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Antibody Crystallization on Phospholipid Films: Dynamics and the Effects of Antibody Conformation

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Monoclonal antibodies form two-dimensional (2-D) crystals when bound to haptinated phospholipid monolayers in physiological conditions and at ambient temperatures. IgG_1 forms two crystal phases: a linear strand phase and a high-order hexagonal phase. The relative distribution of these two phases is dependent on temperature, pH, and salt concentration. This dependence is one that is associated with protein intramolecular interactions rather than lipid-lipid or lipid-protein interactions for a number of reasons: 1) Polyclonal antibodies against the hapten DNP do not organize into any crystal structure for any of the experimental conditions used. 2) Slightly denatured IgG (through storage at 4°C, for example) does not readily crystallize and a shift in the temperature dependence for forming the hexagonal phase is observed. 3) There is no pH driven transition in crystallization tendency for IgE anti-DNP but a transition to disorder is observed at above 30°C. No such transition exists for IgG_1. Observation of the dynamics of crystal growth shows a clear and marked dependence on pH and temperature that is in accord with the results of long-term incubations. It is found that high pH retards crystal growth very significantly for IgG_1 but not for IgE. Also, the crystal growth rate of 4°C-stored IgG_1 is greatly reduced over fresh IgG_1 (−80°C stored). Furthermore, it is found that the linear phase of IgG_1 is an extremely rapidly forming phase but one that is metastable against the hexagonal phase.

Key words: monoclonal antibody, 2-D ordering, phospholipid monolayers

The induction of two-dimensional ordering of monoclonal antibodies bound to phospholipid films was described previously and is of interest as a general technique in structural biology [1]. In Uzgiris [1], the principles of the two-dimensional ordering technique were indicated and the initial results of the antibody experiments were described. Briefly, two-dimensional ordering was achieved by binding antibodies to a phospholipid monolayer in an oriented manner. The fluidity of the phospholipid monolayer at ambient temperatures allows for rearrangement of the bound antibody. Orientation through binding to a hapten derivatized to the phospholipid polar heads (dinitrophenylaminocaproylphosphatidylethanolamine) ensures the equivalence of

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molecular interactions. These are evidently the necessary conditions in two-dimensional ordering, although certain exceptions for spherical protein molecules evidently exist. For example, ferratin may be able to order somewhat without orientation or even on solid surfaces [2,3].

For mouse monoclonal IgG, two crystal phases have been observed: a linear strand phase and a highly ordered hexagonal phase. The latter structure was analyzed by image reconstruction and a detailed three-dimensional analysis of this structure has been completed and a preliminary analysis was reported [4]. The nature of the linear strand phase has not been described in detail. More importantly, the relationship of one phase to the other has not been described and there has not been a systematic investigation of crystal growth parameters.

In this paper, I deal with a number of key issues in two-dimensional crystallization that heretofore have remained obscure. What is the linear strand phase and how is it related to the hexagonal phase? Is there a conformational change in the antibody molecule that is associated with each phase? What are the dynamics of crystal growth? How is crystal growth retarded? Are the dynamics important in achieving large, highly ordered crystals? How can the antibodies be stored to maximize their crystallization tendencies? Are there reversible effects associated with antibody storage?

The insights gained in a systematic study of this antibody system may be generally applicable to other macromolecular assemblies. Indeed, the goal of the initial work was to use the monoclonal antibody as a model system. Finally, the ability to induce large areas of high-order crystals may be used to investigate high resolution structure of the antibody molecule itself (by low temperature electron microscopy [5]) and to ask what the ultimate structural resolution of such a technique might be.

MATERIALS AND METHODS

Antibody

Mouse monoclonal antibody, IgG_1, anti-DNP (Clone 29-B5) was isolated from the ascites in a manner previously described [6] and was a gift from R. Kornberg and L. Herzenberg. Two separate antibody lots were used in these experiments. Lot 1 was isolated from fresh ascites and lot 2 was isolated from ascites that had been stored at $-80°C$. The stock antibody was at ~ 1 mg/ml in 150 mM NaCl, 50 mM Tris, pH 7.4 for lot 2 and pH 8.1 for lot 1. Each of these lots was stored in a variety of ways, either at $-80°C$ or at $4°C$ or at $\sim 0°C$. The thawed antibody was stored for various lengths of time at $4°C$. The stored antibody is identified by lot number and a letter delineating the length of storage: lot 1, fresh isolated antibody, stored at $4°C$, and used within 1 month; lot 1b, isolated antibody stored at $-80°C$, thawed, and used within 2 weeks; lot 1c, thawed and stored for 3 months at $4°C$; lot 1d, unfrozen, stored at $\sim 0°C$ for 9 months; lot 1f, same as lot 1d but frozen and rethawed before use; lot 2, antibody isolated from thawed ascites, frozen at $-80°C$, thawed, and used within several days; lot 2b, thawed and stored 2 weeks at $4°C$; lot 2c, stored for 3 months at $4°C$.

Lipids

N-Dinitrophenylaminocaproyl phosphatidylethanolamine was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). The phosphatidylethanolamine is derived

from egg lecithin by a transphosphaditylation reaction. The lipid was diluted tenfold in chloroform and spread on an air-water interface in a small rectangular Teflon trough by adding a 20-λ drop of the diluted lipid (500 μg/ml) onto the water surface. Transfer of a monolayer to an electron microscope grid was accomplished by immersion of a suitably prepared grid through the interface and then a withdrawal into air again [7]. This resulted in a pickup of one monolayer with the hydrocarbon tails of the lipid abutting the hydrophobic carbon layer on the grid. The deposition of the monolayer was assayed by short-term, high-density binding of antibody to the haptinated monolayer. This type of high-density binding was absent for lipids without hapten, phosphatidylcholine, or phosphatidylethanolamine alone, or for carbon grids alone. In the former case, no binding was observed at all and in the latter the binding to a hydrophobic carbon was diffuse and limited compared to the haptinated lipid. In particular, there was an absence of molecular clusters at high density that was present in the hapten-bound case. Poor phospholipid transfer or poor lipid spreading behavior could be readily diagnosed by the use of this short-term binding assay.

Electron Microscope Grids

Silver electron microscope grids (Ladd Research, Inc. Burlington, VT) were coated with nitrocellulose in a standard way and then shadowed with carbon in a relatively low vacuum. Such grids were generally sufficiently hydrophobic for the lipid transfer procedure described above to be successful as judged by the antibody binding assay. However, the above lipid transfer procedure was less successful for grids stored beyond ~ 12 hr of carbon shadowing.

Incubation

Phospholipid-coated electron microscope grids were floated on 20-λ drops of antibody solution in microtiter wells. The antibody was usually at 50 to 100 μg/ml in 150 mM NaCl, 50 mM Tris,and at pH 7.4. Departures from these conditions are explicitly noted in the text where they occurred. During the incubations, the microtiter plates were placed in humid conditions at the temperatures indicated.

Staining and Electron Microscopy

After a suitable period of time, the incubated grids were lifted off of the antibody solution with tweezers and either washed with one drop of water or one drop of 1% uranyl acetate solution. Then a drop of 1% uranyl acetate was held on the grid for ~ 30 sec; the drop was then slowly drained with a tissue, and the grid was allowed to dry. The electron microscope examinations were done on a Hitachi, Model 600 EM microscope.

Area Measurements

To monitor the relative degree of ordering and the relative distribution of the two crystal phases, low magnification images (15,000 to 20,000) were obtained. An area integration program was then used to measure domain areas (Digital Paintbrush on an Apple II+).

Polyclonal Antibody

Polyclonal anti-DNP IgG (goat) was obtained from Gateway Imunosera, Inc. The antibody was purified on a DNP affinity column and was used without further processing.

RESULTS

Figure 1 is an electron micrograph of two-dimensional (2-D) IgG arrays. Both hexagonal and linear-type domains formed in this particular experiment. Such multi-domain coverage is common and, in the analysis that follows, an attempt is made to correlate the relative domain areas of the two phases with a variety of incubation conditions. An electron micrograph from an incubation that resulted in a dominance of linear-type arrays is shown in Figure 1, lower part. The molecular stacking along each linear strand is clearly visible. There is a periodicity of ~40 Å associated with

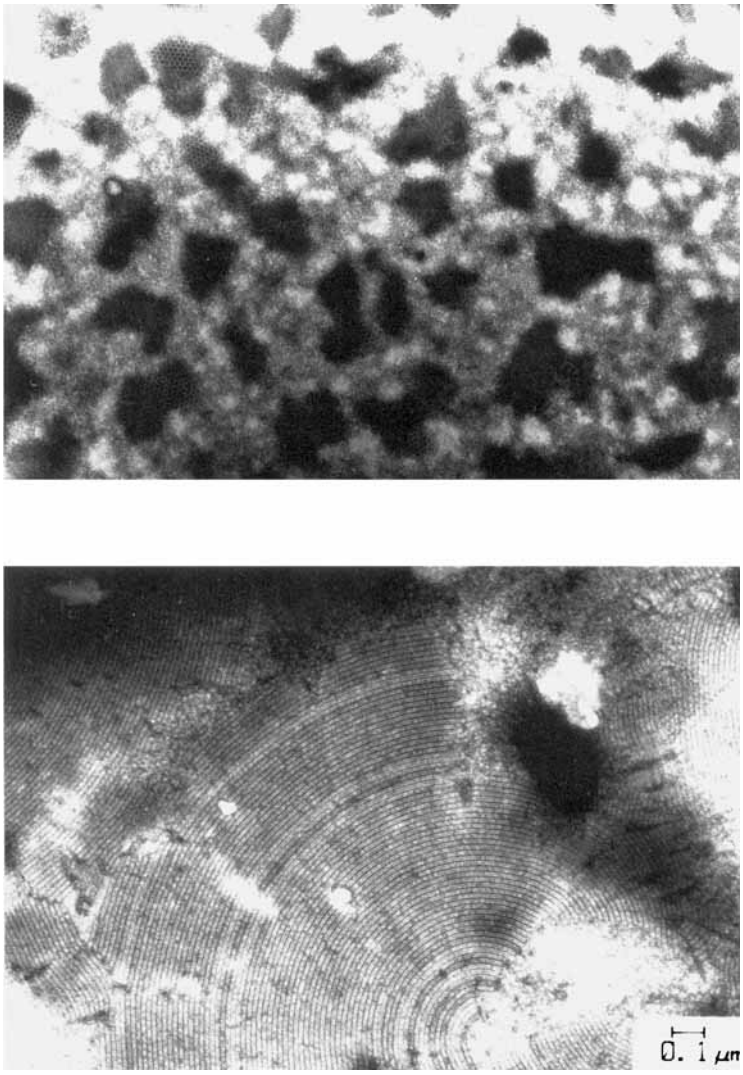

Fig. 1. Upper) electron micrograph of multidomain IgG crystal growth of both linear and hexagonal phase domains. Lot 1a, 18°C, pH 7.4. Lower) electron micrograph of a large linear domain. Linear chain periodicity is 150 Å.

this stacking as shown by optical diffraction. Furthermore, each chain is bisected by a thin filament of stain as if the stacking is with objects that are composed of two linked domains. This two-domain stacking is clearly visible in Figure 1 and is consistent with a view of the antibody molecules as orientating vertically to the grid surface and packing tightly along each chain with the two F_{ab} arms viewed from the top in projection on the grid. The periodicity along a chain is ~40 Å and the chain-to-chain periodicity is 150 Å, both consistent with the model proposed above and the dimensions of the antibody molecule [8]. The position of the F_c portion of the molecule is not clear in these images. The usual image reconstruction techniques are not very helpful because of limited diffraction information from such arrays. The molecular packing along each chain is not well linked to the packing in adjacent chains; hence, one has quasi one-dimensional behavior in this phase.

The hexagonal domains shown in Figure 2 are very well ordered and give extensive optical diffraction patterns. A detailed analysis of this type lattice is given elsewhere [4]. As guidance for the discussion to follow, we consider some aspects of this lattice. The vertex-to-vertex distance between clusters, the large stain exclusion regions, is 150 Å, suggesting that each vertex is linked by a number of antibody molecules. In fact, an examination of this lattice at higher magnification shows six arms protruding from each vertex; hence, a vertex is formed by the clustering of six antibody molecule arms, the F_{ab} domains. The F_c portion of the molecules are visible in the open regions of the lattice as composing the interconnecting filament-like structures. Finally, we note that this lattice is more open than the linear chain lattice; there are ~70% more molecules per unit area in the linear domains than in the hexagonal arrays. This now gives us a physical feel for the two-dimensional arrays that form when monoclonal antibodies bind to a phospholipid monolayer. The question remains as to what drives one phase over the other in the two-dimensional ordering process.

In Figure 3 is shown the dependence of hexagonal ordering on temperature for two different pH values and for various antibody states. The data are a summary of many experiments over a period of several months. The scatter in the data for lot 1 antibody is due to variations in some of the incubation conditions (concentration, exact incubation temperature, and storage effects over a period of 1 month). At the time, the sensitivity of the hexagonal ordering to these types of variables was unappreciated. Nevertheless, the data for lot 1 antibody, taken as a whole, are consistent with the more careful measurements done for lot 2 and lot 1b antibodies. In particular, the scatter in the former data would be consistent with the presence of a steep temperature response curve at around ambient temperatures as is indeed found for lot 2 and lot 1b experiments, both of which utilized "fresh" monoclonal antibodies.

The hexagonal fraction of ordering is a useful way of parameterizing the data, as variations in surface coverage are factored out (a problem for the earlier experiments in particular). Nevertheless, there is very substantial ordering over most of the grid areas, a point explicitly considered below. For lot 2 and lot 1b, the total fractional ordering ranged from 30 to 90%, depending on the temperatures and pH of the incubations. For lot 1, the total fractional order was typically about 20 for 30% owing to more variable lipid transfer.

In Figure 3, data are shown for which no order was obtained of any type. These data represent a form of control experiment in which heavy specific binding of antibody was observed but in which no 2-D structures evolved for any temperature

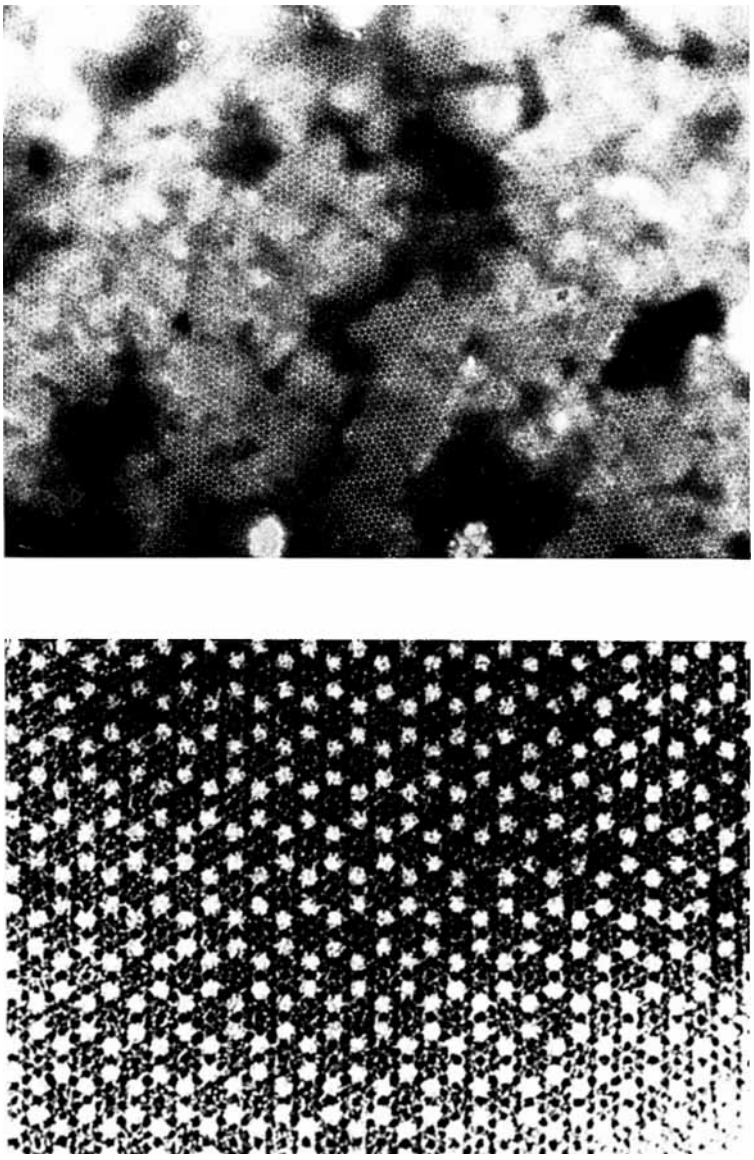

Fig. 2. Upper) electron micrograph of hexagonal IgG crystal growth. Lot 1B, 37°C, pH 7.4. Lower) expanded view from one such domain. Distance between large stain exclusion regions is 150 Å.

or pH condition. These experiments involved polyclonal antibody against DNP and monoclonal anti-DNP IgG (lot 1f) in the presence of 2 mM dithiothreitol (DTT).

For the same lipid lot, a comparison of new antibody and old antibody was done and is shown in Figure 4. The temperature for observing 50% hexagonal and 50% linear domains (by area) is defined as $T_{1/2}$, for identical incubation conditions, ie, 10–12 hr, and standard buffer (150 mM NaCl, 50 mM Tris). The $T_{1/2}$ for lot 2 and lot 1b is seen to be 21°C and 25°C, respectively (Fig. 3). However, for the stored

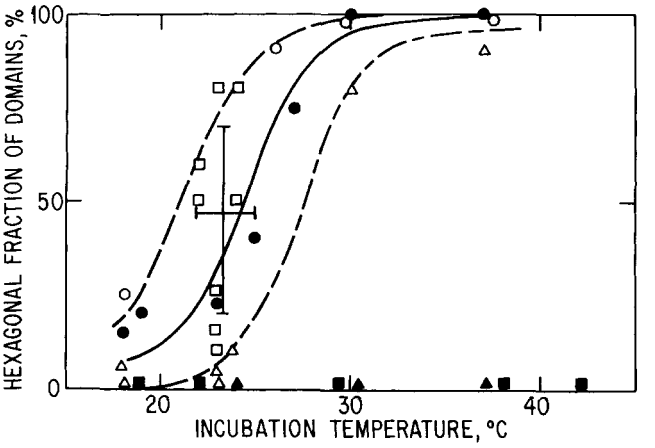

Fig. 3. IgG ordering into hexagonal domains as a function of temperature for several lots of new antibody. ○, Lot 2, 25 μg/ml, pH 6.9; ●, lot 1b, 50 μg/ml, pH 7.2; △, lot 1b, 50 μg/ml, pH 8.1; □, lot 1, pH 8.1 (Actual temperature was not monitored or controlled in this series—range from 22 to 25°C—and the concentration was from 25 μg/ml to 50 μg/ml. The dispersion for this series is indicated by error bars); ■, polyclonal anti-DNP; ▲, lot 1f, pH 7.2, and 2 mM DTT.

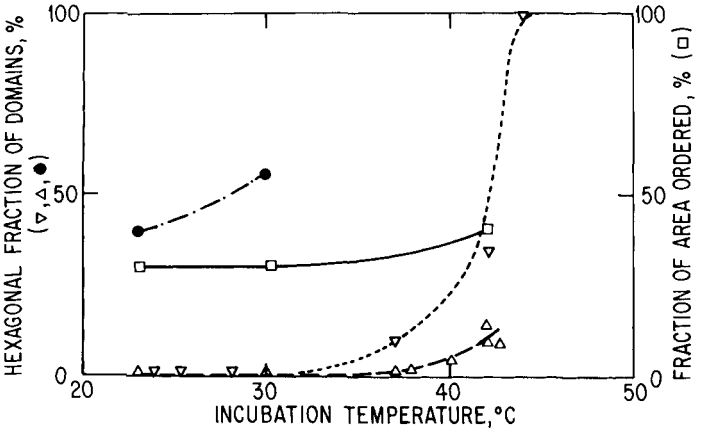

Fig. 4. Ordering vs temperature for stored antibody. □, △, Lot 1d; ●, lot 1d with 1/2 mM DTT and 1/4 mM DTT; ▽, lot 1f.

antibody, for the same source of phospholipid, there is a markedly shifted $T_{1/2}$—to 42°C for lot 1f and to at least that for lot 1c and lot 1d. The ordering is mainly in the linear phase for the temperature range below 40°C as is shown. Up to 30% of total area is ordered even for the stored antibody after a 12-hr incubation for lot 1d and 1f, for example. The addition of DTT produces an increase of the hexagonal fraction (or a decrease in the linear fraction). This result is described in more detail below.

The observations shown in Figures 3 and 4 suggest that a possible conformation change in the antibody is associated with the hex/linear phase distribution. The shift in $T_{1/2}$ with antibody lot, for the same lipid lot, rules out lipid effects as the driving

force. Indeed, there are no lipid phase transitions to be expected in this temperature range for egg lecithin-derived fatty acid chains [9]. The possibility of antibody-associated lipid-driven phase transitions is also ruled out by the experiments utilizing polyclonal antibodies shown in Figure 3. These antibodies show very extensive binding and clustering on the monolayers but fail to show either hexagonal or linear ordering for any of the experimental temperatures or for any concentration used (50–250 μg/ml). Likewise, monoclonal antibodies treated with 1 mM DTT fail to order but are observed to bind and to cluster on the monolayer.

The effects of pH on tendency to hexagonal ordering are shown in Figures 3 and 5 for both fresh antibody and stored antibody at various temperatures. The effects of salt concentration are shown in Figure 6.

The kinetics of hexagonal phase crystal growth, ie, hexagonal relative to linear domains, weighted by area, is indicated in Figure 7 for three different antibody conditions and for the same source of phospholipid. At slightly elevated temperatures fresh antibody (lot 2) shows a very rapid tendency to hexagonal order. This tendency is diminished after 2 weeks storage at 4°C and is absent for antibody stored for an extended period at 4°C and incubated at room temperature.

The fractional ordering into the hexagonal phase is only a partial picture of the phenomena involved. What about total ordering and crystallization rates? The trends for the combined crystal growth rates of both the hexagonal and linear phases is indicated in Figure 8. The ordering tendency is very rapid for fresh antibody with significant ordering in 15 min. The rapid kinetics is somewhat variable from experiment to experiment. The data indicated by the open-triangle symbols are also for lot 2, fresh antibody, but done at another time, with a slightly different history after thawing. It is likely that the crystal nucleation processes are affected by systematic variables that are not controlled well in these experiments. Nevertheless, the retardation of crystal nucleation by long-term storage effects on the antibody is striking. For such antibody, lot 1c, no nucleation occurs even after 10 hr, unless the temperature is raised to at least 37°C (Fig. 4).

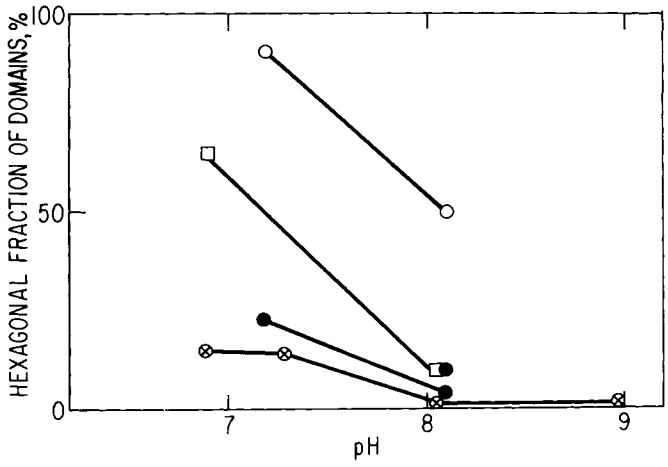

Fig. 5. Hexagonal ordering as a function of pH for new and stored antibody. □, Lot 2, 23°C; ⊗, lot 1f, 37°C; ●, lot 1b, 23°C; ○, Lot 1a, 25°C.

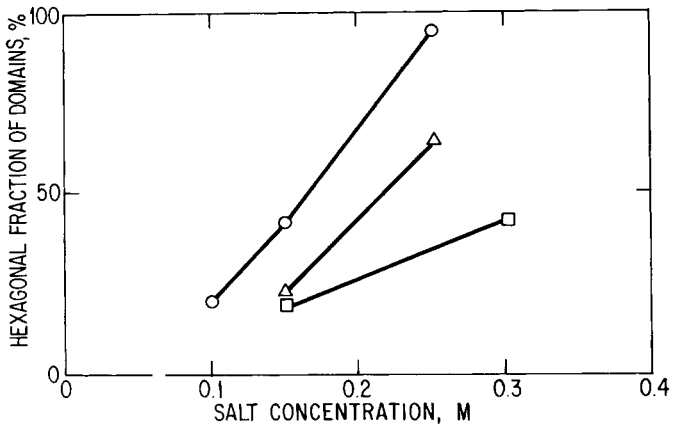

Fig. 6. Hexagonal ordering is a function of NaCl concentration for new and stored antibody. ○, Lot 1f, 30 μg/ml, pH 7.3, 42°C; △, lot 1, 50 μg/ml, pH 8.1, 23°C; □, lot 1, 50 μg/ml, pH 8.3, 23°C.

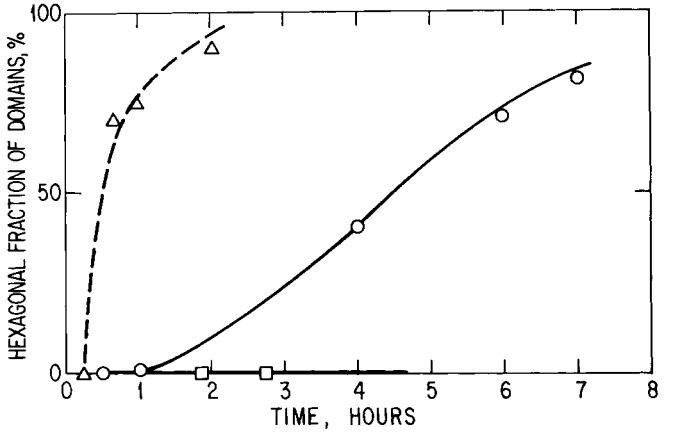

Fig. 7. Kinetics of hexagonal ordering for new and stored antibody. △, Lot 2, pH 6.9, 25 μg/ml, 28°C; ○, lot 2b, pH 6.9, 25 μg/ml, 23°C; □, lot 1c, 50 μg/ml, pH 6.9, 23°C.

It was observed that lot 1d antibody formed only linear arrays at all temperatures up to 40°C but that upon freezing and rethawing the tendency to hexagonal order was recaptured, at least to a partial degree. A similar recovery was observed for lot 2b antibody, shown in Figure 9. After thawing the refrozen aliquot, the rate of ordering was markedly increased and approached the initial rate (data not shown).

The increased tendency to hexagonal order with temperature for IgG is in marked contrast to what was observed for monoclonal IgE. In the latter case, hexagonal order could be induced in the range of 18 to 30°C (data not shown). Above 30°C, the hexagonal order was lost, and in its place emerged an amorphous close-pack arrangement of clusters. Presumably, a melting-out of the two-dimensional lattice had occurred. The IgI_1 hexagonal lattice order persisted to as high as 55°C,

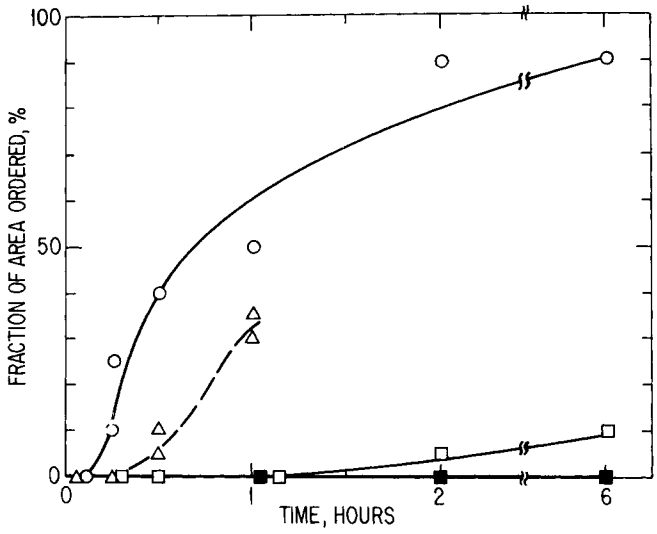

Fig. 8. Kinetics of total ordering (both hexagonal and linear phases) for new and stored antibody. ○, Lot 2, 23°C, 50 μg/ml, pH 6.9; △, same as for lot 2; □, lot 2b, 50 μg/ml, 23°C, pH 6.9; ■, lot 1c, 50 μg/ml, pH 6.9, 23°C.

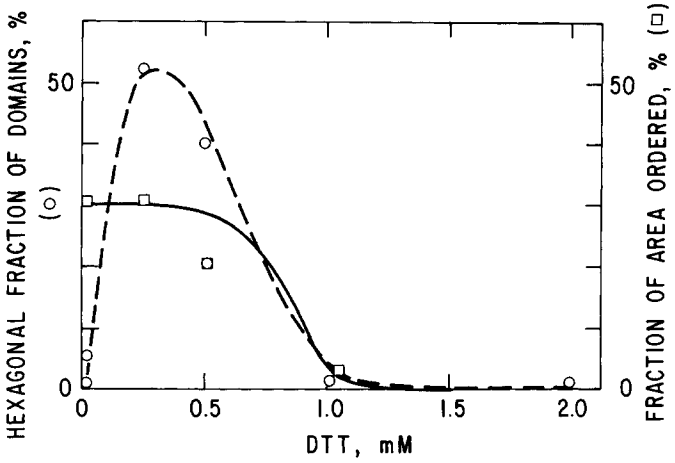

Fig. 9. Effects of DTT on IgG ordering. Lot 1d, 23°C incubation at 50 μg/ml, pH 7.4.

the highest incubation temperature used. It was possible to eliminate order by preincubating the antibody for 1 hr at 65°C before a normal incubation procedure at 42°C.

In Figure 9 is shown the effects of DTT on IgG ordering. The antibody, lot 1d, had a negligible tendency to nucleate hexagonal crystals in the temperature range below 40°C, but a substantial ordering into the linear phase was achieved. With the addition of DTT, the relative distribution of hexagonal domains (weighed by area) increased markedly before all ordering tendency was lost at about 1 mM DTT concentration.

The dependence of IgG ordering on antibody concentration is shown in Figure 10. Note that most experiments cited above were done either at 50 μg/ml or 100 μg/ml. The decline in total ordering at the low antibody concentrations is assumed to be due to lipid monolayer loss—the ordered domains were small and island-like in an empty featureless sea as viewed by EM after staining. At high antibody concentration, the high packing density favors the formation of the linear strand phase of two-dimensional order.

DISCUSSION

The two-dimensional crystallization of IgG can be understood in terms of certain incubation conditions. It is evident that temperature, pH, and salt concentration are important factors in the growth of two-dimensional crystals. The data of Figures 3 and 4 are suggestive of a conformational change that may favor one crystal phase over the other for the same antibody concentration, pH, and salt concentration, and the same source of phospholipid. This is further supported by the data of Figures 5 and 6 in which the involvement of ionizable groups is shown to be a factor in the crystallization interactions.

No lipid phase transitions are expected for egg lecithin-derived phospholipids in the temperature range investigated [9]. The observed $T_{1/2}$ is shifted by the condition of the antibody, ranging from nearly 21°C for fresh antibody to 42°C for extensively stored antibody. Furthermore, antibody stored for a very long time in ice was incapable of forming a hexagonal lattice for temperatures below 40°C. No ordering was observed, for any temperature, for polyclonal anti-DNP antibody and for antibody in the presence of DTT in excess of 1 mM. Finally, to support further the notion that there is no lipid-driven crystallization process, the behavior of IgE monoclonal antibody as a function of temperature for the same lipid (data not shown) is completely different from the IgG: Hexagonal lattices form at ambient temperatures, but the lattices fail to form at all for temperatures above 30°C. The IgE crystallization process also fails to show any dependence on pH, in contrast to IgG.

Of course, it is interesting to speculate what conformation change may be involved. One natural candidate in the crystallization process is the flexibility of the molecule at the hinge [10]. One can imagine the formation of the linear stripes by upright and stiff molecules, packed close together, side by side, to give the kind of

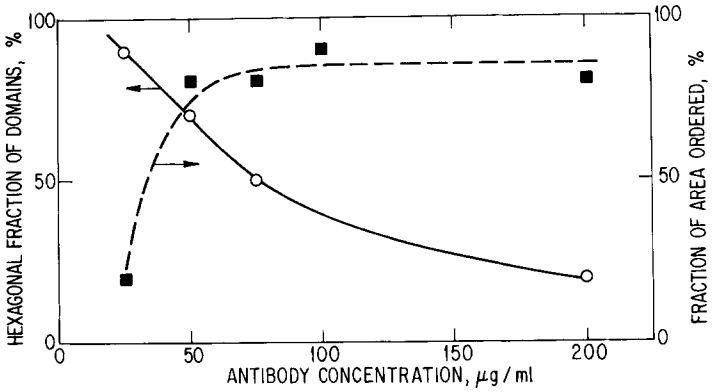

Fig. 10. IgG ordering as a function of concentration Lot 2, 28°C, pH 6.9.

images shown in Figures 1. However, the hexagonal packing requires the antibody molecule to bend over at shallow angle to the surface and to form, perhaps, intimate F_c-F_c contacts with other molecules of the lattice.

Attempts to demonstrate this point with the addition of low levels of DTT to reduce preferentially the disulfide bonds at the hinge [10] have been vey suggestive (Figs. 4, 9). At low levels of DTT a substantial enhancement in hexagonal nucleation over linear was observed. However, it was masked by a reduction in ordering tendency: At DTT levels of 1 mM and above, very little crystallization took place.

The decline in crystallization tendency after storage of antibody at 4°C is quite pronounced. It is paticularly clearly demonstrated in the kinetic data shown in Figures 7 and 8. The decline in crystal nucleation and growth rate leads to a diminution in overall order after a standard incubation procedure (Fig. 4). This crystallization decline can be compensated for in part by increasing the temperature of the incubation. It is important to emphasize that the decline in ordering occurs for both phases (Fig. 8).

We can only speculate on the mechanisms of the decline. Monoclonal antibodies are known to aggregate after prolonged storage at 4°C. It is possible that some preaggregate condition hinders two-dimensional crystal nucleation and growth. This condition may be reversed by mild heating. Observations on the optical clearing effect of mild heating on partially aggregated samples suppport this idea (data not shown).

We note one other variable of importance—antibody concentration. Over what range of concentration will adequate nucleation and crystal growth occur? Figure 10 shows that crystal growth occurs over a wide concentration range, but there is a pronounced bias toward hexagonal growth at lower concentrations. Linear domains nucleate much more readily than hexagonal domains at ambient temperatures. Hexagonal domains are more open; the molecular density of linear domain is $\sim 70\%$ higher than in the hexagonal domains. Thus, at high concentrations, the hexagonal domains do not have the opportunity to nucleate, and there is a bias toward the formation of linear domains as is observed in Figure 10.

The processes associated with a decline in total order at low concentrations are less clear. The domains tend to be isolated and discontinuous with extensive open, featureless areas on the grid. This suggests that there may be lipid loss during the incubation procedure either owing to slow binding rates or to depletion of the antibody. However, at 20 μg/ml, there should be sufficient antibody for a monolayer of ~ 50 cm^2. Rather than being a fundamental issue of dynamics, this loss is an issue of specifics—perhaps owing to contaminants in the antibody stock or in the buffers used or to lipid monolayer instabilities.

The central conclusion of these studies remains that by a judicious choice of incubation parameters it is possible to achieve high crystal growth rates even for somewhat degraded antibody. This understanding may be useful in future studies of antibody-lipid systems and for the attainment of high resolution structure information on the antibody molecule by the two-dimensional crystallization technique. The present studies also indicate that dynamic variables (eg, concentration, rate of binding) as well as solution conditions (eg, pH, salt, temperature) will be important in two-dimensional crystallization processes.

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