NATIVE AND REGENERATED BOVINE ALBUMIN

II. Immunological Properties*‡

By DONALD S. MARTIN, JOHN O. ERICKSON, FRANK W. PUTNAM, and HANS NEURATH

(*From the Departments of Bacteriology and Biochemistry, Duke University, School of Medicine and Duke Hospital, Durham*)

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Previous experiments have shown (2) that regeneration[1] of urea-denatured *horse* serum albumin yielded a material which, in comparison with the native protein, exhibited a significantly lower ability to stimulate precipitins in the rabbit. The regenerated protein, however, was capable of precipitating to full titer the precipitins produced by immunization of rabbits with the native material.

Similar experiments have now been carried out on whole *bovine* albumin, a protein of recognized lower antigenic activity than horse albumin. The preparation and properties of the native and regenerated proteins have been described in the preceding paper of this series (3).

EXPERIMENTAL

Methods

1. Antigens.—The materials used for injection and for titrating the serum for the presence of antibodies were: (1) native whole bovine albumin, (2) albumin regenerated from 8 M urea solutions, and (3) albumin regenerated from 8 M solutions of guanidine hydrochloride (3).

2. Immunization of Animals.—Each antigen was injected intravenously into twenty-four rabbits of varying weights and breeds, each animal receiving six doses, given twice weekly over a 3 week period. To compare the effects of dosage, each group of twenty-four rabbits was divided into four groups of six each. The native and urea-regenerated whole bovine albumin antigens were administered in total doses of 10 mg., 20 mg., 40 mg., and 80 mg. per kilo body weight, while, due to a misunderstanding, the corresponding doses of whole bovine albumin regenerated from guanidine hydrochloride, were 6 mg., 12 mg., 24 mg., and 48 mg. per kilo. For each group, the first two doses were one-tenth of the total dose, the last four injections each containing

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‡ A preliminary report has already been published (1).

[1] "Regeneration" of a protein refers to the reversal of the process applied for denaturation and isolation of that protein fraction most closely resembling the native protein in chemical and physicochemical properties.

one-fifth of the total dose. The rabbits were bled from the heart 7 days after the last injection.

3. Precipitin Titration.—The serum dilution method, described by one of us (4) was used. 0.5 ml. quantities of twofold dilutions of immune serum in saline, and 0.5 ml. quantities of each antigen solution, containing 8 μg. of antigen per ml., were mixed, shaken, and examined for visible precipitation after standing for 2 hours at room temperature. The antigen concentration of 8 μg. per ml. was selected after preliminary determinations had shown that a final concentration of 4 μg. per ml. was the smallest amount of antigen capable of yielding a definitely visible precipitate under the conditions (Table I).

TABLE I

Protocol of Preliminary Experiments to Determine the Smallest Amount of Antigen Capable of Yielding a Visible Precipitate in a Bovine Albumin Anti-Bovine Albumin Rabbit Serum System

Rabbit serum No.	Final serum dilution	Final concentration of antigen, *µg. per ml.*										Antibody units per ml. of undiluted serum
		1000	500	250	125	62.5	31.3	15.7	7.8	3.9	2.0	
3	1–4	+	+	+	+	+	+	+	+	+	–	256
6	1–4	+	+	+	+	+	+	+	+	+	–	128
9	1–8	±	+	+	+	+	+	+	+	+	–	512
10	1–4	+	+	+	+	+	+	+	+	+	–	256
18	1–8	+	+	+	+	+	+	+	+	+	–	1024
20	1–8	+	+	+	+	+	+	+	+	+	–	1024
21	1–8	+	+	+	+	+	+	+	+	+	–	512
23	1–64	–	±	+	+	+	+	+	+	+	–	2048
24	1–8	+	+	+	+	+	+	+	+	+	–	256

+, denotes visible precipitation; –, no precipitation; and ±, doubtful reaction.

Column 1 of Table I lists the serum employed and column 2 the final dilution of serum used in these constant antibody-varying antigen series. The following ten columns show the various final antigen concentrations, expressed in micrograms per milliliter. The table shows that the antigen dilution method yields constant endpoints which have no relationship to the precipitin titer as obtained by the serum dilution method (last column).

Similar titrations showed the concentration of 4 μg. per ml. to be minimal also for observation of visible precipitation of the regenerated antigens with their respective homologous antisera.[2]

The accuracy of this method has already been demonstrated for the Pneumococcus, Type I SSS-antipneumococcus rabbit serum system (4). Its application to a protein-

[2] The results of precipitin titrations are expressed arbitrarily in terms of antigen concentration, following Marrack's suggestion (5) that the unit of antibody be defined as the amount reacting optimally with 1 μg. of antigen. The titration values given for each serum in Table I were obtained by subsequent titration by the serum dilution procedure described above.

antiprotein rabbit serum system was checked by independent titration of a rabbit serum containing antibodies for the horse serum albumin fraction A, prepared by the method of Kekwick (6). The equivalence point, determined by the quantitative method of Heidelberger and Kendall (7), was found to be attained with 1.23 mg. of antigen; by Culbertson's method (8) a value of 1.0 mg. was obtained. The highest serum dilution yielding a visible precipitate, when mixed with an equal volume of antigen containing 8 μg. per ml., was 1–128. In terms of final concentration, a serum dilution of 1–256 produced a precipitate with 4 μg. of antigen, indicating that the undiluted serum was capable of completely precipitating 1.024 mg. of antigen (corresponding to 1024 antibody units).

4. Anaphylaxis.—10 days after the last injection of antigen the rabbits were injected intravenously with homologous or heterologous antigens, in a dose of 100 mg. per kilo body weight.

RESULTS

1. Anaphylaxis.—A few rabbits died during the immunization procedure; of the remainder only one rabbit (No. 23) showed any evidence of shock. However, it recovered completely within 15 minutes.

2. Precipitin Titers.—The results of the precipitin titrations, obtained with the homologous antigens are summarized in Table II.

Column 1 of Table II refers to the nature of the antigen and the total immunizing dose, column 2 to the number of animals receiving this dose. The following columns give the number of antisera that were found to contain the number of antibody units listed in the top row of the table. Mean values for the antibody units, obtained in response to the administration of each immunizing dose, are listed in the last column of the table.

3. Relation between the Immunizing Dose and Precipitin Content.—Inspection of Table II shows a marked variation in antibody titer within each group of antisera. Statistical analysis failed to reveal any significant correlation between the dose injected and the degree of precipitin response.

4. Comparison of the Antigenicity of Native and Regenerated Whole Bovine Albumin.—Calculations of mean values for the antibody content of the first three groups of sera listed in Table II indicate that the antigenic activity of these three antigens decreases in the following order: native whole bovine albumin, urea-regenerated albumin, and guanidine hydrochloride-regenerated albumin. The corresponding antibody values, expressed in antibody units per milliliter of serum are, respectively, 383, 274, and 114. Computation of t values, however, reveals that the differences in antibody response to the native and urea-regenerated proteins were not significant ($p < 0.4 > 0.3$).[3] In these

[3] p is the probability that a given deviation from expectation (based upon hypothesis) shall occur by the action of pure chance. Thus a value of $p = 0.2$ indicates that in 20 per cent of the total number of observations, an event will occur by mere coincidence.

computations, all values were considered to carry equal weight, irrespective of the immunizing dose. A similar comparison of the responses to the native and guanidine hydrochloride-regenerated whole bovine albumin showed the differences in antigenic activity to be statistically significant ($p < 0.02 > 0.01$).

TABLE II

Results of Precipitin Titrations of Rabbit Antisera to Native and Regenerated Bovine Albumins with their Respective Homologous Antigens

Antigen* total dose	No. of rabbits injected	Antibody units per ml.											Mean titer (antibody units per ml.)
		<4	4	8	16	32	64	128	256	512	1024	2048	
mg.													
N-10	6	1			1	1	1	1	1				83
N-20	4								1	3			448
N-40	5					2	1		1		1		282
N-80	6								3	1	1	1	725
Total.......	21	1			1	3	2	1	6	4	2	1	383
U-10	5							3	2				175
U-20	4					1		1	1	1			232
U-40	6					1	1	2	2				144
U-80	6							2	3			1	555
Total.......	21					2	1	8	8	1		1	274
G-6	6		1			1	1	1	1	1			167
G-12	6					1	2	3					91
G-24	6			1		2	1	2					67
G-48	6						4		2				128
Total.......	24		1	1		4	8	6	3	1			114
C-80	6	1	3				2						26

* *N*, denotes whole albumin; *U*, albumin regenerated from 8 M urea; *G*, albumin regenerated from 8 M guanidine hydrochloride; and *C*, native crystalbumin.

Although the mean antibody titer obtained in the series of antisera to guanidine hydrochloride-regenerated albumin may have been due to the smaller immunizing doses employed, comparison of the antigenicities of these two proteins appears to be justified in view of the observation that variation in antibody response to various doses of antigen lacked statistical significance.

Hence, regeneration from 8 M guanidine hydrochloride, but not from 8 M urea, resulted in a significant change in the antigenic property when compared to the antigenicity of the original whole bovine albumin.

5. *Antigenicity of Crystalbumin.*—A generous supply of crystalline, carbohydrate-free bovine albumin, obtained through the courtesy of the Department of Physical Chemistry, Harvard University Medical School, enabled us to compare the immunological properties of our preparations with those of a standardized preparation of a highly purified material (hereinafter referred to as crystalbumin). Six rabbits were immunized with a standard dose of 80 mg. of crystalbumin, the highest immunizing dose employed in the preceding experiments. The antibody titers, given in the last row of Table II, show that the mean titer of these six rabbits was 26 antibody units per ml. The numerical difference between this titer and the mean antibody response elicited by the administration of 80 mg. of native, whole bovine albumin (725 antibody units per kilo body weight), is statistically significant ($p < 0.05 > 0.02$). However, no statistically significant difference could be found when the mean titer of antisera to crystalbumin was compared with that of all antisera to whole bovine albumin regenerated from guanidine hydrochloride ($p < 0.1 > 0.05$), although a numerical difference is apparent. In these computations, the difference in the size of the immunizing dose has been neglected, for reasons already stated in the preceding paragraph. It appears, therefore, that the loss in antigenicity resulting from further chemical purification of whole albumin, yielding crystalbumin, is comparable to, if not greater than, that occasioned by regeneration of native whole bovine albumin from 8 M guanidine hydrochloride.

6. *Serological Specificity.*—Each of the antisera to whole bovine albumin, in the native state (N) and after regeneration from urea (U) and guanidine hydrochloride (G), were titrated with the respective heterologous antigens. Antisera from rabbits that had received the highest immunizing dose of each antigen were titrated also with crystalbumin (C). In addition, all anticrystalbumin rabbit sera were titrated with native whole bovine albumin. In all cases the heterologous and homologous titers were found to be identical. Immunological equivalence of the four antigens, *i.e.* native whole bovine albumin, albumin regenerated from urea and from guanidine hydrochloride, and crystalbumin, appears, therefore, to be established although quantitative precipitin measurements may yet reveal small differences in the degree of cross-reaction.

DISCUSSION

Comparison of the immunological properties of native and regenerated whole bovine albumin reveals their mean antigenic activities to decrease numerically in the following order: (1) native albumin, (2) albumin regenerated from 8 M urea, and (3) albumin regenerated from 8 M guanidine hydrochloride. All antigens proved to be immunologically equivalent with each other as well as with native bovine crystalbumin.

The decrease in antigenicity achieved by regeneration of native albumin by

urea was statistically not significant. However, regeneration from guanidine hydrochloride proved effective in rendering albumin less antigenic, resulting in about a 70 per cent reduction in activity.

It may be of interest to compare these findings with those previously reported for native, and urea-regenerated horse serum albumin (2). While differences in species specificity between the albumin constituents of horse and beef serum, as well as the relatively lower degree of purity of whole bovine albumin, preclude a strict comparison, certain differences in behavior are apparent. With horse serum albumin, fraction A, more than 90 per cent of the initially high antigenic power could be eliminated by regeneration from urea, while the same treatment resulted only in a small reduction in antigenicity of the naturally less antigenic bovine albumin. The more powerful denaturing action of guanidine hydrochloride was required to achieve a significant reduction in the antigenicity of bovine albumin.

An explanation for the different behavior of these two proteins may possibly be found in two factors which have been considered influential in determining the antigenicity of a protein:

The first of these is the presence of carbohydrate constituents. It has been pointed out (2) that carbohydrate groups appear to confer a high degree of antigenic activity on bacteria as well as on certain serum proteins. The data on hand indicate a similar parallelism between carbohydrate content and antigenic power, the order of decreasing antigenicity being as follows: horse serum albumin, fraction A (1.95 per cent carbohydrate), whole bovine albumin (0.4 per cent carbohydrate), and bovine crystalbumin (no carbohydrate). However, the degree of antigenic activity should not be attributed solely to the presence of carbohydrate residues; for, with both horse serum albumin and whole bovine albumin, the regenerated materials, in spite of unchanged carbohydrate content, exhibited a lower antigenicity than the native proteins from which they were derived.

A second factor to be considered in this connection is the contribution of the intact, specific configuration of the native protein molecule. Elsewhere it has been suggested (2) that processes bringing about partial or complete abolition of antigenic activity are those which break down the internal structure of the molecule. Different processes, when applied to a protein, may vary from each other in the extent to which they affect the internal configuration of the protein, guanidine hydrochloride, for instance, being more effective in some respects than urea (9, 10). Conversely, a given denaturing agent may affect different proteins to varying degrees (11). The experimental evidence presented previously (3, 9) suggests strongly that horse serum albumin, fraction A, is more extensively denatured by urea, and is less susceptible to regeneration, than is whole bovine albumin. The present immunological data are indeed indicative of the existence of a close relation between loss of antigenicity and the extent to which denaturation has occurred.

The low antigenic power of native bovine crystalbumin may be ascribed to the combined influence of both of the aforementioned factors. This protein is devoid of carbohydrate; in addition, it is more susceptible to denaturation than either horse serum albumin or whole bovine albumin.

The fact that all four antigens proved to be immunologically equivalent, as revealed by the present method of titration, is very significant. It indicates that the observed differences in antigenic activity are probably not due to the production of different types of antibodies. It reveals that neither denaturation and regeneration, nor chemical purification, produced decisive changes in the chemical structure of bovine albumin. Finally, it adds to the evidence that the carbohydrate residues in these large protein molecules, though a contributing factor in establishing the degree of antigenicity, play no significant rôle in the determination of serological specificity.

SUMMARY

1. The effects of regeneration of whole bovine albumin on antigenic activity and serological specificity were determined by precipitin measurements on rabbit antisera to (1) native whole albumin, (2) albumin regenerated from 8 M urea, and (3) albumin regenerated from 8 M guanidine hydrochloride.

2. While numerically the mean antibody response to these three antigens was found to decrease in the order named, only the difference in antigenic activity between native and guanidine hydrochloride-regenerated albumin was statistically significant. Native, crystalline, carbohydrate-free albumin (crystalbumin) was considerably less antigenic than native whole bovine albumin, its activity being comparable to, if not less than, that observed for guanidine hydrochloride-regenerated whole albumin.

3. All four antigens were immunologically equivalent.

4. The antigenic activity of these proteins is discussed in terms of protein structure and carbohydrate content.

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