this decline was inverse to the rising number of dead robins found. Meanwhile the robin population in Norwich showed no net change. During June 1963, the population in Hanover increased through influx of new robins (*7, 17*), but did not return to the prenesting level. Robin population trends in Hanover and Norwich were similar to each other in 1964.

A comparable population divergence between Hanover and Norwich occurred during 1963 for bark feeders—that is, chickadees, nuthatches, creepers, and woodpeckers. The numbers of bark feeders in Hanover and Norwich study areas were about equal at the time of spraying. Within 3 to 4 weeks, Hanover areas contained one-third as many of these birds as did Norwich areas; dead and tremoring birds of some of these species were found in Hanover. No divergence of bark-feeder populations occurred in 1964.

Evidence indicates that the concentration of DDT residues in the brain is a good criterion for establishing cause of death (*8, 9, 18*). Any dead robin containing more than 50 parts per million in the brain probably died of DDT poisoning (*9*); prior observation of tremors in such a bird would leave little doubt that DDT was the cause of death.

All 16 robins found with tremors (1963), and 20 robins found dead, contained more than 50 ppm of DDT residues (DDT plus DDE plus DDD) in the brain, indicating these birds died of DDT poisoning. Four other robins found dead, and the five reference robins, contained 0 to 5 ppm and died of other causes.

When analyzing whole birds, we suspected DDT poisoning when the residue concentration was 30 ppm or more, since the minimum amount in a tremoring bird was 30.3 ppm (a robin with 70 ppm in the brain); this would assume a similar tolerance among the species represented. Among 99 Hanover birds analyzed, 65 most probably died of DDT poisoning, since all contained more than 30 ppm in the whole bird, and many were seen with tremors or contained more than 50 ppm in the brain. Some of the species represented (for example, myrtle warbler, *Dendroica coronata*, and tree swallow, *Iridoprocne bicolor*) arrived in Hanover several weeks after the time of spraying and feed primarily on living insects in treetops and in the air. We therefore suspect the toxicant was acquired by eating living insects carrying DDT, presenting a paradox of survival of the intended DDT victims and death, instead, of insectivorous birds. Thirty-three other birds, including all those showing injuries, and all Norwich birds, contained 0 to 10 ppm and presumably died of other causes.

Mortality was reduced after the application of Methoxychlor in 1964. Six robins were observed with tremors and others were found dead; these losses are believed caused by residual DDT in soil (*8, 10, 19*).

From the number of dead birds found, the many birds observed with tremors, chemical analyses of these birds, and a population decline among certain species, we conclude that DDT caused severe mortality of resident and migrant birds in Hanover during the spring of 1963.

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References and Notes

1. DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DDD, also known as TDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; Methoxychlor, 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane.
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12. We thank Hans W. Weber for participating in the bird counts during 1963.
13. Analyses by Wisconsin Alumni Research Foundation, Madison, Wisconsin. Method: electron capture gas chromatography [A. K. Klein, J. O. Watts, J. N. Damico, *J. Assoc. Offic. Agr. Chemists* **46**, 165 (1963)], with modification. We are indebted to the Patuxent Wildlife Research Center, Laurel, Maryland, for defraying the cost of these analyses.
14. Of this total, 103 were found in 1963; three robins were collected in 1964 for reference purposes.
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Lattice Formation in Complement Fixation: Studies with Univalent Rabbit Antibody

Abstract. *Hybrid univalent 6.5S antibody molecules, formed by recombination of half-molecules of rabbit antibody to ovalbumin with those of normal rabbit γ_G-globulin, fail to fix complement in reactions with homologous antigen. Such hybrid molecules, however, block complement fixation by intact antibody to ovalbumin. Molecules of antibody reconstituted in the absence of other protein retain the capacity to fix complement. The data suggest that small complexes containing excess univalent antibody do not fix complement and that lattice formation is required for fixation.*

After mild reduction at *p*H 5 and subsequent acidification to *p*H 2.5, rabbit γ_G-globulin (*1*) dissociates into half-molecules (*2*) consisting of one heavy and one light chain (*3*). On neutralization, such half-molecules recombine in pairs to form a product that has the same sedimentation coefficient, diffusion constant, and molecular weight as untreated γ_G-globulin (*2*,

4, 5). After neutralization, most of the antigen-combining sites of such recombined antibodies remain intact (*2, 4, 6*). Recombination of half-molecules of specifically purified antibody to ovalbumin (EA) produces whole molecules which form specific precipitates (*6*). Half-molecules of anti-EA (*1*) can also recombine with half-molecules of normal γ_G-globulin to form mixed (hybrid) 6.5*S* molecules (*4*). Most of the anti-EA combining sites of the hybrid molecules retain specific activity. Such mixed molecules evidently are univalent, since they fail to precipitate when EA is added and specifically block its precipitation by untreated anti-EA (*6*). We now report on complement fixation by recombined half-molecules of anti-EA and by hybrid molecules formed through combination of half-molecules of anti-EA with half-molecules of normal γ_G-globulin.

Because of their very low solubility, the capacity of small complexes containing protein antigen and excess rabbit antibody to fix complement is not known. This question is relevant to the role of lattice formation in complement fixation. The fact that small soluble complexes comprising excess antigen do not fix complement is not necessarily germane because the surface characteristics of the two types of complex differ.

The ability of the aforementioned hybrid 6.5*S* molecules (anti-EA and normal γ_G-globulin) to combine with antigen without the formation of a precipitate allowed us to investigate complement fixation by soluble complexes containing an excess of such hybrid molecules. This approach provided an indirect means of determining whether lattice formation is a prerequisite to complement fixation and whether inhibition with excess antigen is attributable to the absence of lattice formation or to the surface properties of the soluble antigen-antibody complex. The results indicate that soluble complexes made with a large excess of univalent 6.5*S* antibody do not fix guinea pig complement and that such hybrid molecules, when added to antigen, can prevent complement fixation by untreated antibody. In contrast, half-molecules of anti-EA, when allowed to recombine in the absence of other protein, form precipitating 6.5*S* bivalent antibody that retains some complement-fixing capacity.

Half-molecules were prepared (*4*) from (rabbit) anti-EA (40 mg) (*6*) and (rabbit) normal γ_G-globulin (475 mg). The proteins were reduced separately with 0.1*M* 2-mercaptoethylamine hydrochloride at *p*H 5; after removal of the reducing agent by ion-exchange chromatography, the liberated -SH groups were inactivated with *p*-mercuribenzoate. The reduced proteins were dialyzed at 4°C against 0.025*M* NaCl; the *p*H was then lowered to 2.4, the temperature being maintained at 10°C. Sedimentation coefficients, measured at 10°C and corrected to 20°C ($s_{20,w}$), were 3.1*S* for normal γ_G-globulin and 3.0*S* for purified anti-EA, values characteristic of half-molecules (*4*). Portions of the dissociated proteins were mixed in an 11:1 ratio (330 mg normal γ_G-globulin and 30 mg anti-EA) and allowed to recombine by dialysis against saline-borate buffer, *p*H 8, ionic strength 0.16. Similarly, 10 mg of the dissociated, purified antibody was permitted to recombine in the absence of normal γ_G-globulin, and 130 mg of normal γ_G-globulin was allowed to recombine in the absence of anti-EA. More than 84 percent of each neutralized preparation consisted of 6.0 to 6.2*S* material. About 10 percent of the material had an approximate sedimentation coefficient of 10*S*; less than 8 percent of material with a sedimentation coefficient less than 6*S* was observed. The molecular weight of the 6*S* product is the same as that of γ_G-globulin (*5*).

When 300-μg portions of the anti-EA, dissociated and recombined in the absence of added normal γ_G-globulin, were tested with varying concentrations of antigen, precipitates formed; a typical precipitin curve was obtained. With an optimum concentration of antigen, 86 percent of the recombined anti-EA was precipitable. The 11:1 hybrid preparation of normal γ_G-globulin and anti-EA failed to form precipitates when tested with EA in a wide range of concentrations. Furthermore, the hybrid preparation blocked precipitation in the homologous (EA) antigen-antibody system; 15 mg of the hybrid (of which 1/12 or 1.25 mg was derived from antibody) completely inhibited precipitation of 0.2 mg of untreated anti-EA with an approximately optimum amount of antigen. Inhibition by this preparation is specific for the homologous antigen-antibody system (*6*), and the results of the precipitation and inhibition tests agree with those of the earlier study (*6*).

Dissociated, recombined anti-EA fixes guinea pig complement, as shown by inhibition of immune hemolysis (Table 1). The optimal weight ratio of EA to its homologous antibody (both the purified and reconstituted preparations) was 1:8 to 1:10. When added to EA, 0.01 to 0.02 mg of the reconstituted anti-EA consistently inhibited subsequent immune hemolysis (less than 25 percent of the cells were lysed) when two 50-percent hemolytic units of guinea pig complement were present in the test mixture. In comparison with untreated anti-EA, the dissociated, recombined antibody showed some loss in complement-fixing ability.

In contrast, the hybrid preparation in concentrations 100-fold greater failed to fix significant amounts of complement, even though 1/12 of the total protein in the mixed molecules was derived from specific antibody. The hybrid preparation did not fix complement when tested with ovalbumin in a wide range of concentrations (0.0005 to 0.12 mg) with antigen to hybrid weight

Table 1. Complement fixation by rabbit antibody to ovalbumin, either native or reconstituted from half-molecules, expressed as percentage of inhibition of specific hemolysis. Mixtures containing 0.1 ml of antigen solution, 0.1 ml of antibody solution, 0.2 ml of guinea pig complement (2 $C'H_{50}$ units), and 0.1 ml of buffer were incubated at 37°C for 60 minutes; 1.0 ml of a 1-percent suspension of sheep erythrocytes, sensitized by four units of hemolysin, was added. After 1 hour the mixtures were centrifuged and the percentage of hemolysis was measured by the optical density of the supernatants at 540 mμ. The percentage of inhibition of hemolysis is expressed at 100 minus the percentage of hemolysis and is an indirect index of the degree of complement fixation.

Prep* (mg)	Conc. of ovalbumin (mg)			Saline control (%)
	0.01 (%)	0.001 (%)	0.0001 (%)	
Antibody to ovalbumin: Native				
0.1	96	97		0
.01	89	97		0
.001	0	0	16	0
Antibody to ovalbumin: Reconstituted				
0.1	93	97	0	0
.01	21	77	15	14
.001	0	9	4	0
Hybrid				
1.0†	0‡			
Reconstituted normal γ-globulin				
1.0	0§			
0.1	0§			
.01	0§			

* Before use, all preparations were heated at 56°C for 30 minutes and absorbed with nonsensitized erythrocytes to remove naturally occurring hemolysins for sheep erythrocytes. † 1.0 mg of the hybrid preparation contained 0.08 mg of protein derived from antiovalbumin. ‡ No significant complement fixation with 0.12, 0.02, 0.01, 0.0025, or 0.0005 mg of ovalbumin. § No complement fixation with 0.1, 0.01, 0.001, or 0.0001 mg of ovalbumin.

ratios ranging from 1:8 to 1:2000.

The hybrid preparation inhibited complement fixation in the homologous antigen-antibody system (Table 2). The calculated ratio of univalent to bivalent molecules, corresponding to 85-percent inhibition of complement fixation (row 2, Table 2), is approximately 4 to 1. Of the 1.25 mg of hybrid used in the test, 1/12 or 0.104 mg was derived from specific antibody; on the assumption of random recombination (*4*), 11/12 of the 0.104 mg was in the form of univalent molecules and 1/12 in the form of bivalent molecules. The hybrid did not inhibit the complement-fixing activity of a heterologous precipitating system (specifically purified antibody to bovine γ_G-globulin and an approximately optimum amount of bovine γ_G-globulin) (Table 2). Recombined normal rabbit γ_G-globulin inhibited complement fixation to a slight degree (Table 2). Weak inhibition by a normal γ_G-globulin fraction has been described (*7*).

Table 2. Inhibition of complement fixation by reconstituted rabbit γ_G-globulin. A solution containing the hybrid or recombined normal γ-globulin preparations (0.1 ml) was incubated with antigen (ovalbumin or bovine γ-globulin) solution (0.1 ml) for 60 minutes at 37°C. Bivalent intact antibody (0.1 ml) was added, and the mixture was incubated for 60 minutes. A solution (0.2 ml) containing two $C'H_{50}$ units of guinea pig complement was added. After an additional 60 minutes, 1.0 ml of a 1-percent suspension of sensitized sheep erythrocytes was added. After incubation for 45 minutes at 37°C, the tubes were centrifuged and optical densities of the supernatants were read at 540 mμ. HP, hybrid preparation; RNG, recombined normal γ-globulin.

Inhibitor	Amount* (mg)		Lysis (%)	Blocking activity†
Homologous system: 0.002 mg ovalbumin and 0.02 mg anti-EA used in each test				
HP	0	(0)	1	
HP	1.25	(0.10)	86	85
HP	1.00	(0.08)	60	59
HP	0.75	(0.06)	44	43
HP	0.50	(0.04)	10	9
HP	0.25	(0.02)	3	2
HP	0.10	(0.01)	1	0
RNG	0	0	6	
RNG	1.25	0	28	22
RNG	1.00	0	23	17
RNG	0.75	0	23	17
RNG	0.50	0	14	8
RNG	0.25	0	14	8
Heterologous system: 0.0032 mg bovine γ-globulin and 0.02 mg γ-globulin to bovine antigen				
HP	0	(0)	5	
HP	1.25	(0.10)	5	0
HP	1.00	(0.08)	5	0
HP	0.75	(0.06)	5	0
HP	0.50	(0.04)	5	0
HP	0.25	(0.02)	5	0

* Figures in parentheses are equal to the number of milligrams of antiovalbumin protein present in the amount of hybrid tested. † The degree of inhibition of complement, as expressed by the percentage of lysis in the presence of inhibitor minus the percentage of lysis in the absence of inhibitor (buffer control).

Blocking of complement fixation did not occur when untreated antibody was incubated with antigen, and the hybrid preparation was added 1 hour later. Addition of reactants in this order failed to cause a significant decrease in the amount of complement fixed, as compared to that fixed in control tubes to which hybrid was not added. Thus the reaction of antigen with untreated antibody is not readily reversible by hybrid molecules.

The observed inhibition of specific precipitation in the homologous system by hybrid molecules indicates that they form soluble complexes with antigen. With a deficiency of antigen (0.0025 or 0.005 mg, Table 1), such complexes should contain an excess of antibody; however, these mixtures did not fix complement. Also, when a large excess of the hybrid preparation was added to the antigen before the addition of intact antibody, complement fixation was specifically inhibited, again indicating that complexes containing an excess of hybrid molecules do not fix complement.

Since dissociated, recombined anti-EA retains the capacity to fix complement, the procedures used to effect dissociation and recombination apparently do not destroy the complement-fixing sites of the antibody, although complement-fixing ability was decreased to some extent. Therefore, the failure of the hybrid to fix complement probably is not attributable to denaturation. The data do not exclude the alternative possibility that recombination of two like half-molecules effects the re-formation of an active complement-fixing site, whereas, recombination of two unlike half-molecules fails to do so. However, since the property of complement fixation is common to antibodies of varying specificity, the site of complement fixation appears to be independent of the antibody-combining site. Furthermore, fragment III of γ_G-globulin, which lacks an antigen-combining site, fixes guinea pig complement when aggregated (*8*), although aggregation of some proteins other than γ-globulin does not result in acquisition of complement-fixing properties (*9*).

Thus, the failure of hybrid molecules to fix complement probably results from the small size of the lattice formed when antigen is added. It also seems probable that soluble complexes containing a large excess of antibody cannot fix complement. Alternatively, complement fixation may require certain conformational changes in the antibody that cannot occur if only one antigen-combining site interacts with antigen. Complement fixation by subagglutinating amounts of antibody to particulate antigens, such as viruses, bacteria, or erythrocytes, might be an argument against the requirement for lattice formation. In such instances, however, complement fixation may be attributable to the formation of small aggregates that are not visible or to a different mechanism of complement fixation. In any event, the massing or aggregating of antibody related to the complement-fixing potency of an immune serum (*10*) requires that both combining sites of the antibody molecule react with antigen; complexes of a protein antigen and antibody involving only one combining site of each antibody molecule do not appear to fix complement.

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