ported here for HeLa materials further supports the conclusion that the particles reach equilibrium after 5 hours.

The utility of this method depends on its ability to separate particles of similar sedimentation rate but different density. It is therefore critical to show that the glutaraldehyde does not nonspecifically aggregate particles. To do this, advantage was taken of the observation that phenol-extracted poliovirus RNA, when added to a cytoplasmic extract, sediments in a sucrose gradient at about the same rate as ribosomes (*9*). When a fraction from the sucrose gradient containing both ribosomes and viral RNA was fixed with glutaraldehyde and centrifuged to equilibrium in CsCl, the two species separated completely. The ribosomes banded at 1.54 g/cm^3, whereas the viral RNA banded at 1.40 to 1.42 g/cm^3 (because of proteins bound to it). Thus, the two species were fixed separately by the glutaraldehyde and were not aggregated.

Cytoplasmic extracts as usually prepared (*6*) cannot be analyzed by the fixation and banding procedure prior to their fractionation on sucrose gradients because, owing to the high concentration of proteins, glutaraldehyde links proteins to particles and the resultant CsCl profiles are meaningless. The particles must be sedimented some distance through sucrose to separate them from soluble proteins.

A further demonstration of the utility of this method comes from analysis of the polyribosome region of a sucrose gradient of the cytoplasm from poliovirus-infected HeLa cells (*10*) which were labeled with H^3-uridine for 30 minutes (Fig. 2A). The fractions in the polyribosome region marked *B* gave three peaks in CsCl (Fig. 2B). The heaviest peak corresponds to the viral polyribosomes and contains all of the label after an amino acid pulse. It has the same density as normal ribosomes. The middle peak (at 1.44 g/cm^3) represents the replication complex, the site of poliovirus RNA synthesis (*11*). It has been shown previously that the replication complex sediments in the region of polyribosomes in sucrose gradients (*11*). Infected cells labeled for only 5 minutes with uridine show only this peak in the polyribosome region, and in CsCl all of the label is at 1.44 g/cm^3; treatment with ethylenediaminetetraacetic acid does not alter the position of the peak in sucrose or CsCl gradients. The third peak, at 1.34 g/cm^3, represents virions. This has been verified by analysis of purified virions and is consistent with published values for the density of unfixed poliovirus (*12*). The finding of virions sedimenting in the 350*S* region of the sucrose gradient is surprising in light of the fact that the peak of virions is at about 150*S*. This result has been found repeatedly and must be due to virions binding to other materials or to their sliding down the walls of the centrifuge tubes.

The experiment shown in Fig. 2 demonstrates that co-sedimenting species of RNA can be easily separated and quantitated by the fixation and isopycnic centrifugation technique. The rapidity of the method and the small number of necessary manipulations recommends it over the more laborious formaldehyde method (*1, 2*).

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References and Notes

1. A. S. Spirin, N. V. Balitsina, M. I. Lerman, *J. Mol. Biol.* **14**, 611 (1965).
2. R. P. Perry and D. E. Kelly, *ibid.* **16**, 255 (1966).
3. M. Meselson, M. Nomura, S. Brenner, C. Davern, D. Schlessinger, *ibid.* **9**, 696 (1964).
4. D. D. Sabatini, K. G. Bensch, R. J. Barrnett, *Anat. Record* **142**, 274 (1962).
5. More detailed discussions of its application to specific problems are in preparation.
6. S. Penman, Y. Becker, J. E. Darnell, *J. Mol. Biol.* **8**, 541 (1964).
7. R. J. Britten and R. B. Roberts, *Science* **131**, 32 (1960).
8. J. E. Darnell, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 149 (1962).
9. M. Girard and D. Baltimore, *Proc. Nat. Acad. Sci. U.S.* **56**, 999 (1966).
10. S. Penman, K. Scherrer, Y. Becker, J. E. Darnell, *ibid.* **46**, 654 (1963).
11. M. Girard, D. Baltimore, J. E. Darnell, *J. Mol. Biol.* **24**, 59 (1967).
12. C. F. T. Mattern, *Virology* **17**, 520 (1962).
13. H. Eagle, *Science* **130**, 432 (1959).
14. A kind gift of Merck, Sharp and Dohme.
15. D. Baltimore, M. Girard, J. E. Darnell, *Virology* **29**, 179 (1966).
16. Supported by PHS grants CA-07592, AI-08388, and F2-AI-35,110. We thank Laurel Gonsalves, Virginia Lee, and Sharron Gentry for technical assistance. Part of this work was carried out at the Salk Institute for Biological Studies.

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Immunoglobulin M Antibodies with Ten Combining Sites

Abstract. *Immunoglobulin M rabbit antibodies to a hapten are shown to have ten binding sites per molecule. The affinity for the specific hapten is approximately 100 times greater for one-half of the sites than for the other half. All sites are retained in the five 7S subunits produced by reduction and alkylation of the immunoglobulin M. Each of the 7S subunits of the IgM molecule apparently has one strong and one weak site.*

Our studies (*1*) on the combining sites of rabbit IgM-class antibody to a hapten led to the detection of five sites per molecule of IgM on the basis of its having a molecular weight of 900,000. No additional sites were detected when the IgM molecule was separated into its subunits by reduction and alkylation. The average binding constant of the sites in intact IgM or in the subunits was of the order of 1 to 5×10^5 liter/mole, as measured with the homologous hapten, *p*-iodobenzenearsonate. Except for Merler *et al.* (*2*), other workers have also reported five sites per molecule (see *3, 4*).

Studies on the structure of human IgM by Miller and Metzger (*5*) indicated that the molecule was composed of five subunits, each apparently composed of two light chains and two heavy chains (*6*). These authors proposed that IgM antibody possesses ten potential combining sites per intact molecule with only half being effective in binding antigen. Onoue *et al.* showed that there were ten Fab-like fragments per molecule (*7*) and suggested that half of the potential sites might not be detected because of their low affinity for antigen.

We have examined the situation with IgM antibody against a haptenic group different from that used earlier. The antiserums used were against the 1-azonaphthalene-4-sulfonate (azo-NS) group and were obtained from rabbits injected with *Salmonella typhimurium* to which azonaphthalene sulfonate groups had been coupled. This particulate type of antigen is particularly effective for the production of IgM-class antibodies to haptens (*8*).

Antibodies to azo-NS were specifically purified by adsorbing them on an immunoadsorbent prepared by coupling diazotized 1-aminonaphthalene-4-sulfonate to an insoluble polymer of rabbit serum albumin (azo-NS-poly RSA) (*9*), and then eluting the antibodies with 0.2*M* naphthalene-1-sulfonate. The free hapten and the antibodies of IgM and IgG classes were separated by gel filtration on Sephadex G-200 before the hapten was removed. The antibody of each class was further purified by a second gel filtration and then the hapten bound to antibody was removed by

extensive dialysis. The subunits of IgM antibody were prepared by reduction and alkylation of the purified antibody (*10*). For the study of nonspecific binding of hapten, the IgM was also separated from normal rabbit serum by gel filtration on Sephadex G-200 and zone electrophoresis.

The hapten-binding activities of the IgM and IgG antibodies and the IgM subunits were measured by equilibrium dialysis (*11*) with ^{125}I-labeled 1-iodonaphthalene-4-sulfonate (Table 1) as the hapten. The binding curves are shown in Fig. 1. There are three important results: (i) The amount of the hapten bound per mole of protein by the IgM antibody preparation exceeds 5 moles and reaches 8 moles at the highest concentration of the free hapten. Extrapolation of the binding curve indicates about ten combining sites per molecule of IgM. (ii) The binding curve for IgM indicates two major groups of combining sites with respect to affinity for the hapten, one of high affinity with an average binding constant of the order of 10^7 liter/mole and the other with an average constant of the order of 10^5 liter/mole. (iii) The IgM subunits bound the hapten to the same extent as the native IgM antibody did and the binding constants were the same.

Essentially the same results were obtained with an IgM antibody preparation from antiserum to azo-NS from a different group of rabbits (*12*). Since the purity of the IgM antibody preparation was very critical in these investigations, it was carefully examined by several methods.

The antibody content of the preparation was determined from the amount of protein specifically adsorbable on the immunoadsorbent mentioned earlier (*13*). At least 82 percent of the intact IgM protein was specifically adsorbable on the immunoadsorbent, and the adsorption was inhibited by 0.05*M* naphthalene-1-sulfonate. With the reduced and alkylated preparation 59 percent of the protein was adsorbed.

The preparations were tested by immunoelectrophoresis with sheep and goat antiserums containing antibodies to rabbit IgG, IgM, IgA, and a few other α- and β-globulins; no arc other than the arc corresponding to IgM was observed with preparations either before or after reduction and alkylation. The reduced and alkylated IgM antibody also showed a single arc with each of these antiserums. Thus the IgM antibody did not contain more than trace amounts of IgG or IgA. A trace amount of IgG (0.6 percent) was detected by radial diffusion (*14*), that is, by diffusing the reduced and alkylated IgM antibody (*15*) in an agar layer containing goat antiserum against the Fc-fragment of rabbit IgG.

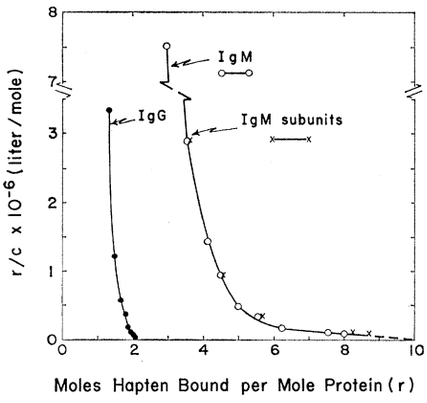

Fig. 1. Binding of 1-iodonaphthalene-4-sulfonate by IgM and IgG antibodies and IgM subunits calculated as moles of hapten bound per 900,000*g* IgM or its subunits or per 160,000*g* IgG. ○, IgM Antibody; x, IgM antibody subunits; ●, IgG antibody; *c*, concentration of free hapten; *r*, moles of hapten bound per mole of protein.

By ultracentrifugation, the native IgM antibody showed a major peak with a sedimentation constant ($S_{20,w}$) of 18.8*S* (not corrected for protein concentration, 6.9 mg/ml), and there was a trace amount of faster-sedimenting material.

The specific binding of the hapten even at the highest concentration of free hapten is still in the range where the experimental values are quite accurate. Since the hapten bound at this point is 8 moles per mole of IgM protein, the number of combining sites measured is ten, calculated on the basis that the protein is 82 percent antibody as determined by specific absorption. If a higher proportion of the protein is antibody, then extrapolation of the binding curve would still indicate ten binding sites. There was very little IgG antibody in this IgM preparation. Furthermore, the experiment carried out simultaneously with the IgG antibody preparation purified from the same pool of antiserums showed two combining sites per molecule. This gives further assurance of the reliability of the experiment with IgM. The affinity of the IgG antibody appeared to be the same as that of the IgM antibody sites of higher affinity.

Table 1. Binding of iodonaphthalene-4-sulfonate by IgM and IgG antibodies and by IgM subunits.

Free hapten (molar × 10^6)	Protein (mg/ml)	Hapten bound (molar × 10^6)*		Hapten molecules bound per molecule protein†
		By antibody protein	By normal protein	
		IgM		
0.393	0.458	1.522	0.009	2.97
1.23	2.465	9.76	0.07	3.54
2.86	0.458	2.27	0.03	4.40
2.86	2.465	11.49	0.17	4.13
4.81	2.465	12.61	0.28	4.50
10.01	2.465	14.23	0.59	4.98
16.86	2.465	16.14	0.99	5.53
35.87	2.465	19.11	2.11	6.21
69.68	2.465	24.78	4.09	7.55
85.93	2.465	26.94	5.04	8.00
		Subunits (reduced—alkylated IgM)		
1.23	2.785	11.14	0.08	3.57
4.81	2.785	14.38	0.32	4.53
16.86	2.785	18.52	1.12	5.62
69.68	2.785	30.12	4.61	8.24
85.93	2.785	32.66	5.69	8.72
		IgG		
0.393	0.235	1.937	0.002	1.32
1.23	1.265	11.92	0.03	1.50
2.86	0.235	2.63	0.01	1.79
2.86	1.265	13.35	0.07	1.68
4.81	1.265	14.18	0.11	1.78
10.01	1.265	14.83	0.24	1.85
16.86	1.265	15.93	0.40	1.96
35.87	1.265	16.79	0.84	2.02
69.68	1.265	17.58	1.64	2.02
85.93	1.265	18.32	2.02	2.06

* Molar concentrations are means of duplicate determinations of the concentration of bound hapten in samples containing protein at the concentration given in column 2, with average deviation from the mean of ± 0.7 percent. † Calculated on the basis of a molecular weight of 900,000 for IgM and of 160,000 for IgG, with the difference between values in columns 3 and 4 used as the value for the concentration of hapten specifically bound to antibody.

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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References and Notes

1. K. Onoue, Y. Yagi, A. Grossberg, D. Pressman, *Immunochemistry* **2**, 401 (1965).
2. E. Merler, L. Karlin, S. Matsumoto, *J. Biol. Chem.* **243**, 386 (1968).
3. K. Lindqvist and D. C. Bauer, *Immunochemistry* **3**, 373 (1966); E. W. Voss, Jr., and H. N. Eisen, *Fed. Proc.* **27**, 2631 (1968); L. W. Clem and P. A. Small, Jr., *ibid.*, p. 2633; M. M. Frank and J. H. Humphrey, *J. Exp. Med.* **127**, 967 (1968); R. E. Schrohenloher and C. B. Barry, *J. Immunol.* **100**, 1006 (1968).
4. H. Metzger, *Proc. Nat. Acad. Sci. U.S.* **57**, 1490 (1967).
5. F. Miller and H. Metzger, *J. Biol. Chem.* **241**, 1732 (1966).
6. This is on the basis that trypsin digestion of the IgM or its subunits yielded two Fab μ fragments per subunit equivalent to the Fab γ fragments of IgG.
7. K. Onoue, T. Kishimoto, Y. Yamamura, *J. Immunol.* **98**, 303 (1967).
8. ———, in preparation. In brief, 2.0 g (wet weight) of *Salmonella typhimurium* at *p*H 9.5 were treated with 1.0 mmole of diazotized 1-aminonaphthalene-4-sulfonate to prepare the azo-NS-salmonella. Each rabbit was injected intravenously first with 3 and then with 9 mg of azo-NS-salmonella with an interval of 2 weeks between injections. Bleedings were made 5, 6, and 7 days after the second injection. The yield of total antibody to hapten ranged from 150 to 400 μg per milliliter of antiserum, and 20 to 40 percent of this antibody was IgM antibody.
9. K. Onoue, Y. Yagi, D. Pressman, *Immunochemistry* **2**, 181 (1965).
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.
11. A. Nisonoff and D. Pressman, *J. Immunol.* **80**, 417 (1958); A. L. Grossberg and D. Pressman, *J. Amer. Chem. Soc.* **82**, 5478 (1960).
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.
14. J. L. Fahey and E. M. McKelvey, *J. Immunol.* **94**, 84 (1965).
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.
17. T. Suzuki and H. F. Deutsch, *J. Biol. Chem.* **242**, 2725 (1967).
18. Supported in part by grant AI-02342 from the NIAID. We thank L. Rendina for technical assistance.
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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-