ON CROSS REACTIONS OF EGG ALBUMIN SERA

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It has been repeatedly reported in work with antiprotein precipitins that exhaustion with a related heterologous protein abolishes the reaction of the serum with this and often also with other cross reacting proteins without much apparent impairment of the ability to precipitate the homologous antigen. A good example, the more valuable since it was carried out with crystallized proteins, is the study by Hooker and Boyd (1) of precipitins for the egg albumins of hen and duck. After duck albumin was added to hen albumin immune sera and the precipitate removed, the sera still reacted with hen albumin to the original antigen titre although no longer with duck albumin, and the converse effect was obtained on anti-duck sera. This result could be explained, as previous observations have been, by assuming at least two determinant groups in each of the antigens, one peculiar to the species, the other identical or similar in both, for each of which an antibody is formed.

In order to provide additional evidence on the unsolved problem of serological protein specificity, experiments similar to those just quoted were undertaken but extended to several heterologous proteins. An attempt was made, furthermore, to characterize antibody fractions by means of inhibition tests. On account of the considerable variety in their properties a fairly large number of antisera to hen ovalbumin was examined.

After the present study was well along a paper appeared by Cole (2) on the precipitin reactions of egg albumin of chicken, guinea hen and two species of pheasants (Amherst and Golden pheasants) in general with experimental results in accordance with our own. From absorption experiments the authors conclude that "the injection of an apparently pure homogeneous antigen may give rise to a number of distinct precipitins." Two explanations are offered, namely that the "ovalbumins contain a number of reacting groups . . . each of which gives rise to a precipitin specific for that group," or, that the proteins examined are not . . . "chemical individuals but a mixture of closely related components. . . ."

Their hen ovalbumin antiserum, unlike ours, did not react with pheasant ovalbumin, while the sera for guinea hen and the pheasants gave reactions with all ovalbumins mentioned.

Still more recently Adair and Hamilton (3) likewise found that immune sera produced with crystalline serum albumin contained a number of distinct precipitins.

EXPERIMENTAL

Immune sera were obtained by injecting rabbits with hen ovalbumin and these were tested against the egg albumins of chicken, turkey, guinea hen, duck and goose.

Chicken and guinea hen ovalbumins were prepared in crystalline form by the Sörensen method, by removal of the globulin fraction of egg white with an equal volume of saturated ammonium sulfate solution and subsequent addition of the required amount of ammonium sulfate and dilute sulfuric acid to the filtrate. Turkey ovalbumin crystallized from a deglobulinated solution without addition of acid, upon standing in the ice box. Turkey and guinea hen albumins were thrice recrystallized, the hen ovalbumin used for the tests and preparation of the immune sera, seven times. The albumin crystals were washed with ammonium sulfate solution each time before recrystallization. Duck and goose ovalbumins were separated by the above method and reprecipitated three times with ammonium sulfate; although not crystalline these two proteins appeared to be homogeneous when examined by the electrophoretic method of Tiselius.[1] After dialysis the ovalbumin solutions were made up to 1 per cent NaCl, and for preservation 0.25 per cent phenol was added.

The immune sera were prepared by intravenous injections of 2 cc. of a 0.6 per cent solution of hen ovalbumin daily for 6 days. One or two, rarely three, further courses were given at intervals of 1 week. The sera were tested 7 days after the last injection. In spite of repeated recrystallization of the ovalbumin, a slight precipitation was observed when the immune sera were tested against chicken serum; therefore, preliminary to the experiments to be described, the ovalbumin antisera were treated with chicken serum to remove the small quantities of the antibodies reacting with chicken serum. Ring tests of the chicken ovalbumin preparations with antiserum to chicken serum were faintly positive with dilutions of 1:8 or 1:4, but entirely negative in higher antigen dilutions.

For the precipitin tests 0.2 cc. of sera was mixed with 0.05 cc. of the antigen solutions, the concentrations in terms of dry weight being given in the tables. The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), tr. (strong trace), ±, ±, +, +±, ++, etc.

For exhaustion the albumins were added to undiluted sera in successive portions, the tubes each time being kept at room temperature for one hour and the precipitates then centrifuged off, until further addition gave no or at most a faint precipitate when the mixture was kept in the room for one hour, then overnight in the ice box.

Two main sorts of experiments were conducted with the hen ovalbumin antisera. First, the sera were exhausted with each of the heterologous albumins and after removal of the precipitates formed were tested against these and hen albumin. Secondly, after exhaustion of antisera with a heterologous albumin inhibition tests were set up by adding the same albumin to precipitating systems of the absorbed immune serum and the albumins of other birds.

[1] For these examinations the authors are indebted to Dr. L. G. Longsworth.

All the albumins gave precipitates with the anti-hen immune sera, the strength of the reactions decreasing with almost every serum in the order hen, turkey, guinea hen, duck, goose,[2] as seen by collecting the precipitates obtained upon complete exhaustion with the respective albumins and estimating their value in tubes ending in a narrow graduated extension (Hopkins' vaccine tube), all the precipitates produced with one serum being centrifuged simultaneously for 15 minutes at 2700 R.P.M. Although these determinations are by no means precise, they serve as an approximate measure of the relative quantities. Some representative examples are given in Table I. The albumins of duck and goose yielded with but one exception (serum 21, which gave unusually weak cross reactions) definitely

TABLE I

Volumetric measurement of precipitates secured with hen egg albumin immune sera by complete precipitation with different egg albumins; the value for chicken ovalbumin is taken as 100.

Ovalbumin immune serum No.	Turkey	Guinea hen	Duck	Goose
1	35	26	9	9
3	67	57	42	31
9	61	51	30	18
13	43	53	25	20
21	19	14	14	12
Mean of 22 sera and standard error.....	50 ± 2.4	42 ± 2.3	25 ± 1.7	19 ± 1.4

smaller amounts of precipitates than those of turkey and guinea hen in accordance with zoological expectation. This grouping (Galliformes, Anseriformes) could often be seen also in absorption experiments.

In general, the strengths of the cross reactions of the various sera showed not too great dissimilarity but in view of results such as those of Adair and Hamilton (3), of Wolfe (4) and of Levine and Moody (5) there very probably would be weaker cross reactions with immune sera produced by a shorter or less intense immunizing procedure.

In spite of marked differences in the quantity of the precipitates upon

[2] A still weaker reaction was seen with an albumin fraction from pigeon egg white. Furthermore, strong reactions occurred with the egg whites of three pheasant species examined, namely, Elliot pheasant, Black Neck pheasant and English Ring Neck pheasant, made available through the courtesy of Dr. C. R. Schroeder of the New York Zoological Park.

complete saturation there was in antigen titrations—made by mixing antigen and immune serum or by means of ring tests—no difference or a difference of only one tube (dilution by halves) in the end titre of the various ovalbumins (see Cole (2)). Clearly, antigen titration failed to reveal the actual marked differences in reactivity of the related antigens, which would follow also from other observations.

TABLE II

Immune serum 15 was exhausted with various ovalbumins. For the tests 0.2 cc. of absorbed serum was added to 0.05 cc. of antigen dilutions expressed in terms of dry weight.

Readings were taken after 1 hour at room temperature (1st line) and after standing overnight in the ice box (2nd line).

Hen ovalbumin immune serum 15 absorbed with ovalbumin of	Ovalbumins from												
	Hen	Turkey			Guinea hen			Duck			Goose		
	1:2000	1:80	1:400	1:2000	1:80	1:400	1:2000	1:80	1:400	1:2000	1:80	1:400	1:2000
Turkey	++±	0	0	0	0	0	0	0	0	0	0	0	0
	++±	0	0	0	0	0	0	0	0	0	0	0	0
Guinea hen	+++			+±	0	0	0	0	0	0	0	0	0
	+++±			+±	0	0	0	0	0	0	0	0	0
Duck	+++±			+++			++±	0	0	0	0	0	0
	+++±			+++			++±	0	0	0	0	0	0
Goose	+++±			+++			++±			0	0	0	0
	+++±			+++			++±			±	0	0	0
Unabsorbed immune serum	+++±			+++			++±			++			++
	++++			+++			++±			++			++

On exhaustion with the heterologous proteins considerable variation was observed among the individual antisera, of which 25 were examined in all. With a part of the sera each protein, practically, removed the precipitating antibodies for itself[3] and for the more weakly reacting proteins (Table II), *e.g.* after absorption with goose albumin the serum still precipitated the other four albumins, whereas on absorption with turkey albumin all reactions disappeared except that with hen albumin. Infre-

[3] Some weak reactions not infrequently occurred, as has been noticed previously (6) on addition of a larger excess of antigen. This may be ascribed to special antibodies which precipitate only relatively high antigen concentrations (*cf.* Heidelberger (7)).

TABLE III

Experiment as in Table II, with immune serum 7. With the unabsorbed serum all albumins, including that of goose, gave strong reactions.

Hen ovalbumin immune serum 7 absorbed with ovalbumin of	Ovalbumins from												
	Hen	Turkey			Guinea hen			Duck			Goose		
	1:2000	1:400	1:2000	1:10,000	1:400	1:2000	1:10,000	1:400	1:2000	1:10,000	1:400	1:2000	1:10,000
Turkey	++±	0	0	0	0	0	tr.	0	+	0	0	0	0
	+++	0	0	0	0	±	±	0	+±	0	0	0	0
Guinea hen	++±		++±	+	0	0	0	±	++	±	0	+±	tr.
	+++		+++	+±	0	0	0	++	++±	±	0	++	±
Duck	+++		+++			+++		0	0	0	0	0	0
	+++		+++			+++		0	±	tr.	0	0	f.tr.
Goose	+++		+++			+++			++	±	0	0	0
	+++		+++			+++			++±	+	0	0	0

TABLE IV

Experiment as in Table II, with immune serum 17. With the unabsorbed serum all albumins, including that of goose, gave strong reactions.

Hen ovalbumin immune serum 17 absorbed with ovalbumin of	Ovalbumins from												
	Hen	Turkey			Guinea hen			Duck			Goose		
	1:2000	1:400	1:2000	1:10,000	1:400	1:2000	1:10,000	1:400	1:2000	1:10,000	1:400	1:2000	1:10,000
Turkey	++±	0	0	0	0	+	+	0	0	±	0	0	±
	+++	0	0	0	0	+±	+	0	±	+±	0	tr.	+
Guinea hen	++±		++±	+	0	0	0	0	+	+	0	±	tr.
	+++		+++	+±	0	0	0	0	+	+	0	+	±
Duck	+++		+++			++±		0	0	0	0	0	0
	+++		+++			++±		0	0	0	0	0	0
Goose	+++		+++			++±			tr.	0	0	0	0
	+++		+++			+++			+	±	0	0	0

quently, treatment with (an excess of?) guinea hen protein resulted in the disappearance of the reaction with turkey, and sometimes the albumin of goose removed the precipitins for duck. Other sera after absorption with a heterologous albumin precipitated not only the albumins of higher,

but also most albumins of lower reactivity though to a lesser extent (Tables III and IV); absorption by duck almost regularly removed the reactivity for goose. Consequently, it is possible by means of suitable anti-chicken albumin sera to identify each of the five proteins here examined, an effect analogous to the result of absorbing normal hemagglutinating sera with

TABLE V

Inhibition test. To 0.05 cc. 1:10,000 hen ovalbumin there was added 0.3 cc. of dilutions of hen or turkey egg albumin, or rabbit or horse serum, and finally 1 drop of anti-hen ovalbumin immune serum 8 which had been exhausted with turkey ovalbumin and then diluted 1:2.

Readings were made after 15 minutes (1st line) and 1 hour (2nd line) at 37°C.

Reaction between hen ovalbumin and immune serum absorbed with turkey, in the presence of																				
Hen ovalbumin (5 per cent)							Turkey ovalbumin (5 per cent)							Rabbit serum			Horse serum			Control saline
1:2	1:4	1:8	1:16	1:32	1:128	1:512	1:2	1:4	1:8	1:16	1:32	1:128	1:512	1:2	1:4	1:8	1:2	1:4	1:8	
0	0	0	0	0	0	tr.	0	0	0	f.tr.	tr.	±	+	+	+	+	+	+	+	+
0	0	0	0	0	0	±	0	0	tr.	±	±	+	+	+	+	+	+	+	+	+

TABLE VI

Inhibition test. To 0.05 cc. 1:10,000 duck ovalbumin there was added 0.2 cc. of dilutions of guinea hen or duck albumin, or rabbit or horse serum, and finally 0.2 cc. of anti-hen ovalbumin immune serum 16 which had been exhausted with guinea hen ovalbumin.

Readings were made after 2 hours at 37°C.

Reaction between duck ovalbumin and hen ovalbumin immune serum after absorption with guinea hen, in the presence of															
Guinea hen ovalbumin (5 per cent)					Duck ovalbumin (5 per cent)					Rabbit serum			Horse serum			Control saline
1:2	1:8	1:32	1:128	1:512	1:2	1:8	1:32	1:128	1:512	1:2	1:4	1:8	1:2	1:4	1:8	
0	0	0	tr.	+	0	0	0	±	+±	±	+	+	±	+	+	+

erythrocytes of different species (see 8) and resembling, also, previous observations on sera for azoproteins (9).

The actual quantities of precipitates secured with sera which had been partially absorbed, when measured volumetrically, were often rather small even in cases where the reactions as shown in the tables were pronounced (*e.g.*, serum absorbed with guinea hen albumin and precipitated with turkey, serum absorbed with goose albumin and precipitated with duck), as well as naturally with weak reactions.

Examples of inhibition tests are given in Tables V and VI. Here it is

seen that following absorption of an immune serum with a given heterologous albumin this, although no longer precipitated by the absorbed serum, still showed noticeable inhibition of the precipitation of other albumins. The effect was definite under certain conditions only, namely when the precipitin reactions were not too strong and the ratio of antibody to antigen not high.

COMMENT

The selective absorption as shown in Tables III and IV is not explicable on the assumption of a single antibody only, and the same conclusion can be drawn from those experiments in which absorption with a heterologous albumin abolishes the reactions with all antigens of weaker reactivity. A result at first sight somewhat similar is obtained when tests are made with successively increased dilutions of an immune serum, for here also the reactions with various antigens will disappear, naturally, in the inverse order of their strength. But there is a sharp difference between negative and markedly positive reactions in the tests with absorbed sera, whereas the dilution experiments show a gradual diminution in the reactions with all antigens, and one sees that the results in the former instance cannot be attributed merely to a reduction in the amount of one antibody. While thus the inference is inevitable that the sera examined contain multiple, perhaps numerous, qualitatively distinct antibodies it is not possible to tell their actual number. Additional information may be gleaned from the use of a greater number of heterologous antigens. In antibacterial sera antibodies differing in avidity have been found in several instances (Hooker and Boyd (1), Heidelberger and Kendall (10), Goodner and Horsfall (11)), and recently specifically different antibodies in immune sera for pneumococci (Goodner (12)).

In crystallized hen egg albumin (not in the other albumins here used) Longsworth (13 *a*) has detected by means of the Tiselius apparatus a second component very similar to the main protein electrophoretically but having a slightly slower mobility throughout the pH range investigated. No definite decision has been reached as yet by this author whether it is a special protein or is formed by alteration (denaturation) of the major constituent. In a paper just published, Tiselius and Eriksson-Quensel also make mention of a second component found in solutions of crystallized egg albumin (13 *b*). At any rate, this observation will not occasion a material change in the interpretation of the present results (*cf.* also 13 *c*).

As regards the antigenic structures involved the reactions of the sera after partial absorption cannot be accounted for—as could prior observations with chicken and duck albumins—by two different groups in hen

albumin, one chicken specific and another shared by the albumins of many birds. Extending this manner of reasoning one would be led to suppose (for instance from the experiment in Table II) that hen egg albumin contains four determinant groups three of which are present in turkey albumin, and two in guinea hen albumin. However such an explanation in terms of discrete, unlike determinant groups and corresponding antibodies seems rather forced, even more so if, as is probable, experiments with a larger variety of antigens would necessitate a still greater complication in the hypothesis. It is true that the absorption experiments and some of the inhibition tests suggest the presence of multiple determinant groups in the albumin molecule each of which may give rise to special antibodies (*cf.* 14, 15); even so, there may be structural similarities between these groups due to a repetition of somewhat similar amino acid patterns (*cf.* 16, 17, 18). Moreover, one may reasonably suppose that any determinant group will vary according to zoological relationships. Such gradual variations, rather than the presence or absence of determinants, more or less invariable, together with the formation of qualitatively different antibodies in response to single determinant structures (9), seem adequate to explain the complexity of the immune sera and the observed absorption phenomena. In fact, inhibition reactions with partially absorbed sera show that the specificity of the various antibodies is not as sharp as might be inferred from the precipitin tests. For example, addition of turkey albumin to a serum which had been exhausted with this protein, until further addition led to no more precipitation, still inhibited the precipitation of hen albumin. If the precipitin reactions of the latter were due to specific groups of its own bearing no resemblance to structures in turkey albumin this should inhibit to no greater extent than any unrelated protein, but as we have noted this is not the case. One may conclude that the antibodies which remain after absorption have some affinity for the proteins used in absorbing, but not sufficient to cause precipitation. In this connection the experiment of Haurowitz (19) may be mentioned in which precipitation of azoproteins did not occur unless the antigens contained a number of specific groups.

The idea sometimes advanced to explain overlapping reactions, namely that a certain protein, as serum globulin, is a mixture of molecular species some of which are contained in the globulins of zoologically related animals, is not only very improbable in itself (*cf.* (8), page 22) but is not tenable in view of results obtained with electrophoresis (20). In such experiments, hen and guinea hen albumins could be clearly separated from a mixture of the two by the difference in electrophoretic mobilities.

To come to a definite opinion as to the groupings in proteins which define the specificity of the corresponding antibodies is difficult, particularly since it is not yet known how large a structure may serve as a determinant, and what the size of the combining site of an antibody may be. That small groups in the antigen are sufficient is common experience in working with synthetic antigens while, on the other hand, with immune sera for polypeptides it was found that the specificity may depend upon a pentapeptide in its entirety (21); however, larger synthetic peptides have not been investigated. The problem might be simplified to some degree by observations on dissociation of proteins and the serological reactivity of hydrolytic split products. In studies with the ultracentrifuge it has been found that protein molecules can dissociate, for instance serum albumin into units possibly one-eighth of the size of the original molecule (Pedersen (22))[4]; in such a case there would still remain structures consisting of some 70 amino acids, and it is not certain whether these units would be identical.[5] Other results which appear to limit the size of the specific structure have been obtained with protein split products when it was found that precipitin reactions of proteins could specifically be inhibited by proteoses (Landsteiner and Chase (25); *cf.* Holiday (26)). Whatever the final answer will be, if one takes into consideration those antibodies in partially absorbed antisera which differentiate one protein from those closely related, one cannot but assume determinants of considerable complexity (*cf.* Marrack (27)), sufficient to afford a pattern characteristic for a single species. While the possibility may be entertained, also, that antiprotein sera contain a number of antibodies each directed towards a different small group, as would be common to unrelated proteins, no evidence has so far been produced to substantiate this view.

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SUMMARY

Experiments are presented on the cross reactions of hen egg albumin immune sera with egg albumins of other species by means of exhaustion with heterologous proteins and by inhibition tests. From the results it can be concluded that the sera contain multiple, qualitatively distinct antibodies. For this, two not mutually exclusive explanations come into

[4] On the dissociation of ovalbumin see (23).

[5] The participation of carbohydrate groupings (24) in the species differentiation of egg albumins would seem improbable (8, pages 32, 33).

consideration: the presence in proteins of a number of different, perhaps similar, complex determinants, and the fact, established by previous results, that one antigenic grouping can call forth the formation of diverse antibodies.

It is inferred that cross reactions between proteins of kindred species are ascribable, in general, to similarity in determinant structures, and not to the distribution of identical determinant groups among the related proteins.

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