The Effect of *p*-Mercuribenzoate on the Ultracentrifugal Behavior of Rat Liver Ribosomes*

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ABSTRACT: When rat liver ribosomes were isolated in the presence of 0.006 M β-mercaptoethanol and dialyzed against 0.05 M Tris–0.025 M KCl–2.5 mM $MgCl_2$ (pH 8.3 at 5°), most of them aggregated to dimers and larger particles. They were then treated with 0.5–5.0 mM *p*-mercuribenzoate for 1–25 hr, at 5 or 25°, and examined in the analytical ultracentrifuge at 5°. As the reaction progressed ribosomal subunits appeared, and the sedimentation coefficients of the various components increased; the final values of *s* for the small subunit, large subunit, and ribosome were 13, 3, and 6% above the initial values. At 5°, after 6 hr in 5.0 mM *p*-mercuribenzoate, most of the larger particles and dimers had been disaggregated; the preparation consisted of a 108S component, single ribosomes, and about 40% of subunits. Liver ribosomes were much less sensitive than bacterial ribosomes. At 25° both the increase in sedimentation coefficients and the dissociation were more rapid, and the dissociation was much more extensive, than at 5°. Dissociation was only partially reversed by dialysis against 0.02 M β-mercaptoethanol.

The contribution of their protein constituents to the structural integrity of animal ribosomes is still not understood. Although the *in vitro* dissociation or association of ribosomes and their subunits is correlated with the amount of magnesium or polyamines bound (Petermann, 1964), their dissociation on warming, with no change in bound magnesium (Petermann and Pavlovec, 1967) or in the presence of urea or formamide (Petermann and Pavlovec, 1968) suggests that other factors may be involved. It has recently been shown that *Escherichia coli* ribosomes are dissociated to subunits by sulfhydryl reagents (Wang and Matheson, 1967; Tamaoki and Miyazawa, 1967) and that the dissociation caused by PMB[1] can be reversed to some degree by ME (Miyazawa and Tamaoki, 1967). The inhibition of polyphenylalanine synthesis that results from incubation of *Escherichia coli* ribosomes with dithiobis(2-nitrobenzoic acid) can also be reversed by ME (Traut and Haenni, 1967). These findings suggest that with bacterial ribosomes the sulfhydryl groups of the proteins are involved in ribosomal stabilization and perhaps in protein synthesis.

Since animal ribosomes have a higher protein content than those of bacteria, and are less easily dissociated to subunits, we wished to learn whether sulfhydryl groups were important for their integrity also. We have found that rat liver ribosomes do dissociate when their sulfhydryl groups are masked by treatment with PMB, but that they are much less sensitive than *E. coli* ribosomes. Also, in contrast to the bacterial ribosomes, only a fraction of the treated particles from liver reassociate in the presence of ME.

Materials and Methods

Reagents. ME was purchased from Eastman Organic Chemicals, and PMB from Mann Research Laboratories. The acid (15–20 mg) was dissolved in 0.2 ml of N KOH and diluted to approximately 0.01 M with Tris-KCl buffer;[1] the pH was readjusted to 8.3 with 1 N HCl. The solution was centrifuged immediately before use and the PMB concentration of the supernatant was determined spectrophotometrically (Boyer, 1954).

Ribosomes. Ribosomes were isolated from rat liver cytoplasm in the presence of bentonite, as described by Petermann and Pavlovec (1967), except that the first wash was in 0.1 M $KHCO_3$–0.5 mM $MgCl_2$ (pH 8.3) and all solutions except the final dialysis buffer contained 0.006 M ME. The final solution, containing 10 mg of ribosomes/ml in 2% sucrose, was frozen and stored under nitrogen at −20°. The ribosome concentration was estimated from the absorbance at 260 mμ with an extinction coefficient, $E^{1\%}_{1cm}$, of 140 (Reboud *et al.*, 1969).

For each experiment ribosomes were thawed rapidly and dialyzed overnight against Tris-KCl buffer. One part of the dialyzed solution was used as control; to the other PMB was added to a final concentration of

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[1] Abbreviations are: ME, β-mercaptoethanol; PMB, *p*-mercuribenzoate; Tris-KCl buffer, 0.05 M Tris–0.025 M KCl–2.5 mM $MgCl_2$ (pH 8.3 at 5°).

0.5 to 5.0 mM. Both samples were kept at 25 or 5° for the same periods of time, after which the 25° samples were chilled to 5°. Their ribosome concentrations, which varied from 2 to 4 mg per ml, were adjusted to 1.6 mg/ml with Tris-KCl buffer before ultracentrifugal analysis.

To study the reversibility of the PMB effects, ribosomes[2] treated with 5.0 mM PMB at 5° for 1, 3, or 6 hr were first dialyzed for 1 hr at 5° against Tris-KCl buffer to remove excess PMB and then dialyzed overnight against buffer containing 0.02 M ME.

Ultracentrifugal Analyses. The PMB-treated samples were examined in analytical ultracentrifuges in 30-mm double-sector cells with schlieren optics at 5° and 44,000 rpm. The controls were analyzed at the same time in wedge-window cells. All the sedimentation coefficients were corrected to water at 20°, and the values for single ribosomes and subunits were extrapolated to infinite dilution by means of the concentration-dependence relationships described by Petermann and Pavlovec (1969). The amounts of the various components were calculated from the areas under the schlieren boundaries and corrected for radial dilution. At this low concentration, less than 2 mg/ml, the effect described by Johnston and Ogston (1946) is negligible.

Results

Ribosomes. In buffer containing 0.001 M potassium phosphate and 0.2 mM $MgCl_2$ at pH 7.3 the ultracentrifugal pattern of these ribosomes showed chiefly 82S, with some 110S and larger aggregates. It resembled the pattern of sonicated ribosomes described by Petermann and Pavlovec (1967), except that the small 100S boundary seen in the usual ribosome preparation was completely absent. After dialysis against the Tris-KCl buffer, the preparation contained only 25% of 82 S; the rest consisted of 120S, 150S, and larger particles (Figure 2A, no PMB).

Changes in Sedimentation Coefficients. The sedimentation coefficients of the PMB-treated ribosomes and subunits were plotted with an abscissa that represents the product of the PMB concentration in millimoles per liter and the time of treatment in hours (Figure 1). Each curve rose and then leveled off, as if the reaction had gone to completion. For the small subunit, no boundary appeared until some reaction had taken place; if we assume that its initial sedimentation coefficient was 35.5 S, the value found for compact small subunits obtained in other ways (Petermann and Pavlovec, 1969), the increase on PMB treatment was 13%. For the large subunit the initial sedimentation coefficient was 60 S, the usual value for the most compact form of the large subunit (Petermann and Pavlovec, 1969). The increase after PMB treatment was only 3%. For the whole ribosome the initial value, 82S, was the same as that of the controls kept for 1–25 hr at 5°. The increase after PMB treatment was 6%, close to the

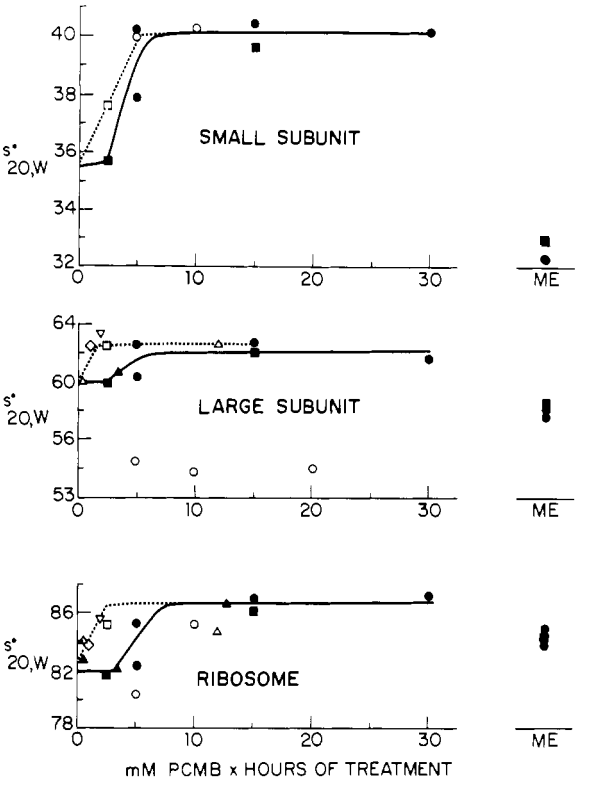

FIGURE 1: Changes in the sedimentation coefficients of subunits and ribosomes following PMB treatment. ▲, △ = 0.5 mM; ◇ = 1.0 mM; ▽ = 2.0 mM; ■, □ = 2.5 mM; ●, ○ = 5.0 mM PMB. Solid symbols show treatment at 5°; open symbols show treatment at 25°. Sedimentation coefficients are values at infinite dilution. The abscissa represents the PMB concentration in millimoles per liter multiplied by the hours of treatment. The points on the right show samples that had been treated with PMB for 3 or 6 hr and then dialyzed against 0.02 M ME.

weighted average for the two subunits. After dialysis against ME the sedimentation coefficient of the ribosomes dropped to 84 S. The subunits had sedimentation coefficients that were lower than the initial values, probably because they had assumed less compact forms (Petermann and Pavlovec, 1969).

If the increases in sedimentation coefficient represent the extent of reaction with PMB, one can use Figure 1 to estimate the rates of reaction. At 5° these rates appeared to be roughly proportional to the PMB concentration, since all the points for each type of particle fell close to the same curve. All three curves show an initial lag period followed by a rise. At 25° there was no lag period, and the reaction was completed in a shorter time. In three experiments the treated large subunits sedimented more slowly than the controls; as mentioned above, they had probably become less compact.

Since the sedimentation coefficients of the dimers and larger aggregates were not corrected for concentration dependence they were rather variable, but they, too, increased somewhat after PMB treatment. In describing the changes in the amounts of the various components we have arbitrarily identified the ribosomes and sub-

[2] The ribosomes used in the 3- and 6-hr experiments, which were the gift of Mrs. Amalia Pavlovec, had been prepared as described by Petermann and Pavlovec (1967), in the absence of ME.

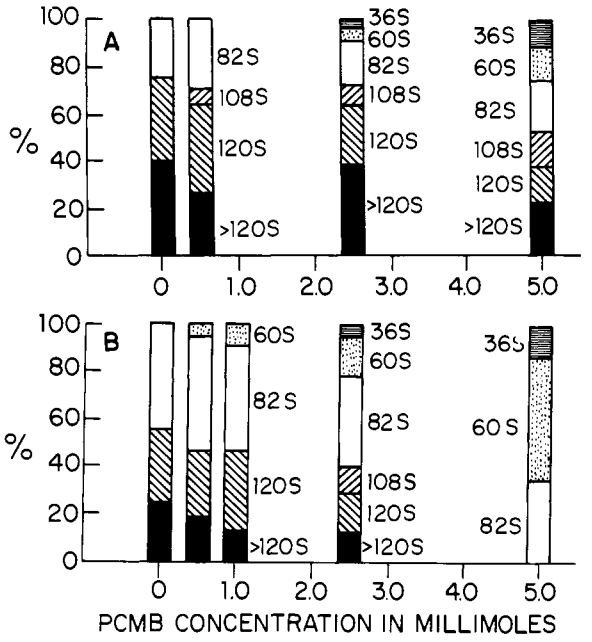

FIGURE 2: The relative amounts of the various ultracentrifugal components of control and treated ribosomes. The samples had been kept for 1 hr at 5 (A) or 25° (B) in Tris-KCl buffer alone or with varying concentrations of PMB and then analyzed at 5°. The sedimentation coefficients are arbitrary values (see text); >120 S includes all aggregates larger than dimers.

units by their initial s° values, 82, 60, and 36 S. We have called the ribosome dimer 120 S, and referred to faster sedimenting material as aggregates. Additional boundaries, seen chiefly in the PMB-treated samples, have been called 108 S, or in one case, 99 S.

Changes in Ultracentrifugal Patterns after PMB Treatment. Ribosomes exposed to PMB also showed changes in the amounts of the various components (Figures 2 and 3). Treatment for 1 hr at 5° with 0.5 mM PMB produced a small amount of 108S material (Figure 2A). With 2.5 mM PMB the sample also showed 10% of subunits, and with 5.0 mM PMB, with the chemical reaction about two-thirds completed, half of the larger aggregates and 120S particles had dissociated and the amounts of 108, 60, and 36S had increased further. The control ribosomes kept for 1 hr at 25°, then analyzed at 5°, did not show subunits, although some of the 120S and larger aggregates had been converted into 82 S (Figure 2B). Greater changes occurred when PMB was present. At the lower concentrations (0.5 and 1.0 mM) some large subunits appeared, and the amount of aggregates decreased. In 2.5 mM PMB, with more extensive chemical reaction, more subunits appeared, more of the larger aggregates and dimers dissociated, and the 108S component appeared. In 5.0 mM PMB even greater changes were observed; all the larger aggregates and 120 S had been converted into 82 S or into subunits, which made up 64% of the preparation.

The effect of time was also studied. Ribosomes were treated with 0.5 or 5.0 mM PMB at 5 or 25° for periods of 1–25 hr and then analyzed at 5° (Figure 3). After treatment with 0.5 mM PMB at 5° the ultracentrifugal pattern showed little change. A small 108S boundary

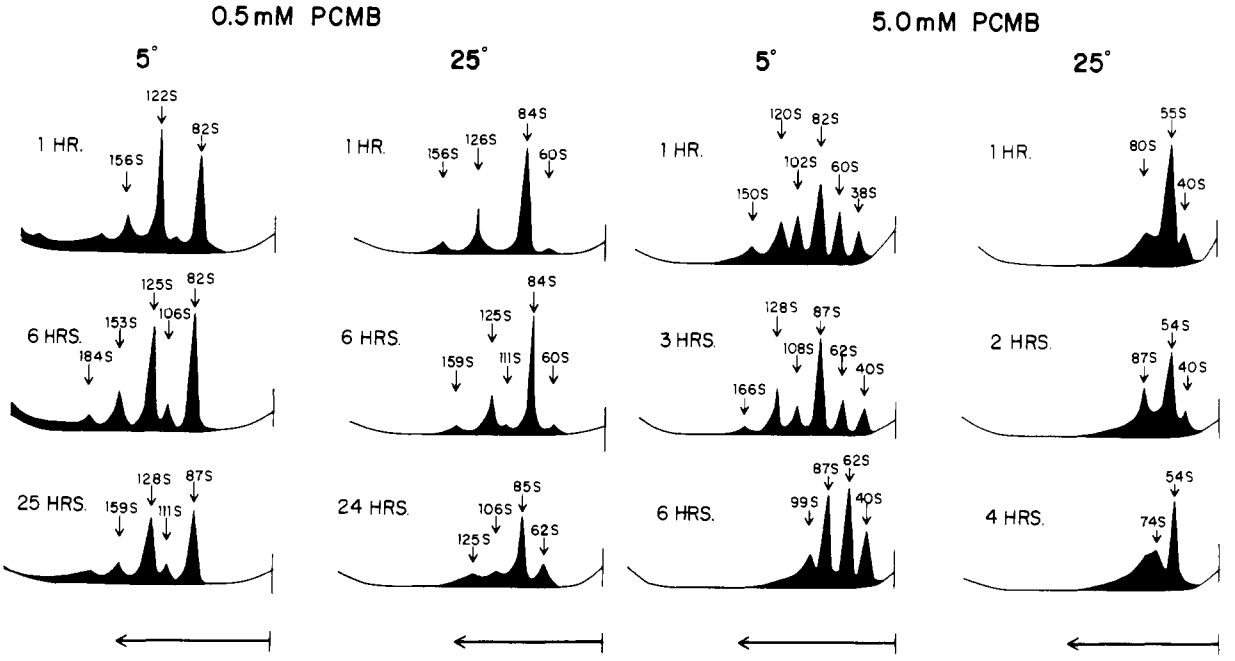

FIGURE 3: Ultracentrifugal patterns of ribosomes treated with 0.5 or 5.0 mM PMB for various periods of time at 5 or 25° and then analyzed at 5°. The sedimentation coefficients of the subunits and ribosomes are values at infinite dilution; the values for the faster components have not been extrapolated.

appeared in the 1-hr sample, but this increased only slightly with time, and subunits were absent even after 25 hr, when the chemical reaction appeared to be complete. In 0.5 mM PMB at 25° a small 60S boundary was present after 1 hr; after 24 hr this peak had increased to 14%, the 108S component was present, the larger aggregates were gone, and the amount of 120S had decreased. In 5.0 mM PMB, in spite of the high magnesium concentration of the buffer, marked changes occurred in the schlieren patterns of the treated ribosomes, even in the cold. At 5° noticeable changes had already taken place after 1 hr, as described above. After 3 hr, when the chemical reaction was complete, the pattern showed little additional change; but after 6 hr all the large species, including 120 S, had dissociated to 99 S, 82 S, and subunits. The control ribosomes, kept at 5° for the same periods as the treated samples, showed no change in their schlieren patterns. At 25° the dissociation was very rapid, and the boundaries were less sharp than those of the ribosomes treated at 5°. After 1 or 2 hr the chemical reaction was probably complete. All the large particles had already dissociated, leaving ribosomes, small subunits, and slowly sedimenting large subunits (54 S). After 4 hr at 25° a 74S boundary had appeared between the 54S peak and the remaining 82 S. The 36 S boundary had disappeared, but no small fragments could be seen. The controls showed some change during the first hour, as described above, but no additional change after this time, whether they were analyzed at 5 or 25°.

Reversibility of the PMB Effect. In these experiments the ribosomes for the 1-hr PMB treatment had been stored at −20° for 2 months, and the control showed a small amount of 108 S (Figure 4). Treatment with 5.0 mM PMB for 1 hr at 5° produced the usual changes, with fewer aggregates and 120 S, more 108 S, and some 60 and 36 S. After dialysis for 22 hr against buffer containing 0.02 M ME the amount of aggregates had decreased farther, and the 108S boundary was also much smaller. The 60S boundary had increased in size, but the 36S boundary had disappeared.

The 3- and 6-hr PMB treatments were carried out on ribosomes[2] that had been prepared in the absence of ME, and their controls also contained a small amount of the 108 S component. The PMB samples again showed more dissociation than material treated for only 1 hr, and these changes, too, were only partially reversed. There was some reassociation of subunits to 82 S, but the amount of 108 S changed very little, and large aggregates that had survived PMB treatment were converted into 120S or slower particles during the 22-hr dialysis. They also disappeared from the pattern of a control dialyzed with ME for the same length of time.

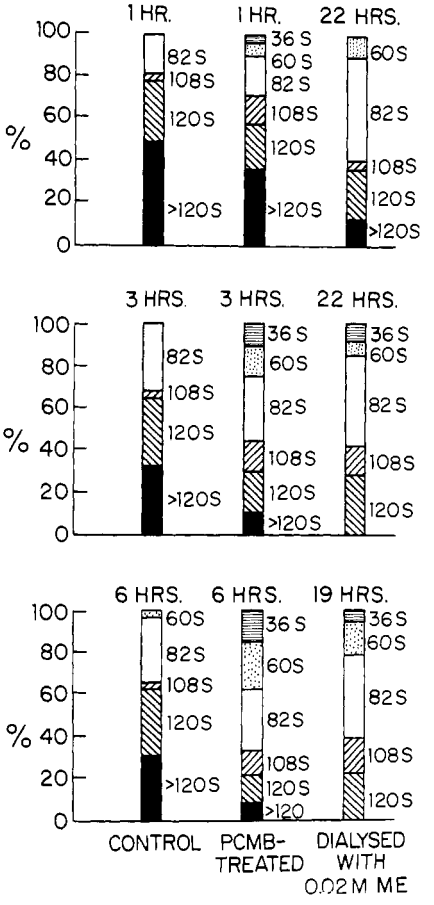

FIGURE 4: Partial reversal of the PMB effect by ME. Ribosomes were treated with 5.0 mM PMB at 5° for 1 hr (top row), 3 hr (middle row), or 6 hr (bottom row). A sample of each was analyzed (center) and the rest was dialyzed overnight in the presence of 0.02 M ME (right). The controls (left) were kept in Tris-KCl buffer at 5° for 1, 3, or 6 hr before analysis. The ordinate shows the relative amounts of the various ultracentrifugal components. The sedimentation coefficients are arbitrary values (see text).

Discussion

These ribosomes were isolated by a large-scale method that minimizes damage by nucleases and removes ferritin. Whether reducing agents are present during the isolation or not, the ribosomes have a high endogenous ability to incorporate phenylalanine in the presence of liver cell sap and are further stimulated by poly U (Petermann *et al.*, 1969). Since the material consists mainly of monosomes and disomes, only fragments of mRNA remain. The preparation contains the expected amount of 5S RNA, and 1.8 molecules of tRNA/ribosome (M. L. Petermann, A. Pavlovec, and I. B. Weinstein, in preparation).

Since the studies of Wang and Matheson (1967) on *E. coli* ribosomes had indicated that the chief effect of PMB was the disaggregation of ribosome dimers, we chose a buffer in which liver ribosomes are largely dimerized (Petermann and Pavlovec, 1967). The ribosomes isolated in the presence of ME did not show the 110S boundary that has usually been found in liver preparations in this laboratory (Petermann, 1964, p 114), until they had been stored for 2 months. When such a boundary appeared after PMB treatment it was as sharp as the dimer boundary, although its sedimentation coefficient varied from 99 to 111 S in different experiments. (These values were not corrected to infinite dilution.) The exact nature of these species is not known; they may be ribosomes with extra subunits attached to them.

Since the *in vitro* dissociation of liver ribosomes is usually correlated with a decrease in magnesium binding or an increase in temperature (Petermann and Pavlovec, 1967), it was important to relate the changes in the PMB-treated ribosomes to the effect of the sulfhydryl reagent and to rule out other factors. Because of the need for excess alkali to dissolve the reagent, a small amount of extra KCl was added with the PMB. Even with 5.0 mM PMB, however, the total monobasic cation concentration ($Tris^+$ and K^+) was only twice as high in the treated samples as in the controls. This would not have affected magnesium binding enough to cause dissociation to subunits; in this buffer the ratio of potassium to magnesium must increase threefold before magnesium binding falls below 0.4 equiv/mole of RNA phosphate and dissociation begins to occur (Petermann and Pavlovec, 1967). A second possibility, the preferential binding of magnesium by PMB, was also eliminated; addition of PMB to the buffer caused no decrease in Mg^{2+} activity, as measured with a dibasic ion electrode.

The ultracentrifugal patterns of the control ribosomes, kept at 5 or 25° with the PMB-treated particles and analyzed simultaneously with them, showed that little change had taken place during storage at either temperature; if a trace of RNase was present, it had been inhibited by the high magnesium concentration of the buffer or complexed with the liver RNase inhibitor. Although this inhibitor is inactivated by 0.1 mM PMB (Roth, 1958), RNase action on the treated ribosomes does not seem to have been extensive. It cannot account for the progressive effects of increasing the PMB concentration from 0.5 to 5 mM, in samples treated for only 1 hr at 5°. Since the particles with sedimentation coefficients greater than 120 S were artificial complexes, not polysomes, their disaggregation cannot be a direct result of RNase action. At 25°, although no breakdown to slowly sedimenting material was noted even after 24 hr, the broadening of the boundaries may have been due to limited RNase action.

The changes produced by PMB were dependent upon time, temperature, and the concentration of the reagent, as Tamaoki and Miyazawa (1967) had found with *E. coli* ribosomes. The liver ribosomes, however, were much less sensitive. In 0.5 mM PMB the bacterial ribosomes dissociated almost completely in 12 hr in the cold. After the liver ribosomes had been treated with 0.5 mM PMB at 5° for 25 hr the chemical reaction seemed to be complete, since the sedimentation coefficient of the monomer had increased to 87 S; but the only change in the ultracentrifugal pattern was the conversion of part of the large aggregates into monomers or into 108 S. Since newly dissociated subunits may have adhered to the aggregates or formed the 108S component, their absence from the schlieren pattern may be misleading. The ribosomes would probably have been more sensitive to PMB treatment if they had bound less magnesium; Matheson and Wang (1968) found that *E. coli* ribosome dimers were more easily dissociated by PMB in the presence of KCl, which probably acted by decreasing the magnesium binding (Petermann, 1964). A test for activity in the presence of PMB was not attempted, since rat liver transferase II contains sensitive sulfhydryl groups (Moldave and Skogerson, 1967).

At 25° the control preparation already contained fewer aggregates and more monomers; this apparently facilitated the access of the PMB to some of the sulfhydryl groups. The chemical reaction was more rapid, dissociation occurred faster, and the subunits remained free in the solution. After treatment for 1 hr with 0.5 mM PMB most of the large particles and dimers had been converted into monomers, but very few subunits had appeared. In this respect the liver ribosomes did behave like the *E. coli* ribosomes studied by Wang and Matheson (1967). After 1 hr in 5.0 mM PMB the dissociation had proceeded much farther, and the preparation consisted mainly of subunits.

The changes produced by PMB were only partially reversible. The ribosomes that had been treated with PMB for 1 hr showed an increase of about 30% in the concentration of 82S after dialysis with ME, but this increase resulted mainly from the dissociation of large aggregates to monomers, and perhaps from the reassociation of the small subunits with some of the large subunits derived from the 108S component. For the ribosomes treated with PMB for 6 hr the increase in 82 S on reversal was only 10%; these particles were formed by the reassociation of the subunits. The extent of reversal was much less than the 65% observed by Miyazawa and Tamaoki (1967) with *E. coli* ribosomes that had been treated with 0.5 mM PMB at 37° for 30 min. Also, unlike the PMB-treated *E. coli* ribosomes of Wang and Matheson (1967), which showed a marked increase in the amount of dimers after 1-hr exposure to ME at 25°, the liver ribosomes showed no change in dimer concentration. The activity was not tested, but would probably have been very low, since the reversal of the physical changes was so slight.

Each rat liver ribosome contains 2.0×10^6 daltons of protein (Hamilton and Ruth, 1969) or about 2.0×10^4 amino acid residues. The half-cystine content is 1.09 moles/100 moles of amino acid (Petermann, 1964) or 218 moles. How much of this is cysteine is not known. If 218 molecules of PMB were added to a ribosome of molecular weight 4.3×10^6 (Hamilton and Ruth, 1969), its mass would increase 218×321 daltons or 1.6%. Its density would increase about the same amount, and the buoyancy factor, $1 - \bar{v}\rho$, would increase about 3%. Since the sedimentation coefficient did increase 6%, it appears that most of the sulfhydryl groups must have reacted. The distribution of half-cystines between the two subunits has not been determined. The greater increase in the sedimentation coefficient of the small subunit suggests that it contains relatively more half-cystine; the gel electrophoretic pattern of its proteins also changes more after reduction and carboxymethylation than the pattern of the large-subunit proteins (M. G. Hamilton, unpublished data). The two *E. coli* subunits contain only 6.0×10^5 and 3.0×10^5 daltons of protein, and both have low half-cystine contents, 0.5% (Petermann, 1964); thus the large subunit contains only 29 half-cystines, and the small one only 16. These low values, only one-fifth the

number in liver ribosomes, may explain the greater sensitivity of bacterial ribosomes to PMB treatment.

Although liver ribosomes are more resistant, our data suggest that for animal ribosomes, as well as those of bacteria (Tamaoki and Miyazawa, 1967; Wang and Matheson, 1967; Traut and Haenni, 1967), the sulfhydryl groups of the proteins are somehow involved in holding the two subunits together. The location of these sulfhydryl groups and the type of bond they form is still unknown, but the separation of ribosomal subunits must involve the dissociation of the small subunit–mRNA–tRNA–large subunit complex. Whether the tRNAs were completely detached, or remained with one of the subunits, was not determined. The tRNA binding site on the reticulocyte ribosome is 50% inactivated by 10^{-5} M PMB (McAllister and Schweet, 1968). Some of the sulfhydryl groups may be involved in hydrogen bonding with amino groups (Cecil, 1963) located on a protein or the rRNA of an adjacent particle. This type of bonding could help to explain why the subunits must assume particular conformations before they are able to associate. Its specificity may be important in protein synthesis since ribosomes apparently exchange subunits between successive rounds of translation (Kaempfer *et al.*, 1968).

Acknowledgment

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Added in Proof

Parish *et al.* (1968) have noted that thiouridylic acid is found only in those bacterial ribosomes that are sensitive to dissociation by *p*-mercuribenzoate, and does not occur in rat liver rRNA.

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