The Essential Role of Specific Conformation in the Antigenicity of Ribonuclease*

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ABSTRACT: The reaction of ribonuclease A, ribonuclease S, and ribonuclease S-protein with their respective antisera has been employed to demonstrate the essential role of specific conformation in maintaining the antigenic combining sites of these proteins. Heating or exposure to urea, sufficient to reversibly denature RNAases S and S-protein, induced complete, but reversible, loss of precipitin formation between these RNAase derivatives and antiRNAase A serum. It was demonstrated that the effects were not due to alteration of the antibody as there was unchanged reactivity between RNAase and antiRNAase A serum with the denaturing conditions sufficient to abolish the antigenic reactivity of the RNAase S preparations.

The three-dimensional character of the antigenic combining site in antigen–antibody reactions has been assumed since the work of Landsteiner (1936). Studies concerned with the role of conformation, as well as other factors, in the immunologic function of polypeptide antigens has been recently reviewed and extended by Gould *et al.* (1964).

Since native bovine pancreatic ribonuclease (RNAase A) has a well-characterized covalent (Smyth *et al.*, 1963) and folded or tertiary structure (Scheraga and Rupley, 1962) and has been extensively evaluated immunologically, it was a useful protein for study of the relationship of conformation to antigenic function. The studies of Brown (1959) had shown that performic acid oxidized or reduced carboxymethylated ribonuclease, lacking disulfide bonds, did not precipitate antibody directed against the native RNAase molecule. Mills and Haber (1963) demonstrated furthermore that RNAase A lost reactivity against antiRNAase A antiserum after randomization of the disulfide bonds of the enzyme during reduction and reoxidation. These studies with RNAase supported the general view that the antigenic combining sites in globular proteins consisted of specific groupings of amino acid residues brought together by the proper disulfide bonds from originally remote positions in the sequence of the extended chain. The short-range electrostatic and hydrophobic interactions between adjacent amino acid residues, responsible for much of the specific conformation of RNAase, can be reversibly disrupted by heat and urea without interference with normal disulfide bond pairing; demonstration of the importance of these interactions for immunological reactivity would provide additional evidence for the presumed highly specific three-dimensional character of antigenic combining sites. Evidence in support of this concept for ribonuclease was gathered in studies by Bennett and Haber (1963), who successfully employed heat and acid to separate the enzyme from its antibody without disruption of disulfide bonds. There was good correlation during heating between decline in reactivity and the thermally induced rotatory transition of RNAase. However, it was difficult to completely exclude the possibility that reversible changes in the antibody contributed to the loss of precipitin reactivity. Gould *et al.* (1964), in their study of the effects of heat and acid with precipitin systems of synthetic polypeptide antigens and their antibodies, demonstrated a decline in the reactivity of antibody after exposure to denaturants insufficient to produce changes in the rotatory dispersion constants of the γ-globulin.

Singer and Richards (1959) have demonstrated that RNAase S (resulting from cleavage of the peptide bond in RNAase between residues 20 and 21) and RNAase S-protein (the larger fragment of RNAase S, residues 21–124, containing the intact disulfide bonds) strongly react with antibody directed against RNAase A. However, the subtilisin-produced derivatives did not react as strongly with antiRNAase A serum as did the native molecule; this was attributed to the conformational differences between the RNAase S derivatives and RNAase A. It seemed valuable to extend these studies by performing immunodiffusion analysis to define possible reactions of partial identity between RNAase A, S, and S-protein. In addition, further information could be gained concerning the immunologic differences between the closely related RNAase molecules by preparing antiserum against RNAase S and S-protein. Most importantly, the immunologic cross reactions between RNAase A and the RNAase S

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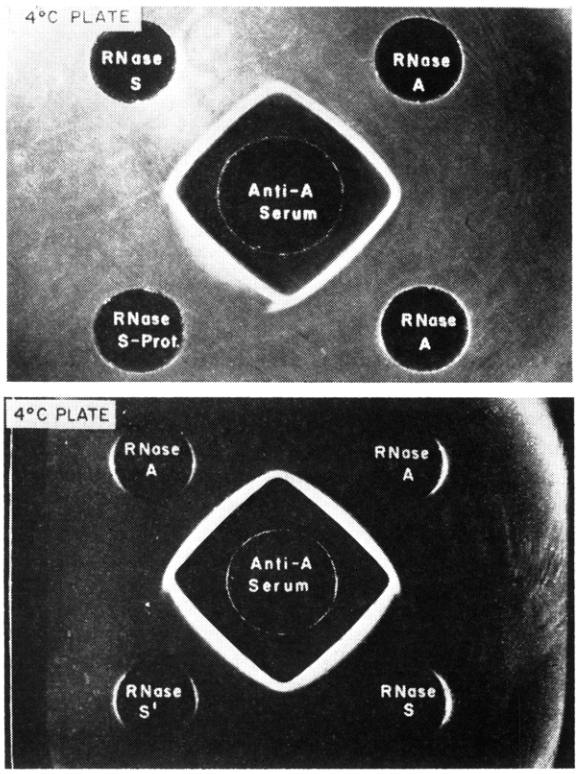

FIGURE 1: Immunodiffusion study in which undiluted antiribonuclease A serum is developed against: (1A) ribonuclease A, S, and S-protein; and (1B) ribonuclease A, S, and S′ (antigen concentration 1 mg/ml).

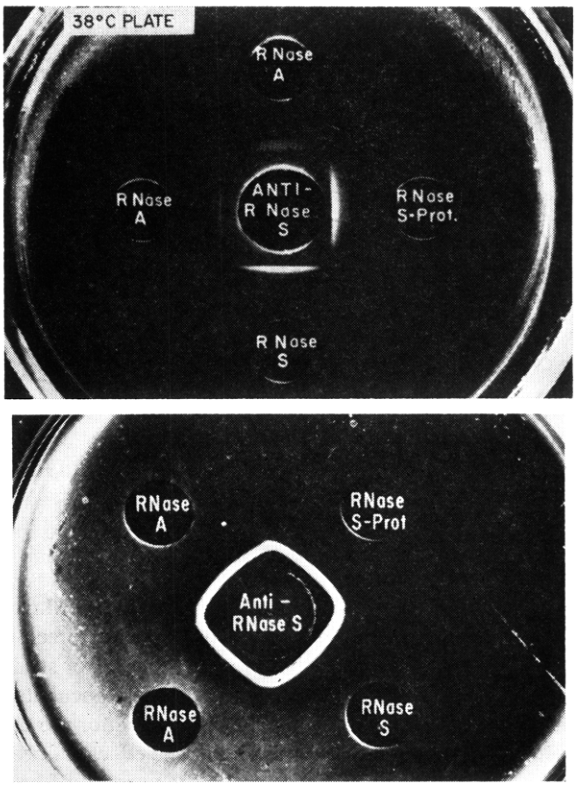

FIGURE 2: Immunodiffusion study in which undiluted antiribonuclease S serum is developed against ribonuclease A, S, and S-protein for (1A) 24 hr, and (1B) 4 days (antigen concentration 1 mg/ml).

antigens and their corresponding antisera made available an experimental immune system which permitted evaluation of changes in immunologic reactivity effected exclusively by alterations in the structure of the RNAase antigen. RNAase S and RNAase S-protein are much more susceptible to denaturation by heat or urea than the native RNAase A (Richards and Vithayathil, 1959; Sherwood and Potts, 1965a). Conditions were found which would denature RNAase S and S-protein but not RNAase A (Sherwood and Potts, 1965A) and which did not decrease the reaction of RNAase A with its antiserum. The observed, reversible reduction in the extent of precipitin reaction between RNAase S or RNAase S-protein and antiRNAase A serum under such conditions could be interpreted as occurring solely through alterations in the conformation of the antigen species.

Materials and Methods

RNAase A was prepared by chromatography on IRC-50 (Hirs *et al.*, 1953); RNAase S and RNAase S-protein were prepared and assayed by methods previously reported (Richards and Vithayathil, 1959; Potts *et al.*, 1963). All antisera were prepared in rabbits employing complete Freund's adjuvant with added *Myobacterium butylicum*. Adjuvant (0.5 ml) was mixed with 0.5 ml of normal saline containing 5 mg of protein until a stable emulsion was obtained. This preparation was then injected into a toe pad in each of the four extremities (0.2 ml) and the final 0.2 ml was injected intramuscularly. In addition, employing the method of Cohn (1952), 1 mg of alum precipitated protein antigen was given intravenously the following day. In all, three animals were immunized with RNAase A, one animal with RNAase S, and two animals with RNAase S-protein. All rabbits were bled every third day for 2 weeks starting at the fourth week after immunization. Serum from these bleedings were combined to give three antiserum pools, antiRNAase A, antiRNAase S, and antiRNAase S-protein. To facilitate comparison of the studies of immunologic activity of ribonuclease with independent physicochemical studies of the RNAase molecules, the antisera were dialyzed against several changes of 0.8% NaCl containing 0.01 M phosphate buffer, pH 6.8 (see Figure 8).

Double diffusion in agar was carried out by the method of Ouchterlony (1948), employing flat-bottom glass plates and Feinberg-type agar gel cutters obtained from Consolidated Laboratories. Noble agar (0.8%) in 0.9% saline was used throughout these studies. All antigens were added to their respective wells at concentrations of 1.0 mg/ml and undiluted antiserum was added simultaneously to the antibody well. Quantitative precipitin curves and the amount of protein in the precipitate

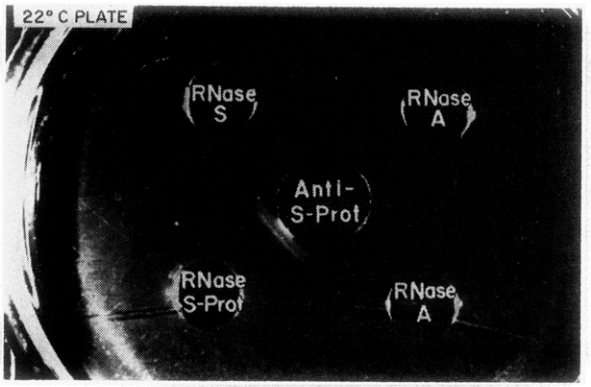

FIGURE 3: Immunodiffusion study in which undiluted antiribonuclease S protein serum is developed against ribonuclease A, S, and S-protein (antigen concentration 1 mg/ml).

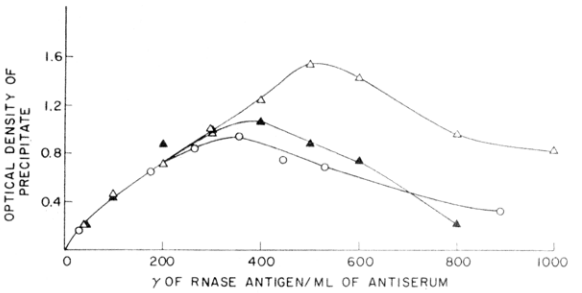

FIGURE 4: Quantitative precipitin curves where antiribonuclease A serum is allowed to react with ribonuclease A (Δ), ribonuclease S (▲), and ribonuclease S-protein (○). The studies, conducted with 0.5 ml of antiserum in phosphate buffer, are expressed as micrograms of antigen per milliliter of undiluted antiserum.

were determined by the method of Kabat and Mayer (1961), employing 0.25 N acetic acid to dissolve precipitates and measuring absorbance at λ 280 mμ. For study of heat denaturation, diffusion plates were incubated for periods from 12 hr to 6 days as required for the development of strong precipitin lines. The effects of heat were also evaluated by determining the amount of precipitate formed during precipitin reactions conducted at elevated temperatures. After 15 min of preincubation of antigen and antibody solutions separately at the chosen temperature, the antigen was rapidly added to the antibody solution. Following a 1-hr incubation, the tubes were transferred to a clinical centrifuge which had been heated in an oven to a temperature 5° warmer than the incubation temperature. The tubes were spun for 10 min at 2000 rpm, and the supernatant serum was poured off quickly before any substantial cooling of the mixture occurred. The precipitate was washed three times with the phosphate-buffered saline at 4°. The incubations in the presence of urea were carried out for 72 hr at 4°. All urea was freshly recrystallized from 95% ethanol.

In performing the quantitative precipitin curves with RNAase A antiserum and the three ribonuclease preparations (RNAase A, RNAase S, and RNAase S-protein), 0.5 ml of undiluted antiserum was mixed with 0.8 ml of phosphate-buffered saline. The antigen solutions were prepared at a concentration of 10 mg/ml in phosphate-buffered saline, the concentration being determined from the absorbance of each solution of the ribonuclease preparations. The incubations conducted at a higher temperature or in the presence of urea employed the same quantity of antiserum diluted with phosphate-buffered saline and 0.2 mg of RNAase A, RNAase S, or RNAase S-protein, a quantity of antigen close to the equivalence point for the three precipitin systems.

Results

Characterization of Antiserum. The RNAase A antiserum appeared to react with RNAase A as a single antigen–antibody system. In Figure 1A, it can be seen that RNAase S failed to bind all antibody molecules directed against RNAase A. This derivative had a reaction of incomplete identity with RNAase A. This spur can be somewhat better seen in Figure 1B where the plate has been allowed to incubate for a longer time. In addition, RNAase S-protein bound even less anti-RNAase A antibody, forming a stronger spur with ribonuclease A (Figure 1A). RNAase S′ (reconstituted RNAase S, made by mixing molar equivalent amounts of separated ribonuclease S-protein and S-peptide) cannot be immunologically distinguished from unseparated RNAase S (Figure 2). RNAase S was a poorer antigen and produced lower concentrations of antibody when it was used as the immunizing antigen. AntiRNAase S serum showed a stronger reaction with RNAase S and RNAase S-protein than it did with RNAase A, as can be seen in Figure 2A and 2B. RNAase A always gave fainter lines appearing later than the lines directed against RNAase S or RNAase S-protein with this antiserum. RNAase S-protein, when it was used as an immunizing antigen, produced an antiserum of very low precipitin titer. No reaction on Ouchterlony plates could be seen between antiRNAase S-protein serum and RNAase A; a distinct precipitin line was seen against RNAase S-protein and a just barely discernible line was seen against RNAase S (Figure 3).

The results of the quantitative precipitin curves were similar to those observed by Singer and Richards (1959) in that at equivalence, RNAase S produced less precipitate than RNAase A, and RNAase S-protein had the lowest amount of precipitate (Figure 4).

Heat Denaturation. The effects of heat on the antigenic reactivity of ribonuclease A and the two ribonuclease derivatives was surveyed, initially employing double diffusion in agar at various temperatures. There were no discernible effects during incubations on agar plates performed at 4, 15, 25, 45, and 55°, except for an increase in the rate of formation of the precipitin lines with the higher temperatures. However, at 65°,

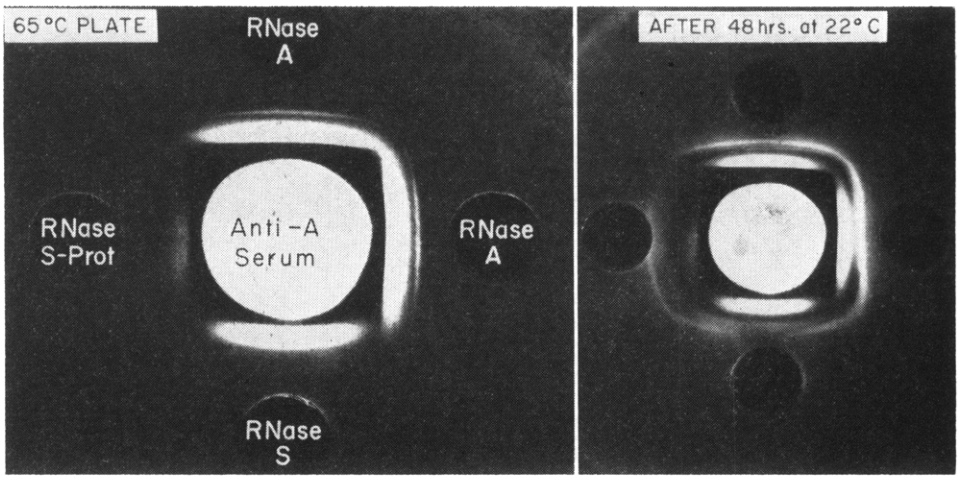

FIGURE 5: Immunodiffusion study in which undiluted antiribonuclease A serum is allowed to react with ribonuclease A, S, and S-protein at 65° and then for 48 hr at 22°.

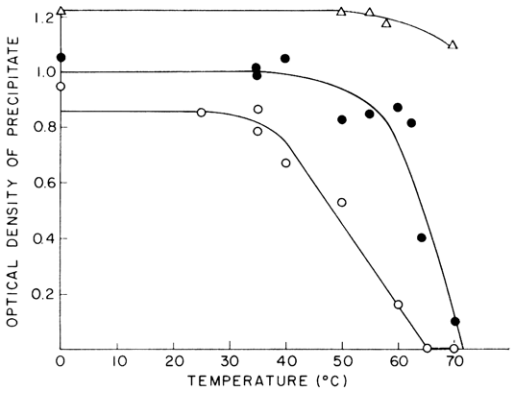

FIGURE 6: Reaction of antiribonuclease A serum in phosphate buffer with ribonuclease A (Δ), ribonuclease S (▲), and ribonuclease S-protein (○) at progressively increasing temperatures. In each incubation 200 μg of antigen is treated with 0.5 ml of antiserum; as in Figure 4, the amount of precipitate formed is expressed in terms of the quantity of precipitate formed per milliliter of antiserum.

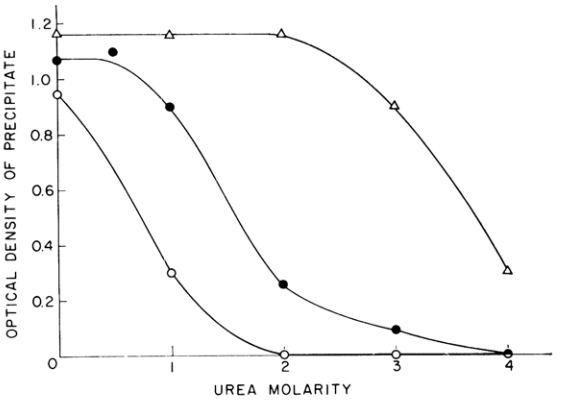

FIGURE 7: Reaction of antiribonuclease A serum with ribonuclease A (Δ), ribonulease S (●), and ribonuclease S-protein (○) in solutions of progressively increasing molarity of urea. Results expressed as quantity of precipitate formed per milliliter of antiserum by 400 μg of each antigen.

the ability of ribonuclease S-protein to precipitate antibody was almost completely lost and the capacity of the ribonuclease S to precipitate antiRNAase A was decreased; the antiS precipitin line shifted toward the antiserum well (Figure 5).[1]

These qualitative effects observed with Ouchterlony plates were more precisely determined by precipitin reactions in solution. A solution of 0.5 ml of anti-RNAase A serum diluted with 1 ml of buffer incubated at 65 and 70° revealed no nonspecific precipitation of serum protein. Figure 6 reveals the studies performed with the three ribonuclease preparations at the various temperatures. There was no decrease in the quantity of antigen–antibody precipitate formed in the reaction between RNAase A and antiRNAase A below 60°, with only a slight decrease being detected between 60 and 65°. In contrast, the ability of RNAase S to bind antibody diminished rapidly above 55°. The antigenic combining power of RNAase S-protein was even more sensitive to heat. Here, decrease in the quantity of precipitate was noted around 40°, and at 65° there was a complete absence of precipitation. When the clear supernatant, removed after centrifugation at high temperature, was allowed to cool and react at 4° in a cold

[1] When the plates were removed from the oven at 65° and kept at 22° for 48 hr, precipitin bands then formed on the antigen side of the previous precipitin line. These lines are of uncertain significance, but may represent effects due to antigen which refolded at the lower temperature, and then could react with antibody.

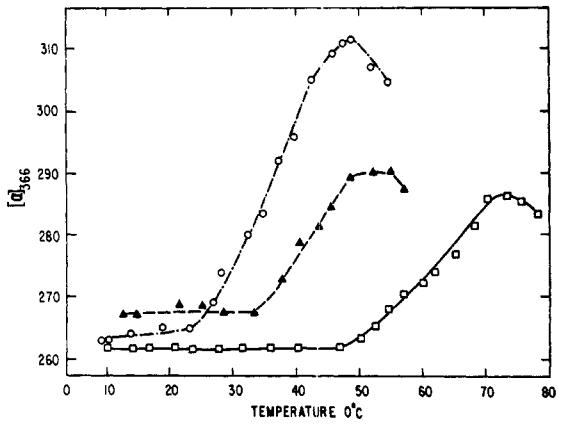

FIGURE 8: Effects of heat on the specific rotation of ribonuclease A (□), ribonuclease S (▲), and ribonuclease S-protein (○). Measurements of rotation were made at λ 366 mμ, evaluating the protein at a concentration of 3 mg/ml in solutions of 0.01 M phosphate, pH 6.8, containing 0.85% NaCl.

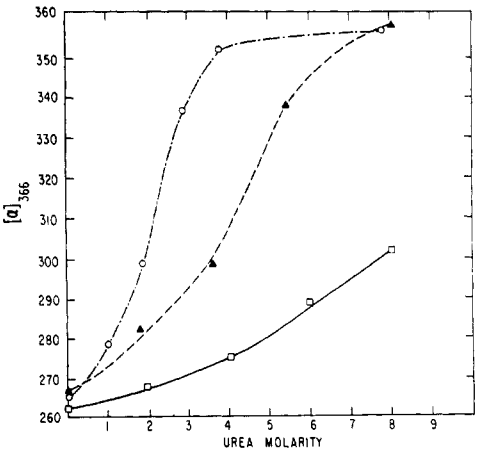

FIGURE 9: Effects of urea on the specific rotation of ribonuclease A (□), ribonuclease S (▲), and ribonuclease S-protein (○). Protein concentration was 3 mg/ml.

room, precipitate formed. The quantity of precipitate forming under these conditions closely approximated the amount obtained in a control mixture of RNAase S or RNAase S-protein with antiRNAase A antiserum incubated entirely at 4°.

Incubations were also performed at elevated temperatures employing the same three ribonuclease preparations and antiRNAase S antiserum. RNAase S forms less immune precipitate than RNAase A at elevated temperatures, even when the two antigens are assayed with antiRNAase S serum. The conditions of incubation and estimation of the quantity of precipitate using this antiserum were identical with those employed in the studies with antiRNAase A serum. With equivalent amounts of enzyme, the quantity of precipitate at each temperature was: RNAase S, 25°, 0.43; RNAase A, 25°, 0.36; RNAase S, 66°, 0.30; RNAase A, 66°, 0.44. (That is, the ratio of the quantity of precipitate formed by RNAase S *vs.* RNAase A declines 57% between 25 and 66°.)[2]

Denaturation with Urea. Figure 7 shows the results of incubating equivalent amounts of RNAase S-protein, RNAase S, and RNAase A at 4° against 0.5 ml of antiserum, in solutions of 0.01 M phosphate buffer containing 0.9% NaCl and various concentrations of urea. Urea (1 M) largely inhibited, and 2 M urea completely inhibited, the formation of immune precipitate between RNAase S-protein and antiRNAase A serum. RNAase S itself was somewhat inhibited in its antigenic combining power by 1 M urea. Again, the effects of urea do not seem to be related to alterations of the antibody, since the amount of precipitate formed in the reaction between RNAase A and antiRNAase A was still unchanged at a concentration of 2 M urea. At concentrations of 3 M urea, however, inhibition of precipitate formation is seen even with the reaction of RNAase A and antiRNAase antiserum.

Discussion

AntiRNAase A antiserum distinguished RNAase A, RNAase S, and RNAase S-protein on double diffusion in agar. RNAase S, although nearly identical in covalent structure with RNAase A, failed to precipitate all of the antibody directed against RNAase A. This is shown by the spur formation during immunodiffusion (Figure 1A). The results with quantitative precipitin curves confirmed the observations on Ouchterlony plates. All three ribonuclease preparations, A, S, and S-protein, strongly reacted with antiRNAase A serum, but the amount of immune precipitate and the equivalence point was lower for RNAase S than for ribonuclease A, and lowest for RNAase S-protein. While these observed differences in immune reactivity of the closely related RNAase preparations suggested the importance of conformation in antigen reactivity, the influence of the covalent differences between the three molecules could not be separately evaluated by this approach.

There are great differences in the behavior of the three ribonuclease molecules when they are employed as antigens in rabbits. Antibody made against ribonuclease S is of much lower titer than that made against RNAase A. Some fraction of antibody made against RNAase S is not bound by native RNAase A. With the antiserum made in two rabbits against ribonuclease S-protein, only a very low titer of precipitating antibody was obtained.

The principal evidence for the importance of precise folded structure in the antigenic species of the RNAase–antiRNAase system was afforded by the studies of the

[2] With the incubations performed for only 1 hr, RNAase A forms more immune precipitate at 66° than at 25°, as was noted in the results using antiRNAase A serum and the three RNAase antigens. However, the quantity of precipitate formed by RNAase S still declines at the higher temperature in reaction with its homologous antiserum.

immune reaction under denaturing conditions. By both immunodiffusion and antigen–antibody reactions in solution, reversible inhibition of precipitation was demonstrated in the reaction of RNAase S and RNAase S-protein with antiserum at elevated temperatures and concentrations of urea insufficient to affect the antibody species, as shown by the full precipitin reactivity retained by the RNAase A–antiRNAase A system. As noted in the studies of Sherwood and Potts (1965a,b) (Figures 8 and 9 are replotted from the data in those studies), RNAase S-protein is 50% denatured by heating to 35° or exposure to 2 M urea and RNAase S by heating to 41° or exposure to 4 M urea. The conformation of RNAase A is but little altered under these conditions; there is no marked change in its optical rotation (likewise, absorbance is little altered; Sherwood and Potts, 1965b). This marked discrepancy in response to denaturing agents results in a selective loss of ordered structure in the RNAase S derivatives when incubated at elevated temperature or in urea at concentrations of 0.5–4.0 M prior to their reaction with antibody.

The loss of precipitin reactivity with denaturation occurred with the reaction of RNAase S, not only with antiserum to RNAase A, but also with its homologous antiserum antiRNAase S. This indicated that we were not dealing merely with a greater heat sensitivity of the heterologous reaction, the derivatives *vs.* antiRNAase A. Rather RNAase S was the sensitive antigen under denaturing conditions whether evaluated against antiRNAase A or antiRNAase S, its homologous antiserum.

There is a selective, reversible, loss of the reactivity of RNAase S and S-protein against antiRNAase A antiserum under these conditions of denaturation. There is correlation between loss of immune reactivity and ease of denaturation in that RNAase S-protein is most easily affected, RNAase S intermediate in susceptibility, and the native molecule, RNAase A, quite resistant to loss of immune reactivity due to heating or incubation in urea. The covalent structure in the macromolecular disulfide containing region of the three molecules is identical and cross-chain groupings of amino acid residues constrained by a given set of disulfide bonds are maintained. The immunologic effectiveness of the antiserum was not altered, and the loss of precipitin reaction was fully reversible when the heated solutions were cooled.[3] This set of observations strongly suggested that the loss of immune reactivity of RNAase S-protein and RNAase S was due to disruption of the conformation of these RNAase derivatives. Conversely, the preservation of the folded structure of the native molecule results in persistent strong reaction of RNAase A with antibody in the presence of the same denaturing conditions which abolish the immune reactivity of the RNAase S derivatives.

The earlier studies with RNAase (Brown, 1959; Mills and Haber, 1963; Bennett and Haber, 1963; Singer and Richards, 1959) and the present report all indicated the importance of cross-chain groupings of amino acid residues, stabilized by precise interresidue associations into highly specific three-dimensional sites which confer antigenic reactivity on the protein.

Gerstein *et al.* (1963) have demonstrated that progressive denaturation by heat or alkali of pepsin and pepsinogen can reveal and subsequently destroy antigenic combining sites, using antisera prepared against native and denatured forms of the proteins. The specificity of these antisera clearly varied for different conformational states of the enzyme. Denaturation of the antigen was performed separately, then it was rapidly added to antibody. At higher temperatures, much of the loss of immune reactivity was irreversible.

The studies of Gould *et al.*, with synthetic polypeptides and their respective antigens (1964), the variation in antigenic reactivity of covalently similar insulin preparations attributed to conformational differences (Berson and Yalow, 1961), the influence of acid on dissociability of various protein antigen–antibody combinations (Givol *et al.*, 1962), and several studies with other protein precipitin reactions in response to solvent changes (reviewed by Gould *et al.*, 1964) indicate the growing generality of the concept that specific conformation is closely related to antigenic reactivity with protein antigens. As a further demonstration of the importance of conformation in antigenic reactivity, the present studies permitted a comparison under identical conditions of the reversible loss of reactivity of the antigen with its reversible unfolding as assessed by independent methods. The unchanged results in the control reaction, RNAase A with antiRNAase A serum, excluded effects on antibody in explaining the influence of denaturants on precipitin reactivity.

[3] In addition to the reappearance of precipitate after cooling, there was essentially complete (90–100%) preservation of enzymic activity when activity was measured with samples of the RNAase preparations which had been heated or exposed to concentrations of urea in a manner similar to the conditions used in the immunologic studies. This provided further indication that disulfide interchange or irreversible structural changes were not responsible for the loss of immunologic reactivity of the RNAase derivatives.

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Glutamine Synthetase. Determination of Its Distribution in Brain during Development*

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ABSTRACT: Glutamine synthetase and glutamyl transferase activity have been assayed in the neocortex, hippocampus, cerebellum, diencephalon, and pons–medulla of developing kittens and mature animals. The method used for synthetase assay enabled the determination of enzyme activity in homogenates of whole brain tissue. The five brain areas showed different patterns of development for the enzyme. The neocortex had the sharpest increase in enzyme activity and achieved the highest values. In the neocortex the rate of enzyme development coincided with the pattern of development of the compartmentation of glutamic acid metabolism (Berl, 1965); this was not true for the other brain areas (Berl and Purpura, 1965).

The presence in brain of metabolic compartments or pools of glutamic acid which are not in rapid equilibrium with each other has been established by several investigations (Berl *et al.*, 1961 Berl *et al.*, 1962a; 1962b; Waelsch *et al.*, 1964). However, evidence for a separate pool of glutamic acid preferentially used for glutamine formation appears in various parts of the brain at different periods during ontogenesis (Berl, 1965; Berl and Purpura, 1965). Since glutamine synthetase activity may contribute to the emergence of the glutamic acid–glutamine compartmentation system, the changes in levels of this enzyme in the neocortex (cerebral cortex), hippocampus, cerebellum, diencephalon, and brain stem (pons–medulla) of the postnatal animal was followed to determine the extent of its coincidental or correlative development with the development of such compartmentation.

The determination of glutamine synthetase activity in brain tissue homogenates is hampered by the inhibitory effect of adenosine diphosphate (ADP) (Elliott, 1951). It has also been described that the presence of adenosine triphosphate (ATP) and Mg^{2+} offers at least 85% protection against enzyme inactivation at 60° for 10 min (Pamiljans, *et al.*, 1962). A method is herein described which takes advantage of the latter finding for the inactivation of ATPase in an assay for glutamine synthetase activity in whole brain tissue. Glutamyl transferase activity was determined for comparison as an aid in the evaluation of the method; there is little doubt that glutamine synthetase and glutamyl transferase activities are associated with the same enzyme (Meister, 1962). The activity of the enzyme in sixteen brain areas of the adult cat are also reported.

Methods

The glutamine synthetase method is based on the elimination of the inhibitory effect of ADP. This is accomplished by heat inactivation of ATPase (10 min at 58–60°). The addition of ATP and Mg^{2+} protects the synthetase enzyme from inactivation (Pamiljans *et al.*,

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