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Structural Studies on the Duck 5.7S and 7.8S Immunoglobulins*

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ABSTRACT: Structural studies on the duck 5.7S and 7.8S immunoglobulins revealed the former to have a molecular weight of 118,000, an Fab fragment of 48,000, and component heavy and light chains of 35,000 and 23,000, respectively. The carboxy-terminal residue on the heavy chain was phenylalanine and the molecule had a relatively low total carbohydrate content of 0.6%, which was localized to the Fab fragment. The 7.8S immunoglobulin was shown to have a molecular weight of 178,000 with a heavy chain of 62,000–66,000. The total carbohydrate content was 5.0%. Both proteins are structurally distinct from previously described mammalian immunoglobulins. The phylogenetic implications of these findings with respect to immunoglobulin evolution are discussed.

The study of nonmammalian γ-globulins has provided a useful tool for the analysis of immunoglobulin evolution. Avian antibodies which differ in certain respects from mammalian have been studied extensively in two species, the duck and the chicken (Leslie and Clem, 1969; Dreesman and Benedict, 1965; Tenenhouse and Deutsch, 1966). In the duck two classes of low molecular weight immunoglobulins exist, the biologic properties of which have been investigated and reported previously (Grey, 1967a,b). In brief, following immunization with bovine serum albumin, both 7.8S and 5.7S antibovine serum albumin antibodies were found, although late in the immune response the 5.7S antibody predominated. The 7.8S antibodies fixed duck complement and fixed to duck skin whereas the 5.7S antibody gave negative results in both these systems. Experiments were also performed to rule out generation of the 5.7 S molecule through degradation of the 7.8 S. Injection into ducks of isotopically labeled 7.8S protein demonstrated that the 5.7S immunoglobulin was not a breakdown product of the larger protein.

In this paper we describe some of the major structural characteristics of these two low molecular weight immunoglobulins.

Materials and Methods

Protein Isolation. Adult Pekin and Muscovy male ducks were immunized by weekly intramuscular injections of 1 mg of bovine serum albumin in complete Freund's adjuvant. Sera from these ducks were fractionated in two steps (Grey, 1967a,b): starch block electrophoresis followed by application of the γ-globulins to an upward-flow Sephadex G-200 or Bio-Gel P300 column from which they were eluted with phosphate-buffered saline.

Antigenic Analysis of γ-Globulins. Antisera were prepared in rabbits against whole duck serum and purified γ-globulins by repeated footpad injections of 1 mg of the antigen in complete Freund's adjuvant. Ouchterlony gel diffusion (Ouchterlony, 1953) and immunoelectrophoresis (Scheidegger,

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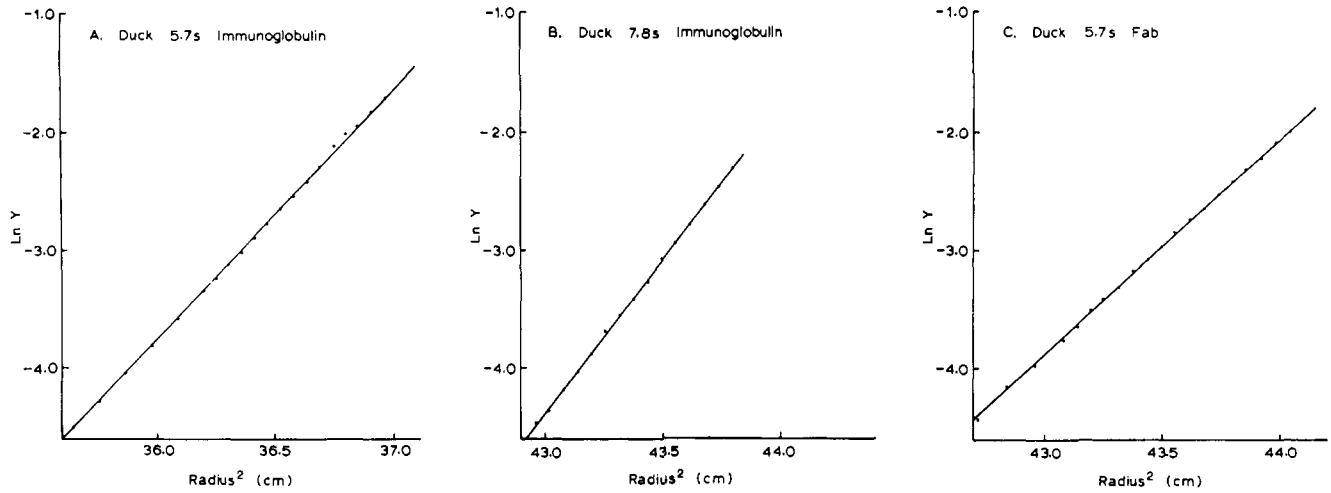

FIGURE 1: Representative plots of the logarithm of concentration (ln of vertical fringe displacement from the base line in centimeters) against the square of the distance from the center of rotation (radius²): (A) duck 5.7S immunoglobulin; (B) duck 7.8S immunoglobulin; (C) duck 5.7S Fab. The solvent used was phosphate-buffered saline.

1955) were used to analyze the various immunoglobulin preparations. Both the antiwhole serum and the anti-7.8S immunoglobulin were capable of recognizing antigenic differences between the 7.8S and 5.7S immunoglobulins such that the 7.8S protein spurred over the 5.7S on immunodiffusion analysis. However, even after repeated immunization with 5.7S material, it was not possible to obtain an antiserum recognizing determinants specific for the 5.7S protein.

Enzymatic Digestion of γ-Globulins. Papain digestion was performed using a 1% by weight enzyme:protein ratio with an incubation period of 4 hr at 37° in the presence of 0.01 M cysteine and 0.002 M EDTA. Proteolysis was stopped by alkylation with a fivefold molar excess of iodoacetamide for 1 hr at 0°, followed by dialysis against phosphate-buffered saline.

Reductive Cleavage of γ-Globulins. Mild reduction was performed by treating 60–80 mg of protein in 0.55 M Tris, pH 8.2, for 1 hr at room temperature with 0.2 M 2-mercaptoethanol. Alkylation was accomplished by the addition of 0.3 M recrystallized iodoacetamide and allowing the sample to stand for 1 hr in the cold. The products were then dialyzed 2–3 hr against phosphate-buffered saline to remove the reagents and finally dialyzed overnight against 8 M urea in 0.05 M propionic acid. Separation of heavy and light chains was achieved by passage through a G-200 Sephadex column (2 × 100 cm) equilibrated with 8 M urea in 0.05 M propionic acid.

Extensive reduction of these proteins for determination of molecular weights was done on 60–80 mg of material dialyzed against 8 M urea in 0.1 M Tris buffer, pH 8.2. The remainder of the procedure was the same as for the mild reduction method except that after alkylation the sample was immediately dialyzed overnight against 8 M urea in 0.05 M propionic acid.

Molecular Weight Determinations. Molecular weights of the intact proteins and fragments were determined by high-speed sedimentation equilibrium (Yphantis, 1964) in a Spinco Model E ultracentrifuge utilizing interference optics and a temperature control unit. Fringe patterns were recorded and analyzed on a comparator. The molecular weights were calculated as described by Yphantis (1964) using a partial specific volume ($\bar{V}$) calculated from the amino acid and carbohydrate composition of the protein (Cohn and Edsall, 1943).

Molecular weights of extensively reduced heavy and light chains were calculated by their elution volumes on an upward flow (2 × 100 cm) Sephadex G-200 column equilibrated with 8 M urea in 0.05 M propionic acid. Prior to reduction of the intact proteins an internal marker of ^{125}I-labeled human γ-G was added to the sample. By plotting the square root of the distribution coefficient $\sqrt{K_d}$ of the marker polypeptide chains on the column against the square root of their respective molecular weights, the $\sqrt{K_d}$ of the experimental material could be placed and the unknown molecular weights determined (Andrews, 1964). K_d for a given protein is calculated by the formula: $K_d = (V_e - V_0)/V_i$ in which V_0 = excluded volume; V_e = elution volume of a particular substance; V_i = included volume = total volume − excluded volume.

Carbohydrate Determinations. Hexoses were determined by the Orcinol reaction (Svennerholm, 1956) with the modifications previously described (Abel *et al.*, 1968). Hexosamines were determined on an amino acid analyzer (Abel *et al.*, 1968), L-fucose by the cysteine reaction after sulfuric acid hydrolysis (Dische and Shettles, 1948), and sialic acid by the thiobarbituric acid assay (Warren, 1959).

Amino Acid Analysis. Carboxy-terminal residues of isolated heavy chains were analyzed by hydrazinolysis and carboxypeptidase A digestion (Abel and Grey, 1967).

Amino acid composition of the immunoglobulins and their chains were determined on a Beckman Model 120-C amino acid analyzer.

Starch Gel Electrophoresis. Vertical starch gel electrophoresis (Smithies, 1959) was performed using an acid urea–formate buffer, pH 3.0 (Poulik, 1960) or a glycine buffer, pH 8.8 (Fahey, 1963).

Results

Isolation of Duck Immunoglobulins. γ-Globulins applied to a Sephadex G-200 or Bio-Gel P300 column eluted in three peaks, the first of which emerged in the void volume, and contained the duck γ-M; the second peak consisted of the 7.8S immunoglobulin and the third, 5.7 S (Grey, 1967a).

The distribution of γ-globulins in nonimmune serum

TABLE I: Sedimentation Equilibrium Data for Intact Duck 5.7S and 7.8S Protein and the 5.7S Fab Fragment.[a]

Protein	Number of Determinations	Protein Concentration (mg/ml)	$\bar{V}$[b]	rpm	Average Molecular Weight
5.7 S	3	0.1–0.4	0.725	17,250	118,000 (116,000–120,000)
5.7 S Fab	2	0.15–0.6	0.720	25,630	48,000 (47,000–48,000)
5.7 S partially reduced	3	0.17–0.7	0.725	25,980	58,000 (56,000–59,000)
7.8 S	3	0.15–0.6	0.716	15,220	178,000 (175,000–179,000)

[a] Samples in phosphate-buffered saline (pH 7.2) were run at controlled temperature of 20° in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Molecular weights were calculated from the plot of ln C *vs.* r^2 (Figure 1). The values presented are averages of the number of determinations shown. The range of values is indicated in parentheses. Solvent density (ρ) was taken as 1.0 for all calculations. [b] Partial specific volume ($\bar{V}$) was calculated from amino acid and sugar composition (Cohn and Edsall, 1943).

differed between Pekin and Moscovy sera. The former contained 7.8S and 5.7S γ-globulin in equal amounts while the latter demonstrated greater quantities of 7.8 S. However, repeated immunizations led to a marked preponderance of 5.7 S in both species. Pure samples of each γ-globulin were obtained by pooling the leading edge of the second column peak and the center of the third. When necessary the recovered proteins were recycled over the columns.

Molecular Weights of Intact Proteins and Fragments. Prior to all molecular weight determinations by the Yphantis method, sedimentation velocity runs were routinely performed in order to verify sample homogeneity. Immediately following the sedimentation velocity, the high-speed equilibrium determinations were performed at three different sample dilutions. In no instance was concentration dependence noted. Representative plots of ln C *vs.* r^2 are shown in Figure 1 (A and B) for both intact immunoglobulins. The resultant straight lines verified the homogeneity of the preparations. The calculations based on the respective slopes gave molecular weights of 118,000 for the 5.7S protein and 178,000 for the 7.8S protein (Table I).

Papain digestion of these proteins, under a variety of conditions, yielded an unequivocal Fc fragment only in the 7.8S preparation. Fab fragments as well as some undigested material were identified in the papain digest of both 5.7S and 7.8S proteins by immunodiffusion and starch gel electrophoresis. The Fab was recognized by its faster migration than whole protein on starch gel, its reaction of identity with pepsin-digested material and its antigenic deficiency compared to whole protein when analyzed by immunodiffusion.

The Fc fragment cleaved from the 7.8S γ-globulin was characterized by its greater anodal migration on immunoelectrophoresis when compared with either the Fab or whole protein. No such precipitin band was seen on immunoelectrophoresis of the 5.7S digest either before or after removal of undigested whole protein by passage through a Sephadex G-100 column.

The 5.7S Fab fragment was isolated with a 75% yield and its molecular weight as determined by Yphantis sedimentation equilibrium was 48,000 (Figure 1c, Table I).

Molecular Weights of the Polypeptide Chains. The molecular weights of the component polypeptide chains of the duck 5.7S and 7.8S immunoglobulins were determined on Sephadex G-200 columns equilibrated with 8 M urea in 0.05 M propionic acid using simultaneously reduced and alkylated ^{125}I-labeled human γ-globulin in trace amounts as a marker. This allowed comparison of the elution volumes of the duck immunoglobulin chains with human γ-globulin heavy and light chains of known molecular weight (human γ-globulin heavy = 50,000 and light = 23,000). After extensive reduction, the duck 5.7 S eluted in two peaks, the heavier of which emerged later than human heavy chain and the lighter cochromatographed with human light chains. The duck 7.8 S also gave two peaks but the heavy chain emerged ahead of human γ chains while the light chains again coincided with human light chains. The 5.7S heavy chains were recovered with a yield of 58% of the starting material and were calculated

TABLE II: Molecular Weights of 5.7S and 7.8S Polypeptide Chains as Estimated from Their Distribution on Sephadex.[a]

Protein	$\sqrt{K_d}$	Molecular Weight (Literature Value)	Calculated Molecular Weight
γ-Chain (human)	0.3943	50,000	
L-Chain (human)	0.5955	23,000	
5.7S H-Chain	0.4994		35,000
5.7S L-Chain	0.5955		23,000
γ-Chain (human)	0.4717	50,000	
L-Chain (human)	0.6460	23,000	
7.8S H-Chain	0.4164		62,000
7.8S L-Chain	0.6460		23,000

[a] Gel filtration of completely reduced and alkylated 5.7 and 7.8S light and heavy chains done on two different Sephadex G-200 columns each equilibrated with 8 M urea in 0.05 M propionic acid. The differences in $\sqrt{K_d}$ of human γ-chains and light chains are due to differences in the void volumes of the two columns.

TABLE III: Amino Acid Composition of Duck 5.7S and 7.8S Immunoglobulins.

	5.7S H		7.8S H		Human γ-G H[a]		5.7S L		7.8S L		5.7S Fab	
Amino Acids	g %[b]	Mole/ Mole	g %[b]	Mole/ Mole	g %	Mole/ Mole	g %[b]	Mole/ Mole	g %[b]	Mole/ Mole	g %[b]	Mole/ Mole
Lysine	4.23	11.6	4.27	19.3	7.06	29.0	5.28	9.5	5.72	10.3	5.61	21.0
Histidine	3.48	8.9	3.85	16.3	2.44	9.6	1.67	2.8	1.61	2.7	2.82	9.9
Arginine	5.68	12.7	5.50	20.5	4.02	12.0	3.68	5.4	2.97	4.4	4.33	13.3
Aspartic acid	6.02	18.3	5.92	29.8	7.77	33.0	8.12	16.2	8.06	16.1	6.95	29.0
Threonine	9.58	33.2	9.30	53.4	7.04	34.0	10.37	23.6	10.37	23.6	9.77	46.4
Serine	8.82	35.5	8.72	58.1	9.13	50.0	11.77	31.1	12.40	32.7	11.71	64.5
Glutamic acid	11.28	30.6	11.64	52.3	11.18	39.0	11.83	21.1	14.24	25.4	11.66	43.3
Proline	6.54	23.6	8.02	47.1	6.40	33.0	5.48	13.0	5.28	12.5	5.38	17.0
Glycine	6.49	39.8	5.58	56.7	3.37	28.0	5.37	21.7	5.05	20.4	7.21	60.5
Alanine	5.88	28.9	5.66	46.2	3.29	19.0	4.35	14.1	4.08	13.2	5.19	35.0
Valine	7.57	26.7	6.67	39.0	7.92	41.0	7.65	17.7	8.09	19.3	7.02	33.9
Methionine	1.09	2.9	1.31	5.8	0.93	3.9	0.93	1.6	0.83	1.5	1.01	3.7
Isoleucine	2.24	6.9	2.40	12.3	2.16	8.2	3.26	6.6	2.94	6.0	2.81	11.9
Leucine	6.31	19.5	7.66	39.3	7.40	31.0	5.73	11.7	5.04	10.3	5.28	22.4
Tyrosine	4.54	9.7	3.62	12.9	5.76	17.0	6.89	9.7	6.19	8.7	5.43	16.0
Phenylalanine	5.08	12.1	5.49	21.6	4.07	13.0	3.73	5.8	2.75	4.3	4.01	13.1
CM cysteine												
Total reduction	5.14	11.2	4.38	15.8	2.07	6.9	3.88	5.5	4.35	6.2	3.80	11.3
Partial reduction		5.2				3.6		1.7				5.2
Total	99.97	332.1	99.99	546.4	92.01	407.6	99.99	217.1	99.97	217.6	99.99	452.2

[a] Chaplin *et al.* (1965). [b] Calculated using the total micrograms of amino acid residues recovered as 100%.

to have a molecular weight of 35,000 (Table II). The 7.8S heavy chain had a molecular weight of 62,000 and the light chain again was 23,000. On the basis of a molecular formula of H_2L_2 the molecular weights of the intact proteins would be 116,000 for the 5.7S immunoglobulin and 170,000 for the 7.8S immunoglobulin. Both figures are in quite good agreement with the data obtained from sedimentation equilibrium.

Interchain Bond Structure. Neither the 5.7S nor 7.8S immunoglobulins when applied in the unreduced state to an 8 M urea–formate, pH 3, starch gel, released light chains or light-chain dimers indicating the presence of both L–H and H–H disulfide bridges in the proteins.

In an earlier study it was found that after mild reduction, the 5.7S immunoglobulin in the absence of denaturing agents, had a sedimentation coefficient of 3.5 S (Grey, 1967a). It was suggested that rupture of the interchain disulfides resulted in half-molecule formation in the absence of denaturants. However, conformational changes leading to an increase in the frictional coefficient without a change in molecular weight could not be ruled out. Additional studies were undertaken to further evaluate these results. After mild reduction with 0.2 M 2-mercaptoethanol, a homogeneous protein resulted which did not display evidence of free light-chain release when analyzed by starch gel electrophoresis, immunoelectrophoresis, and double diffusion in agar using antisera capable of recognizing free light chains. By sedimentation equilibrium the mildly reduced protein had a molecular weight of 58,000 one-half the figure obtained for the unreduced 5.7S protein, thereby proving that partial reduction of this protein created stable L–H half-molecules (Table I).

Amino Acid and Carbohydrate Analysis. Carboxy-terminal analyses of both the 5.7S and 7.8S heavy chains were performed by hydrazinolysis and carboxypeptidase A digestion. Repeated determinations by both methods yielded phenylalanine as the C-terminal residue of the 5.7S chain. No other amino acids were released even after prolonged digestion with carboxypeptidase. Cyanogen bromide cleavage failed to yield a small C-terminal peptide suitable for amino acid sequence analysis. In the case of the 7.8S protein, both methods were unsatisfactory in identifying the C-terminal residue. Carboxy-terminal analyses of isolated light chains were not done.

Amino acid compositions for heavy and light chains of both the duck immunoglobulins and 5.7S Fab are shown in Table III. Values from the literature for human γ chain are included for comparative purposes. When calculated as gram per cent, the amino acid compositions of the duck 5.7S and 7.8S heavy chains were very similar but differed significantly at certain residues from human γ chains; notably in lysine, glycine, and alanine.

Half-cystines were calculated as the carboxymethyl derivatives following partial as well as complete reduction of the polypeptide chains and Fab fragment. Following partial reduction and alkylation, 5.2 carboxymethylcysteines (CMCys) were found in the 5.7S heavy chain and 1.7 CMCys per light chain. These figures are somewhat higher than those obtained for mammalian γ-G polypeptide chains, especially considering the shorter chain length of the 5.7S heavy chain. Similarly, the 5.7S Fab fragment contained 5.2 CMCys after partial reduction and alkylation. This is considerably higher than the value of 2 obtained with mammalian γ-GFab fragments and suggests that labile intrachain disulfides were split within the Fd fragment. After complete reduction and al-

TABLE IV: Carbohydrate Composition of Duck 5.7S and 7.8S Immunoglobulins.

	5.7S		5.7S FAB		7.8S		Human γ-G[a]	
	g/100 g	Mole/ Mole	g/100 g	Mole/ Mole	g/100 g	Mole/ Mole	g/100 g	Mole/ Mole
N-Acetyl-glucosamine	0.28	1.4	0.11	0.2	1.60	12.5	1.46	10.1
Hexose	0.28	1.9	0.55	1.5	3.11	31.0	1.28	11.4
Sialic acid	0.09	0.3			0.26	1.5	0.15	0.8
L-Fucose	0.12	1.0			0.42	4.4	0.23	2.2

[a] Abel *et al.* (1968).

kylation, 11.2 CMCys were found in the 5.7S heavy chain and 15.8 in the 7.8S heavy chain. This latter figure is higher than that obtained with mammalian γ-G proteins and is in the range found for mammalian α chains.

The carbohydrate composition, that is, the content of *N*-acetylhexosamine neutral hexose, L-fucose, and sialic acid is shown in Table IV. The 5.7S γ-globulin had a very low total carbohydrate of 0.6% with individual values for *N*-acetylglucosamine of 1.4 moles/mole of protein; hexose 1.9 moles/mole of protein; fucose 1.0 mole/mole of protein and sialic acid 0.3 mole/mole of protein. Sugar determinations on the H and L chains and Fab fragment indicated that the carbohydrate was localized to the Fd portion of the heavy chain and to the light chain. The 7.8S immunoglobulin had a total carbohydrate content of 5% with a relatively high hexose value of 31.0 moles/mole of protein, 12.5 moles/mole of *N*-acetylglucosamine, 4.4 moles/mole of fucose, and 1.5 moles/mole of sialic acid.

Antigenic Studies. Possible homology between the duck immunoglobulins and the immunoglobulin of the other well-studied avian species, the chicken, was studied by immunodiffusion analysis. Antisera prepared in rabbits against the duck 7.8S immunoglobulin, the 5.7S immunoglobulin, and chicken γ-globulin were used in these studies. Antisera to the 5.7S immunoglobulin, when absorbed with the 7.8S protein, failed to react with any of the immunoglobulins tested and therefore no further studies were done with this antiserum. When the antiserum to the 7.8S protein was used, both the 7.8S and 5.7S duck immunoglobulins spurred over the chicken γ-globulin. However, this antiserum recognized antigenic determinants on the chicken γ-globulin that were not present on the duck 5.7S protein but were shared with determinants on the 7.8S protein, indicating some antigenic homology between the chicken γ-globulin and the 7.8S protein not shared by the 5.7S protein. Similar results were obtained with a rabbit antichicken γ-globulin antiserum. This is shown in Figure 2. Again, precipitin arcs were developed against all three proteins. Both duck immunoglobulins were antigenically deficient compared to the chicken γ-globulin. However, whereas the 5.7S protein reacted very poorly and was markedly deficient to the chicken γ-globulin, the 7.8S protein gave a strong line which markedly spurred over that developed by the 5.7S protein and formed a line of partial identity with the chicken γ-globulin. These antigenic studies are suggestive that the 7.8S immunoglobulin and the chicken γ-globulin are homologous proteins.

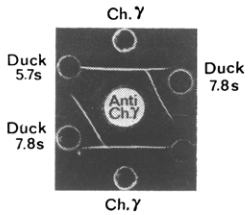

FIGURE 2: Antigenic relationship between duck and chicken immunoglobulins (Ch-γ) developed with rabbit antichicken γ-globulin serum. There was a reaction of partial identity between chicken γ-globulin and duck 7.8S immunoglobulin. A barely visible reaction was obtained with the 5.7S protein which was strongly spurred over by the other two proteins. All antigens were at a concentration of 2 mg/ml.

Discussion

Both the 5.7S immunoglobulin and 7.8S immunoglobulin described above have certain structural features which suggest that these proteins are different than previously described mammalian immunoglobulins. The 5.7S protein has a molecular weight of 118,000 with a heavy chain of 35,000–36,000, a light chain of 23,000 and an Fab fragment of 48,000. Since the molecular weight and yield of the Fab fragment indicated the presence of 2 Fab fragments per intact molecule, the molecular weight of the Fc fragment can be calculated to be 118,000 − (2 × 48,000) = 22,000. Similarly, the Fd fragment can be calculated to have a molecular weight of 25,000 on the basis of an Fab fragment of 48,000 and light chain of 23,000. This information together with the molecular weight of 35,000 for the intact heavy chain result in a figure of 10,000 for the Fc piece of a single heavy chain or 20,000 for the dimer Fc fragment.

When the 5.7S immunoglobulin was partially reduced, the molecular weight dropped from 118,000 to 58,000. These data together with antigenic analysis led to the conclusion that mild reduction resulted in the formation of LH half-molecules. Presumably the relatively small Fc fragment did not possess sufficient noncovalent binding to maintain a 4-polypeptide chain structure in the absence of HH disulfide bridges.

The 5.7S immunoglobulin was also characterized by an extremely low carbohydrate content of 0.6%. The small amount of carbohydrate together with its localization to the Fab fragment suggest that a situation may exist similar to that found in the Fab fragment of human γ-G where the carbohydrate is found on the variable region of heavy

or light chain but only on 10–15% of the molecules (Abel *et al.*, 1968).

There were a total of 11–12 half-cystine residues per heavy chain, 6 to 7 of which were in the Fd fragment. Unlike mammalian γ-G Fab fragments, in which partial reduction leads to the cleavage of a single disulfide bridge linking the heavy and light chains together, in the case of the 5.7S immunoglobulin similar conditions of reduction led to the reduction of at least one other disulfide bridge within the Fd fragment besides the one involved in the HL disulfide bond. On the basis of these findings it is possible to explain some of the effects of partial reduction and alkylation on the binding characteristics of the 5.7S antibody that are not observed with mammalian γ-G antibodies (Grey, 1967b). On the one hand, the production of half-molecules following reduction would explain the complete loss of hemagglutination activity of the 5.7S immunoglobulin with retention of 50–70% of the antibody activity as measured by primary binding tests. The cleavage of a labile intrachain disulfide bridge within the Fd fragment might also explain this 30–50% decrease in the primary binding capacity of the 5.7S immunoglobulin with a concomitant decrease in the affinity of the antibody if the disulfide bridge involved affected the conformation of the antibody combining site.

The salient features of the 7.8S immunoglobulin were the following. The molecular weight of the intact protein was 178,000. The molecular weight of its heavy chain was determined to be 62,000 by gel filtration and on the basis of the molecular weight of the intact protein to be 66,000. As in the case of the 5.7S immunoglobulin the component polypeptide chains were held together by disulfide bridges. The total carbohydrate content was 5.0%. It was of interest that this relatively high carbohydrate content compared with that of mammalian γ-G immunoglobulin was composed primarily of an increase in the hexose content whereas the hexosamine content was similar to that found in mammalian γ-G proteins. Antigenic analysis of the duck immunoglobulin indicated that the 7.8S immunoglobulin was an homologous protein to the chicken 7S immunoglobulin. Previous biochemical studies are consistent with the hypothesis that these proteins may be homologous to one another. Intact chicken gamma globulin has a molecular weight of 170,000 with a heavy chain of 67,000 molecular weight and a hexose content reported at 2–3% (Leslie and Clem, 1969; Hersh *et al.*, 1969; Tenenhouse and Deutsch, 1966).

If the carbohydrate content of the 7.8S protein is taken into account, the molecular weight of the peptide portion can be calculated to be approximately 58,000–61,000. This is quite similar to the molecular weight of mammalian μ chains which have a molecular weight of 70,000 but when carbohydrate is subtracted from this figure a molecular weight of 59,000 is calculated for the peptide portion. It has been postulated (Hill *et al.*, 1966) on the basis of internal homologies in the sequence data that light and heavy chains evolved from a precursor gene coding for a protein of half-light-chain size, *i.e.*, 11,000 to 12,000 molecular weight, which by gene duplication gave rise to a gene coding for a light chain of 23,000 and finally a primitive heavy chain of 50,000. This latter consists of a variable region and three homologous constant region domains, C1–C3, equal in size to the original precursor polypeptide chain. This theory has been modified more recently to include the two gene hypothesis, one coding for the variable region and the other, the constant region. The duck 5.7S heavy chain of 35,000 molecular weight is compatible with either evolution by deletions

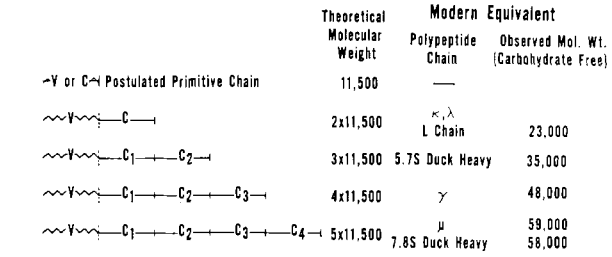

FIGURE 3: Immunoglobulin polypeptide chains arranged as increasing multiples of a postulated primative chain equal in size to a half-light-chain molecule.

in the gene coding for the constant region or, a more interesting speculation, total absence of one of the homologous regions of the Fc, perhaps the carboxy-terminal region, the region in which most of the noncovalent bonding occurs between heavy chains of rabbit γ-globulin (Charlwood and Utsumi, 1969). By recalculating the molecular weights of several heavy-chain types (human γ, μ, α, and δ chains and duck 7.8S and 5.7S heavy chains) as carbohydrate-free proteins, it becomes possible to arrange the polypeptide chains as near multiples of the orginal 11,000 to 12,000 molecular weight precursor (Figure 3). Assuming all the carbohydrate to be on the heavy chain, γ-chain protein has a sugar-free molecular weight of 48,000, α chain of 49,000 (C. A. Abel and H. M. Grey, 1970, unpublished data), δ chain of 49,000 (Spiegelberg *et al.*, 1970), μ chain of 59,000, and duck 7.8S heavy chain of 58,000–61,000. A series of polypeptide chains progressively increasing by one domain can be developed beginning with the theoretical precursor (one domain), light chain (two domains), 5.7S heavy chain (three domains), γ, α, δ chain (four domains), μ chain, and duck 7.8S heavy chain (five domains). This concept of evolution by progressive additions of domains may not be in keeping with current phylogenetic data which suggest a γ-M-like immunoglobulin as the most primitive immunoglobulin. However, recent studies on lower vertebrates have indicated that the 5.7S immunoglobulin that we have described in the duck may also be found in a variety of primitive species, such as the Australian lungfish (Marchalonis, 1969), the grouper (L. W. Clem, 1970, personal communication), the sea turtle A. A. (Benedict, 1970, personal communication), and perhaps a homologue of the 5.7S immunoglobulin may exist in mammals as well (Steward *et al.*, 1969). It is apparent from these recent studies that the 5.7S immunoglobulin is not unique to the duck but may be observed in widely divergent species and may yet be described, perhaps as a minor component, in the most primitive vertebrate species, such as the cyclostome and elasmobranchs.

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Chromatographic Comparison of the Transfer Ribonucleic Acids of Rat Livers and Morris Hepatomas*

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ABSTRACT: tRNAs from hepatomas 5123D and 3924A have been aminoacylated and compared with the corresponding aminoacyl-tRNA from normal rat liver by cochromatography on reversed-phase columns. The iso-accepting species of Leu-, Lys-, and Tyr-tRNA from both hepatomas cochromatographed at approximately the same salt concentrations as the corresponding species from normal liver. The Ser-, Phe-, and His-tRNAs from hepatoma 3924A also showed no deviation from normality. Hepatoma 5123D exhibited two more Ser-tRNA species, one more Phe-tRNA species, and two fewer His-tRNA species than Buffalo rat liver. These chromatographic alterations appeared to be due to new or altered species of tRNA. They were not due to differences in the aminoacyl-tRNA synthetases, nor were they aggregates of the tRNA. The altered tRNAs were a property of the malignant cells, and were not due to an alteration in the environment or to their increased growth rate.

Alterations in the tRNAs of systems undergoing, or having undergone, changes in metabolic control processes have been reported to occur in numerous systems. These alterations have recently been the subject of an excellent review (Sueoka and Kano-Sueoka, 1970). The tRNAs of neoplastic tissues have been examined by several workers. Axel *et al.* (1967) reported differences in hepatic tumor tRNAs induced by ethionine feeding. Taylor *et al.* (1967, 1968) studied the methylated albumin kieselguhr elution profiles of the tRNAs from a number of sources. They reported that most of the aminoacyl-tRNAs studied showed a remarkable similarity. However, differences in the profiles of a number of aminoacyl-tRNAs, particularly Tyr-tRNA, from a variety of tumors as compared with the corresponding normal tissues were observed. In addition to this, the Leu-tRNAs of mouse plasma cell tumors have been shown to elute differently from reversed-phase columns as compared with the Leu-tRNAs from liver cells (Mushinski and Potter, 1969). Further work with mouse plasma cell tumors (Yang and Novelli, 1968) has indicated that a tumor which produces immunoglobulin A contains an extra species of Ser-tRNA as compared with tumors producing immunoglobulin G. A comparison of the tRNAs from Novikoff hepatoma with the corresponding tRNAs from normal rat liver has been carried out by two groups. Baliga *et al.* (1969) compared the elution of eighteen aminoacyl-tRNAs from methylated albumin keiselguhr columns, and reported that new species of His-, Tyr-, and Asp-tRNA were present in the hepatoma. No differences were observed in the Phe-tRNA. However, Goldman *et al.* (1969) reported that, using reversed-phase chromatography, only two Phe-tRNA peaks were observed in the hepatoma, while the rat liver Phe-tRNA appeared to contain three iso-accepting species. Gonano and Chiarugi (1969) Gonano *et al.* (1971); examined several tRNAs from the Morris hepatoma 5123, and reported the presence of a new species of Phe-tRNA in the hepatoma as compared with normal rat liver.

In an attempt to distinguish essential changes in tRNAs which may be connected with the carcinogenic process from nonessential changes brought about in tRNAs as a result of carcinogenesis, we have initiated a comparison of the tRNAs of three rat hepatomas, namely, 9618A, 5123D, and 3924A. Hepatoma 9618A is an extremely slow-growing tumor which is morphologically and histologically very similar to normal hepatocytes, and which contains the normal complement

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