AN IMMUNOLOGICAL STUDY OF NATIVE, DENATURED, AND REVERSED SERUM ALBUMIN*

By BENJAMIN F. MILLER, M. D.

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston)

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The denaturation of proteins has long been considered an irreversible process. This view is still generally prevalent in spite of evidence which has accumulated showing that denatured protein may, under appropriate conditions, be reversed into soluble material which exhibits the same physical and chemical properties as the original native protein. The transformation of denatured hemoglobin and globin to their native states has been extensively studied by Anson and Mirsky (1). In no case could they detect any physical or chemical differences between their original substance and the one obtained by the reversal of the thoroughly denatured protein. In 1931 they extended their observations to serum albumin (2) and showed that it was possible to prepare a soluble, heat-coagulable, crystalline protein from albumin which had been denatured by heat, acetone, or trichloracetic acid. The reversal of heat-denatured serum albumin from acid solution had been observed as early as 1910 by Michaelis and Rona (3). These investigators did not crystallize the soluble material recovered from the denatured protein, nor did they attempt to apply any other criteria of reversal. Recently Northrop has reported the reversal of denatured pepsin (4) and trypsin (5). The reversal of denatured egg albumin has not been accomplished by the present methods.

The technique involved in the reversal process is simple, especially with serum albumin. Anson and Mirsky (2) dissolved denatured serum albumin, prepared by the action of heat, acetone, or trichlorace-

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tic acid, in an acid solution. When this solution was neutralized with sodium hydroxide at room temperature, it yielded about 65 per cent soluble protein at the isoelectric point. All this soluble protein could be crystallized into a form resembling native albumin.

In view of the fundamental importance of the reversal process in understanding the mechanism of denaturation it appeared desirable to apply another criterion of reversibility. This we felt could be accomplished better by immunological, than by any but the most exhaustive chemical and physical methods at present available for protein characterization.

A study of the reversal of heat-denatured horse serum albumin by means of the precipitin reaction was therefore undertaken. It had been shown by Obermayer and Pick (6) that heated serum proteins lose most of their ability to react with precipitin antibodies obtained by the injection of the native protein. Later Schmidt (7) obtained substantially the same results. It thus appeared feasible to employ this method to determine the relationship of the reversed albumin to its native and denatured precursors.

Experimental Methods

Native, heat-denatured, and reversed heat-denatured horse serum albumins were prepared by the technique of Anson and Mirsky (2) modified, in certain respects, for an immunological study. Amorphous serum albumin was chosen, as it had been studied by Anson and Mirsky. Moreover, Svedberg (8) has shown that repeated crystallization of serum albumin gives an increasingly heterogeneous mixture; and it has been known to immunologists that the crystalline albumin is a poorer antigen than the amorphous form.

The specific sera were obtained by sensitizing rabbits to each of the three antigens. These sera were tested against the three antigens by means of the ring test precipitin method. The antigen dilutions were made on a comparable basis by expressing weight of total protein per volume of saline. A detailed description of the antigen preparations and the immunological procedures is given below.

Native Serum Albumin.—Prepared by half saturating horse serum with $(NH_4)_2SO_4$ and discarding the globulin precipitate. Solid $(NH_4)_2SO_4$ was then added to the filtrate (200 gm. per liter) for precipitation of the albumin fraction. This

amorphous albumin was dialyzed against cold distilled water in cellophane membranes for 24 to 48 hours until practically salt-free. A 2 per cent solution was used for sensitization of animals and for dilutions in the precipitin tests, as well as for the preparations below.

Heat-Denatured Albumin.—Prepared as follows: 100 cc. of 2 per cent native albumin were acidified with 30 cc. of N/10 HCl. The solution was stirred in a bath of boiling water until it reached 90°C. The heating was then continued for exactly 3 minutes, during which time the temperature usually rose to 97°C. The hot solution was neutralized with sufficient N/10 NaOH to give maximum coagulation (about 22.5 cc.) and then cooled at once to room temperature. Upon neutralization, a heavy precipitate of coagulated albumin appeared. It was separated by centrifugation, suspended and thoroughly stirred in 100 cc. of 0.9 per cent NaCl, and again centrifuged. This washing was repeated 5 times with 100 cc. portions of 0.9 per cent saline in order to remove any undenatured serum protein which might have been present.

The coagulated albumin dissolved incompletely at pH 7 by addition of NaOH at room temperature, but it was readily rendered soluble by suspending the denatured albumin in 75 cc. of distilled water, warming to 90°C., and dissolving with sufficient N/10 NaOH to give a clear solution. It was then immediately neutralized to pH 7 with the required amount of N/10 HCl, and cooled at once to room temperature by immersion in an ice bath. The amount of HCl needed was previously determined by means of an aliquot. This procedure did not subject the denatured albumin to such conditions of pH or temperature as would favor reversal; and resulted in a slightly opalescent solution which was analyzed for total protein, and was satisfactory for injections and for the precipitin tests. Although there was an additional manipulation involved in this process, it was deemed permissible since the rate of heat denaturation at reactions slightly alkaline to the isoelectric point is negligible, in comparison with the rate in acid solution (9).

Reversed Heated Albumin.—Prepared from 100 cc. of 2 per cent native albumin which had been heated, coagulated, and washed as described above. The denatured protein precipitate was dissolved in 15 cc. of N/5 HCl and diluted to 60 cc. This clear solution of denatured albumin was then reversed at room temperature by addition of sufficient N/5 NaOH to bring the pH to 4.7 (determined by methyl red as indicator). The reversed solution was only slightly turbid at this pH, indicating that most of the denatured albumin had regained solubility at the isoelectric point. Half saturation with $(NH_4)_2SO_4$ resulted in considerable precipitation. This procedure is recommended by Anson and Mirsky to remove any denatured albumin held in solution. It by no means follows that all of the precipitated protein is denatured, since even the solubility of native serum albumin in half saturated $(NH_4)_2SO_4$ near the isoelectric pH is distinctly limited. The filtrate from the half saturated solution containing the soluble, reversed albumin was dialyzed in a cellophane membrane against cold distilled water until practically salt-free. It was then concentrated in the cold by means of a Sørensen type of negative pressure dialyzer (10) to approximately 1 per cent. After analysis for

total protein this solution was used for the injection and immunological procedures. For convenience in the tables, it has been designated as reversed heated albumin.

Preparation of Antisera.—Three rabbits were injected with each of the above albumins both intravenously and subcutaneously until they gave a precipitating titer of at least 1:100,000. Eight of the nine animals showed a satisfactory response. Usually three injections of 5 to 20 mg. of total protein were given each week for 3 to 5 weeks.

Precipitin Tests.—The three antigens were diluted with 0.9 per cent saline. The dilutions are on a quantitative basis, all being reducible to grams of total protein (Kjeldahl nitrogen multiplied by 6.25) per cubic centimeter of saline. Thus a 1:100,000 dilution of antigen corresponds to 1 gm. of albumin in 100,000 cc. of saline, and is not directly comparable with the total protein in dilutions of whole serum unless one corrects for the original approximately 1:12 dilution of protein in horse serum.

The tests were made by layering about 0.3 cc. of the antigen dilutions over an equal volume of the antisera and reading the ring which formed at the interface. The following notation was used throughout in order to put the tests on a semi-quantitative basis.

++++ = heavy ring which formed within 2 minutes.
+++ = heavy ring at 20 minutes.
++ = moderate ring at 20 minutes.
+ = faint ring at 20 minutes.

Thus readings were taken at 2 minutes and the 4 plus rings recorded. The remainder were read at the end of 20 minutes. The unknowns were always compared with control solution of (1) saline + specific antisera, and (2) 1:1,000 dilutions of the antigens + normal rabbit serum.

RESULTS

The reactions of the native, heat-denatured, and reversed heated albumins with sera prepared by injection of native albumin are shown in Table I. The results show a marked decrease in the reactivity of the heat-denatured albumin toward the native precipitin, confirming the results of Obermayer and Pick (6) and of Schmidt (7). The table also indicates a striking increase in the precipitability of the reversed heated albumin by native precipitin as compared with the denatured antigen. The reactivity is approximately equal to that of native serum albumin since both react in dilutions of 1:100,000 which, as previously explained, contain the same weight of total protein for both antigens.

The reactions of the reversed protein with native precipitin were not considered sufficient evidence for this study because it might be argued

TABLE I

Precipitin Reactions with Anti-Native Albumin Sera

Rabbit No.	Antigen	Antigen dilutions						
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
78	Native serum albumin	++++	++++	++++	++	++	+	−	−
	Heat-denatured albumin	++	+	−	−	−	−	−	−
	Reversed heated albumin	++++	++++	+++	++	+	+	−	−
79	Native serum albumin	++++	++++	++++	++	++	+	−	−
	Heat-denatured albumin	+	+	+	−	−	−	−	−
	Reversed heated albumin	++++	++++	++++	++	++	+	−	−

Controls: 0.9 per cent saline + Sera 78 and 79, negative.
1:1,000 dilutions of the antigens + normal rabbit serum, negative.

TABLE II

Precipitin Reactions with Anti-Heat-Denatured Albumin Sera

Rabbit No.	Antigen	Antigen dilutions						
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
83	Native serum albumin	++++	++++	++	++	++	+	−	−
	Heat-denatured albumin	++++	++++	++	++	++	+	−	−
	Reversed heated albumin	++++	++++	++	+	+	+	−	−
84	Native serum albumin	++++	++	++	+	+	+	−	−
	Heat-denatured albumin	++++	++	++	+	+	+	−	−
	Reversed heated albumin	++++	++	++	+	+	+	−	−
85	Native serum albumin	++++	+++	+++	++	+	+	−	−
	Heat-denatured albumin	++++	++++	++++	++	++	+	−	−
	Reversed heated albumin	++++	++++	++++	++	++	+	−	−

Controls: 0.9 per cent saline + Sera 83, 84, and 85, negative.
1:1,000 dilutions of the three antigens *vs.* normal rabbit serum, negative.

that the reversed protein was acting as a partial antigen. To guard against this possibility we obtained additional results which show the

reactions of the various antigens with sera obtained by injecting both denatured and reversed protein. The fact that the reversed protein gave rise to antibodies is evidence that it is a complete and not a partial antigen.

First let us consider the reactions of the three antigens with sera obtained by the injection of heat-denatured albumin, as set forth in Table II. They show an equal reactivity of all three antigens with the specific sera. The phenomenon that heat-denatured serum pro-

TABLE III

Precipitin Reaction with Anti-Reversed Heated Albumin Sera

Rabbit No.	Antigens	Antigen dilutions							
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
88	Native serum albumin	++++	++++	++++	+++	+++	++	+	−
	Heat-denatured albumin	+++	+	+	−	−	−	−	−
	Reversed heated albumin	++++	++++	++++	+++	+++	++	+	−
89	Native serum albumin	++++	++++	+++	+++	+	+	−	−
	Heat-denatured albumin	+++	++	+	−	−	−	−	−
	Reversed heated albumin	++++	++++	++++	+++	+	+	−	−
90	Native serum albumin	++++	++++	++++	++++	++	++	+	−
	Heat-denatured albumin	++++	+++	++	+	−	−	−	−
	Reversed heated albumin	++++	++++	++++	++++	++	++	+	−

Controls: 0.9 per cent saline + Sera 88, 89, and 90, negative.
1:1,000 dilutions of the three antigens *vs.* normal rabbit serum, negative.

teins produced an antibody which reacted equally well with both native and heat-denatured serum was observed by Obermayer and Pick (6). This type of antibody is now well known in immunology by the name coctoprecipitin. Our chief interest in the coctoprecipitin was whether or not the reversed heated antigen would give rise to this peculiar antibody.

The reactions of the same three antigens with specific sera obtained by injecting reversed albumin are given in Table III. A comparison

of Tables I and III shows that the reversed heated albumin did not act like the denatured albumin and give rise to a coctoprecipitin; but produced an antibody indistinguishable from the native precipitin.

A considerable interaction of the heat-denatured albumin with the native and reversed antisera is indicated at the lower dilutions. This apparently is a partial function of the severity of the denaturation process, for, as shown by Schmidt (7), the cross-reaction varies inversely with the temperature and duration of the heating. Also, sera with high titers against native protein show more reaction with heated antigen than sera with low titers (6). Our results were all obtained with sera of high titer.

We accordingly wished to study the immunological effect of the reversal process on native albumin which had been treated more drastically than the heat-denatured protein. We therefore denatured serum albumin by exposure to acidified acetone for 24 hours, and reversed it in the usual manner as detailed below.

Acetone-Denatured Albumin.—Prepared after Anson and Mirsky (2) with certain departures necessitated by our special needs. 70 cc. of 4.5 per cent native horse albumin (prepared as before) were acidified to 0.05 normal by the addition of 5 N HCl. The albumin was precipitated by the addition of 700 cc. of acetone containing 1.4 cc. of 5 N HCl. The mixture was allowed to stand at room temperature for 24 hours with occasional shaking. The coagulated albumin was then filtered off and dried between filter papers for 2 hours. After pulverization, it was dissolved in 150 cc. of distilled water. The denatured albumin hydrochloride went into solution readily giving a pH of 3.5 to 4. It was a solution prepared by this method that Anson and Mirsky neutralized at room temperature to obtain reversal of acetone-denatured albumin. Since we wished to obtain a denatured albumin at pH 7.0 for comparison with the reversed acetone-denatured albumin, we found it necessary to approach the isoelectric point at a high temperature where reversal could not take place. This was accomplished by warming the acid solution of denatured albumin to 90°C. and neutralizing with N/10 NaOH until all the protein precipitated out. This precipitate was then washed 6 times with 100 cc. portions of 0.9 per cent NaCl. A portion was brought into solution by warming to 90°C. and bringing to pH 7.0 exactly as described before with the heat-denatured albumin. The protein is referred to as acetone-denatured albumin for convenience in the text; but it should be borne in mind that it had been exposed to dilute acid and heat as well as excess of acetone. Hence it was much more violently denatured than the heat-denatured albumin described above.

Reversed Acetone Albumin.—Prepared from the remainder of the washed precipitate of coagulated albumin. This was dissolved in N/5 HCl and reversed and

dialyzed exactly as described above for the reversed heated antigen (page 627). This protein is designated reversed acetone albumin in the tables.

Results with Acetone-Denatured and Reversed Acetone Albumins

These two antigens were tested against one of the sera obtained by injecting native albumin. The technique and nomenclature of the precipitin reactions are the same as previously used except that all readings were taken at the end of 90 minutes. The results are set forth in Table IV where, even more strikingly than before, the contrast between the reactivity of the denatured albumin and its reversed derivative is demonstrated. This drastically treated denatured

TABLE IV

Precipitin Reactions with Anti-Native Albumin Serum

Rabbit No.	Antigen	Antigen dilutions							
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
78	Native serum albumin	+++	+++	+++	++	++	+	+	−
	Acetone-denatured albumin	−	−	−	−	−	−	−	−
	Reversed acetone albumin	+++	+++	++	++	++	+	+	−

Controls: 0.9 per cent saline + Serum 79, negative.
1:1,000 dilutions of antigens *vs.* normal rabbit serum, negative.

albumin showed no reactivity with native precipitin even in the lowest dilution, whereas the reversed material prepared from it reacted in a dilution of 1:200,000 exactly as did the same weight of native albumin.

Because of the general agreement of the data obtained, we did not sensitize animals to the acetone-denatured and reversed acetone albumins and study their interactions.

DISCUSSION

These data indicate that, as far as can be shown by the precipitin reaction, there is no difference between native and reversed serum albumin. This is not proof, of course, that the two proteins are identical, but is evidence that they do not differ in the portions of the molecule which exert the specific influence in the precipitin reaction.

It seems reasonable when one considers the delicacy of the biological method employed to suppose that, if the reversed protein is not identical in all parts of its complex molecule with the native albumin, it is closer, at least, to the native than the denatured state. This is suggested by the failure to form a coctoprecipitin by the reversed albumin and its production of a precipitin indistinguishable from that produced by the native albumin.

There are also possible applications of these results in immunology. It is apparent how carefully any antigen such as horse serum must be described in terms of hydrogen ion activity, for it would be obviously useless to compare a native with a denatured serum if the latter had been exposed to such conditions of pH as to result in reversal of the denaturation. Furthermore, the marked changes in antigenic properties produced by the chemical manipulations involved in the reversal process may give some insight into the nature of immunological specificity, especially when more exact chemical and physical characterizations of native and denatured proteins become available.

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CONCLUSION

Native and reversed horse serum albumin are indistinguishable when tested immunologically by means of the precipitin reaction.

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