ribosome association-dissociation. What we have found, as illustrated in figs. 8–10, is that for glutaraldehyde concentrations of 0.4% and below, fixation is too slow to freeze the ribosome system at its initial equilibrium composition, whenever the Mg^{2+} concentration allows reequilibration between 70 S ribosomes and subunits. On the other hand, glutaraldehyde concentrations as great as 0.25% have been reported to cause cross-linking aggregation reactions to occur between individual subunits and also between assembled ribosomes [2]. If one chooses to avoid the possibility of such aggregation reactions by selecting glutaraldehyde concentrations of 0.25% or less, then the bias in analyses by the fixation method is predicted from our present research to be always in the same direction. Ordinarily, every analysis which is affected by a reequilibration of ribosomes with subunits during the period of fixation will probably indicate that the preparations contain more dissociated subunits than were really present in the original ribosome solution prior to fixation. As shown in fig. 8, even at 6 mM Mg^{2+}, there is still time for a slight reequilibration of tight-couple 70 S ribosomes to subunits when the glutaraldehyde concentration is 0.156%. Consequently, the results of this research would indicate a strong preference for performing the fixation with glutaraldehyde at Mg^{2+} concentrations no lower than 6 mM.

6. Summary

Stopped-flow measurements with a light-scattering detector were used to follow the formation or dissociation of ribosomes with sudden changes of Mg^{2+} concentration. The perturbation of such mixing experiments by the addition of glutaraldehyde to the mixing buffer system was used to assess the relative speeds of the ribosome reequilibration and the fixation process. In order to control the Mg^{2+} concentration, an Mg^{2+} buffer of malonate ion was found to be successful at pH 7.8, for moderate ribosome concentrations. The validity of the fixation-ultracentrifugation analysis of ribosome purity has been assessed, and it has been indicated that when the rate of fixation is too slow, this procedure tends to assign too high a concentration of subunits to the original preparation of ribosomes. It is recommended that fixation be performed at Mg^{2+} concentrations no lower than 6 mM.

It is cautioned that these conclusions were derived in phosphate buffers, since Tris buffers could not be used in quantitative light-scattering observations. Tris buffers are at risk in general during fixation, because of the generation of high molecular weight contaminants caused by glutaraldehyde interaction with Tris.

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