Immunochemical Studies on Oxidized Ovalbumin

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In the preceding paper it was described that crystalline ovalbumin can, without giving any coagulation, be oxidized in the molar ratio of 10-30 moles of periodate per mole of protein[1]. The oxidized ovalbumin thus obtained was soluble in water at the isoelectric point; accordingly it appears to have been undenatured[3a]. But it is obvious that some groups on protein surface were decomposed by oxidation. Therefore, the present experiment was made with a view to elucidating what rôle those decomposed groups would, as determinant molecular ones, take in connection with the antigenicity of ovalbumin.

Experimental

1. Immune Sera Antisera were prepared by applying ovalbumin and oxi.-ovalbumin (oxidized with 30 moles of periodate) in the following way, after recrystallizing them several times (1). The aqueous protein solution (10 mg./cc.) containing phenol (0.5%) were injected into the auricular vein of rabbits four times at the interval of four days each. The initial dose was 0.5 cc. and each successive one was increased by 0.5 cc., thus the fourth and last one reaching 2 cc. After a week from the last injection, the blood of each rabbit was exsanguinated by cardiocentesia, and each serum was prepared from the blood by the usual method[2].

2. Analytical Methods (*a*) *Quantitative precipitin reaction.* Quantitative analyses in anti-ovalbumin sera and anti-oxi.-ovalbumin system were carried out as follows. Accurately measured solutions of antigens and sera were mixed and allowed to stand at 37° for 3 hours[3b]. The precipitates were centrifuged at room temperature and washed twice with 2 cc. of 1% aq. NaCl solution at 0° and centrifuged again.

(*b*) *Determination of nitrogen in the specific precipitates.* Nitrogen in the precipitates was determined as following. After 1 cc. conc. H_2SO_4 and 20 mg. K_2SO_4 (without addition of $CuSO_4$) were poured directly in the conical centrifuge tube containing precipitates, decomposition was conducted by heating for one hour. On the digested solution, ammonia was determined by the nesslerization according to modified Thompson and Morrison method[4]. Namely, the solution was poured into a 50 cc. measuring flask, neutralized completely with NaOH, and then filled up with water to the mark. To 20 cc. of this solution, were added 0.2 cc. of 50% Rochelle salt solution and 0.2 cc. of Nessler solution. After the solution was allowed to stand at 20° for 20 min., the transmittancy of light was measured by a photoelectric colorimeter using filter S 44. Previous adjustment is necessary to keep the ammonia content within a range of 1 γ—3.5 γ/cc. When the solution becomes turbid, after adding Nessler solution and warming, this sample is too conc. as to ammonia and therefore must be further diluted to repeat the same process as mentioned above.

(*c*) *Assay of supernatant solutions.* After precipitates of antigen-antibody complex were centrifuged, the supernatant solutions were tested qualitatively for both excess antigen and antibody. Approximately one-third of the solution was assayed for excess antigen and the rest for excess antibody by the addition of small amounts of antiserum and antigen respectively[5,6].

1) The foregoing report; reported in the meeting of the *Agr. Chem. Soc. Japan.* (at Kyoto, May 1951).

2) J. A. Kolmer and F. Boerner, "Approved Laboratory Technic." (New York) 1945, p. 616.

3a) P. H. Maurer and M. Heidelberger, *J. Am. Chem. Soc.*, **73**, 2070 (1951).

3b) P. H. Maurer and M. Heidelberger, *J. Am. Chem. Soc.*, **73**, 2076 (1951).

4) J. F. Thompson and G. R. Morrison, *Anal. Chem.*, **23**, 1153 (1951).

5) L. R. Wetter and H. F. Deutsch, *J. Biol. Chem.*, **192**, 237 (1951).

6) H. F. Deutsch, *J. Biol. Chem.*, **185**, 377 (1950).

TABLE I

ADDITION OF INCREASING AMOUNTS OF OVALB. AND OXI.-OVALB. TO 1.0 cc. OF ANTI-OVALBUMIN SERUM

Kind of antigen	Antigen N added, mg.	Total N pptd., mg.	Antibody N by diff., mg.	Antibody N / Antigen N	Supernatant tests
Ovalbumin	1.5760	0.0752			Excess antigen
	0.1576	0.5024	0.3448	2.2	,,
	0.0628	0.6531	0.5903	9.4	Excess antibody
	0.0314	0.4710	0.4396	14.0	,,
	0.0157	0.3014	0.2857	18.2	,,
	0.0063	0.1342	0.1279	20.3	,,
	0.0031	0.0688	0.0657	21.2	,,
Oxi. ovalbumin	1.5760	0.0837			Excess antigen
	0.1576	0.5495	0.3919	2.5	,,
	0.0628	0.6154	0.5526	8.8	Excess antibody
	0.0314	0.4145	0.3831	12.2	,,
	0.0157	0.2434	0.2277	14.5	,,
	0.0063	0.1071	0.1008	16.0	,,
	0.0031	0.0539	0.0508	16.4	,,

TABLE II

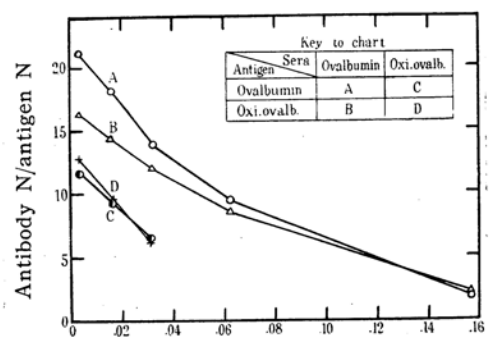
ADDITION OF INCREASING AMOUNTS OF OVALBUMIN AND OXI.-OVALBUMIN TO 1.0 cc. OF ANTI-OXI.-OVALBUMIN SERUM

Kind of antigen	Antigen N added, mg.	Total N pptd., mg.	Antibody N by diff., mg.	Antibody N / Antigen N	Supernatant tests
Ovalbumin	0.1576	0.0523			Excess antigen
	0.0314	0.2449	0.2135	6.8	,,
	0.0157	0.1649	0.1492	9.5	Excess antibody
	0.0063	0.0775	0.0712	11.3	,,
	0.0031	0.0400	0.0369	11.9	,,
Oxi. ovalbumin	0.1576	0.0542			Excess antigen
	0.0314	0.2355	0.2041	6.5	,,
	0.0157	0.1696	0.1539	9.8	Excess antibody
	0.0063	0.0832	0.0769	12.2	,,
	0.0031	0.0434	0.0403	13.0	,,

Results and Discussion

The results are shown in Tables I and II. It is apparent that there is no difference between the homogeneous reaction and the cross one in precipitin reaction upon ovalbumin-serum, except in the case of the less antigen region.

The slight difference which was found in the less antigen region, suggests that there is some loss on antigen surface which should be combined complementarily. On the other hand, there is also no difference between homogeneous and cross reaction in the reaction upon oxi.-ovalbumin-serum, except in the case of the less antigen region. In the less antigen region, the precipitate is formed somewhat more in the case of homogeneous reaction. The relation between the ratio of the antibody N to antigen N in the precipitates and the amount of antigen N increasingly added are shown in the Figure 1. As



Mg. antigen N added per cc. of serum dilution

Fig 1. Relation between the ratio of antibody N to antigen N in the precipitates and antigen N added to the serum dilution.

shown in the figure, it would seem that the oxi.-ovalbumin is not serologically heterogeneous, since the curve concerning serum of oxi.-ovalbumin is in straight line[6].

From the comparison between Tables I and II, it is clear that serological specificity was diminished considerably by the oxidation. Heidelberger and his collaborator[3b] reported that the deaminated proteins, even though one third of free NH_2-group was eliminated off, retain their serological specificity, so long as protein was not denatured.

The oxi.-ovalbumin reported here can be regarded as not denatured[3a]. Therefore, this difference between those facts is to be ascribed to the difference in the contribution to serological specificity between end-NH_2-group[7] and those groups on protein surface which were oxidized by periodate. In other words it seems that the specificity is more attributable to tryptophan, hydroxylamino acids and tyrosine, situated on protein surface, than to end NH_2-group.

7) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

The decrease in serological specificity of oxi.-protein probably is not due to the fact that the surface, as in case of denatured protein, is random and has no definite folding, but to the fact that some determinant groups on the surface is decomposed by oxidation.

Summary

Using the oxidized ovalbumin, quantitative precipitin reactions were brought forward. There was some difference between the homogeneous reaction and the cross one in the precipitin reaction upon ovalbumin- and oxidized ovalbumin-serum only in the case of the less antigen region. The serological specificity of ovalbumin diminished considerably by the periodate-oxidation.

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