Anti-AMP antibody precipitation of multiply adenylylated forms of glutamine synthetase from *Escherichia coli*: A model relating both concentration and density of antigenic sites with the antibody–antigen interaction

(anti-nucleotide antibody/anti-hapten antibody)

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Contributed by E. R. Stadtman, September 12, 1980

ABSTRACT

Sheep antibodies directed against an AMP-bovine serum albumin conjugate exhibit highly specific binding toward AMP. These antibodies bind to the AMP moiety of adenylylated glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.12] from *Escherichia coli* and to no other antigenic determinant on the protein. *E. coli* glutamine synthetase can exist in various modified (isomeric) forms that differ with respect to the number (0–12) and the distribution of identically adenylylated subunits [Ciardi, J. E., Cimino, F. & Stadtman, E. R. (1973) Biochemistry 12, 4331–4330]. Using this enzyme, together with the AMP-specific antibodies, we have investigated the effects of the total concentration, population density, and topographical distribution of multiple identical antigenic determinants on the antigen–antibody interaction. Stopped-flow fluorescence measurements show that the rate and extent of initial binding of the antibodies to the antigen are a function of the total concentration of AMP groups and are independent of the number of AMP groups per dodecamer. However, the rate of lattice formation increases with increasing epitope density, and the maximal amount of glutamine synthetase precipitated is directly proportional to the average number of adenylylated subunits per dodecamer. The data suggest that partially adenylylated enzyme preparations are composed of subpopulations of glutamine synthetase molecules that differ in their tendency to form precipitable aggregates, due presumably to differences in the topographical distribution of antigenic determinants on the surface of the enzyme. The enzyme species that form soluble immune complexes do so possibly due to intramolecular crosslinkage of the bivalent antibodies with adenylylated subunits to the exclusion of intermolecular crosslinkage.

Glutamine synthetase (GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.12) from *Escherichia coli* is a multimeric enzyme consisting of 12 identical subunits (Mr 50,000) arranged in two superimposed hexagonal arrays. Regulation of the enzyme involves a cyclic cascade system that catalyzes a covalent attachment of a 5'-AMP moiety to a specific tyrosyl residue in each subunit (1).

Depending on growth conditions, the enzyme in *E. coli* contains, on the average, between 0 and 12 covalently bound AMP groups per dodecamer (2). We previously reported the isolation of sheep antibodies directed against an AMP-bovine serum albumin conjugate that precipitated adenylylated, but not unadenylated GS (3). Thus, for these antibodies, adenylylated GS serves as a large protein antigen with between 0 and 12 precisely defined antigenic determinants per protein molecule.

We report here the results of studies with variously adenylylated forms of GS to determine the effects of the total concentration of antigenic determinants, density of determinants per antigen, and the topological distribution of antigenic determinants, on the initial antibody–antigen binding, lattice formation, and immunoprecipitation. Some of the experimental data were presented in an earlier preliminary communication (3).

MATERIALS AND METHODS

Purification and Assay of GS. Various GS preparations with different 〈n〉 values were obtained from *E. coli* cells that had been cultured under various degrees of nitrogen availability. GS was purified by Zn2+ precipitation (4), followed by the acetone and acid (NH4)2SO4 steps described by Woolfolk et al. (5). Total enzyme activity and the average state of adenylylation, 〈n〉, were determined at pH 7.5 by means of the γ-glutamyltransferase reaction (6). The presence of normal sheep serum or anti-AMP antibodies in the assay mixture had no effect on enzyme activity or measurements of the state of adenylylation.

Preparation of Antiserum and Immunoprecipitation Tests. Preparation of sheep anti-AMP antiserum and the procedure for immunoprecipitation tests have been described (3).

Purification of Antibodies. Partially purified antibody preparations used for immunoprecipitation experiments were obtained by (NH4)2SO4 fractionation (7) followed by dialysis against 10 mM imidazole/150 mM KCl at pH 7.3. The serum was sterilized by filtration and stored at −20°C.

The AMP-specific antibodies used in light scattering and stopped-flow experiments were purified by immunoabsorbant chromatography on a gel consisting of fully adenylylated GS covalently coupled to AH-Sepharose 4B (Pharmacia). Partially purified serum was added to this gel, which had been equilibrated with 10 mM imidazole/150 mM KCl (pH 7.3), and incubated for 12 hr at 4°C with gentle mixing. The Sepharose then was washed on a coarse sintered glass filter funnel with cold 20 mM imidazole/500 mM KCl (pH 7.3) until the effluent was free of protein. The washed gel was packed into a 0.9 × 10 cm column and AMP-specific antibodies were eluted with 10

Abbreviations: GS, glutamine synthetase; GSn, a glutamine synthetase preparation containing on the average 〈n〉 adenylylated subunits per dodecameric molecule. The value of 〈n〉 varies between 0 and 12 and is referred to as the average state of adenylylation.

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"Concentration of antigenic determinants" refers to the total concentration of adenylylated subunits, in the reaction mixture, irrespective of the state of adenylylation. "Concentration of antigenic determinants per antigen" refers to the number of adenylylated subunits per GS dodecamer and is synonymous with the terms "epitope density" or "state of adenylylation."
mM imidazole/250 mM KCl/50 mM AMP, pH 7.3. The fractions containing protein were pooled, concentrated by ultrafiltration, and dialyzed extensively against 10 mM imidazole/150 mM KCl, pH 7.3. The purified antibodies were stored aseptically at 4°C until used.

Radioimmunoassay. Binding data for the Sips plot and competition experiments were obtained by the Farr technique (8). The experiments were performed by adding, in the following order: buffer (10 mM imidazole/150 mM KCl, pH 7.3), 1.81 μM [14C]AMP, nonradioactive competing ligand (if any), and antiserum. Samples then were incubated for 18 hr at 4°C. The radioactivities of duplicate samples were measured in 10 ml of quasial, NEA (anti-sheep IgG) was used as a control.

Rapid Mixing Experiments. A stopped-flow apparatus was used to mix purified antibodies and GS (2 msec) into a spectrophotometer with excitation and emission monochromators set at 300 nm and 340 nm, respectively. Initial rates of light scattering were observed by setting both excitation and emission monochromators to 400 nm and measuring the rate of increase of scattered light at a 90° angle to the incident beam.

Large-Scale Immunoprecipitation and Repurification of Partially Adenylylated GS. Eight milligrams of GS in 10 mM imidazole/100 mM KCl/1 mM MnCl₂ (pH 7.0) was added to a sufficient amount of partially purified antiserum to give maximal precipitation without approaching antibody excess. After incubation at 37°C for 25 min, followed by 18 hr at 4°C, precipitated and soluble immune complexes were separated by centrifugation. The precipitated and supernatant enzyme fractions were further treated separately, but identically. To dissociate the immune complexes, 1% by volume of 0.1 M AMP was added to each fraction, and the fractions were incubated at 37°C for 20 min. The dissociated mixture of GS and IgG was loaded onto a DEAE-agarose column that had been equilibrated with 10 mM imidazole/100 mM KCl/10 mM AMP/1 mM MnCl₂, pH 7.0 (room temperature). IgG, which does not bind to DEAE-agarose, was washed off the column with equilibration buffer. GS was eluted with buffer containing 300 mM KCl. Fractions containing GS were pooled, concentrated by ultrafiltration, and loaded onto an immunoabsorbent column consisting of rabbit anti-sheep IgG coupled to Affigel 10 (Bio-Rad) that had been equilibrated with 10 mM imidazole/100 mM KCl/1 mM MnCl₂/50 mM AMP. The GS that appeared in the void volume was free of IgG. The final yield of enzyme both from the original immunoprecipitate and the soluble immune complexes was 75%.

Each GS preparation was dialyzed extensively to remove AMP and was then reprecipitated with the anti-AMP antibodies under the same conditions as the initial precipitation (see Fig. 6).

Protein Determinations. GS was quantitated spectrophotometrically, using A₂₅₀ = 0.385 (9). Protein concentration of crude serum was determined by the method of Bradford (10), using IgG as a standard. Purified antibodies were quantitated spectrophotometrically, assuming A₂₅₀ = 1.4.

RESULTS

Characterization of Anti-AMP Antibodies. A curved line, typical of all antibody-hapten systems (except monoclonal antibody) is generated when binding data from the radioimmunoassays of anti-AMP antiserum with unconjugated [14C]AMP are represented in a Scatchard plot (Fig. 1 Inset). A Sips plot (11) of the same binding data (Fig. 1) produces a straight line with a half-saturation value (average K₀) of 4.3 μM and a heterogeneity index a = 0.89. Because a heterogeneity index of 1.0 indicates all binding sites have the same affinity for the ligand, the antibodies in the serum have nearly identical intrinsic affinities for AMP.

The specificity of the antibodies for AMP was determined by measuring the competition between [14C]AMP and various ligands for binding sites on the anti-AMP antibodies. From logit plots (12) of data derived from these competition experiments, it was estimated that the affinity of antibodies for 5'-AMP is at least 100 times greater than for ADP, adenine, adenosine, 3'-AMP, 2',3'-cyclic AMP, GMP, or IMP. No significant binding occurred with ATP, CMP, UMP, or TMP.

Effect of the State of Adenylylation on the Primary Antibody-Antigen Reaction. A stopped-flow fluorescence technique (cf. refs. 13, 14, and 15) was used to monitor the initial binding of the anti-AMP antibodies to GS preparations with different average states of adenylylation (epitope density). The curves in Fig. 2A show that, after rapid mixing of adenylylated GS and purified anti-AMP antibodies, there is an immediate and rapid decrease of the intrinsic tryptophan fluorescence of GS, IgG, or both proteins. Because both antibody and GS concentrations are identical for all three curves, the differences observed reflect differences in the concentration of adenylylated subunits. Replots (not shown) of the data show that the rate and amplitude of the quenching reaction are nearly pro-

![Fig. 1. Binding of anti-AMP antibodies to unconjugated [14C]AMP. All experiments were performed at 4°C in 10 mM imidazole/150 mM KCl at pH 7.3. (Inset) Scatchard plot of binding data from radioimmunoassays of partially purified anti-AMP antiserum and [14C]AMP. Straight lines are least-squares fit of data points in the high- and low-affinity regions of the binding curve and represent the two extremes of the range of binding affinity. The value at the x intercept of the extrapolated curve gives the total number of antibody binding sites that was used for the Sips plot (11). The main figure is binding data represented in a Sips plot. r = mol of AMP bound per mol of antibody; n = number of binding sites per antibody molecule = 2. The value of the x intercept of the Sips plot is the logarithm of the free hapten concentration that gives 50% saturation of antibody sites. Due to the heterogeneity of binding affinity of the antisum for AMP, this half-saturation value (4.3 μM) cannot rigorously be referred to as K₀ (which requires normal rectangular hyperbolic binding) but should be considered as an "average K₀" or Kₐ (11). The Sips heterogeneity index (the slope of the Sips plot) indicates the extent to which the binding sites are heterogenous with respect to binding affinity (see text).

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A standard fluorescence spectrophotometer and chart recorder were used to measure the slow increase in light scattering due to lattice formation. Results with three different native GS preparations ($\bar{n} = 4, 8$, and 12) are shown in Fig. 3. Whether compared at identical protein concentration (Fig. 3A) or at identical adenylylated subunit concentration (Fig. 3B), the rate of lattice formation increased as the state of adenylylation was increased. Lattice formation is therefore a function of both the concentration of antigenic determinants and the epitope density. The latter is in contrast to the data in Fig. 2B showing that the initial binding of antibodies to antigen is independent of epitope density.

Immunoprecipitation as a Function of $\bar{n}$ Values. To examine the effect of epitope density on precipitability, GS preparations with states of adenylylation ranging between 4 and 12 were titrated with anti-AMP antiserum.

The data in Fig. 4 show that, at each state of adenylylation, the fraction of GS precipitated increased with increasing concentration of antiserum to a maximal value; and in each case antibody excess led to the formation of soluble immune complexes. Fig. 5 (solid line) shows that the maximal amount of GS that can be precipitated is proportional to the average state of adenylylation over the range $\bar{n} = 4$ to 10, and that there is nearly complete precipitation of GS$_{12}$. As isolated, GS preparations with intermediate states of adenylylation are complex mixtures of enzyme species containing between 0 and 12 adenyl groups per molecule (16). Enzyme species containing fewer than 2 adenyl groups per molecule can not undergo intermolecular cross-linking with bivalent antibodies. However, incomplete precipitation of partially adenylylated enzyme preparations cannot be explained by a high concentration of such species, because the average states of adenylylation (indicated by the numbers on the curves in Fig. 4) of the nonprecipitable fraction are greater than 2. For example, the nonprecipitable fraction of the GS$_{12}$ and GS$_{10}$ preparations had $\bar{n}$ values of 4.7 and 6.9, respectively. That incomplete precipitation reflects the amount of enzyme species with low states of adenylylation ($\bar{n} < 2.0$) is contradicted also by studies with subfractions of native GS preparations. By means of affinity

A stained procedure was also used to follow the rapid increase in 90° light scattering that signals the primary binding of the antibodies to adenylylated GS, as opposed to the slow changes in light scattering described below that monitor lattice formation. The rapid changes in light scattering (data not shown) paralleled those obtained in the fluorescence quenching experiments. Thus, using two independent techniques to monitor the initial binding reaction, we found that the antibodies do not distinguish between GS preparations with different epitope densities.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Intrinsic tryptophan fluorescence decrease due to the initial binding between adenylylated GS and purified anti-AMP antibodies. All experiments were performed at 20°C in 10 mM imidazole/150 mM KCl/1 mM MnCl$_2$ at pH 7.3. (A) A stopped-flow apparatus was used to mix GS$_7$, GS$_8$, or GS$_{12}$ at 0.33 mg/ml with purified anti-AMP antibodies at 0.23 mg/ml in a flow cell in a fluorescent spectrophotometer. The change in tryptophan fluorescence was monitored by means of an oscillograph tracing over a period of 10 sec. (B) Similar to A, but a native GS$_{12}$ preparation (0.58 mg/ml) and an artificial GS$_{12}$ preparation (obtained by premixing equal volumes of GS$_7$ and GS$_{12}$ at 0.58 mg/ml were each mixed with purified anti-AMP antibodies at 0.23 mg/ml (see text). The changes in fluorescence obtained with the native and artificial GS$_{12}$ preparations were identical, as shown by the curve.

Control experiments in which fully adenylylated GS was mixed with nonimmune sheep serum produced no change in tryptophan fluorescence (data not shown).

Fig. 2B shows results of fluorescence quenching experiments using identical concentrations of purified anti-AMP antibodies and two different GS$_{12}$ preparations. Native GS$_{12}$ was isolated from *E. coli* that was grown under conditions such that on the average the enzyme contained 6 adenylylated subunits per dodecamer. The "artificial" GS$_{12}$ preparation was produced by mixing equal concentrations of GS$_7$ and GS$_{12}$ prior to the stopped-flow experiments. Thus, for a given protein concentration, the native GS$_{12}$ and "artificial" GS$_{12}$ preparations have identical concentrations of antigenic determinants (AMP moieties). Because there is no exchange of subunits among enzyme species under these conditions (16), native GS$_{12}$ has an average epitope density of 6, whereas the epitope density of the artificial GS$_{12}$ is essentially 11 (Fig. 2A predicts that the GS$_7$ component of the "artificial" mixture will not contribute significantly to the fluorescence quenching signal). A single curve (Fig. 2B) describes both the rate and extent of the initial antibody-antigen reaction with both preparations. These data and the data in Fig. 2A show that the primary reaction between antibody and antigen is a function of the total concentration of antigenic determinants and is independent of epitope density.

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![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Light scattering increase as a function of total concentration and density of adenylylated subunits. All experiments were performed at 20°C in 10 mM imidazole/150 mM KCl/1 mM MnCl$_2$ at pH 7.3, and in a total volume of 1.5 ml. Two hundred micrograms of purified anti-AMP antibodies was mixed with: 67 μg of GS$_7$, GS$_8$, GS$_{12}$, or GS$_{12}$(A); 70 μg of GS$_{12}$, 105 μg of GS$_8$, or 210 μg of GS$_7$ (B). In B, the total concentration of adenylylated subunits was identical in all three curves.
chromatography on Cibacron blue-Sepharose columns, GS preparations with intermediate states of adenylylation can be resolved into more homogeneous subfractions. For example, a GS\(_{6.2}\) preparation can be resolved into subfractions containing on the average 0–3, 4–6, 7–9 adenylylated subunits (unpublished data). Fig. 5 (open circles) shows that the dependence of immunoprecipitation of these partially resolved fractions on the value of \(\bar{n}\) is identical to that of unfractinated GS preparations.

**Differences in Immunoprecipitation Between Isomeric Forms of Partially Adenylylated GS.** To investigate further the incomplete precipitation of partially adenylylated GS, large-scale immunoprecipitations were carried out at an antibody-to-antigen ratio that gave maximal precipitation. After removal of the IgG from both the precipitated and soluble GS fractions (see Materials and Methods and Fig. 6), the GS in both fractions was reisolated and each was subjected to a second immunoprecipitation along with the unfractinated GS. The data in Fig. 6 show that when a GS\(_{6.2}\) preparation was titrated with antibodies, only 55% of the enzyme was precipitated. However, when subjected to a second cycle of precipitation, 77% of the enzyme recovered from the first precipitate was precipitated, whereas only 30% of the enzyme recovered from the soluble fraction was precipitated. It is evident from Fig. 5 (broken lines) that factors other than the average state of adenylylation determine the precipitability of GS, because coordinates of data points for enzyme preparations that had been reisolated from the immunoprecipitated and the supernatant fractions do not fall on the same line as those obtained from unprecipitated enzyme preparations.

**DISCUSSION**

The high affinity of an antibody for a multideterminant antigen is due to multiple binding of the bivalent IgG molecule to identical antigenic determinants on the same or different antigens, and not to a high intrinsic affinity of the antibody for an isolated determinant (17, 18, 19). The independence of the initial antibody–antigen binding on the density of antigenic determinants that we observed suggests that this enhancement operates exclusively at the level of lattice formation.

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**FIG. 4.** Immunoprecipitation as a function of the state of adenylylation. Immunoprecipitations were performed as previously described (3). Both the total GS concentration and the average state of adenylylation was determined by assaying the supernatant solutions from each immunoprecipitation tube after centrifugation. GS was used at 100 μg/ml for all points. Numbers above each data point refer to the \(\bar{n}\) value of the GS in the supernatant. The average state of adenylylation of all supernatants from the GSP preparation was 12. The average state of all GS preparations prior to immunoprecipitation was: O, 5.1; Δ, 6.0; ●, 6.2; ■, 9.0; ▲, 12.

**FIG. 5.** Maximum precipitability as a function of \(\bar{n}\) value. Values for the ordinate were obtained from the minimum point in immunoprecipitation curves (as in Fig. 4). ● represent native GS preparations and were obtained from Fig. 4. Os represent GS that has been fractionated on a Cibacron blue column (see text). Broken lines connecting squares and triangles represent the data from immunoprecipitation, resolation, and reimmunoprecipitation experiments described in Fig. 6. ■ and ▲ represent the starting GS preparations used for two separate experiments. Os and ▲ above and below the solid line are the minimum in the immunoprecipitation curves obtained from the second antibody titration of the reisolated enzyme from the “pellet” and “supernatant” fractions of the initial immunoprecipitation, respectively.

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**FIG. 6.** Immunoprecipitation, resolation, and reimmunoprecipitation of a GS\(_{6.2}\) preparation. The first set of \(\bar{n}\) values (6.9 and 4.7) refers to the state of adenylylation of the enzyme that has formed precipicable (55%) and soluble (45%) immune complexes, respectively, during the first immunotitration. The values under pellet fractions after the second immunoprecipitation (77% and 30%) refer to the maximal amount of enzyme precipitated during the second immunoprecipitation, and the \(\bar{n}\) values under the supernatant fractions (6.3 and 4) represent the average state of adenylylation of the enzyme forming soluble immune complexes during the second immunotitration starting with the repurified fractions (\(\bar{n}\) = 6.9 and 4.7) from the initial immunoprecipitation. These data are represented by triangles in Fig. 5.
Adenylylated GS and anti-AMP antibody have several distinct advantages over most other antigen–antibody systems. Most importantly, the adenylyl groups can be attached to only one or more of 12 precisely defined positions on the GS molecule; moreover, the number of adenylyl groups per enzyme dodecamer can be varied experimentally. Furthermore, this system offers all of the advantages of anti-hapten systems, including ease of quantitation and purification.

A statistical analysis shows that native GS preparations are complex mixtures of up to 382 uniquely different enzyme species (M. S. Raff and W. C. Blackwelder, personal communication) that differ from one another with respect to both the number (0–12) and distribution of adenylylated subunits. Therefore, with the aid of highly specific anti-AMP antibodies, this enzyme offers a unique opportunity to investigate the effect of epitope density, topographical distribution, and concentration of antigenic sites on the antibody–antigen reaction. The present study confirms that at a minimum three steps are involved in the reaction of anti-AMP antibodies with the enzyme: (i) rapid initial binding of antibody to the antigen, (ii) slow rearrangement of bonds to form an intermolecular lattice network, and (iii) precipitation of the lattice when it reaches sufficient complexity and size.

Results from stopped-flow fluorescence experiments (Fig. 2) and rapid light scattering measurements show that both the rate and extent of the initial binding reactions are proportional to the total concentration of adenylylated subunits and to this parameter only. Epitope density plays no role in this initial binding reaction; antibodies bind equally well to an AMP moiety whether it is part of a GS or GS$_3$ subunit. Light scattering measurements show that the initial antibody–antigen reaction is followed by slow lattice formation, which is influenced by the total concentration of antigenic determinants (AMP groups) and also by the epitope density. Clearly, these two processes (initial binding and lattice formation) operate under different constraints.

In theory, any antigen that contains two or more antigenic sites can undergo intermolecular crosslinkage with bivalent antibodies to yield a lattice network that will precipitate (20). It is therefore curious that with optimal ratios of GS to antibody, the fraction of GS molecules that can be precipitated increases almost linearly as a function of the average state of adenylylation, and that throughout the range of $\bar{n} = 4$–9, the nonprecipitable molecules contain an average of between 2.8 and 6.9 adenylyl groups per molecule (Fig. 4). Furthermore, it is apparent that factors other than the number of antigenic sites govern the ability to form precipitable complexes, because the $\bar{n}$ values of GS in the soluble immune complexes formed during the second immunotitration (Fig. 4) are nearly the same as those of the starting material (6.3 vs. 6.9 and 4.1 vs. 4.7). Incomplete precipitation of these multiply adenylylated GS species may be explained by the fact that GS molecules containing 2–10 adenylylated subunits may exist in many isomeric forms that differ with respect to the arrangement of the adenylylated subunits. The distribution of adenylylated subunits in some enzyme species might favor monogamous interactions of bivalent antibodies with two adenylylated subunits within the same GS molecule and thereby preclude the availability of these subunits for intermolecular crosslinking to form precipitable lattices.

The flexible “hinge” at the base of the Fab arms of an IgG molecule permits the distance between the two antigen binding sites to vary. The minimal distance between adjacent sites on an IgG molecule has recently been determined by fluorescence energy transfer to be about 60 Å, with a minimal angle between the two Fab arms of 40–50° (21). Coleman et al. (22) have demonstrated by x-ray crystallographic studies that the maximal angle between antigen binding sites on one IgG molecule is 180°, which corresponds to a maximal separation of binding sites of about 150 Å. Therefore, with a GS subunit diameter of about 45 Å and one AMP moiety per subunit, the IgG molecule can easily form intramolecular bivalent binding with a single GS dodecamer, as well as intermolecular crosslinking between GS molecules.

Finally, it is noteworthy that because of the rigid specificity for 5'-adenyl groups, these anti-AMP antibodies have been useful in establishing that the GS from Azotobacter vinelandii (23) and Pseudomonas fluorescens (unpublished data) undergo an adenylylation/deadenylylation reaction similar to that in E. coli.

The authors thank Drs. Andrew Sh rake and Michael Maurizi for many helpful discussions during the course of these experiments. This work is partial fulfillment of requirements for a Ph.D. degree from the University of Maryland.