Use of Quantitative Micro-Complement Fixation for Detection of Small Differences in Protein Structure

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[redacted]

Abstract

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Three techniques (the quantitative precipitin reaction, macro-complement fixation and micro-complement fixation) have been compared for their capacity to distinguish chicken from turkey H_4 lactic dehydrogenase, human from chimpanzee serum albumin, and chicken from turkey ovalbumin. With each immune system, greater serological differences were found by micro-complement fixation. It is suggested that the greater sensitivity exhibited by micro-complement fixation may reflect the measurement of a more avid fraction of the antibody population. Hence, even a slightly different conformation in a heterologous antigen might be expected to lead to dissociation of the antigen-antibody aggregates that are required for complement fixation.

Using quantitative micro-complement (C') fixation, rabbit antisera to human hemoglobin A detected differences between hemoglobin A and the mutant hemoglobins S and C (1) which differ in only one of 146 amino acids in the beta chain (2). The same rabbit antisera did not discriminate between these hemoglobins when measured by the quantitative precipitin technique of Heidelberger and Kendall (3) or by the C' fixation technique of Mayer, et al. (4).

To determine whether micro-C' fixation is always more sensitive to differences in protein structure, other immune systems were examined by the same three serological techniques. The rabbit antisera used were anti-chicken H_4 lactic dehydrogenase, anti-human serum albumin and anti-chicken ovalbumin. The proteins that were examined serologically were chicken and turkey H_4 lactic dehydrogenase, human and chimpanzee serum albumin, and chicken and turkey ovalbumin. With each antiserum, it was found that micro-C' fixation was a more delicate procedure for demonstrating differences in protein structure.

MATERIALS AND METHODS

Preparation of Immunizing Antigens. Human serum albumin (HSA) was crystallized three times as the mercury dimer by the method of Hughes (5). Mercaptoalbumin monomer was prepared by dialysis of the dimer against an excess of cysteine to remove the mercury and, finally, against several changes of water. Crystalline, chicken heart lactic dehydrogenase (LDH) was prepared according to Pesce et al. (6). The enzyme was electrophoretically and ultracentrifugally homogeneous and corresponded to the H_4 form described by Cahn et al. (7). Chicken ovalbumin, five times crystallized, was obtained from Pentex Corp.

Preparation of Antisera. Rabbit anti-HSA (No. 669-1) was prepared by the intravenous injection of increasing doses of alum-precipitated antigen on alternate days for five weeks. A total of 60 mg of protein was administered. The rabbit was bled six days after the last injection. For production of anti-chicken H_4 LDH (175B6) and anti-chicken ovalbumin (95B3), 10 mg of protein in a volume of 1.0-1.5 ml mixed with an equal volume of complete Freund's adjuvant was injected into the toepads and intramuscularly. Three weeks later the rabbits were boosted with 5 mg of the antigen intravenously. After a further three weeks they were boosted again. Bleedings were taken seven days following the last intravenous injection.

Preparation of Antigens for Serological Analysis. In the LDH studies, crystalline chicken and turkey heart LDH's, prepared and characterized by the same procedures as described in the preparation of immunizing antigens, were used. ~~For HSA and chimpanzee serum albumin (CSA),~~ Whole human serum and a chimpanzee serum obtained from Dr. Morris Goodman were used as sources of serum albumin. The albumin content of these sera was assumed to be 40 mg/ml. For serological analysis of the ovalbumin systems, both crystallized chicken ovalbumin (used for immunization) and chicken and turkey egg whites were used. The ovalbumin content of egg white was assumed to be 60%.

Serological Methods. For quantitative precipitin analysis, the method of Heidelberger and Kendall (3) was used. The twice-washed immune precipitates were dissolved in 2.0 ml of 0.1 N NaOH and the protein concentration measured at 287 mμ. For quantitative C' fixation using 100 C' H_{50} (macro-C' fixation), the method of Mayer et al. (4) was used. For micro-C' fixation, the procedure of Wasserman and Levine (8) was used with the modification they describe in

footnote 3. Agar diffusion analyses were performed by the method of Ouchterlony (9).

RESULTS

Reaction of Chicken and Turkey H_4 LDH with Anti-chicken H_4 LDH. When tested by double diffusion in agar, performed in a triangular pattern, both chicken and turkey H_4 LDH (10 µg) gave a single band of precipitation and a reaction of identity with undiluted anti-chicken LDH. If any antigenic difference between chicken and turkey LDH did exist, it was not detected by this double diffusion technique.

The results of quantitative precipitin, macro-C' fixation and micro-C' fixation with chicken or turkey LDH and anti-chicken H_4 LDH are shown in Fig. 1. For the quantitative precipitin analyses, the constant amount of antiserum used was 1.0 ml of a 1/3 dilution. Supernate analyses of both homologous and heterologous precipitin reaction mixtures revealed no overlapping zones of precipitation. If the antibody content is calculated from the antigen-excess side of the equivalence zone after substraction of the added antigen, chicken and turkey LDH precipitated 301 µg and 274 µg antibody N respectively/.33 ml of undiluted antiserum, a difference of 9%. In macro-C' fixation, using as the constant quantity of antiserum 1.0 ml of a 1/250 dilution (3.6 µg antibody N/reaction mixture), the heterologous LDH reacted more effectively than the homologous LDH, 58 and 52 C' H_{50} maximum C' fixation respectively. By micro-C' fixation, however, using 1.0 ml of a 1/20,000 dilution of antiserum (0.045 µg antibody N/reaction mixture) the difference between the chicken and turkey LDH was easily detected. The two enzymes can also be distinguished by starch gel electrophoresis (Wilson, unpublished observation).

Reaction of Human and Chimpanzee SA with anti-HSA. In a study of the immunological relationship of primate plasma proteins, Goodman (10) using chicken anti-HSA did discriminate between CSA and HSA as measured by a turbidometric technique. The difference was of the order of ~~about~~ 5%. The rabbit anti-HSA used in the present study which gave a single band of precipitation with whole human serum and whole chimpanzee serum (0.1 ml of a 1/200 dilution of each serum used as antigen) gave a pattern of identity when tested by the Ouchterlony procedure. The data obtained when this rabbit-anti-HSA reacted with HSA and CSA by the quantitative precipitin, macro and micro-C' fixation techniques are shown in Fig. 2. Supernate analysis of the HSA and CSA precipitin reaction mixture suggested immunological homogeneity as judged by the absence of any overlapping precipitation. The antibody content of this serum was calculated to be 611 µg antibody N/ml of undiluted serum. CSA precipitated 540 µg antibody N when calculated from a similar region of the precipitin curve (antigen excess side of the equivalence zone), and assuming that the same amount of CSA was required to reach this point of the curve, a difference of 11%. By macro-C' fixation, using 10 µg antibody N/reaction mixtures, HSA and CSA fixed 59 and 57 C' H_{50}, ~~mixture, and~~ respectively. Again, micro-C' fixation was the most discriminating technique, as shown by the percent C' fixation using 0.7 µg antibody N/reaction mixture. HSA and CSA fixed C' maximally at values of 68 and 32% respectively, a difference of 52%.

Reaction of Chicken and Turkey Ovalbumins with Anti-chicken Ovalbumin. A reaction of partial identity was seen when chicken egg white, turkey egg white and undiluted anti-chicken ovalbumin was tested by agar diffusion set up in a triangular pattern. Only

one band of precipitation was observed with 16 µg of chicken egg white. A second band of precipitation was seen, however, when 200 µg of chicken egg white were used. This immunochemical heterogeneity can also be seen by inspection of the quantitative precipitin curve in which the contaminating immune system is reflected by the rise in precipitable N in the antigen excess side of the precipitin curve, both with chicken and turkey egg white (Fig. 3). The heterogeneity, however, was not seen by analysis of the precipitin reaction mixture supernates. Calculation from the antigen excess side of the equivalence zone showed that chicken egg white, turkey egg white and crystalline ovalbumin precipitated 606, 580, and 540 µg antibody N, respectively. The increased antibody N precipitated by the chicken egg white reflects, in part, antibody to the contaiminating antigen which is probably at a higher concentration in the egg white. If we compare the antibody N values obtained with the egg white preparations, the difference between the chicken and turkey antigens is only 4%. Using the antibody content of anti-ovalbumin as 540 µg antibody N, the macro-C' fixation was performed with 2.7 µg antibody N/reaction mixture and the micro-C' fixation with 0.07 µg antibody N/reaction mixture. In the macro- and micro-C' fixation procedures, the contaminating immune system was not seen. Probably it has either been diluted out or it has less efficient C' fixing properties than the major antibody, anti-ovalbumin. When measured by macro-C' fixation, turkey ovalbumin reacted 89% as effectively as chicken ovalbumin. By micro-C' fixation no reaction with the heterologous antigen was observed at the antiserum dilution employed to demonstrate the homologous reaction.

DISCUSSION

The percent of cross reaction as measured by quantitative precipitin, macro- and micro-C' fixation is summarized in Table 1.

In order to explain the greater sensitivity of micro-C' fixation, the following experimental findings must be considered. Wallace et al. (11) have shown that rabbit antisera to bovine serum albumin, when compared at equal levels of antibody N, varied in C' fixing capacity from serum to serum. This variation in C' fixing capacity was large in antisera after one course of immunization. By micro C' fixation, we also find a variation in C' fixing capacity. Equal C' fixation was obtained with 0.045 µg anti-chicken H4 LDH N, 0.07 µg anti-chicken ovalbumin N, and 0.7 µg anti-HSA N (the anti-HSA is a first course antiserum; the other immune sera are third course antisera). More pertinent, perhaps, is the observation by Hill and Osler (12) that C' fixing capacity varies even among antibodies in a single immune serum. In micro-C' fixation, the concentrations of antigen and antibody are each 2-3 orders of magnitude below those used for the quantitative precipitin reaction and 1-2 orders of magnitude lower than those used by Hill and Osler (macro-C' fixation). Thus, it is possible that at very low concentrations of antibody, only a fraction of the antibody population is being measured. This avid fraction would be expected to have receptor sites of maximum complementarity to each antigenic determinant. Since C' fixation measures antigen-antibody aggregates (12,13) and since the rabbit-immune precipitate can be appreciably soluble (14), even a slight change in conformation of an antigenic determinant may render imperfect the fit between avid antibody and its determinant and lead to a greater dissociation of the aggregate.

Moreover, a slight change in conformation of one antigenic determinant may influence the binding of neighboring antigenic determinants to their antibodies and thus lead to a dissociation of the antigen-antibody aggregate into smaller complexes incapable of fixing C'.

SUMMARY

1. The antigen-antibody interaction of chicken and turkey H_4 LDH with rabbit anti-chicken H_4 LDH as measured by quantitative precipitin, macro- and micro-C' fixation gave 91%, 112% and 32% cross reaction values respectively.

2. The antigen-antibody interaction of human and chimpanzee serum albumin with rabbit anti-HSA as measured by quantitative precipitin, macro- and micro-C' fixation gave 89%, 97% and 46% cross reaction values respectively.

3. The antigen-antibody interaction of chicken and turkey ovalbumin with rabbit anti-chicken ovalbumin as measured by quantitative precipitin, macro- and micro-C' fixation gave 96%, 89% and 3% cross reaction values respectively.

4. A possible explanation for the greater sensitivity of micro-C' fixation to detect differences in protein structure is discussed.

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TABLE 1. Comparison of Sensitivity of Immunological Methods

Antiserum	Heterologous antigen	Cross Reaction*		
		Quantitative precipitin reaction	Macro-complement fixation	Micro-complement fixation
Anti-human hemoglobin A	Human hemoglobin S	100**	86**	41**
Anti-human serum albumin	Chimpanzee serum albumin	89	97	46
Anti-chicken ovalbumin	Turkey ovalbumin	96	89	3
Anti-chicken H_4 LDH	Turkey H_4 LDH	91	112	32

*The heterologous reaction is expressed as a percentage of the homologous reaction.

**Data of Reichlin *et al.* (1).

LEGENDS FOR FIGURES

Fig. 1. Serological activity as measured by the quantitative precipitin reaction with a 1:3 dilution of antiserum (left), macro-C' fixation with a 1:250 dilution of antiserum (center), and a micro-C' fixation with a 1:20,000 dilution of antiserum (right). Antiserum: rabbit-anti-chicken H_4 LDH. Antigens: pure chicken H_4 LDH (o—o) and pure turkey H_4 LDH (•---•).

Fig. 2. Serological activity as measured by the quantitative precipitin reaction with undiluted antiserum (left), macro-C' fixation with a 1:60 dilution of antiserum (center), and micro-C' fixation with a 1:900 dilution of antiserum (right). Antiserum: rabbit-anti-human serum albumin. Antigens: human serum (o— o) and chimpanzee serum (•---•).

Fig. 3. Serological activity as measured by the quantitative precipitin reaction with a 1:2 dilution of antiserum (left), macro-C' fixation with a 1:200 dilution (center), and micro-C' fixation with a 1:8,000 dilution of antiserum (right). Antiserum: rabbit-anti-chicken ovalbumin. Antigens: crystalline chicken ovalbumin (o——o), chicken egg white (■—■), and turkey egg white (•---•).

Footnotes

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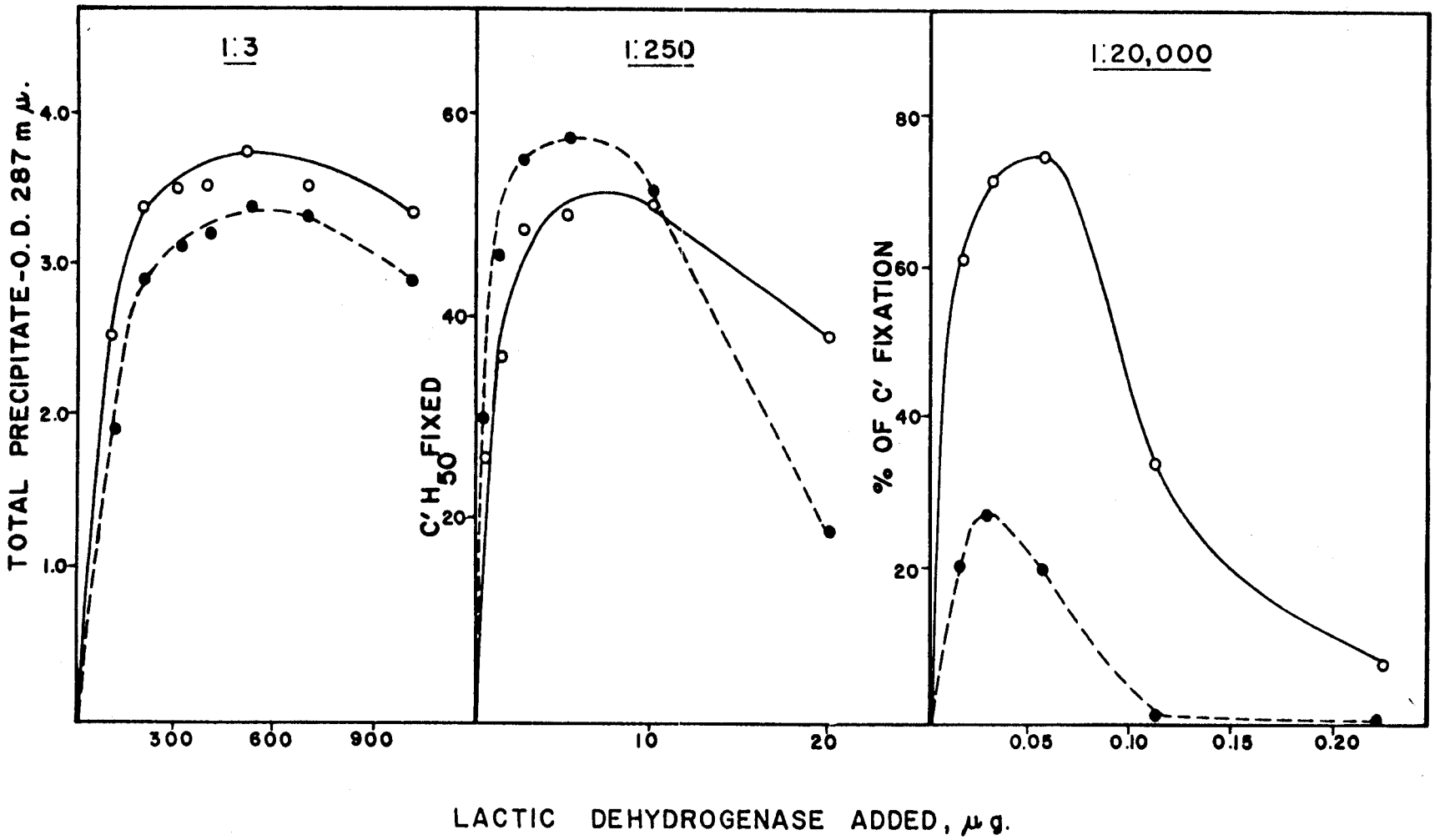
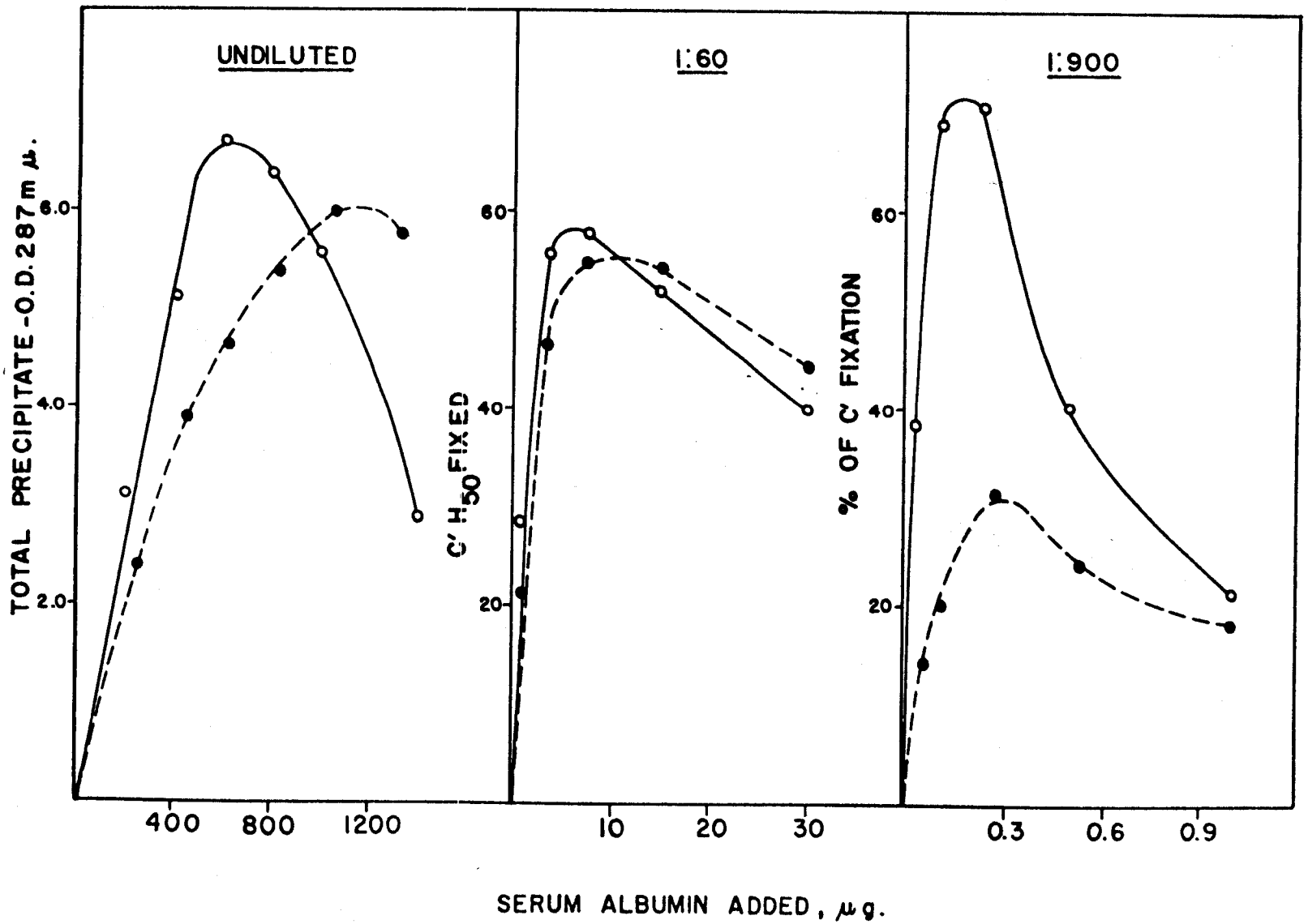
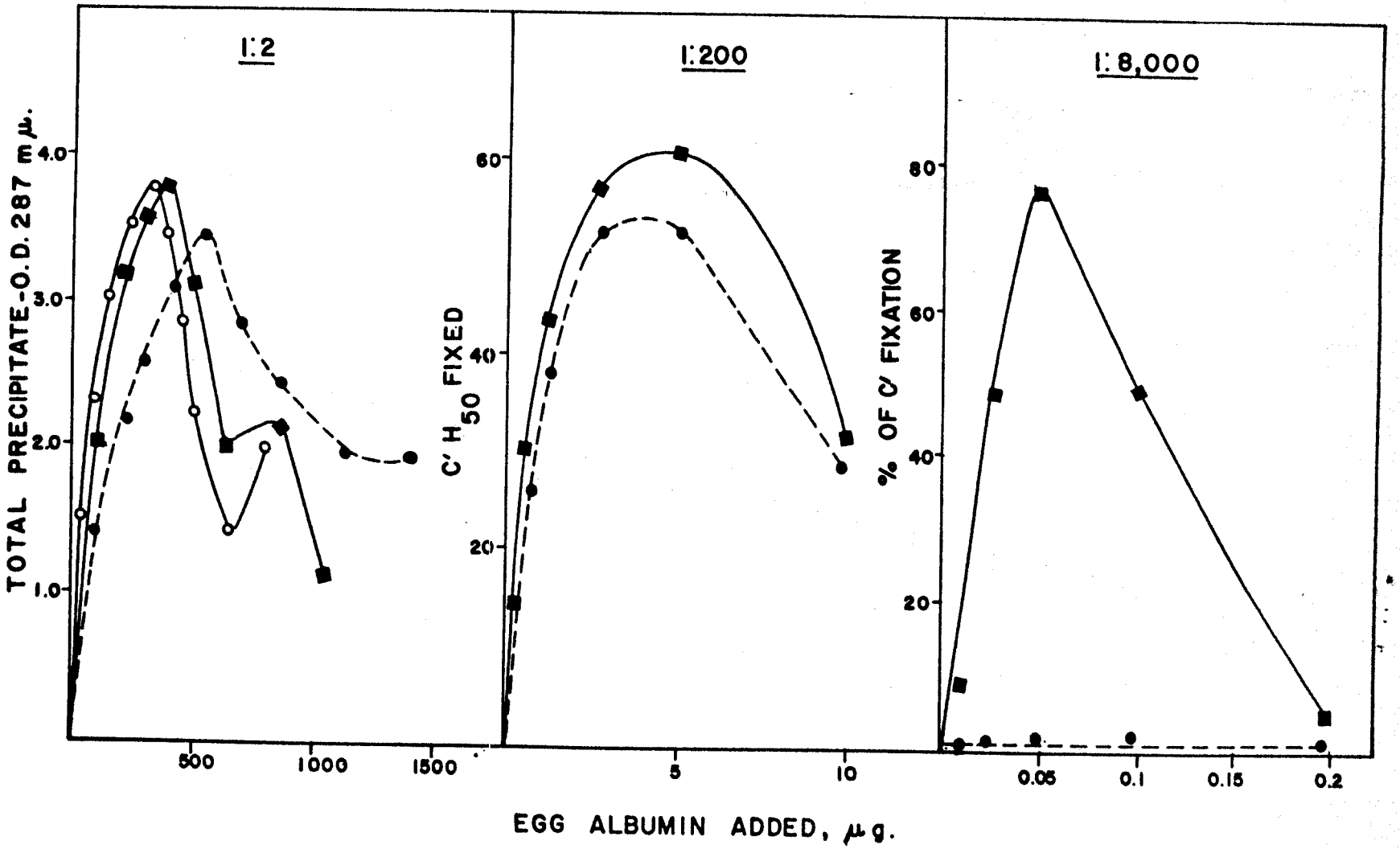

1:3
1:250
1:20,000
TOTAL PRECIPITATE-O.D. 287 mμ.
C'H50 FIXED
% OF C' FIXATION
4.0
3.0
2.0
1.0
60
40
20
80
60
40
20
300
600
900
10
20
0.05
0.10
0.15
0.20
LACTIC DEHYDROGENASE ADDED, μg.

1171


UNDILUTED
1:60
1:900
TOTAL PRECIPITATE - O.D. 287 mμ.
C' H50 FIXED
% OF C' FIXATION
6.0
4.0
2.0
60
40
20
60
40
20
400
800
1200
10
20
30
0.3
0.6
0.9
SERUM ALBUMIN ADDED, μg.


1:2
1:200
1:8,000
TOTAL PRECIPITATE-O.D. 287 mμ.
C' H50 FIXED
% OF C' FIXATION
4.0
3.0
2.0
1.0
60
40
20
80
60
40
20
500
1000
1500
5
10
0.05
0.1
0.15
0.2
EGG ALBUMIN ADDED, μg.