The natural heterohaemagglutinin in the serum of the toad *Bufo regularis,* and its relationship to lower vertebrate immunoglobulins

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Summary. The serum of the toad *Bufo regularis* contains a natural heterohaemagglutinin for human erythrocytes, which appears to have anti-(B + HP) specificity. Results of inhibition and absorption experiments indicate that only one agglutinin is present. The biochemical specificity of the agglutinin may be provisionally described as involving α-D-galactose residues linked (1–3) in the B determinant or (1–4) in the P_1 determinant and β-N-acetyl-D-galactosamine (1–3) in the P determinant, of red cells possessing the H antigen.

Unlike amphibian IgM, the agglutinin was insensitive to 2-mercaptoethanol treatment; moreover, it could be eluted from the α_1 globulin region on cellulose acetate electrophoresis. These results suggest that this naturally occurring heterohaemagglutinin has a structure similar to that of plant and animal lectins. The relationship of this observation to the phylogenetic evolution of immunity is discussed.

INTRODUCTION

Although substances elicited as a response to a foreign stimulus have been observed in certain invertebrate species (Chadwick, 1967; Evans, Painter,

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Evans, Weinheimer and Acton, 1968; Weinheimer, 1970), it is generally accepted that the production of immunoglobulins first occurs in lower vertebrate class animals subsequent to immunization. However, the so-called 'immunoglobulins' found in the serum of the lamprey (*Petromyzon marinus*) and the hagfish (*Eptatretus stouti*) which are representative species of jawless cyclostomes belonging to the most primitive existing vertebrate class, the *Marsipobranchii* (*Agnatha*) bear little resemblance in structure to immunoglobulins of higher animals (Linthicum and Hildemann, 1970; Marchalonis and Edelman, 1968). They are comparable to the plant and animal lectins, being composed of non-covalently bonded subunits; for this reason they have been included as Receptor-specific proteins (RSP) by Gold and Balding, (1975).

The jawed vertebrates on the other hand have all been found to produce typical IgM-type immunoglobulins with classical 2H–2L Porter structure as a result of antigenic stimulation (Litman, Rosenberg, Frommel, Pollara, Finstad and Good, 1971; Marchalonis and Edelman, 1965).

It has been tacitly assumed that the heterohaemagglutinins occurring naturally in the sera of these lower vertebrates also possess a corresponding immunoglobulin structure, even though in the lamprey (Marchalonis and Edelman, 1968), probably also the hagfish (Linthicum and Hildeman, 1970), the shark (Sigel, 1974), the eel (Bezkorovainy, Springer and Desai, 1971) and the trout (Hodgins, Weiser and

Ridgeway, 1967; Holt and Anstee, 1975), they have been shown not to have a typical 2H–2L Porter structure. Since IgG type immunoglobulins are first found in the anuran amphibia (Diener and Marchalonis, 1970; Evans, 1963; Marchalonis, Allen and Saarni, 1970; Marchalonis and Edelman, 1966), although an 'immunoglobulin-like' molecule similar to IgG has been reported in unstimulated (normal) serum of the lungfish, *Neoceratodus forsteri* (Marchalonis, 1969), it seemed possible that an amphibian might also provide a necessary clue to the development of natural heterohaemagglutinin structure.

Previous reports of agglutinins in normal sera of amphibia are sparse (see Gold and Balding, 1975); basically only simple observations of either their presence in (Amaral and Klobusitzky, 1932; Frair, 1963; Noguchi, 1903), or absence from (Ashurst, 1956; Fishbein, 1913), frog or toad serum have been made. We have therefore investigated both the serological specificity and the structure of the natural heterohaemagglutinin present in the serum of the toad *Bufo regularis*. To our knowledge this is the first report of a blood group specific natural heterohaemagglutinin in the serum of an amphibian.

MATERIALS AND METHODS

(1) *Toad serum natural heterohaemagglutinin*
Serum from the toad, *Bufo regularis*, was a gift from Dr T. El-Mekkawi, Cairo University, Egypt. Whole blood was obtained by cardiac puncture. After clotting was complete, the serum was removed, centrifuged to separate contaminating erythrocytes, divided into aliquots of approximately 1·0 ml and frozen at −20°.

(2) *Treatment of serum with 2-mercaptoethanol (2-ME)*
0·5 ml of serum was diluted to 4·0 ml with 10 mM phosphate-buffered isotonic saline, pH 7·3 (PBS) and 1·0-ml aliquots were dialysed at 4° against: (i) PBS for 72 h; (ii) 0·1 M 2-ME (Koch-light Laboratories Ltd) in PBS for 24 h and then against PBS for 48 h; (iii) 0·1 M 2-ME in PBS for 24 h, then against 0·05 M iodoacetamide (BDH Chemicals Ltd) in PBS for 24 h and finally against PBS for 24 h; (iv) 0·05 M iodoacetamide in PBS for 24 h and then against PBS for 48 h. At the same time, the following controls, consisting of 1·0 ml aliquots diluted to the same extent as the toad serum, were similarly treated: (a)

human anti-A serum from an individual with no history of transfusion (IgM anti-A); (b) immune anti-A (IgG + IgM anti-A); (c) anti-A lectin from the albumin gland of the snail *Helix pomatia* (anti-A$_{HP}$). After dialysis, the non-dialysable fractions were collected, their volume measured, and the haemagglutination titres determined using appropriate indicator cells.

(3) *Erythrocytes*
Human red cells were obtained from the Blood Transfusion Centre, Bristol, and were washed three times in PBS before use. Frozen cells were recovered from glycerol by dialysis against warm saline, or from liquid nitrogen by washing in 0·15 M NaCl containing 17·5 per cent sorbitol, followed by three washes in PBS.

(4) *Enzyme treatment of erythrocytes*
Papain treatment of erythrocytes was performed according to methods described in Dunsford and Bowley (1967).

(5) *Determination of H antigen status of erythrocytes*
The H antigen status of erythrocytes was determined using purified anti-H lectin (Hyland; Thetford) from *Ulex europaeus*. The lectin was first standardized against a number of human red cell samples of the various blood groups; the H antigen status of the cell sample in question was then estimated from its titration endpoint using the same lectin anti-H, and could be compared to the values given by red cells of known blood group.

(6) *Substances used in haemagglutinin–inhibition (H/I) tests*
Pneumococcus type XIV polysaccharide, purified P$_1$ substance from hydatid cyst fluid and the chitin oligosaccharides, consisting of *N*-acetylglucosamine saccharide chains of various lengths which strongly inhibited wheat-germ agglutinin, were a gift from Dr D. Anstee. Blood Transfusion Centre, Bristol. Purified B substance was a gift from Professor W. M. Watkins, Lister Institute of Preventive Medicine, London. ABH and Lewis non-secretor saliva was a gift from Dr D. Voak, Blood Transfusion Centre, Cambridge; other saliva samples were obtained from the Blood Transfusion Centre, Bristol. The rare sugars *N*-acetyl-lactosamine, lacto-*N*-biose I and lacto-*N*-tetraose were a gift from Dr A. Gauhe, Max Planck Institute, Heidelberg. Other test substances

were obtained from Sigma Chemical Company or from Koch-light Laboratories Ltd.

(7) Serological techniques

Haemagglutination tests were carried out at 20° using 0·05-ml volumes of serum to which equal volumes of 3 per cent red cell suspensions in PBS were added. Titrations were performed by the 'Master Dilution' technique using automatic pipettes with a new disposable tip for each transfer. The reaction mixture was incubated at 20° for 2 h before reading the results microscopically. H/I tests were performed by incubating 0·05 ml of serum dilution (4 haemagglutinating units, that is a dilution giving a titre of 1 in 4 with the indicator cells) with 0·05 ml of test substance for 30 min before addition of 0·05 ml of a 3 per cent suspension of indicator cells. Results were read microscopically after a further incubation for 2 h at 20°.

Absorption was performed by incubating equal volumes of serum and appropriate washed packed red cells for 18 h at 4°. After this time the serum was separated by centrifugation at 6000 r.p.m. and tested with appropriate indicator cells. (For certain absorption tests the serum was diluted before use with PBS in order to conserve material.)

(8) Cellulose acetate electrophoresis

Electrophoresis was carried out with commercial (Gelman Instrument Company, Ann Arbor, Michigan) apparatus using Sepraphore III^R cellulose poly-acetate electrophoresis strips (2·5 × 15 cm) and 0·14 M baritone buffer, pH 8·6, according to the manufacturer's instructions. The method was basically that of Kohn (1958). Duplicate 5-μl samples of toad serum were run for 45 min at 0·5 mA per strip in parallel with inert human AB serum and a human anti-B serum of equal activity to the toad serum. Strips were stained with Poinceau S to visualize the protein bands.

The position of the band containing haemagglutinating activity was located as follows: 10 μl of serum was applied to duplicate strips and electrophoresis was carried out. One strip was stained with Poinceau S and the other was cut into 0·3 cm sections starting from the point of application of the serum. Each 2·5 × 0·3 cm section was immersed in 0·1 ml of PBS for 12 h at 4° to elute the protein. 0·05 ml of the eluate was then tested with appropriate indicator red cells. An alternative method was to cut the unstained strip into sections corresponding to the pro-

tein bands revealed on staining the duplicate strip with Poinceau S.

RESULTS

Serological properties of the toad serum

Haemagglutination tests using fresh normal red cell samples show the natural heterohaemagglutinin in

Table 1. Results of haemagglutination tests using fresh erythrocytes

	Haemagglutination titres*	
Red cells	Normal cells	Papin-treated cells
O Rh+ve	8	256
O Rh−ve	4	256
A₁ Rh+ve	0	256
A₁ Rh−ve	0	64
A₂ Rh+ve	1	128
B Rh+ve	16	2048
B Rh−ve	32	2048

* Titre expressed as reciprocal of greatest dilution to give agglutination visible under the microscope

Table 2. Serological specificity of *Bufo* serum heterohaemagglutinin

	Haemagglutination titres†	
Red cells*	Normal cells	Papain-treated cells
OP₁	8	512
OP₂	8	256
Opp	1	64
Oh	0	0
Oii	4	n.t.
A₁P₁	0	256
A₁P₂	0	128
A₁pp	0	8
BP₁	32	2048
Bpp	8	2048

n.t. = Not tested.

* All cells used in this series were recovered from the frozen state.

† Titre expressed as reciprocal of the greatest dilution of serum to give agglutination visible under the microscope.

the serum of the toad *Bufo regularis* to have anti-H-like specificity, but with an unusually high titre with group B red cells (Table 1). Further tests using rare red cell samples modify the initial interpretation (Table 2). The failure of Oh (Bombay) erythrocytes to react supports the anti-H specificity, but the results with red cells of various blood group P phenotype indicate an additional specificity. The Opp, OP_1 and OP_2 red cells used had an almost identical H antigen status but Opp red cells were only weakly agglutinated, and no agglutination of A_1P_1 and A_1P_2 red cells was observed, using non-enzyme-treated erythrocytes.

These results suggest that the H and P antigens must be present simultaneously for agglutination of normal red cells to occur. BP_1 red cells gave a significantly higher titre than did OP_1 red cells, which were, however, agglutinated to the same titre as were Bpp red cells. This suggests that an anti-B-like specificity is present in addition to anti-HP. Oii red cells were agglutinated almost as strongly as OI red cells indicating lack of I antigen involvement. Perhaps at present the best serological notation to describe the observed specificity is therefore anti-(B + HP).

The results obtained when papain-treated red cells are used are more difficult to interpret. The anti-B component again gives very high titres with all group B red cells tested. Perhaps a stronger reaction of the P antigen affects the titres obtained with other red cells, group A_1P_1 and A_1P_2 cells then being agglutinated to almost the same titre as OP_1 and OP_2

red cells. A possible explanation for the apparently anomalous weak agglutination of A_1pp red cells and the stronger reaction of Opp red cells after papain treatment is presented later.

Absorption experiments were performed using the erythrocytes shown in Table 3. Unfortunately absorption tests using Bpp red cells were not possible. The specificity determined in haemagglutination tests was confirmed. We were unable to obtain active agglutinin by the usual elution methods described by Dunsford and Bowley (1967).

Results of H/I tests with various mono-, oligo- and polysaccharides and macromolecules are shown in Table 4. Results with normal and papain-treated group B red cells were identical. A surprising result was that the monosaccharide D-galactose inhibited agglutination, albeit only weakly. The dissaccharides containing D-galactose as terminal sugar in β configuration inhibited quite strongly, whereas melibiose which contains D-galactose in α (1–6) anomeric linkage inhibited only weakly. The (1–3) linkage of β-D-galactose to the subterminal sugar is only slightly more favoured than the (1–4) linkage. The relative influence of the subterminal sugar compared to the α or β anomerism is illustrated by the very small difference in inhibition by α and β methyl derivatives of D-galactose, compared to much greater inhibition by disaccharides containing β-D-galactose. These results suggest that the anomerism of D-galactose is relatively unimportant compared to its linkage to the subterminal sugar residue. As with other B-specific

Table 3. Absorption experiments with toad serum

| Indicator red cells | Unabsorbed control | Haemagglutination titres using serum absorbed with: | | | | |
| | | Normal cells | | | Papain-treated cells | |
		OP_1	Opp	A_1P_1	BP_1	A_1P_1
BP_1 normal	16	0	n.t.	8	0	n.t.
BP_1 papain-treated	32*	0	16	16	0	2
A_1P_1 papain-treated	32*	0	16	16	0	0
OP_1 papain-treated	32*	0	16	16	0	1

n.t. = Not tested.

* Serum diluted with PBS to give this titre before absorption
† Titres expressed as reciprocal of greatest dilution to give agglutination visible under the microscope.

Table 4. Inhibition of toad serum heterohaemagglutinin by various mono-oligo- and polysaccharides, and other macromolecular substances

Inhibitor*	Quantity of inhibitor for complete inhibition using as indicator erythrocytes†			
	Normal cells	Papain-treated cells		
	BP_1	A_1P_1	BP_1	OP_1
D-Galactose (μM)	5·0		5·0	
Methyl-α-D-galactose (μM)	10·0			
Methyl-β-D-galactose (μM)	10·0			
N-acetyl-D-galactosamine (μM)	2·5		2.5	
Lactose (μM)	0·156	0·156	0·156	0·078
Melibiose (μM)	2·5		5·0	
N-acetyl-lactosamine (μM)	1·0		0·5	0·063
Lacto-N-biose I (μM)	0·125		0·125	0·031
Lacto-N-tetraose (μM)	0·063		0·063	0·031
Pn XIV polysaccharide (μg)	7·8	7·8	7·8	3·9
B substance (μg)			0·195	0·099
P_1 substance (HCF) (μg)	500.0	3·9	500·0	62·5

* No inhibition by 10 μM of L-fucose, L-rhamnose, D-glucose, D-mannose. N-acetyl-D-glucosamine, D-galactosamine, D-glucosamine, D-fructose, L-arabinose or 500 μg of chitin oligosaccharides.

† The quantity of inhibitor for complete inhibition is the minimum amount of each substance (expressed in μM for the mono- and oligo- saccharides and in μg for PnXIV, B and P_1 substances) necessary to completely prevent agglutination of the indicator erythrocytes by a dilution of serum sufficient to give a titre of 1 in 4 with that red cell sample (4 haemagglutination units).

lectins N-acetyl-D-galactosamine inhibited slightly more than D-galactose.

The inhibition of papain-treated A, B and O red cells and of untreated B red cells by Pn XIV, and the inhibition of both O and B papain-treated cells by B substance supports the view that in the toad serum only one agglutinin is present which has a specificity directed against both α-D-galactose (1–3)—and β-D-galactose (1–4)—the terminal sugar residues of B substance and Pn XIV polysaccharide respectively. However, the stronger inhibition by P_1 substance from hydatid cyst fluid of A_1P_1 papain-treated cells compared to BP_1 and OP_1 red cells is difficult to explain if only one agglutinin is present. Perhaps the agglutinin has a much higher affinity for the receptor on BP_1 red cells than for P_1 substance, but a lower affinity for the receptor on A_1P_1 red cells resulting in the greater inhibition of A_1P_1 red cells by P_1 substance.

The results of inhibition tests using A_1, B and H secretor salivas were inconclusive; no inhibition by non-secretor saliva samples was observed.

Physical properties of the toad serum

The activity of the toad serum agglutinin was unaffected by treatment with 2-mercaptoethanol (2-ME) alone, 2-ME treatment followed by alkylation using iodoacetamide, or by treatment with the iodoacetamide alone.

In contrast, IgM anti-A was completely destroyed, the titre of the immune anti-A (IgG + IgM) was only slightly reduced, and the anti-A_{HP} activity was unaffected by treatment with 2-ME alone, but subsequent alkylation completely abolished activity. This observation has been attributed to the fact that disulphide bonds present in the subunits are able to recombine resulting in active agglutinin (Uhlenbruck and Prokop, 1967), whereas subsequent alkylation with iodoacetamide prevents this recombination, and abolishes activity.

The human anti-B serum and the inert AB serum gave typical patterns on cellulose acetate electrophoresis. Anti-B activity was found solely in the γ-globulin band as expected.

Centimetres
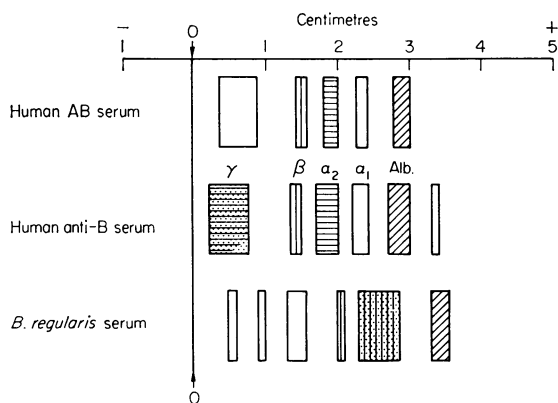
Figure 1. Cellulose acetate electrophoresis of human and toad (B. regularis) serum, showing the globulin regions and the location of haemagglutinin activity (5 μl of serum was applied at 0–0). Staining with Poinceau S. Protein-band stain intensity; open columns, light; horizontally hatched columns, light-medium; vertically hatched columns, dark-medium; diagonally hatched columns, dark; stippled columns, haemagglutinin activity.

The toad serum behaved rather differently as shown in Fig. 1. A fairly strong band migrated at the same rate as the human pre-albumin band, followed by a diffuse but equally intensely staining band in the albumin and α_1 globulin region. Two unidentified bands and bands corresponding in position to the α_2, β and γ globulins were also present. The haemagglutinating activity of the toad serum was located solely in the diffuse region corresponding to the α_1 and part of the albumin, regions.

DISCUSSION

The existence of natural agglutinins in fish and reptile sera with a specificity directed against the ABH antigens of human erythrocytes has been known for some time (Gold and Balding, 1975) but this is the first report of such a specific agglutinin in amphibian sera.

The initially determined anti-(B + H) specificity in *Bufo regularis* serum was not clear cut; this corresponds to reports of anti-B specific agglutinins in extracts of fish ova which Anstee, Holt and Pardoe (1973) later found to be anti-(B + P). A panel of erythrocytes possessing P_1, P_2 and p phenotypes was therefore used in the present study, resulting in the demonstration of the anti-(B + HP) specific agglutinin in toad serum. This finding suggests that the receptors of other haemagglutinins with an ABH specificity

which is not clear cut, e.g. the plant lectin *Sophora japonica* (Balding and Gold, 1973), may also involve the P or P_1 antigens.

The association between the B, H and P antigens as revealed by the toad serum agglutinin is unusual, although both anti-HI (Voak, 1964) and anti-IP (Allen, Marsh, Jensen and Fink, 1974) are well known. Another agglutinin with some similarities to the one reported here has been found in the serum of a patient suffering from a lymphomatous tumour (Tippett, Sanger, Race, Swanson and Busch, 1965). Anti-Luke, as it was called, did not agglutinate p or P^k red cells or 2 per cent of P_1 and P_2 red cell samples, and erythrocytes of group A_1 and A_1B were very significantly more frequently negative in saline tests than were erythrocytes of other ABO groups. However, an association with the H antigen as suggested here for the toad serum agglutinin was thought unlikely since out of four Oh samples available, two gave strongly positive reactions. Perhaps if a sufficient volume of toad serum were available, a survey of red cell samples as carried out for the anti-Luke agglutinin would reveal more similarities between them.

It is difficult to interpret the serological results in biochemical terms. The reaction with group B and P_1 red cells can be understood since α-D-galactose linked (1–3) or (1–4) to the subterminal sugar is the terminal residue of the B and P_1 antigens respectively (Anstee *et al.*, 1973; Cory, Yates, Donald, Watkins and Morgan, 1974). It is well known that both serum anti-(A + B) and lectin anti-(A + B) agglutinins do not distinguish between α-D-galactose (1–3) and α-N-acetyl-D-galactosamine (1–3), the terminal sugars of the B and A antigens respectively. It is therefore not surprising that the toad serum agglutinin can react with β-N-acetyl-D-galactosamine (1–3) terminal residues of the P antigen (Naiki and Marcus, 1974). Alternatively subterminal α-D-galactose (1–4) residues could be responsible for the reaction with the P antigen, similar to the reaction of con A with both terminal and subterminal D-mannose residues in oligosaccharides (Goldstein, Reichert, Misaki and Gorin, 1973).

It is interesting that the anti-(B + P) agglutinin in fish ova also does not distinguish between P_1, P_2 and P^k antigens (Anstee *et al.*, 1973).

Perhaps the H specificity of the toad serum agglutinin may involve the residues of the ABH megalosaccharide backbone in a similar way to the anti-H lectins of *Laburnum alpinum*, *Ulex europaeus II*, and *Cytisus sessilifolius* (Matsumoto and Osawa, 1971),

which are not inhibited by L-fucose, the specific residue of the H antigen, but are inhibited by a disaccharide containing *N*-acetyl-D-glucosamine, a sugar of the megalosaccharide backbone. However, Oh red cells are not agglutinated by these reagents showing that the presence of L-fucose is essential for agglutination to occur.

A participation of β-D-galactose (1–3) or (1–4) residues of incomplete ABH megalosaccharide chains in the determinant for the toad serum agglutinin may possibly be the cause of the very weak agglutination of A_1pp red cells and the stronger agglutination of Opp red cells.

In all reports so far dealing with the immune antibodies of amphibia or higher vertebrates, the active molecule has had typical immunoglobulin structure, with a mobility on electrophoresis comparable to mammalian γ-globulins (Diener and Marchalonis, 1970; Evans, 1963; Marchalonis *et al.*, 1970; Marchalonis and Edelman, 1966). In addition, the lungfish serum fraction migrating at the same rate as the γ-globulins of human serum has been shown to have immunoglobulin structure (Marchalonis, 1969). Conversely, Bezkorovainy *et al.* (1971) have shown that eel anti-H, which does not have immunoglobulin structure, migrates like an α_2 globulin. Thus the observation that the toad serum agglutinin migrates at the same rate as the α_1 globulin region strongly suggests that it differs from normal immunoglobulins.

When the immunoglobulins in stimulated amphibian sera have been treated with 2-ME, the antibody activity has been totally destroyed. This is in agreement with the proposed IgM-type structure of the immunoglobulin (Diener and Marchalonis, 1970). Marchalonis and Edelman (1966) found that both the IgM and IgG fractions of bullfrog (*Rana catesbiana*) serum were not only inactivated, but were reduced to their heavy and light chain components. The observation that the toad serum agglutinin is completely resistant to reduction by 2-ME and to subsequent alkylation suggests a further departure from typical immunoglobulin structure.

Thus although the preliminary structural investigation of the toad serum natural heterohaemagglutinin was hampered by the short supply of serum, these results strongly indicate that it does not possess 2H–2L Porter structure, and is therefore not an immunoglobulin. Further detailed analysis, including amino acid sequencing, is necessary to completely establish the structure of the molecule.

Both the serological and the physical results using the toad serum demonstrate a departure from the generally accepted view of naturally occurring 'antibodies' in vertebrates; this has an important bearing upon the phylogenetic development of antibody structure and function. True immunoglobulin structure is first found in stimulated sera of cartilagenous fish; but the heterohaemagglutinins naturally present in amphibian sera still appear to have a structure similar to that of RSP in invertebrates, and to the heterohaemagglutinins of the lamprey, and eel, and the fructose-specific protein of the shark. In addition Holt and Anstee (1975) have found that trout serum agglutinin is resistant to reduction by 2-ME; Khalap, Phelps and Gold (1972) have shown that the anti-H in the serum of the reptile *Boa constrictor* can be inhibited like eel serum anti-H by the simple sugar L-fucose, in contrast to the naturally occurring anti-H in chicken serum which is not inhibited by L-fucose (Watkins and Morgan, 1955).

We therefore think that substances like the animal lectins with subunit structure and simple sugar specificity confer upon the organism a natural protection against invading parasites, bacteria or other foreign bodies. This 'natural immunity' can be increased in some cases by immunization with the antigen to which the organism is sensitive, e.g. the induction of bacteriocidins (Evans *et al.*, 1968). A remnant of this broad specific natural immunity is perhaps still present in the fish, amphibia and possibly in the reptiles. The immune response first occurs in a simple form in higher invertebrates (Weinheimer, 1970), but the molecules so formed have subunit lectin structure. In the lower vertebrates, probably in animals intermediate between cyclostomes and cartilaginous fish, the broad spectrum specificity is no longer adequate and the ability to form immunoglobulins with their typical specificity and complementarity has developed. In the birds and higher vertebrates lectin-like natural heterohaemagglutinins are no longer prominent. However, sugar-specific proteins have been reported in human sera (Tal, 1965); they may perhaps be a remnant of phylogenetic evolution.

Profitable lines of research therefore appear to be a study of the structure of both the 'immune' substances in invertebrates, and of the natural heterohaemagglutinins in other amphibia, and in reptiles and birds. This knowledge may contribute significantly to the understanding of the phylogenetic development of immunoglobulins. If possible, a com-

parison of anti-Luke and the toad serum agglutinin reported here should be made.

ACKNOWLEDGMENTS

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