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Immunoglobulins consist of heavy (H) and light (L) polypeptide chains including those from primitive vertebrates (Grey, 1969). The lower vertebrates, such as cyclostomes (Marchalonis and Edelman, 1968) and elasmobranchs (Marchalonis and Edelman, 1965; Clem *et al.*, 1967; Clem and Small, 1967), have only one immunoglobulin class and it grossly resembles human IgM. Distinct classes of H chains, which resemble the H chains of human IgM and IgG, occur in amphibians (Marchalonis and Edelman, 1966). Studies on the antibody responses of reptiles have shown the presence of only two molecular species of antibodies (Grey, 1963; Lykakis, 1968); that is, high and low molecular weight antibodies. Marchalonis *et al.* (1969) reported that the tuatara, *Sphenodon punctatum*, a lizardlike "living fossil", had 18S and 7S immunoglobulins, and that they were composed of H and L chains. To date, there are no data reported that reptiles have antigenically different H chains. In the present study on the immunoglobulins of the sea turtle, 17S and 7S antibodies were found and, to our surprise, a third antibody with a $s_{20,w}$ value of 5.7 was demonstrated. Based on antigenic differences, they represented different immunoglobulin classes.

Male and female sea turtles from the local Hawaiian population, weighing approximately 25 to 400 lbs, were maintained in deep salt water tanks at the Waikiki Aquarium. Some animals were given repeated intracardial injections of either 18 to 80 mg of bovine serum albumin (BSA) or 5 – 40 mg of highly substituted 2,4 dinitrophenyl-bovine gamma globulin (DNP-BGG). Other animals were given repeated intramuscular injections in the thighs of the hind legs of these antigens in Freund's complete adjuvant. Intracardial injections and bleedings by heart puncture were made through a 6 mm hole drilled in the ventral pastron at the approximate level of the heart.

Examination of whole turtle serum in the Spinco Model E ultracentrifuge showed major peaks with sedimentation coefficients of 3.8S, 5.3S, 7.0S, and 14.5S. The concentration of 7S was much lower than the concentration of the 5S component. The 17S protein was isolated by successive precipitations of the globulins from serum with Na_2SO_4 followed by recycling through Sephadex G-200 equilibrated with borate buffer (pH 8.2, $r/2 = 0.16$), and the 17S anti-DNP antibody was recovered from this preparation by specific purification by affinity chromatography (Cuatrecasas *et al.*, 1968; Wofsy and Burr, 1969). The 7S was similarly purified, but with difficulty because of the low concentration. The 5S was easily purified and it was

the only protein to be eluted from DEAE-Sephadex equilibrated with 0.04M PO_4, pH 8.0. The sedimentation coefficients *vs.* concentrations of the purified proteins in borate buffer gave $s_{20,w}$ values at infinite dilution of 5.7, 7.3 and 17.6 respectively. In immunoelectrophoresis (IE) the 5.7S formed a long slow-moving arc in the γ-globulin region (Plate 1A); the 7S arc was faster moving and in whole serum it was located "inside" the 5S arc (Plate 1E). The purified 17S usually formed a double precipitin arc resembling mammalian IgM (Plate 1C); however, it was not detected often in whole serum.

The antibody responses to BSA and to DNP each was demonstrated by means of radioimmunoelectrophoresis (Yagi *et al.*, 1962) (RIE). Heavily substituted 2,4 dinitrophenylhuman serum albumin (DNP-HSA) and BSA each labelled with ^{131}I according to the method of Greenwood and Hunter (1963) and rabbit anti-turtle globulin antiserum, were used for RIE. The first antigen-binding (antibody activity) was detected 4 to 8 weeks following injection of 10 turtles with BSA. No obvious differences in the earliest response were noted between animals whether they had been injected intracardially or intramuscularly, or whether they were given single or multiple injections. All turtles showed only 7S binding (Plate 1E). Preimmune sera showed no nonspecific binding of BSA ^{131}I. Twenty-eight days following a second intracardial injection (101 days following the primary infection), the serum of one turtle showed heavy 5.7S binding as well as 7S binding (Plate 1F). Four turtles whose early bleedings had only 7S binding, were maintained for two years, and one year following their last injections both 5.7S and 7S binding were observed. Only one turtle showed weak binding of BSA in the 17S arc. Thus, as detected by RIE, it appeared that the 7S response occurred before the 5.7S response, but the 5.7S binding was intense 2 years following repeated injections. The failure to detect 17S antibody was confirmed by the absence of antibody in Sephadex G-200 17S fractions of anti-BSA sera when the passive hemagglutination test was employed.

In contrast to the BSA response, 4 weeks following a single intracardial injection of DNP-BGG, both the 5.7S and 7S immunoglobulins bound DNP-HSA^{131}I intensely (Plate 1H). Antigen also was weakly bound by an arc on the anodal side of the center well, which tentatively has been identified as α_2-macroglobulin (Plate 1). In preimmune sera the 7S protein and the α_2-macroglobulin both bound DNP-HSA weakly (Plate 1G). This was due to the binding of DNP since HSA^{131}I was not bound nonspecifically. The anti-DNP antibodies were specifically purified by affinity chromatography (Cuatrecasas *et al.*, 1968). The immunoabsorbent was prepared by coupling δ-N-2,4-DNP-ornithine to CNBr-activa ed Sepharose (Wofsy and Burr, 1969). Dinitrophenyl-glycine was used to elute the antibodies. The yields of protein from normal serum was 0.07 mg/ml whole serum; the α_2-macroglobulin was not detected in IE. As an example, 28 days following injection of a turtle with 40 mg of DNP-BGG, there were 0.161 mg of antibody per ml of whole serum. A second injection consisting of 10 mg DNP-BGG in Freund's adjuvant given 9 months later boosted the amount of antibody to 0.432 mg/ml of serum 14 days later, and a third injection given 42 days later boosted the amount to 1.56 mg/ml serum 14 days following injection. Although definite booster responses were obtained in this animal the relative concentrations of the three antibodies remained about the same for this period of time. The per cent relative composition of the antibodies sedimenting in the ultracentrifuge was determined by area measurements. The percentages of the 5.7S, 7S and 17S proteins ranged from 23 to 30, 30 to 50, and 37 to 39, respectively, over this period of time.

In Ouchterlony plates and with rabbit anti-whole turtle serum the 5.7S immuno-

globulin was antigenically deficient with respect to the 7S and 17S immunoglobulins (Plate 2). Specifically purified 17S contained two precipitin bands. One of these showed a closer antigenic relationship to the 7S (faint spur) than to the 5.7S (large spur). The other 17S band had no obvious identity with the 5.7S and seemed to form a line of partial identity with the 7S. In IE the 17S formed a double arc (Plate 1C). Absorption of the antiserum with 5.7S removed the antibodies directed to that 17S band which had shown a close relationship to the 7S. As shown in Plate 2 with the absorbed serum the remaining 17S band had no cross-reactivity with the 7S; and in IE only a single 17S arc was seen (Plate 1D). The 5.7S antibody which had been reduced with 0.1M 2-mercaptoethanol formed a line of complete identity with unreduced 5.7S (Plate 2B). To further resolve these antigenic relationships analyses with isolated H chains are in progress.

It is clear that sea turtles are capable of anamnestic responses; and that they have at least 3 classes of immunoglobulins. In none of the previous studies (Grey, 1963; Lykasis, 1968; Lerch *et al.*, 1967; Ambrosius, 1966; Rothe and Ambrosius, 1968) on reptilian immunoglobulins has the 5.7S antibody been reported. A comparable immunoglobulin occurs in the duck (Grey, 1967a, b), in the Australian lungfish (Marchalonis, 1969) and in the goose (Benedict and Pollard — unpublished results). The molecular weight of the duck and lungfish 5.7S immunoglobulins is about 120,000 (Marchalonis, 1969; Zimmerman *et al.*, 1970). The molecular weight of their L chains is about 20,000 (Marchalonis, 1969; Zimmerman *et al.*, 1970), which is similar to the molecular weight of L chains of higher vertebrates. However, the molecular weight of the H chains is about 35,000 (Marchalonis, 1969; Zimmerman *et al.*, 1970), a value lower than that of any of the other major classes of H chains. The structural characteristics of the sea turtle 5.7S antibody will be reported shortly. It is not known what selective pressures are operating to cause the evolution of an immunoglobulin with a smaller H chain in such taxonomically-unrelated species of animals. Perhaps it has gone unrecognized in other species.

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The plates will be found at the end of the issue.

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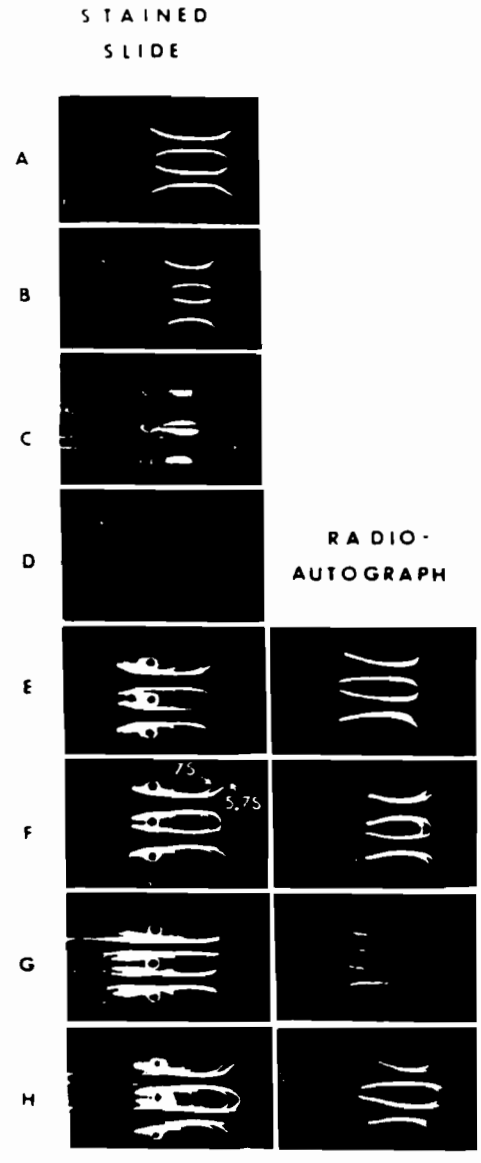

PLATE 1. Immunoelectrophoresis of purified turtle immunoglobulins and radioimmunoelectrophoresis of antisera. A — 5.7S; B — 7S; C — 17S; D — 17S; E — anti-BSA serum; F — anti-BSA serum; G — normal serum; H — anti-DNP serum. Rabbit anti-turtle globulin antiserum was added to all troughs except in D. Rabbit anti-turtle globulin, which had been absorbed with 5.7S protein, was added to D. $BSA^{131}I$ was added to troughs in E, F and G. DNP-$HSA^{131}I$ was added to troughs in C and H.

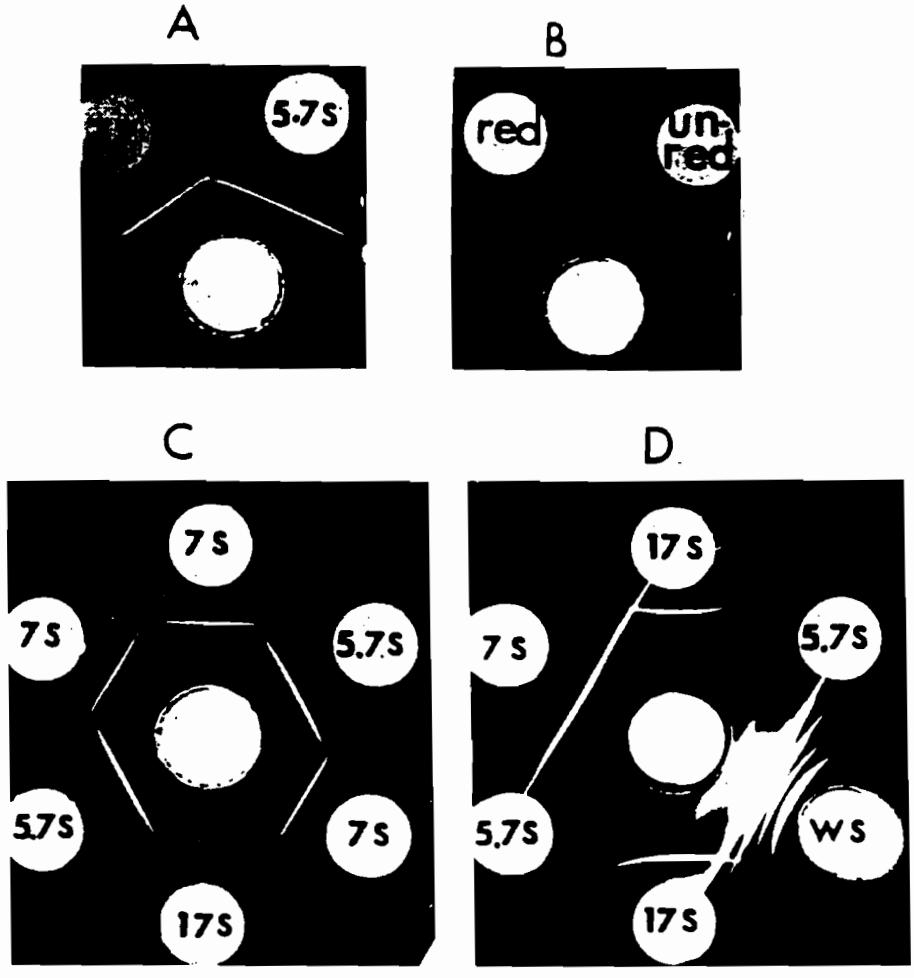

PLATE 2. Immunodiffusion in agar of turtle immunoglobulins. A — purified 5.7S and 7S; B — reduced and unreduced 5—7S; C — 5.7S and 17S; D — 5.7S, 7S, 17S and whole serum. Rabbit anti-turtle globulin was antiserum added to all center wells except in D. Rabbit anti-turtle globulin, which had been absorbed with 5.7S protein, was added to D.

ERRATA

REPRODUCTION FAILS TO SHOW LINE OF IDENTITY IN B AND SPUR BETWEEN 5.7S & 7S IN C.