Hydrogen Exchange Studies on Ribosomes*

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ABSTRACT: The Sephadex and rapid dialysis techniques have been used to observe the hydrogen–tritium exchange behavior of rat liver ribosomes. In addition, the possibility of using hydrogen exchange methods for detecting changes in ribosomal structure has been studied by seeking changes in hydrogen exchange behavior on the removal of Mg^{2+} from ribosomes. The data showed good precision and reproducibility. The large number of slowly exchanging hydrogens per ribosome suggests that ribosomal fine structure is highly hydrogen bonded. A spectrum of exchange rates for these hydrogens was found. The rate of exchange increases with increasing temperature and pH (pH 6.5–8.2), and is catalyzed by Tris buffer. Addition of EDTA to a ribosome solution *during* an exchange-out experiment leads to dissociation into ribosomal subunits and causes a great increase in the exchange-out rate of almost all remaining bound tritium.

That this accelerated exchange does not simply reflect an unmasking of intersubunit exchange sites upon dissociation was shown by the demonstration that the dissociation proceeds much faster (half-time of 20 sec) than does the accelerated exchange. Some results with the isolated large subunit are consistent with the hypothesis that the accelerated exchange represents a general "loosening" of subunit structure, and further suggest that maintenance of the "tight" structure of subunits in the intact ribosome depends upon intersubunit interactions.

A knowledge of structure and of possible functionally important changes in structure of intact ribosomes is fundamental to a detailed understanding of the chemistry of protein biosynthesis. The recently improved hydrogen exchange technique, among other methods, offers the possibility of approaching such problems.

Hydrogen atoms bound to nitrogen, oxygen, and sulfur can exchange with hydrogen atoms of water. These include the hydrogens that participate in protein and nucleic acid hydrogen bonds. It is thought that hydrogen bonds of proteins and nucleic acids engage in continual opening and closing reactions (Hvidt and Nielsen, 1966) which have been termed "breathing" (Printz and von Hippel, 1965). The extent of the breathing of a particular hydrogen bond depends upon the nature and stability of the three-dimensional structure in its immediate vicinity, and regulates the rate of exchange of the hydrogen atom. Under the conditions of our experiments, hydrogens unhindered by structure will exchange "instantaneously,"[1] those involved in or protected by structure may exchange measurably slowly. For example, hydrogen-bonded hydrogens of double-stranded DNA (Printz and von Hippel, 1965) and many hydrogens of sRNA (Englander and Englander, 1965) and proteins (Hvidt and Nielsen, 1966) show substantially decreased rates of exchange.

The realization that hydrogen exchange data could provide information about the three-dimensional structure of proteins led Linderstrøm-Lang and his collaborators initially and others subsequently to develop methods for obtaining this kind of information (Hvidt and Nielsen, 1966). In the experiments on ribosomes reported here, we have used the recently developed Sephadex and rapid dialysis methods (Englander, 1963; Englander and Crowe, 1965). These methods avoid the freezing and drying steps of previous techniques which might lead to spurious results (Englander, 1967), particularly with structures as complex as ribosomes.

In the present experiments, the exchangeable hydrogens of ribosomes were first labeled with tritium by prolonged incubation in tritiated water (THO).[2] Passage through a Sephadex column (or rapid dialysis)

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[1] "Instantaneous" exchange means exchange that is too rapid to measure by the techniques used. Under the conditions used in our experiments, unhindered protein hydrogens would be expected to exchange in less than a second, unhindered nucleic acid hydrogens in less than a millisecond (Englander, 1967).

[2] Abbreviations used: THO, tritiated water; GTP, guanosine triphosphate.

removed free ("excess") THO, yielding tritiated ribosomes in unlabeled solvent. The kinetics of exchange out of tritium from these ribosomes were then followed by repeating the Sephadex (or dialysis) procedure, to measure the amount of tritium remaining bound to the ribosomes as a function of time. Very early points were obtained by combining the two Sephadex steps into a single column run.

In addition to its potential usefulness in the study of fine structure, the hydrogen exchange approach can be used to detect *changes* in structure. Further, it offers the unique opportunity for investigating changes in the structure of a specific macromolecule even though it is only a minor component of a complex macromolecular system. Indeed, these changes can, in principle, be followed while the system is functioning. For example, should a transitory change in the structure of the *tritium-labeled* ribosome occur in its interaction with some component of the system during protein synthesis, say tRNA or GTP, or with some exogenous substance such as streptomycin, the change in structure might be detected as an alteration in the rate at which bound tritium is freed.

This paper reports some exploratory experiments on the hydrogen exchange behavior of ribosomes and on the possible use of this approach in the study of changes in ribosomal structure. As an example of such structural change we have studied the effect of Mg^{2+} withdrawal on the hydrogen exchange character of ribosomes.

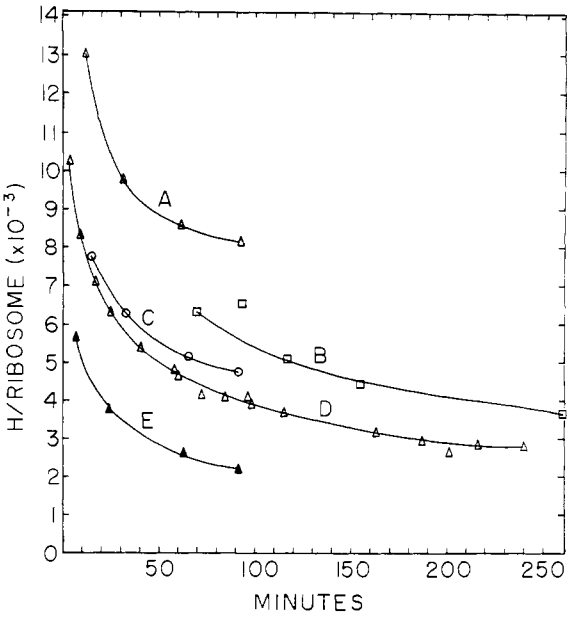

FIGURE 1: Effect of solvent conditions on hydrogen exchange of ribosomes. Curve A, cacodylate, pH 6.5, 4°; curve B, cacodylate, pH 7.3, 4°; curve C, cacodylate, pH 8.2, 4°; curve D, Tris, pH 8.2, 4°; curve E, cacodylate, pH 7.3, 25°. Curves A, B, C, and E, tritium exchange out was followed in a solution containing 0.05 M cacodylate, 0.001 M $MgCl_2$, and 0.05 M KCl. Curve D, 0.05 M Tris substituted for the cacodylate (*i.e.*, solution A). Later points were obtained by the rapid dialysis technique; the Sephadex technique was used to obtain the points earlier than about 60–100 min. The pH of the cacodylate solution at pH 8.2 was very carefully adjusted and rechecked.

Materials and Methods

Materials. Rats were male Wistar or CD strains obtained from the Charles River Laboratories or from Connecticut River Biological Supplies and usually weighed 75–150 g. Nucleoside triphosphates were products of Pabst Laboratories or the Sigma Chemical Co. DL-[1-^{14}C]Leucine (25 mc/mole) and THO (1 c/ml) were obtained from the New England Nuclear Corp. ^{28}Mg was purchased as $MgCl_2$ from Iso-Serve, Inc. Tris was a product of Sigma Chemical Co. Sucrose (Fisher Scientific Co.) was tested and found to be free of RNase activity. Solution A consisted of 0.001 M $MgCl_2$, 0.05 M Tris, pH 7.6 (adjusted at 25°), and 0.05 M KCl. At 4° the pH of this solution is 8.2. Solution B contained the same components except that $MgCl_2$ was omitted. pH adjustments were routinely made after all components of a solution were added.

Temperature. All preparative operations were carried out at 2° as were the tritium equilibrations.

Preparation of Ribosomes. Preparation of ribosomes was carried out essentially as described by Takanami (1961) except that the Mg^{2+}-precipitated ribosomes, after washing, were dialyzed overnight against solution A (see Materials). In one experiment (Figure 1, curve B), cacodylate was substituted for Tris in this solution and the pH was adjusted to 7.3. The ribosomal suspension was then centrifuged at 13,000*g* for 20 min and the equilibration in THO was begun. Ribosomes so prepared possessed an OD_{260} of 24.5 cm^{-1}/mg of ribosomal protein (determined by the biuret method (Layne, 1957)), indicating a composition of approximately 50% protein, and gave a 260-:280-mμ absorbancy ratio of about 1.9. Sedimentation analysis using ultraviolet optics showed a major 82S peak which comprised at least 80% of the total material, and a small 54S peak. It should be noted that salt-washed ribosomes have thus far not been used in this work. The relationship of hydrogen exchange to the removal of ribosomal protein and other ribosomal constituents is a subject for future study.

Preparation of Other Cell Fractions. The pH 5 fraction and DL-[1-^{14}C]leucyl-sRNA were prepared essentially as described by Hoagland *et al.* (1958), except that the DL-[1-^{14}C]leucyl-sRNA preparation was freed of small molecules by passage through Sephadex G-25 and reprecipitation with cold ethanol. The specific activity of different preparations ranged from 33,000 to 45,000 cpm/mg of sRNA. The pH 5 supernatant fluid was immediately adjusted to pH 8.0 (0°) and was stored at −70°.

Assay of Ribosomal Activity. Ribosomes were tested for amino acid incorporating activity using DL-[1-

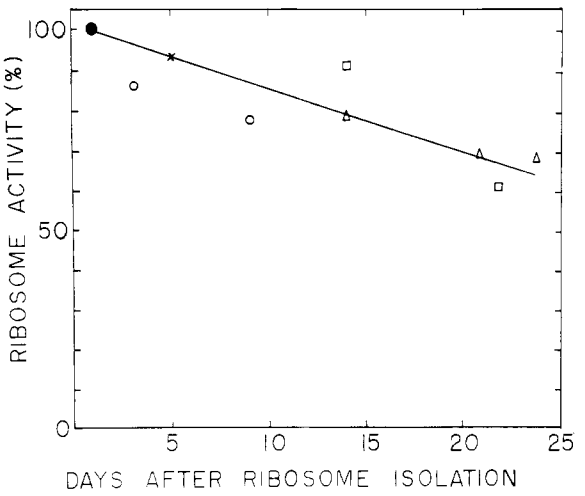

FIGURE 2: Decrease in ribosomal activity with time of preincubation. Preincubation was at 2° in solution A. Ribosomes were assayed by their ability to effect the transfer of [^{14}C]leucine from leucyl-sRNA to polypeptide. Initial activity for each preparation was obtained one day after isolation (immediately after overnight dialysis). These initial values were all normalized to 100% activity, which represents about 50% transfer of leucine. The incubation mixture contained in a 0.25-ml volume: 0.85 μmole of $MgCl_2$, 11.3–12.5 μmoles of KCl, 7.5 μmoles of Tris·HCl, (pH 7.6, at 25°), 0.2 μmole of GTP (pH 5), supernatant fluid containing 0.6–0.7 mg of protein, 0.025 mg of an aminoacyl-sRNA preparation containing 1000 cpm of [1-C^{14}]leucyl-sRNA, and an amount of rat liver ribosomes equivalent to 0.15 mg of ribosomal RNA (0.30 mg was used in some preparations). The reaction mixture was incubated at 37° for 20 min. The different symbols marking points on the curve represent different ribosomal preparations.

^{14}C]leucyl-sRNA in the system described in Figure 2. Incorporation showed an absolute dependence on added pH 5 supernatant fluid and on ribosomes.

Hydrogen Exchange. With one exception (see legend to Figure 1), equilibration of the exchangeable hydrogens of ribosomes with THO was accomplished by incubating ribosomes in solution A at 2° for the times specified. The final concentrations used were about 1.5 mg/ml of ribosomal protein and 10 mc/ml of tritium. At the termination of the equilibration period, unbound tritium was removed by the Sephadex procedure (Englander, 1963) or by rapid dialysis (Englander and Crowe, 1965). For the Sephadex technique, in a single-column run (4-min point, Figure 1), 0.5 ml was applied to the column (8 cm in height, 3 cm in diameter) and samples through the effluent peak were assayed directly for absorbancy at 260 mμ and for tritium content. For the two-column technique, 0.75 ml was used for the first column (6 cm in height, 3 cm in diameter). The ribosome peak emerged in a volume of about 5 ml and was allowed to incubate under conditions described in the figures. Samples of 1 ml were removed at appropriate time intervals and applied to the second Sephadex column (6 cm in height, 3 cm diameter) and the effluent peak was collected in several fractions for assay of absorbancy and radioactivity. Fractions preceding the ribosome peak were used as blanks for the radioactivity and spectrophotometric analyses and were always very low.

The rapid dialysis procedure was carried out as described by Englander and Crowe (1965) using 0.5- to 1.2-ml samples of the THO-equilibrated ribosomal solution. Samples were removed from the dialysis sac at appropriate time intervals and diluted for spectrophotometric and radioactivity assays. Rubber gloves were used to avoid nuclease contamination. Correction for free tritium inside the sac was made by counting a sample of the dialysate removed simultaneously. Corrections rarely exceeded 1–2%.

The choice of technique was dictated by a number of considerations. Points as early as 2 (in single-column runs) or 5 min (in two-column runs) can be obtained by the Sephadex technique, whereas only longer time points can be obtained by the dialysis procedure. On the other hand, rapid dialysis requires fewer preparatory operations and considerably less material per point. Aside from these characteristics, the particular manipulations necessary for an experiment have often been a decisive factor.

Assay of Radioactivity. Tritium samples were counted in Bray's (1960) solution in a Packard Tri-Carb liquid scintillation counter at a counting efficiency of about 8% and a background of about 15 cpm; ^{14}C-labeled protein was washed and counted by the slightly modified (Dietz *et al.*, 1965) paper disk method of Mans and Novelli (1961), with an efficiency of 50% and a background of 12 cpm. ^{28}Mg was counted on a Nuclear-Chicago DS-5 scintillation detector with a counting efficiency of 28% and a background of 220 cpm.

Measurement of Light Scattering. Light scattering at an angle of 90° was measured in a Turner photofluorimeter at 530 mμ (*i.e.*, filters 1-60 and 58 combined) and recorded automatically on a linear scale. The ribosome suspensions used (1 mg/ml) had a transmittance of 97–99% at this wavelength. All operations were carried out at 4° and precautions were taken to keep solutions and glassware free of atmospheric dust. Unless a special holder is constructed for the cuvet, it is important not to move it during any series of measurements.

Isolation of Large Subunit. Convex exponential gradients of sucrose in solution B were made as described by Wettstein and Noll (1965) using 1.3 M sucrose in the reservoir and 0.3 M sucrose in the mixing chamber. The ribosome suspension containing 12.5 mg of ribosomal protein was layered onto 55 ml of this density gradient as an inverted ribosomal concentration gradient (Britten and Roberts, 1960) in 2.7 ml of a 0–2.5 M sucrose density gradient in solution B, containing EDTA in a concentration equimolar to the Mg^{2+} concentration of the ribosome solution. Simultaneous sedimentation of two such samples was accomplished in the SW 25.2 rotor of the Spinco L-2 centrifuge at

25,000 rpm for 20 hr with no braking. The tubes were tapped from the bottom, 1.25-ml samples were removed, and their absorbancies at 260 mμ were determined. The leading edges of the large subunit peaks in two samples were pooled and dialyzed 15 hr against 100 volumes of solution B and 12 hr against multiple changes of 100 volumes each of solution A, with stirring. The 260-:280-mμ ratio of the dialyzed solution was 1.86. This preparation was equilibrated in THO (50 μc/ml) and approximately 1.2-ml samples were analyzed for hydrogen exchange by the rapid dialysis technique.

Calculations and Expression of Data. The parameter, hydrogens per ribosome, was calculated as described by Englander (1963) using standards prepared from the equilibrating solution to determine C_0, the initial specific activity of the THO. The "molar" absorbancy of ribosomes at 260 mμ was taken as 5.56×10^7 cm^{-1} using a nominal value of 4×10^6 for molecular weight and of 139 for $E^{1\%}_{1\,cm.}$.

Zero time for the Sephadex runs was taken as the moment of entry of the equilibrated sample into the first column. In dialysis, zero time was taken as the moment of introduction of the equilibrated sample into the immersed dialysis bag. True zero time, *i.e.*, the time at which sufficient reduction of solvent tritium occurs to render back exchange of tritium insignificant, differs slightly from the above nominal zero time (Englander and Englander, 1965).

In the dialysis technique, gradual dilution of the contents of the bag occurs in long runs, and can occur inadvertently during sampling. Such dilution does not affect the results.

Results

Hydrogen Exchange Character of Ribosome

Hydrogen Exchange Curve. The results in Figure 3 show that it is possible to obtain hydrogen exchange data for rat liver ribosomes. These and other data have shown a high degree of precision within an experiment. Reproducibility from preparation to preparation was excellent although an occasional run was 10% high (*cf.* Figures 3 and 4). Ribosomes from old and young rats yielded identical exchange data. *Escherichia coli* ribosomes showed the same general pattern of exchange.

As with proteins and sRNA, the hydrogens of ribosomes show a spectrum of exchange rates. Some hydrogens exchange extremely slowly; even after 2 days, about 1000 hydrogens have not yet exchanged out.

Equilibration of Ribosomes with THO. In view of the slow exchange rates of some hydrogens, it is necessary, in order to obtain reasonably complete labeling of the ribosome, to incubate in THO for a number of days. Figure 3 shows data obtained from two different rat liver ribosome preparations equilibrated for 4, 7, and 8 days. It can be seen that the 7- and 8-day equilibration periods result in little additional labeling; 14 days of equilibration (Figure 5, curve B) in one preparation showed some increase in labeling of the slowly exchanging hydrogens. The decay in ribosomal amino acid incorporating activity over these time periods is not large (Figure 2).

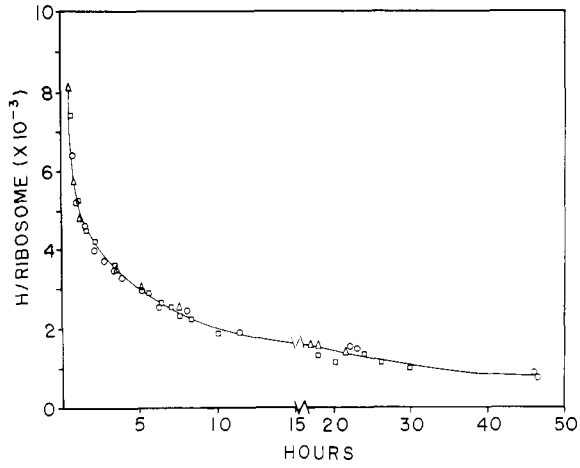

FIGURE 3: Hydrogen exchange curve of rat liver ribosomes. The data were obtained by the rapid dialysis technique. Ribosomes (1.8 mg of protein) were dialyzed for exchange out in 1.2 ml of solution A at 4°. Two independent ribosome preparations were used. □, preparation A, equilibrated with THO for 4 days; ○, preparation A, equilibrated for 8 days; and Δ, preparation B, equilibrated for 7 days.

Effect of Solvent Conditions. The effect of some solvent conditions on the hydrogen exchange rate of ribosomes is shown in Figure 1. Some of these data were obtained by the Sephadex method and some by rapid dialysis; studies on ribonuclease (Englander, 1967) and sRNA (Englander and Crowe, 1965) have shown that the two techniques give identical results. An increase in the rate of exchange is seen as the pH is increased through the physiologically interesting region, from 6.5 to 8.2 (curves A–C). This behavior is qualitatively the same in proteins (Hvidt and Nielsen, 1966) and in sRNA (R. Gantt, S. W. Englander, and M. V. Simpson, unpublished data), through this pH range and presumably results from hydroxide ion catalysis, in part. However, the relationship between rate and hydroxide ion concentration does not seem to be a simple linear one and requires further investigation.

A comparison of curves B and E shows a large temperature effect, a rise of 21° resulting in an approximately tenfold increase in rate, indicating an average Q_{10} of about 3. A Q_{10} of the same order has been observed for sRNA (R. Gantt, S. W. Englander, and M. V. Simpson, unpublished data), proteins and polypeptides (Hvidt and Nielsen, 1966), and *N*-methylacetamide (Klotz and Frank, 1964). Part of this effect very likely stems from an increase in hydroxide ion concentration resulting from the temperature dependence of the ion product of water ($K_{w,\,25°}/K_{w,\,4°} = 6.0$).

Although curves C and D were obtained at identical conditions of pH and temperature, they indicate that hydrogen exchange rates may be influenced by the

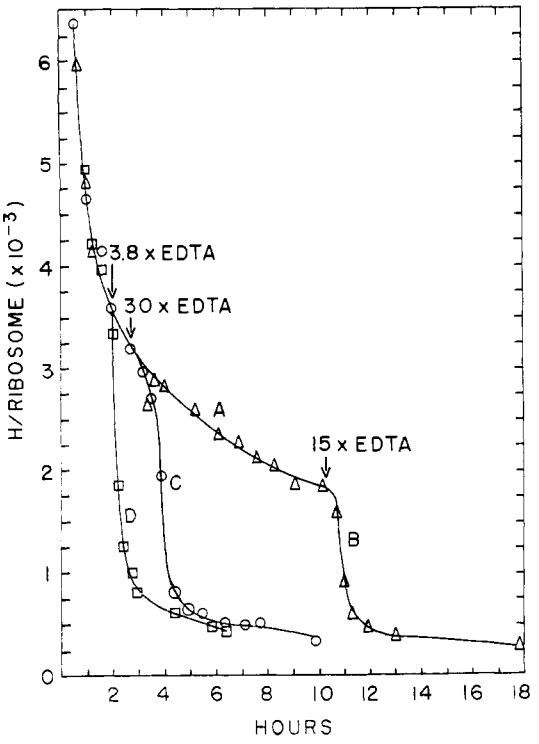

FIGURE 4: The effect of dialysis against EDTA and of direct addition of EDTA on the hydrogen exchange properties of ribosomes. Experimental conditions were identical with those given in Figure 5 with the exception that in curve D, the EDTA was added directly to the ribosomal suspension within the dialysis sac.

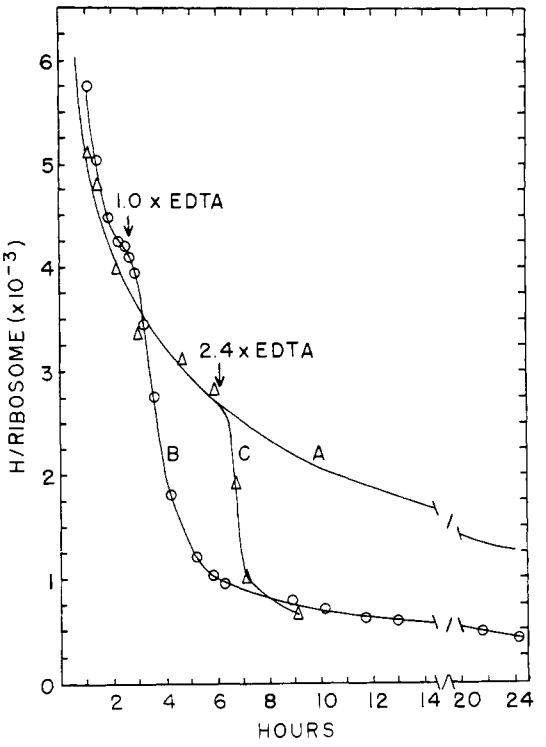

FIGURE 5: The effect of dialysis against EDTA on the hydrogen exchange properties of ribosomes. Data were obtained by the rapid dialysis technique. THO-equilibrated ribosomes were dialyzed for exchange out in the rapid dialysis unit, against solution A (0.05 M Tris, pH 8.2, 0.001 M $MgCl_2$, and 0.05 M KCl). At the time indicated by the arrow, the sac was transferred to a similar solution except that EDTA replaced Mg^{2+}. Dialysis was carried out at 4°. The values given for EDTA represent the molar ratio of the total amount of EDTA in the dialysis solution to the total amount of Mg^{2+} initially within the sac (ignoring ribosome-bound Mg^{2+}). Curve A is taken from Figure 3.

nature of the buffer present. Here, the rate in 0.05 M Tris exceeds that in 0.05 M cacodylate by about 40%. Catalysis of hydrogen exchange by Tris has previously been shown for *N*-methylacetamide (Klotz and Frank, 1965), for sRNA (R. Gantt, S. W. Englander, and M. V. Simpson, unpublished data), and for DNA (M. Printz and P. H. von Hippel, unpublished data).

Effect of Mg Withdrawal. The physical integrity of ribosomes and their activity in protein synthesis are known to depend upon the presence of Mg^{2+} (see Petermann, 1964). Here (Figures 4 and 5), we have studied the effect of withdrawal of Mg^{2+} on the hydrogen exchange properties of ribosomes. Curves A of Figures 4 and 5 show the results of a number of experiments in which hydrogen exchange out of ribosomes was measured in the presence of 0.001 M Mg. In some experiments, the removal of Mg^{2+} (and conceivably some other trace metal) was effected by the transfer of the dialysis sac to a Mg^{2+}-free solution containing EDTA in a total molar ratio to free Mg^{2+} within the sac of 1 (Figure 5, curve B), 2.4 (Figure 5, curve C), 15 (Figure 4, curve B), and 30 (Figure 4, curve C). This treatment leads to a drastic acceleration in the rate of hydrogen exchange so that in a short time, most of the bound tritium is lost. The effect is similar throughout this range of EDTA:Mg^{2+} ratios, though preceptibly slower at the lowest ratio.

The early hydrogen exchange kinetics following EDTA addition were somewhat obscured in these experiments by the slow rate of entry of EDTA from the outer solution into the dialysis sac. The lag after addition of EDTA is abolished by its introduction directly into the sac (Figure 4, curve D). (Tritium, once lost from the ribosome, escapes from the dialysis sac with a half-time of 30 sec. The half-time for dialysis of ^{28}Mg-EDTA is about 5 min. Neither half-time is affected by the presence or absence of ribosomes.) It can now be seen that the effect of the addition of EDTA is immediate. The majority of the accelerated hydrogens exchange with an over-all half-time of about 10 min; the possibility exists, however, that some may exchange much faster.

Structural Changes in Ribosomes. It is known from studies on sRNA (Englander and Englander, 1965) and total cellular RNA (S. W. Englander, unpublished

data) that under salt conditions comparable to those used here, hydrogen exchange is severalfold faster when Mg^{2+} is removed. On the basis of what is known about salt effects on hydrogen exchange of proteins (Benson *et al.*, 1964) and protein models (Klotz and Frank, 1965), it seems very unlikely that 0.001 M Mg^{2+} would exert any significant general effect on protein hydrogen exchange. By contrast, the effect of Mg^{2+} removal on hydrogen exchage of rat liver ribosomes is very large, accelerating the rate by about two orders of magnitude. It therefore seems likely that this large alteration in hydrogen exchange behavior results from a gross change in ribosomal structure rather than simply from an increase of breathing activity alone.

The accelerated hydrogens resulting from this presumed change in ribosomal structure could arise *directly* from the well-known dissociation of ribosomes to subunits, *e.g.*, by the unmasking and liberation of possible intersubunit hydrogens. On the other hand, they may reflect structural changes in the subunits themselves. These possibilities might be distinguished by ascertaining whether or not the rate of loss of the accelerated hydrogens mirrors the rate of ribosome dissociation; the following section contains a description of such kinetic experiments.

Kinetics of Ribosome Dissociation. Inasmuch as information on the early kinetics of ribosome dissociation was not available, experiments were designed to obtain these data. Initial experiments employed band centrifugation in the analytical ultracentrifuge (Vinograd and Bruner, 1966). Here, a band of ribosomal solution was automatically layered on and sedimented into a denser solution containing a total amount of EDTA which was in 1.5-fold excess over the total Mg^{2+}. By 8 min after layering, and in contrast to a no-EDTA control, there were few or no intact ribosomes remaining. Since some of this time was expended in the ribosome band moving away from the Mg^{2+} layer, a reasonable upper estimate of the *half-time* for dissociation is 2 or 3 min. These data are in accord with sedimentation data from other laboratories (see Petermann, 1964, p 134) where, however, the earliest observations were made at least 20 min after EDTA addition.

More rapid observations were made by measuring light scattering, which is sensitive to particle size (Figure 6). Four sequential additions of EDTA were made to the cuvet and readings were taken after each addition. Each volume of solution added contained a total molar amount of EDTA equal to the total number of moles of free Mg^{2+} in the original ribosomal solution. Readings could be taken in less than 20 sec after addition of the EDTA (inclusive of stirring, shutting the door of the instrument, and pen lag).

Each addition of EDTA resulted in a rapid drop in light scattering until a threefold excess of Mg^{2+} over EDTA was attained. Further addition of EDTA caused no further change in light scattering. At this point, the scattering had fallen to just over one-half the initial value, indicating complete dissociation of the ribosome into its two component subunits. Accord-

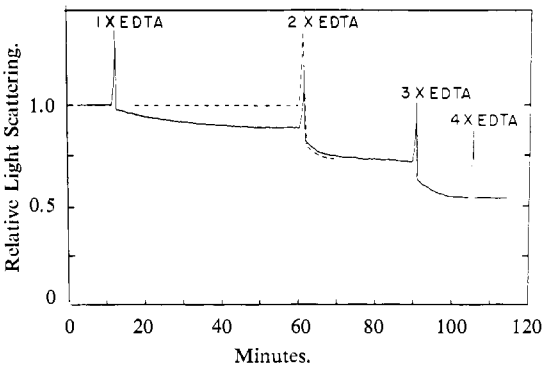

FIGURE 6: Measurement by light scattering of EDTA-induced ribosomal dissociation. The solid lines represent actual tracings. The dotted line is a close approximation to the actual tracing. The spikes represent the points at which the instrument was opened in order to introduce EDTA into the sample cuvet. Subsequent stirring of the sample and closing the instrument door occupy about 10 sec. The molar amount of EDTA added at each spike was equal to the initial molar amount of free Mg^{2+} present, so that the amounts of EDTA indicated are the molar ratios of EDTA:initial Mg^{2+} existing in the sample cuvet after EDTA addition not taking into account the Mg^{2+} introduced as the ribosome-bound ion. The EDTA was added as a component (0.1 M) of solution B. No correction was made for the small (1%) volume changes which occurred on addition of this solution. The dashed line represents the results on initial addition of 2 × EDTA. Ribosomes were present at a concentration of 1 mg/ml in solution A and the temperature was maintained at 4°.

ing to Tashiro and Siekevitz (1965; Petermann, 1964), addition of EDTA to guinea pig liver ribosomes results in a primary dissociation of ribosomes into subunits which then aggregate and which, upon further EDTA addition, disaggregate until the final individual subunits ($s^0_{20,w}$ of 47 and 32 S) are formed at an EDTA:Mg^{2+} ratio of about 3:1. The light-scattering data presented here appear to be in agreement with these results. Monitoring of ribosomes at an EDTA:Mg^{2+} ratio of 2:1 indicates the presence of two components whose $s_{20,w}$ values (ultraviolet optics) are 54 and 32 S, and the complete absence of any intact ribosomes.

The rapidity with which the dissociation of ribosomes occurs after the addition of EDTA is striking; the drop in light scattering is 50% complete in less than 20 sec. When a twofold excess of EDTA was added as a single addition (Figure 6, broken curve), the rate and extent of the change in scattering was almost identical witn that obtained when the same total amount was added in two separate additions. This observation suggests that the result of EDTA addition is independent of the addition schedule.

Following the initial rapid drop in light scattering, a slower continuing drop is not seen once final dissociation to subunits is achieved (at threefold excess of

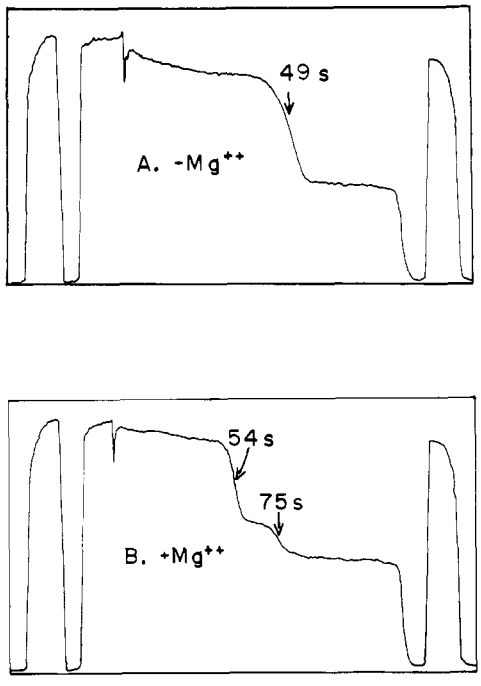

FIGURE 7: Sedimentation pattern of large subunit preparation. The ultraviolet absorption optical system was used and scanning of the photographic plate was done with the Spinco Analytrol. Ribosomes were used at a concentration which gave an $OD_{260\,m\mu}$ of 1.0. (A) 56,100 rpm, 0.05 M Tris–0.05 M KCl, 3°; and (B) 56,100 rpm, 0.05 M Tris–0.05 M KCl–0.001 M Mg, 5.6°.

EDTA). Whether these slow kinetics reflect readjustment of subunit aggregation (Tashiro and Siekevitz, 1965) has not been studied.

Thus, both the band centrifugation results and the light-scattering studies indicate that the dissociation of ribosomes to subunits as a result of exposure to EDTA is a rapid process relative to the "10-min" hydrogen exchange rate. In short, the *dissociation of the ribosome is not the rate-limiting step for the loss of the 10-min hydrogens.* This makes more likely the alternate hypothesis that the appearance of the 10-min hydrogens is caused by a structural change in the subunits, a change which leads to greatly augmented breathing behavior, that is, to a generally "looser" structure. To test this possibility further, the attempt was made to study isolated subunits under the same conditions as the intact ribosomes.

Subunit Hydrogen Exchange. The subunit preparation, about 70% 54 S in the presence of Mg^{2+} (Figure 7), was equilibrated in THO in the usual manner and then was carried through manipulations and measurements identical with those used for intact ribosomes in Figure 5. The results of two experiments are shown in Figure 8. The subunit curves (C and D) can be compared directly to the curves for intact ribosomes (A and B) since for both, ordinate values were calculated to the same bases; *i.e.*, the ordinate is proportional to the parameter, number of hydrogens per optical density unit (260 mμ). Such a direct comparison seems justified because the protein to nucleic acid composition of the subunit is probably similar to that of the whole particle. (Spirin *et al.* (1963) have demonstrated this in *E. coli* ribosomes and we have found the 260-:280-mμ ratios to be identical in the ribosomes and subunits used here.) The results indicate that *even in the presence of* Mg^{2+}, the isolated large subunit shows a hydrogen exchange picture different from that of the intact ribosome. Relatively less tritium is retained by the subunit at any given time, indicating a greater hydrogen exchange rate. Apparently, the structure of the isolated subunit is different from that maintained in the intact ribosome.

The effect of EDTA addition results in a rapid loss of only a small number (as well as a small fraction) of the remaining hydrogens, in comparison with its large effect on the intact ribosome. The contrast is clear even if the comparison is made at equal levels of remaining hydrogens (*cf.* Figure 4, curve B). It is possible that even this small effect of EDTA on the subunit stems from some contamination, visible in Figure 7B, of the subunit preparation by reassociated ribosomes. The absence of a large EDTA effect strengthens the view that the structures of the subunit when isolated and when associated in the intact ribosome are not identical.

Thus far, because of technical difficulties encountered in obtaining stable 32S particles (*i.e.*, particles which remain intact throughout the several-day preincubation period in THO), successful experiments have been done only with the large subunits. It may be noted, however, that the large subunit comprises two-thirds of the mass of the total ribosome.

Discussion

The results obtained indicate that intact ribosomes possess a relatively large number of slowly exchanging hydrogens. Under conditions leading to comparatively slow exchange (Figure 1, curve A), about 13,000 hydrogens have been measured. A rough extrapolation of this curve to zero time suggests that ribosomes possess on the order of 20,000 or more slow hydrogens. A nominal four million molecular weight ribosomal particle, composed of 50% RNA and 50% protein, would contain a grand total of about 50,000 exchangeable hydrogens, of which two-thirds would belong to protein. This number includes side chain and peptide group hydrogens of the protein moiety and base and ribose hydrogens of the RNA. If, however, only peptide hydrogens and the usual Watson–Crick hydrogens are considered, the number would be about 25,000, again about two-thirds from protein. This number of hydrogen bonds would reflect a very highly structured particle. Thus, the extrapolated value of 20,000 slowly exchangeable hydrogens suggests that ribosomes are highly structured particles (see Petermann, 1964, pp 102–103), and the methods used seem capable of monitoring directly an appreciable fraction of the

structure.

Nonhydrogen-Bonded Hydrogens. It is possible that in a particle the size and complexity of the ribosome, an appreciable number of exchangeable hydrogens might be slowed not by hydrogen bonding but simply by being inaccessible to solvent. It is also conceivable that some of the exchanging hydrogens measured here might represent water molecules enclosed within the ribosomal matrix. A number of considerations militate against this point of view. (1) The number of *unhydrogen-bonded* hydrogens that can be protected in this way (either ribosomal hydrogens or water molecules) is severely limited by the large expenditure in thermodynamic energy of destabilization (*ca.* 6 kcal/hydrogen) (Némethy *et al.*, 1963) necessary to "bury" hydrogens. That is, each unhydrogen-bonded hydrogen sequestered within the molecule is at an energy level 6 kcal higher than it would be were it hydrogen bonded. Therefore, as the number of such hydrogens increases, structures containing such sequestered hydrogens become less favored by comparison with alternate structures which permit these hydrogens to be bonded. (2) The previous argument does not speak against the possibility of the occurrence in the ribosome of sequestered water with all of its hydrogens being hydrogen bonded, *e.g.*, to inner surfaces of the ribosome. However, in order to enclose an appreciable number of water molecules and still satisfy all hydrogen-bonding requirements, it would seem that rather severe geometrical restrictions would be imposed on the ribosomal structure. Other arguments against this possibility follow. (3) As mentioned earlier, the Q_{10} for the exchange of both peptide and nucleic acid hydrogens is about 3. This value has also been found for ribosomes, suggesting that peptide and/or nucleic acid hydrogens are being measured rather than included water. (4) The rate of hydrogen exchange of ribosomes, like that of nucleic acids and proteins, depends on pH, and this speaks against at least the uncomplicated exchange of sequestered water which would not be expected to be pH dependent. (5) Tris is known to catalyze hydrogen exchange in sRNA, in DNA, and, under special conditions, in the peptide model *N*-methylacetamide. Its catalysis of hydrogen exchange in ribosomes again suggests that what is being measured here is the exchange of peptide and nucleic acid hydrogen. (6) It can be seen from three-dimensional models of known macromolecules (DNA, collagen, myoglobin, and lysozyme) that few, if any, hydrogens are so protected from the solvent environment that they cannot be brought into contact with water by relatively low energy transconformational ("breathing") reactions. The intuitive notion that more effective shielding is probable in the ribosome may well be misleading. Both low-angle X-ray diffraction and hydrodynamic studies (see Petermann, 1964, pp 124–125) indicate an unusually high degree of hydration, about 3 or 4 g of water/g, for this particle. These studies have led to a picture of the ribosome as a rather spongelike particle, highly permeated with solvent. In this view it seems unlikely that large numbers of hydrogens "inside" the ribosome

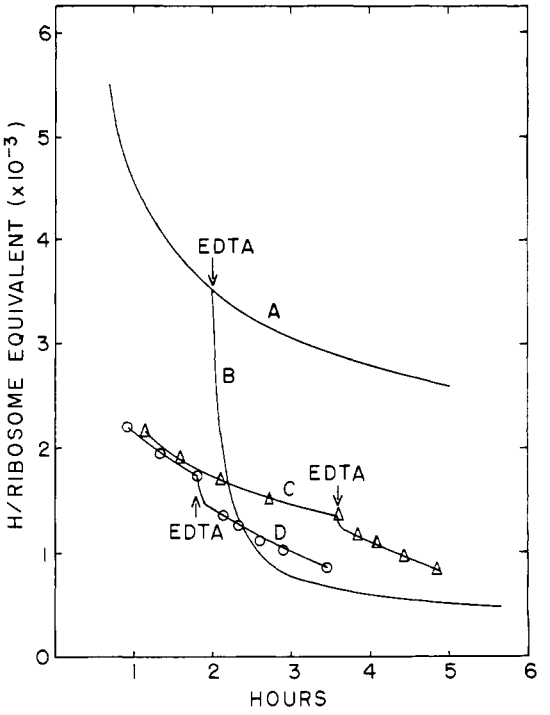

FIGURE 8: Hydrogen exchange properties of the large ribosomal subunit. Conditions of incubation were as given in Figure 5, and the rapid dialysis technique was used. All EDTA additions were directly into the sac. Curves A and B, ribosomes (identical with curves A and D of Figure 4 and duplicated here for comparison); curve C, large ribosomal subunit, EDTA added at EDTA:Mg ratio of 2.2; and curve D, same as curve C except that EDTA was added earlier (and EDTA:Mg ratio was 2.0).

are more shielded from solvent than is true in smaller macromolecules in aqueous solution. Finally, it may be noted that, while the uncertainties discussed above are pertinent to studies of absolute structure of ribosomes, they do not seriously compromise the utility of the exchange method for detecting *changes* in structure.

Structural Changes. It is not yet known how to interpret hydrogen exchange curves directly in terms of specific kinds of structure within macromolecules, *e.g.*, α helix and nucleic acid double helix, so that the present data on ribosomes give no information in these terms. On the other hand, present hydrogen exchange methods seem to hold great promise for detecting structural changes. It is obvious that structural changes may result in a change in the total *number* of slowly exchanging hydrogens. In many instances, however, this parameter may not be easily measurable (*e.g.*, the present case). Fortunately, structural change may also be manifested as an alteration in *rate* of hydrogen exchange.

The rate of exchange of a hydrogen-bonded hydrogen is thought to be regulated by the fraction of time that

the hydrogen bond is open. It should be clear that such local opening–closing reactions depend not only on the stability of the particular hydrogen bond involved in the exchange. Rather, since protein and nucleic acid structure is stabilized by a large number of cooperating bonds, the breathing behavior of a particular hydrogen bond should depend on a sizable segment of surrounding structure. Thus, a change in ribosomal structure should be detectable as an alteration in the exchange rates of some hydrogens.

Experiments have been done here with a view toward developing the capability of hydrogen exchange methods for following and characterizing changes in ribosome structure. It is well known that 80S ribosomes dissociate into a large and a small subunit in low Mg^{2+} concentration. In the experiments reported here, EDTA was used to remove Mg^{2+}, and the hydrogen exchange properties of the ribosomes were monitored before, during, and after the dissociation. A stimulatory effect of Mg^{2+} removal was indeed found. The effect (about two orders of magnitude in rate) is far greater than can be accounted for by the known hydrogen exchange behavior of proteins and nucleic acids.

Two possible causes for this behavior were considered. (1) The accelerated hydrogens are simply unmasked and liberated on exposure of a new surface. (2) Some gross structural change occurs which results in a shift in the opening–closing equilibria of many hydrogen bonds. That is, at least part of the subunit structure is destabilized with respect to breathing reactions, or "loosened." This structural change might be: (a) the dissociation itself, so that the stabilizing factor lost is intersubunit bonding; and (b) an actual change in stable structure within the subunits (*i.e.*, a change in their "X-ray diffraction structure"). Such a change might be brought about either by the removal of Mg^{2+} *per se*, or again, by the loss of intersubunit interactions.

The unmasking hypothesis (alternative 1, above) seems unlikely *a priori* since, upon ribosome dissociation, nearly *all* the remaining slow hydrogens are accelerated. It is difficult to see how a small region could contain such a large fraction of the slowly exchangeable hydrogen of the particle. In fact, the kinetic studies on ribosome dissociation reported here show that most of the EDTA-sensitive hydrogens could not have been freed simply by unmasking of intersubunit surface; while addition of EDTA results in immediate dissociation (less than 1 min) of the ribosome, most of the accelerated hydrogens exchange with a half-time of about 10 min.

This 10-min exchange time would seem to identify groups which, in the dissociated subunits, are still involved in hydrogen bonds, albeit bonds with greatly augmented breathing. Thus, treatment with EDTA "loosens" subunit structure (alternative 2, above).

Isolated Subunits. To gain further insight into this phenomenon, some work has been done with the isolated subunit. While it has not yet been possible to define the nature of the structural change (2a and b, above), preliminary results with the isolated large subunit bear on this problem. When Mg^{2+} was added back to the isolated subunit, it did not regain the tight form characteristic of the intact ribosome. Thus, even in Mg^{2+}, subunit hydrogen exchange remained fast. Furthermore, addition of EDTA had little effect. If the subunit in Mg^{2+} had regained its tight form, then EDTA should have exerted the dramatic effect seen with the intact particles.

These observations are consistent with the previous conclusion that EDTA treatment leads to a generally looser structure. Further, the results indicate that the presence of Mg^{2+} alone is not sufficient for maintaining the tight form. This suggests the interesting hypothesis that the intersubunit interactions themselves influence subunit structure. Such a phenomenon, the modification of subunit structure by subunit–subunit interactions, could provide the physical basis for the recent results of Suzuka and Kaji (1966) who studied sRNA binding to the 30S subunit; on interaction with the 50S subunit, a new binding site was generated. Indeed, this phenomenon in ribosomes may provide an example of a possible mechanism of control involved more generally in allosteric interactions or protein subunits.

Firm demonstration of the validity of this hypothesis in ribosomes requires hydrogen exchange studies on the small subunit, the clear demonstration of the continuing structural integrity of both subunits, including evidence of the recovery of the slow hydrogen exchange character in reconstituted ribosomes, and studies on the possible role of other dissociable factors such as loosely bound protein or trace metals. These studies are in progress.

To our knowledge, there have been no previously published studies comparing the structure of the free subunits with that of the subunit in the intact ribosome, although the "loosening" of structure seen in the present work might explain the observation of an increased susceptibility of dissociated subunits to RNase (Santer, 1963; Rodgers, 1964). However, studies on isolated subunits have been carried out which demonstrate their striking facility for structural change. A number of workers (Petermann and Pavlovec, 1966; Gavrilova *et al.*, 1966; Weller and Horowitz, 1964; Rodgers, 1964) have observed the considerable degree of dependence of the *s* value of the large subunit on Mg^{2+} and salt concentration. Gavrilova *et al.* (1966) were able to obtain a sedimentation constant of 3–6 S in deionized water and Spirin *et al.* (1963) have confirmed such shape changes by electron microscopy.

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Specificity of Serine Transfer Ribonucleic Acids in the Synthesis of Hemoglobin*

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ABSTRACT: Partition chromatography on Sephadex G-25 has been used to separate *Escherichia coli* transfer ribonucleic acids (tRNAs). Three fractions of serine tRNA have been obtained. A rabbit reticulocyte cell-free system has been used to test whether differences in the specificity of these serine tRNAs could be observed in the synthesis of a natural protein. Incorporation studies using [^{14}C]serine attached to tRNA$^{Ser\ I}$ and tRNA$^{Ser\ II}$ as a source of this amino acid indicated that serine is selectively incorporated into some of the tryptic peptides of hemoglobin. As has been shown in the case of leucine tRNAs, the specificity of transfer of serine into various positions of hemoglobin depends on a particular tRNA. This indicates that in a natural messenger there are different code words for the same amino acid. At least three distinct serine tRNAs are present in *E. coli* and are able to recognize different degenerate code words.

It has been clearly established from the work of Nirenberg (Nirenberg *et al.*, 1965) and Khorana (Söll *et al.*, 1965) that the genetic code is highly degenerate. For the majority of the amino acids there are at least two coding triplets that differ in the third base (or in some cases the first).

Evidence has been presented by Bernfield and Nirenberg (1965) that a single species of Phe-tRNA recognizes both UpUpU and UpUpC. The mechanism by which a single tRNA molecule is able to recognize more than one triplet has not been experimentally determined. The "wobble hypothesis" of Crick[1] suggests that certain anticodons can match more than one synonym codon because of a "certain amount of wobble in the base pairing of the third letter."

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[1] F. H. C. Crick (1965), personal communication to Informational Exchange Group No. 7.