That our earlier results showed only five combining sites per molecule of IgM requires some explanation. It now appears that the affinity for hapten in half the sites is 100 times greater than the other half. If the factor beween high- and low-affinity sites was 100 in the preparations studied earlier (*1*, *4*), then the low-affinity sites would have had a binding constant of about 10^3 and would not have been detected by equilibrium dialysis (*16*).

Each subunit's having one high-affinity site and one low-affinity site would be consistent with the observation that about half of the sites show high affinity and half show low affinity, with the observed lack of hemagglutinating and precipitating activity of the IgM subunits, and with the observation that more than half of the subunits derived from the active IgM antibody ($59/82 = 0.72$) were adsorbed on the specific immunoadsorbent.

The observed proportion of high- and low-affinity sites is probably not due to the presence of equal numbers of molecules that have ten sites of high affinity and ten sites of low affinity because it is not likely that such an equimolar mixture occurred in each of the earlier preparations showing five combining sites (*1*, *3*, *4*).

Although no change was observed in the hapten-binding property of the combining sites after reduction and alkylation of the IgM antibody, the reduced and alkylated IgM antibody was partially separated by ultracentrifugation into approximately equal amounts of two components, one somewhat heavier and lighter than 5.5*S*. The slower-sedimenting component did not seem to be released light chains, as observed by Suzuki and Deutsch (*17*) with human IgM proteins, because its amount was too great even if all the light chains had been released by the reduction and alkylation.

Because the total number of combining sites are retained in the reduced and alkylated materials, it is therefore more likely that some subunits tend to dissociate into split subunits (a single pair of heavy and light chains) and that the binding activity is still retained in the split subunits as well as in the intact subunits. However, the split subunits containing low-affinity sites may not be capable of being adsorbed to the immunoadsorbent, thus accounting for the observed decrease of about 28 percent in the amount of IgM protein specifically adsorbable after reduction and alkylation. Further investigations are required to establish the actual existence of one high-affinity site and one low-affinity site on each 7*S* subunit.

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10. The IgM antibody was reduced with 0.1*M* 2-mercaptoethanol (20°C for 3 hours) and alkylated with iodoacetamide (10 percent excess over 2-mercaptoethanol; 1 hour in an ice bath). The excess reagents were removed by dialysis in the cold.
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12. This IgM preparation differed from the previous one in that it was eluted from the immunoadsorbent by 1*M* propionic acid rather than by hapten.
13. The antibody content was determined by the method described (*1*) except that the antibody preparation was labeled with ^{125}I, and the amounts of protein were determined by the measurement of radioactivity rather than by measurement of optical density.
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15. We found that reduction and alkylation of the IgM preparation facilitated the detection of IgG, which was apparently aggregated when present.
16. Merler *et al.* (*2*) have apparently been able to detect ten sites on a human IgM antibody to *Salmonella typhi* 012 antigen. These sites were measured by combination with a tetrasaccharide derived from the bacterial lipopolysaccharide. The experimentally determined binding constant was about 3×10^4 on the assumption of a homogeneous population of sites, but the data are not sufficient to permit a decision concerning the possible presence of both weaker and stronger affinity sites in their preparation.
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Erythromycin-Resistant Mutant of Escherichia coli with Altered Ribosomal Protein Component

Abstract. *Erythromycin combines with 50S ribosomal subunit of an erythromycin-sensitive* Escherichia coli *(strain Q13), while ribosomes from an erythromycin-resistant mutant from this strain have little affinity for the antibiotic. A protein component of the 50S subunit of the mutant strain is distinct from that of the parent Q13 strain.*

Erythromycin which inhibits protein but not nucleic acids synthesis in bacteria has been shown to inhibit polypeptide synthesis in vitro (*1*, *2*). Erythromycin has also been shown to bind to ribosomes in vitro, suggesting that the effect of the antibiotic is mediated through the ribosome (*3–7*). The 50*S* ribosomes from erythromycin-resistant mutant from *Bacillus subtilis* have been shown to have reduced erythromycin binding capacity (*5*) and to be less sensitive to this antibiotic in cell-free peptides synthesis (*6*) as compared with those from sensitive strain.

We have isolated from *Escherichia coli* a mutant which is highly resistant to erythromycin. We now report some biological and chemical properties of the ribosomes of the resistant strain.

The erythromycin-resistant mutant was obtained by treating *E. coli* Q13 cells with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine. This mutant could be cultured in nutrient agar broth containing as much as 1.1 mg of erythromycin per milliliter, whereas the parent strain (Q13) failed to grow in as little as 40 μg/ml. The cell-free polylysine synthesizing system obtained from the resistant cells was much less sensitive to erythromycin than the corresponding system from the parent Q13. The ribosomes from the resistant and sensitive (Q13) cells were then treated with C^{14}-erythromycin in vitro and the complex formed was adsorbed on a cellulose nitrate filter (HA Millipore filter) (*4*). The Q13 ribosomes formed a fairly stable complex with erythromycin, and maximum binding was observed at a very low concentration of antibiotic (~0.6 μg/ml). Under the same conditions, however, only about 1/10th the amount of erythromycin was bound by the ribosomes from the resistant mutant. Dissociation of the C^{14}-erythromycin-bound Q13 ribosomes into 50*S* and 30*S* ribosomal subunits in 0.01*M* phosphate buffer at *p*H 7.0 did not yield signifi-

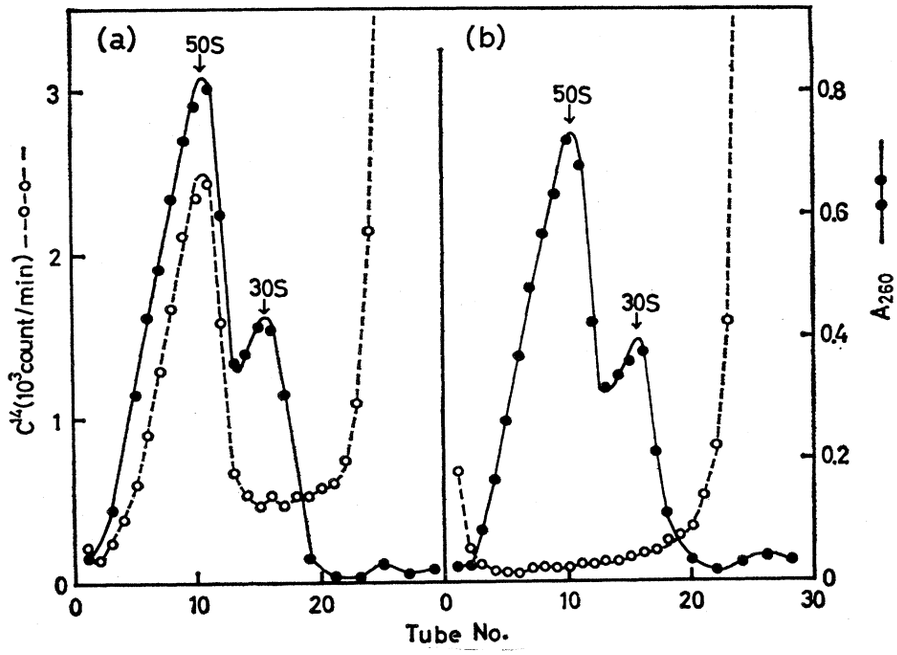

Fig. 1. Binding of C^{14}-erythromycin to ribosomes. Ribosomes (3.3 mg protein) of *Escherichia coli* Q13 or its erythromycin-resistant mutant were incubated (in final volume of 500 μl) with C^{14}-erythromycin (6 μg; 183,000 count/min), 50 μmole of tris-acetate (*p*H 7.2), 10 μmole of magnesium acetate and 25 μmole of KCl. After incubation at 24°C for 20 minutes, the reaction mixture was diluted with 100 μl of 0.2*M* phosphate buffer to dissociate the 70*S* ribosomes to 50*S* and 30*S* ribosomal subunits. It was then centrifuged in a sucrose gradient (5 to 20 percent in 15 ml of 0.01*M* phosphate, *p*H 7.0, containing $10^{-3}M$ Mg^{++}) at 24,000 rev/min for 9 hours and fractionated. Each 0.4-ml fraction was counted (- - - - ○ - - - -). The absorbance at 260 nm (—— ● ——) was read after a 21-fold dilution with water. Sedimentation profiles of ribosomes of Q13 (a), and of the erythromycin-resistant strain (b).

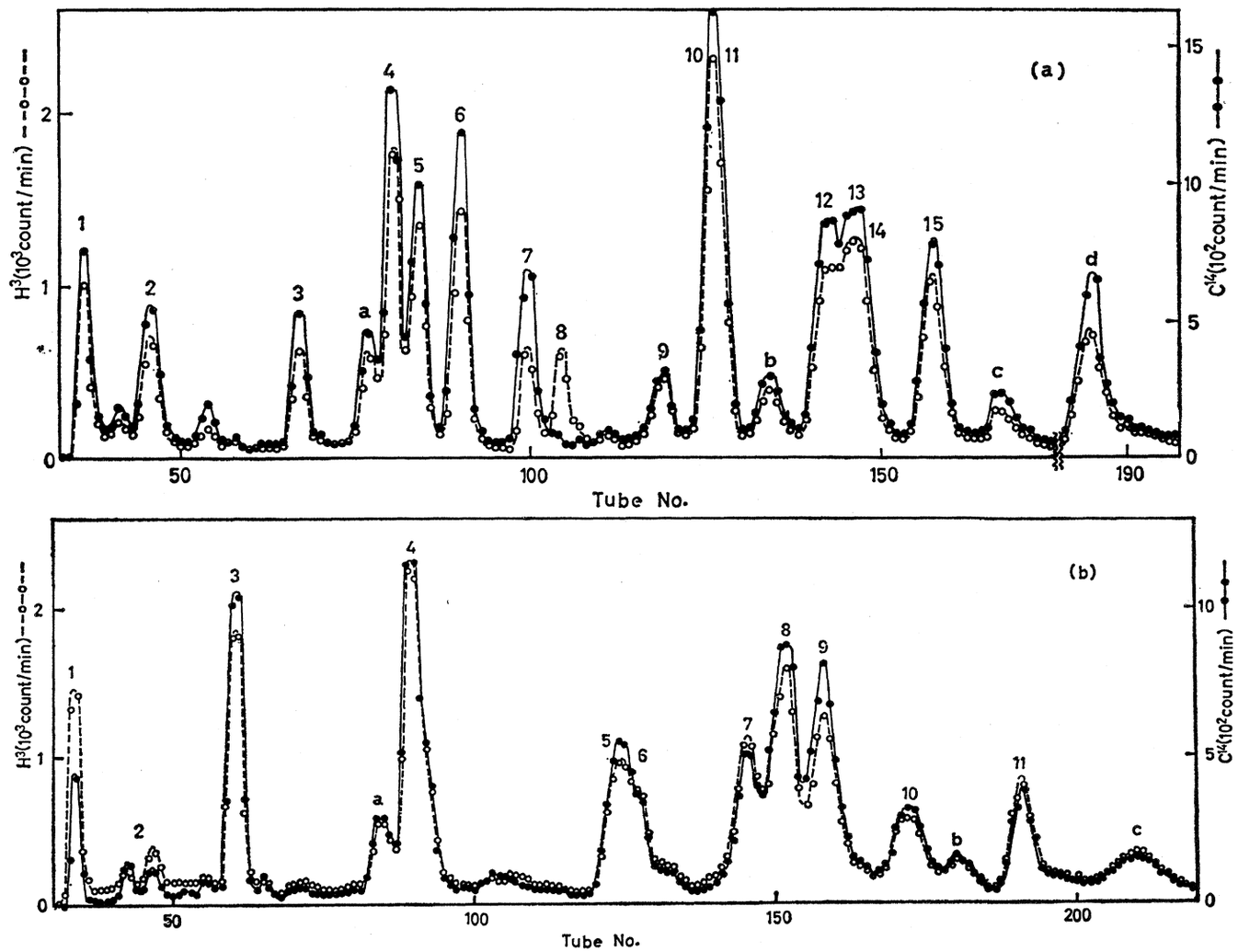

Fig. 2. Chromatography on carboxymethyl-cellulose columns of ribosomal proteins from Q13 and the erythromycin-resistant strain of *Escherichia coli*; (a) 50*S* protein; (b) 30*S* protein. (- - - - ○ - - - -) H^3-Lysine-labeled Q13 protein. (—— ● ——) C^{14}-Lysine-labeled protein of the resistant strain. For the preparation and fractionation of ribosomal protein, see reference (*7*).

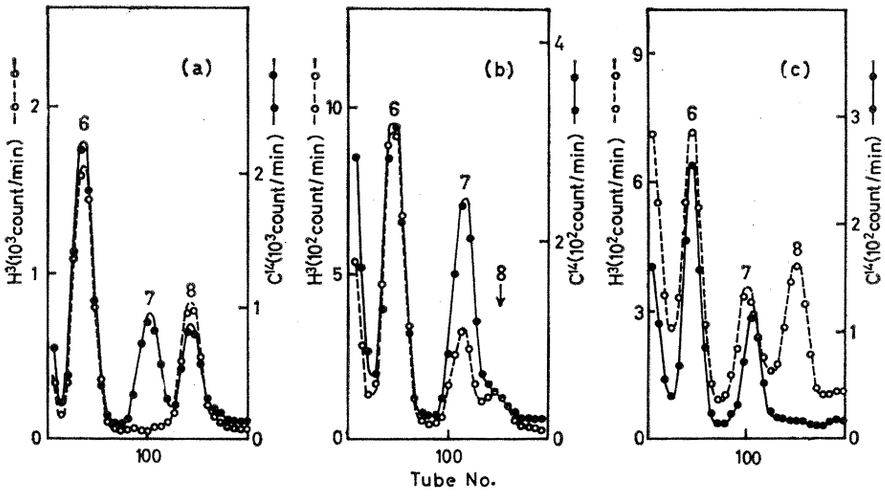

Fig. 3. Chromatography on carboxymethyl-cellulose columns of ribosomal proteins of *Escherichia coli*. (a) C^{14}-Lysine-labeled Q13 50*S* protein (—— ● ——); H^3-tryptophan-labeled Q13 50*S* protein (- - - - ○ - - - -). (b) C^{14}-Lysine-labeled 50*S* protein of the resistant strain (—— ● ——); H^3-tryptophan-labeled 50*S* protein of the resistant strain (- - - - ○ - - - -). (c) H^3-Lysine-labeled Q13 50*S* protein (- - - - ○ - - - -); C^{14}-lysine-labeled protein of 40*S* Li-particles prepared by exposing 50*S* ribosomal subunit of the resistant cells to 1.25*M* LiCl (*9*) (—— ● ——). Only parts of chromatograms are shown.

cant amounts of the antibiotic. Sucrose-gradient centrifugation of such dissociated Q13 ribosomes revealed that the erythromycin was bound almost exclusively to the 50*S* subunit (Fig. 1a). Neither the 50*S* nor the 30*S* subunit from the resistant cells bound the antibiotic (Fig. 1b).

In order to detect alterations of ribosomal constituents due to the aforesaid mutation, sample of 30*S* or 50*S* ribosomal protein obtained from H^3-lysine-labeled Q13 cells and samples from C^{14}-lysine-labeled resistant cells were mixed and simultaneously chromatographed on a carboxymethyl-cellulose (CMC) column (*7*). We found no difference in the 30*S* proteins of these two strains (Fig. 2b). However, examination of the protein components of the 50*S* subunits of the parent strain and mutant indicated differences (Fig. 2a); no significant amount of protein of the resistant mutant was detected at the chromatographic position of the No. 8 component of the parent strain Q13. Instead, the component around No. 7 position of the resistant strain was nearly twice that of the parent Q13 No. 7 component.

Two interpretations are possible. One is that the resistant 50*S* subunit lacks No. 8 component and contains twice the amount of No. 7 component. Another is that the chemical structure (amino acid sequence) of the No. 8 component of the resistant 50*S* subunit has been modified so that it has a weaker affinity for the CMC-column and is eluted at nearly the same position as the No. 7 component. Both No. 7 and No. 8 components of strain Q13 showed a single band on polyacrylamide-gel electrophoresis (*7*). These two components were chemically distinct; the No. 8 component contains tryptophan which is, however, missing in the No. 7 component (Fig. 3a). Thus the C^{14}-lysine-labeled resistant 50*S* protein was chromatographically compared with the H^3-tryptophan-labeled resistant 50*S* protein (*8*). As seen in Fig. 3b there are materials containing tryptophan at about the position of No. 7, and the ratio of C^{14} to H^3 of this region is nearly twice that of the No. 6 component of strain Q13 (Fig. 3a) or of the No. 6 component of the resistant strain (Fig. 3b). These results suggest that the components at the No. 7 region of the resistant 50*S* subunit are a mixture of unaltered No. 7 component containing no tryptophan plus another component that resembles the No. 8 component of Q13 in that it contains tryptophan.

Exposure of 50*S* subunits from Q13 to 1.25*M* LiCl solution results in production of 40*S* ribonucleoproteins (Li-particles) (*9*). During this conversion, No. 7 component is released from the 50*S* subunit, while No. 8 protein remains undissociated (*9*). If the components at the No. 7 region of the resistant 50*S* subunit are a mixture of unaltered No. 7 and altered No. 8 components, then the true No. 7 component should be released from the 50*S* subunit during conversion to 40*S* Li-particles, but the modified No. 8 protein, if present, may be detected at the No. 7 region as a constituent of the Li-particles. We thus prepared the 40*S* Li-particles by exposing C^{14}-lysine-labeled 50*S* subunits of the resistant strain to 1.25*M* LiCl solution as described (*9*), and their protein composition was chromatographically analyzed together with H^3-lysine-labeled 50*S* protein of Q13 as a reference. The amount of protein at the No. 7 region (Fig. 3c) was reduced in the 40*S* Li-particles to about one half as compared with that of the original 50*S* subunit (Figs. 2a and 3b), and the position of the remaining component was shifted slightly from that of the No. 7 component of Q13. These facts suggest that the true No. 7 component has been released during the preparation of the 40*S* Li-particles just as in the case of strain Q13. The remaining component at the No. 7 region may well be the mutationally altered counterpart of the No. 8 component of strain Q13. It remains to be seen whether the No. 8 protein of Q13 50*S* subunit is responsible for the binding of erythromycin to ribosomes, and whether this binding is affected by the alteration of the chemical structure of the protein upon mutation.

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