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# Electron Microscopic Studies of Mouse Immunoglobulin M; Structure and Reconstitution Following Reduction

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**Summary.** Immunoglobulin M formed by mouse plasmacytoma MOPC 104E was examined by electron microscopy of negatively stained material. The protein displayed a central core to which were attached five and very occasionally six, radially arranged subunits. The subunits were Y-shaped with the branches of the Y presumably representing the two Fab fragments per 7S subunit. The distance from the centre of the molecule to the point of branching was 100–110 Å, and the length of each branch was 60–80 Å. IgM subunits (obtained by partial reduction of IgM with dithioerithritol) could be reconstituted after removal of the sulphydryl reagent. The reconstitution was confirmed by electrophoresis on polyacrylamide gel and by electron microscopy; assembly of molecules with the characteristic radial symmetry was observed.

## INTRODUCTION

High molecular weight (19S) immunoglobulin M (IgM) has been demonstrated in all vertebrate species which have been studied. Most extensive work has been carried out on human IgM, where the available evidence suggests that there are five 7S subunits(IgMs), linked via disulphide bonds (Miller and Metzger, 1965a, b) located in the Fc part of the molecule (Beale and Buttress, 1969). Confirmation of this type of ring structure has come from direct visualization of the molecule by electron microscopy showing molecules with five arms attached to a central ring (Chesebro, Bloth and Svehag, 1968). Valentine and Binaghi (1969) demonstrated five-fold symmetry in a picture of rat IgM, which showed greatly improved resolution as the result of the application of Valentine's technique for spreading molecules on carbon films.

Our interest in the structure of IgM stems from studying biosynthesis of IgM in mouse plasma cell tumour MOPC 104E (McIntire, Asofsky, Potter and Kuff, 1965). Using this model system, polymerization of 7S subunits (IgMs) to IgM was shown to occur shortly before, or simultaneously with, secretion of the molecules (Parkhouse and Askonas, 1969). In addition, treatment of extracellular IgM or intracellular IgMs with sulphydryl reagent followed by removal of the reducing agent led to the formation of material which behaved as IgM on polyacrylamide gel electrophoresis (Askonas and Parkhouse, to be published).

To fully assess the fidelity of reconstitution of IgM from its subunits, an alternative criterion to electrophoresis was thought to be necessary. The morphology of reduced and reconstituted IgM was, therefore, compared with that of the native molecule by electron microscopic examination using recent advances in specimen preparation (Valentine, Shapiro and Stadtman, 1968).

## MATERIALS AND METHODS

*Preparation of serum IgM*

The MOPC 104E plasma cell tumour secreting IgM and $\lambda$ type light chains (McIntire *et al.*, 1965) was kindly provided by Dr M. Potter and maintained in BALB/c mice by subcutaneous transfer of 1-mm$^3$ pieces. IgM was purified from the serum of tumour bearing mice as described by Parkhouse and Askonas (1969) by fractionating serum on a 10–30 per cent (w/v) linear sucrose gradient in phosphate buffered saline (PBS), pH 7·4 (Spinco No. 30 rotor, 16½ hours at 27,500 rev/min) followed by preparative polyacrylamide electrophoresis [3 per cent (w/v) acrylamide, 0·015 M Tris, 0·05 M glycine buffer, pH 8·7] (Brownstone, 1969).

An alternative, more rapid, method was used to obtain IgM for prompt electron microscopic analysis. Serum (0·4 ml) was fractionated on a 7–30 per cent (w/v) sucrose gradient in PBS for 24 hours at 41,000 rev/min in a Spinco SW 41 Ti rotor. The leading edge of the fastest moving peak contained IgM and trace amounts of $\alpha_2$-macroglobulin. This fraction was immediately freed of sucrose by passage over Sephadex G-25, equilibrated with PBS, and examined in the electron microscope within 30 minutes.

*Analytical polyacrylamide gel electrophoresis*

Electrophoresis was carried out in 8-cm gel columns in glass tubes (0·6 cm internal diameter) on 4·25 per cent (w/v) acrylamide, with 2·7 per cent cross linking, for 4 hours (10 mA/tube). The buffer contained 0·1 per cent sodium dodecyl sulphate (SDS), 0·5 M urea and 0·1 M sodium phosphate buffer, pH 7·2 (Summers, Maizel and Darnell, 1965). The presence of SDS in the gels results in a separation based primarily on the molecular size of the proteins analysed.

*Reduction and reconstitution of IgM*

Pure IgM (3 mg/ml) was reduced with various concentrations of dithioerithritol (DTE) for 1 hour at room temperature in 0·05 M Tris–HCl, pH 8·0, and 0·001 M ethylenediamine tetra-acetic acid. A sample was immediately alkylated with a 50 per cent molar excess of iodoacetamide in 0·25 M Tris buffer, pH 8·0 at 0°.

For reconstitution of the 19S molecules the remainder was filtered through Sephadex G-25 equilibrated with 0·05 M sodium phosphate, pH 7·2 (column volume equal to fifteen times the sample volume) to separate the protein from the DTE. The extent of reduction or reconstitution could be analysed by polyacrylamide gel electrophoresis. To remove last traces of DTE the G-25 protein peak was filtered through Sephadex G-200 equilibrated with PBS (column volume equal to 100 times the sample volume) before electron microscopic examination.

*Electron microscopy*

Protein samples for electron microscopy were prepared by Valentine's procedure for negative staining (Valentine *et al.*, 1968). Carbon films were floated from a mica substrate on solutions of protein adjusted to about 100 $\mu$g/ml. After a few seconds had been allowed for adsorption of protein to the recently exposed carbon film, the latter was lifted out on the mica sheet and transferred to 2 per cent (w/v) sodium phosphotungstate. A grid recently made adhesive with 'Glauert's glue' ('Cellotape' paste dissolved in chloroform) was dropped on the floating carbon film which was then picked up on a piece of clean newspaper. The specimen was then dried on filter paper to obtain a thinly spread layer of negative stain.

An alternative procedure, yielding a thicker layer of stain, was to pick up the grid with forceps and allow it to dry without blotting.

To facilitate separation of carbon from the mica, freshly cleaved sheets of mica were first coated with glycerine by evaporation from the electrodes in a vacuum of 50 $\mu$mHg. The vacuum was then reduced to 0·1 $\mu$mHg, and carbon evaporation was carried out using a freshly sharpened carbon rod.

FIG. 1. Electron micrograph of mouse IgM purified by sucrose density gradient centrifugation. × 450,000.

A Phillips electron microscope EM 200 was used at 60 kV with disposable 50-$\mu$ objective apertures. The electron micrographs were taken at a magnification of 55,000.

## RESULTS

### MORPHOLOGY OF IgM

The best electron micrographs were obtained by examining freshly prepared IgM as soon as possible. Using a single sucrose density centrifugation (see 'Materials and methods') electron microscopy (Figs. 1 and 2) shows that these IgM molecules consist of a central core to which are attached a number of radially arranged Y-shaped subunits. The distance from the centre of the molecule to the point of branching is 100–110 Å, and the length of each branch is 60–80 Å. The overall diameter of the molecule ranges from 300 to 375 Å. Where molecules were aggregated, interpretation of the structure was not attempted. The large majority of the molecules whose structure could be seen clearly, showed five subunits, but six subunits were discernible on some molecules (Fig. 2). The 7S subunits mostly show a Y-branching with the arms presumbly representing the two Fab pieces per 7S subunit.

The presence of molecules with six discernible subunits is of interest since *Xenopus laevis* IgM molecules all appear to have six subunits (Fig. 3). (Parkhouse to be published in detail).

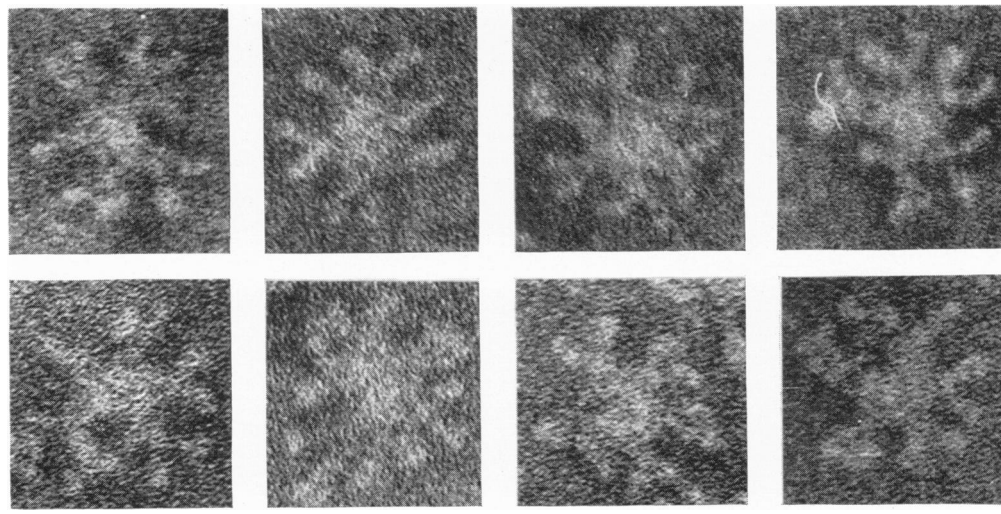

FIG. 2. Electron micrographs of mouse IgM purified by sucrose density gradient centrifugation. ×660,000.

### REDUCTION AND RECONSTITUTION

When IgM was reduced with $5\times10^{-4}$M DTE, polyacrylamide gel electrophoresis of the reduced and alkylated sample showed that all of the IgM (Fig. 4a) had been reduced to smaller fragments, the major components being IgMs and HL (a half-molecule of one heavy and one light chain) (Fig. 4b). On passage of the reduced protein over G-25 the

leading edge of the protein peak was predominantly IgM (Fig. 4c) whilst the trailing edge contained relatively more of the smaller molecular weight material (Fig. 4d). The subsequent filtration over G-200 yielded a homogeneous component with the electrophoretic characteristics of IgM (Fig. 4g).

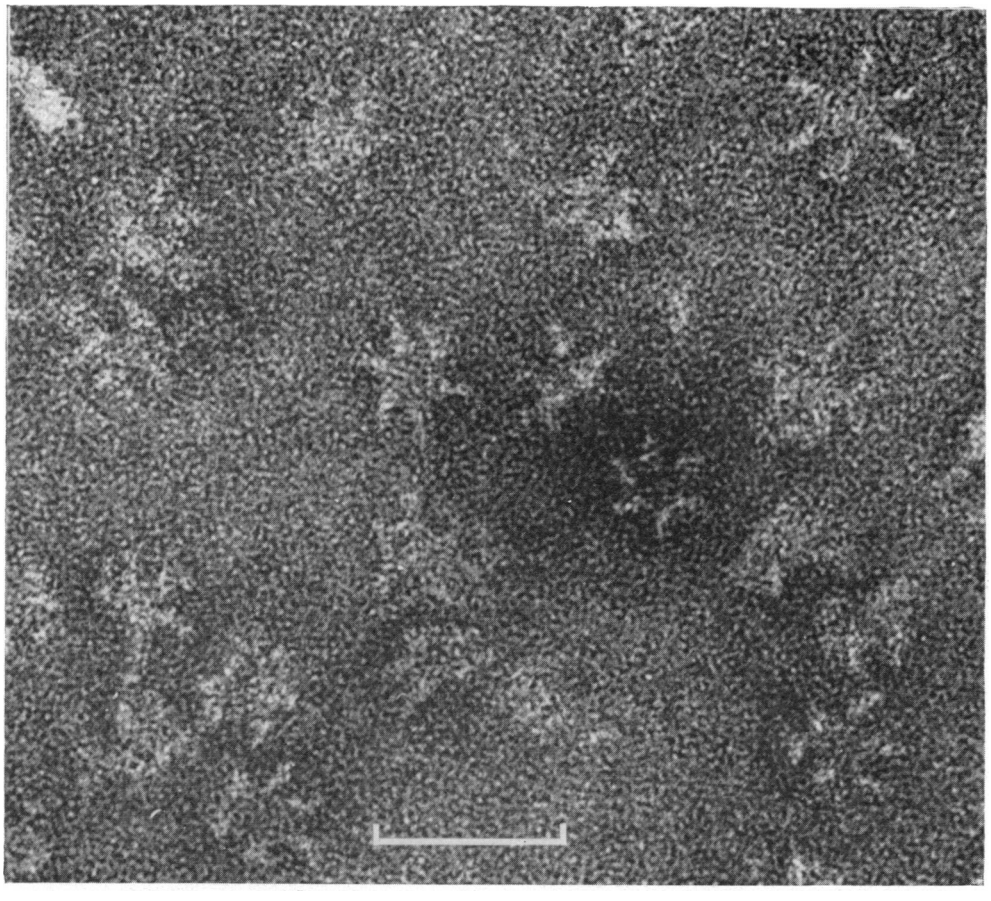

FIG. 3. *Xenopus* IgM. × 500,000. Bar represents 500Å.

Reduction with $5 \times 10^{-3}$M DTE resulted in almost complete splitting to heavy and light chains (Fig. 4e). Passage of this reduction mixture through Sephadex G-25 caused a large measure of reconstitution to material with the electrophoretic properties of IgM (Fig. 4f).

Electron microscopy of the alkylated reduction mixture ($5 \times 10^{-4}$M DTE) of IgM showed most of the material to be small fragments 50 × 50–150 Å in dimension (Fig. 5a), with a few subunit polymers present. The starting material for the reduction-reconstitution experiments was IgM purified by our usual method, i.e. sucrose gradient centrifugation, polyacrylamide gel electrophoresis, and storage at 4°. Electron micrographs of this material (Fig. 5b) lacked definition compared to the freshly prepared IgM illustrated in Figs. 1 and 2, but the same central core with subunits radially arranged and an overall diameter of 370 Å can be seen. In many cases five arms are discernible. Preparations of IgM reduced

with $5\times10^{-4}$ or $5\times10^{-3}$ M DTE and then reconstituted after passage through columns of Sephadex G-25 and G-200 contained many similar structures showing the characteristic radial symmetry (Fig. 6a and b). After all these manipulations many molecules of indeterminate morphology or random aggregates were also present.

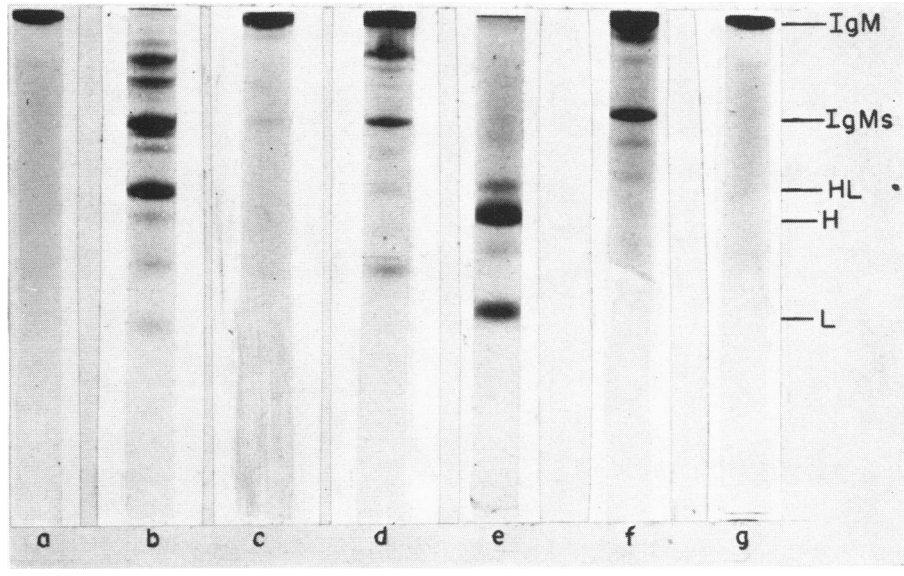


FIG. 4. Reduction and reconstitution of mouse IgM analysed by polyacrylamide gel electrophoresis in 4·25 per cent (w/v) polyacrylamide gel in SDS-urea as described under 'Materials and methods'. (a) IgM; (b) IgM reduced with $5\times10^{-4}$ M DTE and alkylated; (c) reconstituted IgM after reduction with $5\times10^{-4}$ M DTE and Sephadex G-25 filtration. Leading edge of protein peak; (d) as in (c) except that the trailing edge of the Sephadex G-25 protein peak was analysed; (e) IgM reduced with $5\times10^{-3}$ M DTE and alkylated; (f) reconstituted after reduction with $5\times10^{-3}$ M DTE and Sephadex G-25 filtration; and (g) reconstituted after reduction with $5\times10^{-4}$ M DTE and filtration first through a Sephadex G-25 column and then Sephadex G-200.

## DISCUSSION

Our electron micrographs show that the mouse IgM formed by plasmacytoma MOPC 104E has a radially arranged structure with usually five, but sometimes six, subunits. It is conceivable that the structural arrangement allows a slightly variable number of subunits per molecule. Should this prove to be generally true for IgM, then determination of accurate molecular weights would pose problems. On the other hand, the variation might be explained by disturbances in assembly related to the neoplastic origins of the material. It is interesting, however, that six binding sites per rabbit IgM molecule have been reported on the basis of equilibrium dialysis studies (Onoue, Yagi, Grossberg and Pressman, 1965). The variable appearance of the mouse IgM contrasts sharply with the uniform morphology of *Xenopus* IgM where six subunits per molecule are invariably found.

The overall structure and size of the molecule is similar to that reported by Chesebro *et al.* (1968) for normal rabbit and human IgM, and for human pathological IgM. Their conclusions regarding the flexible nature of the molecule as judged by its appearance in electron micrographs may also be drawn from our studies. In our electron micrographs,

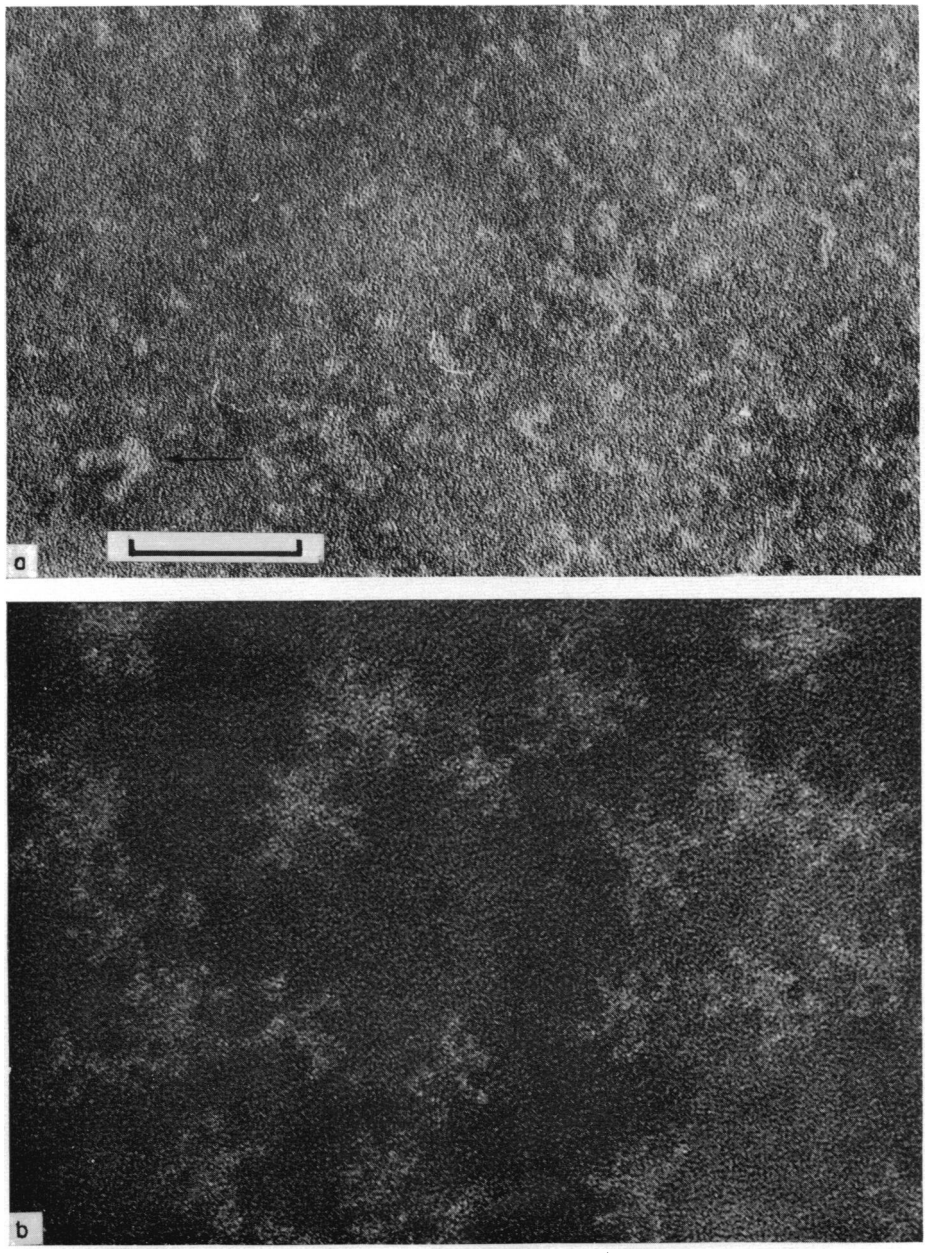


FIG 5. (a) Mouse IgM after reduction with $5 \times 10^{-4}$ M DTE and alkylation with iodoacetamide. Arrow indicates a possible IgMs dimer. × 450,000. (b) Mouse IgM purified by polyacrylamide gel electrophoresis and stored at 4°. This was the starting material for the reduction and reconstitution experiments. × 346,500.

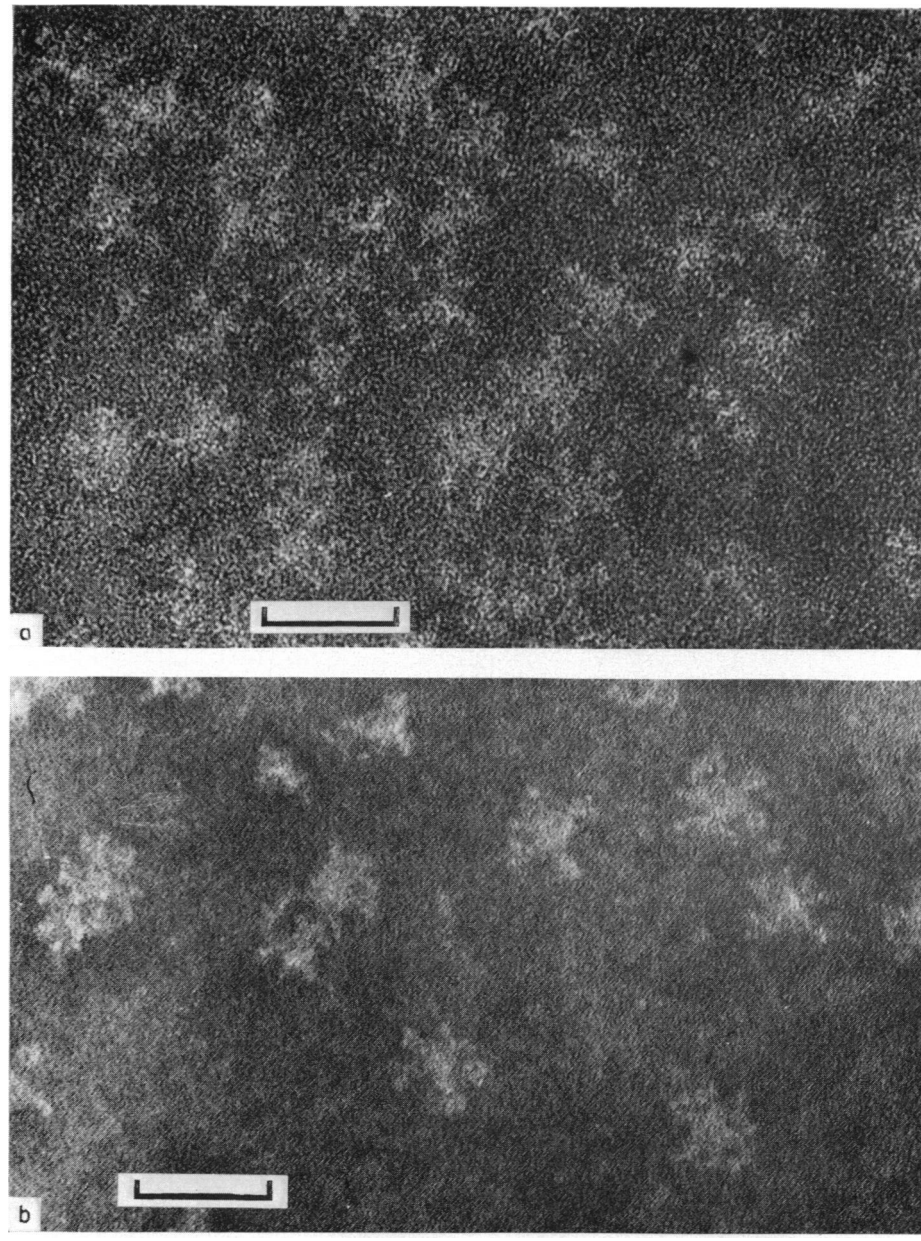


FIG. 6. Mouse IgM reconstituted (a) after reduction with $5 \times 10^{-4}$ M DTE. × 346,500. (b) Mouse IgM reconstituted after reduction with $5 \times 10^{-3}$ M DTE. × 346,500.

however, the central area of the molecule is more of a disc than a ring. In addition, the mouse IgM subunits are clearly branched, the Y-shaped branches presumbably representing the two Fab fragments per 7S subunit. This has not been previously demonstrated for IgM. The mouse preparations contained no structures resembling the round or ovoid particles described for human IgM (Höglund and Levin, 1965). An important factor in the preservation of symmetry of IgM molecules, was that the preparations for electron microscopy should be made shortly after purifying the IgM on a sucrose gradient, without storage.

The reconstitution of IgM following reduction with DTE is confirmed in a general way by electron microscopy in that assembly to molecules with the characteristic radial symmetry is obtained. Since preparations of mouse IgM deteriorate from the point of view of their electron microscopic appearance simply by storing at 4°, it is not surprising that the reduced and reconstituted IgM appears less homogeneous than the original material. In addition, the apparently less efficient assembly at higher DTE concentrations may be due to incomplete removal of the reducing agent. We are currently engaged in attempts to improve the quality of the reconstituted material. In a recent report (Chesebro and Svehag, 1969) recovery of about 50 per cent of the antibody activity of reduced rabbit IgM antibody to polio virus was associated with the presence of numerous aggregates of different sizes in electron micrographs. This may be partly due to the heterogeneity of the antibody, so that a mixture of different subunits might polymerize. The same may be true for the variable recovery of IgM antibody activity to red blood cells following reduction (Jacot-Guillarmod and Isliker, 1962; Harboe, 1967; Frank and Humphrey, 1969). In this report we are dealing with a homogeneous IgM formed by a plasmacytoma and this problem does not arise.

The subunits derived from reduction ($5\times10^{-4}$ M DTE) and alkylation of IgM failed to show Fab branches. However, these are only demonstrable in IgG under special conditions involving cross-linking of Fab sites by a divalent hapten reagent (Valentine and Green, 1967). Consequently it is difficult to know the extent to which the structure of IgM subunits is determined by their environment in the whole IgM molecule.

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