XXVI. CHEMICAL STRUCTURE AND ANTIGENIC SPECIFICITY. A COMPARISON OF THE CRYSTALLINE EGG-ALBUMINS OF THE HEN AND THE DUCK.

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Any rational theory of immunity involves a conception of the relation between an antigen and its corresponding antibody. The side-chain theory of Ehrlich, to which immunological investigation in the past two decades has largely owed its direction, gave a purely chemical conception of this relation. More recently attempts have been made to explain certain immunity reactions, such as specific precipitation and agglutination, on purely physical lines, as the mutual precipitation of oppositely charged colloids. This latter hypothesis, which would make the properties of an immune serum depend upon the electric charge on its proteins, and enable these to react with any oppositely charged amphoteric colloid, clearly provides no explanation for the specific discrimination which is the characteristic of immunity reactions. There is evidence, on the other hand, to show that, whatever be the exact nature of the relation between antigen and antibody, it is dependent on the stereochemical structure of the molecule of the antigen. Ten Broek [1914] showed that a racemised protein, which Dakin and Dudley [1913] had found to be quite unaffected by proteolytic enzymes, had also lost all power of evoking the production of antibodies. It was immunologically an inert substance.

This observation suggests a possibility of accounting for the hitherto puzzling phenomenon of the antigenic disparity between proteins, which to ordinary methods of physical and chemical investigation seem to be identical. Corresponding proteins from different species often yield, to an ordinary hydrolytic analysis, the same amino-acids in apparently identical proportions; yet an immunity reaction, such as specific precipitation or anaphylaxis, will discriminate clearly between them. Though the amino-acids are the same, however, and present in identical proportions, it is evident that change in the order of their linkage in the molecule makes possible an immense number of variations in its structure. That specific differences, between corresponding proteins from allied species, might be due to such differences in the intimate pattern of the molecule, was indicated by some recent results.

When a protein is racemised as far as possible by Kossel's method, and subsequently subjected to acid hydrolysis, it is found that certain of the amino-acids have partly or wholly retained their natural optical activity. Dakin [1912] gave reason for believing that the amino-acids thus escaping racemisation are those occupying the terminal positions in the peptide chains, of which the protein molecule is built. By this method Dudley and Woodman [1915] were able to obtain a first indication of structural difference between the caseinogen of cow's and sheep's milk. There was clearly a possibility that this type of structural difference, of which the method only gives a partial indication, might account for the existence of antigenic distinction in cases where an explanation was hitherto wanting.

It seemed to be worth while, therefore, to take two such pure similar proteins, with good antigenic properties, and to examine, on the one hand, their structure, with the help of the racemisation method, and to test, on the other hand, the possibility of distinguishing between them by the highly specific anaphylactic reaction. In the case of the proteins we chose for this purpose the crystalline albumins from the eggs of the domestic fowl and the duck. Preliminary experiments, recorded by Dale and Hartley [1916], had given reason to expect that no difference would be detected by the anaphylactic reaction. If this had been confirmed, and if evidence of structural difference had been obtained, the possibility of explaining antigenic specificity along these lines would have been seriously weakened. The outcome of our experiments, however, has been to give definite evidence of difference in the arrangement of the amino-acids, and, with a more careful and extended series of experiments than those of Dale and Hartley [1916], a clear discrimination between the two proteins by the anaphylactic reaction.

The preparation of the pure, crystalline albumins, and the comparison by the chemical method, were carried out by Dakin, whose share in the work was completed many months ago, before his recently published method for separation of amino-acids was available. Samples of the albumins were sent at the same time to Dale, who was prevented, however, by pressure of other work, from undertaking until recently the comparison by means of the anaphylactic reaction, for which he is responsible. It was thought better to hold back the chemical findings until the biological results were available.

Chemical Examination.

The albumins were prepared from the whites of hens' and ducks' eggs of unquestionable origin. The method employed for the crystallisation of the albumins was that described by Hopkins [1901] and the products were twice crystallised. In the case of the duck albumin the initial crystallisation was induced by adding rather more acetic acid than that necessary for the crystallisation of hen albumin. Rather over 300 g. of each of the purified proteins were dissolved in water and then dialysed so as to remove most of the adhering

ammonium sulphate. The resulting solutions containing 8—10 per cent. of protein were incubated for 23 days at 37° with the addition of the requisite quantity of caustic soda to bring the concentration of alkali up to half normal. At the end of the incubation period, the alkaline mixtures were hydrolysed by boiling with sulphuric acid and the resulting amino-acids were separated according to the usual methods previously employed in experiments on the racemisation of proteins.

The results as to the relative quantitative yields of individual amino-acids from the two proteins are not considered sufficiently accurate for reproduction, but in general it may be said that they appeared remarkably similar, especially as regards the high yield of phenylalanine and arginine, and relatively low yield of histidine, aspartic acid and leucine.

The results follow:

Amino acid	"Racemised" hen albumin	"Racemised" duck albumin	Comments
Alanine (1)	Not racemised	Not racemised	No difference
Valine (2)	Partly racemised	Partly racemised	,, ,,
Leucine (3)	Mostly racemised	Mostly active	*A definite difference*
Proline (4)	,, ,,	Mostly racemised	No difference
Phenylalanine	Completely inactive	Completely inactive	,, ,,
Tyrosine (5)	Inactive	Inactive	,, ,,
Aspartic acid (6)	Mostly inactive, some active	Completely inactive	*Definite difference*
Glutamic acid	Completely inactive	,, ,,	No difference
Histidine (7)	,, ,,	Mostly active	*Definite difference*
Arginine	Active	Active	No difference
Lysine (8)	Inactive	Inactive	,, ,,

(1) The specific rotations for the alanine hydrochlorides were + 6° and + 7·5° respectively compared with a theoretical value of + 10° but this represents no greater racemisation than usually occurs in the separation of the active alanine from proteins. No racemisation of the alanine group in the proteins by the alkali is indicated.

(2) The valine fraction was not obtained perfectly free from leucine and in both cases was mostly racemised.

(3) A marked difference was noted here. The first large fraction of pure leucine (9 g.) crystals was absolutely inactive in the case of the hen albumin while the corresponding fraction (7 g.) from the duck albumin was almost exclusively the *l* form, $[\alpha]_D = + 15°$ in 20 per cent. hydrochloric acid. Theoretical value = + 15·6°. The whole of the leucine from the duck albumin had a mean rotation in hydrochloric acid of + 13·3° indicating about 85 per cent. of the active variety, while the corresponding rotation of the hen albumin leucine was + 4·3°, and this is probably partly due to contaminating valine. Certainly not more than 27 per cent. of the leucine from the racemised hen albumin was in the active form.

(4) Most of the proline from both preparations was inactive. The hen product had a rotation in water of − 5° while the proline from duck egg

albumin showed a value of − 9·9°. Pure *l*-proline has a specific rotation in water of − 77·4°, and while much racemisation ordinarily occurs in the process of isolation, the above values are much lower than those previously observed even from other racemised proteins, so that it appears that most of the proline groups in both proteins were inactivated by the action of alkali.

(5) No perfectly pure specimens of tyrosine were obtained, since the more soluble inactive acid in small amounts is hard to separate from leucine and other impurities. No laevo-rotation was observed in any case.

(6) The whole of the aspartic acid from the duck egg albumin was optically inactive as well as the mother-liquors from which aspartic acid was separated, so that it is certain that none of the active acid was present. From the hen egg albumin, in addition to a considerable amount of racemic aspartic acid, a small amount (0·3 g.) of the pure laevo-acid was separated. It decomposed around 270°, was faintly laevo-rotatory in alkaline solution, and had $[\alpha]_D^{20^\circ} = + 24{\cdot}5^\circ$ in hydrochloric acid solution (3 mols). Titration with standard alkali using litmus as indicator proved the purity of the product and freedom from leucine. Optically pure aspartic acid under similar conditions shows a specific rotation of + 25·7°. Since in any case *l*-aspartic acid is largely racemised during its separation by the ester method, the detection of a little of the laevo-acid must be taken to indicate the original presence of considerably more of the active acid.

(7) The whole of the histidine fraction from the hen egg albumin was completely inactive both as free base and as salts. On the other hand the histidine from the duck egg albumin, though small in quantity, was strongly laevo-rotatory, having $[\alpha]_D^{20^\circ} = - 27{\cdot}2^\circ$, compared with − 39° for the optically pure base. 70 per cent. of the histidine was therefore made up of the laevo-variety. In each case the histidine was successively separated and identified as phosphotungstate, silver salt, precipitation with mercuric sulphate, and picrolonate.

(8) In neither case could any dextro-lysine be detected, but it is noteworthy that in each case the lysine fraction was feebly laevo-rotatory. The cause of this is obscure, but it was certainly not due to contamination with proline.

Anaphylactic Reactions.

The experiments were all made on guinea-pigs, each of which received a preparatory injection of 1 mg. of one or other of the albumins. After intervals varying from 19 to 31 days they were tested for sensitiveness. Most of the tests were made, by the now familiar method, on the isolated uterine muscle, young virgin females being chosen for this purpose. The use of this method enabled sensitiveness to both albumins to be tested on the same animal. In most cases some degree of sensitiveness to both was present. In many a high degree of sensitiveness to the albumin not used for the preparatory injection was observed. In all cases, however, a preferential sensitisation

to the antigen given in the preliminary injection could be detected. Even when a small first dose of the non-specific antigen caused an apparently maximal contraction, the uterine muscle could be completely desensitised to this and yet retain sensitiveness to the specific one; whereas desensitisation to the specific antigen left the muscle completely insensitive to the other. In some cases the specificity was apparently absolute, the muscle being indifferent to a large dose of the non-specific and subsequently reacting typically with the specific antigen.

A small confirmatory series of experiments was made on intact animals, the doses being injected into the jugular vein.

The albumins were always dissolved in physiological saline solution, and the greatest care was exercised, by the use of separate sets of measures, pipettes, etc., to avoid any possibility of contaminating one solution with traces of the other.

Experiments on the Isolated Uterus.

The following are characteristic records.

Expt. 1. Sensitised with 1 mg. of duck albumin. 19th day.

1*st horn.*	(1) 0·1 mg. Hen. No reaction.
	Change Ringer's solution.
	(2) 0·1 mg. Duck. Good but not maximal contraction.
	Change Ringer.
	(3) 1 mg. Hen. Small reaction.
	Change Ringer.
	(4) 1 mg. Hen. Nil. (Desensitised to hen.)
	(5) 1 mg. Duck. Good but not maximal reaction.
2*nd horn.*	Not tested.

Expt. 2. Sensitised with 1 mg. hen albumin. 21st day.

1*st horn.*	(1) 0·1 mg. Duck. Nil.
	(2) 0·1 mg. Hen. Maximal contraction.
	Change Ringer.
	(3) 0·1 mg. Hen. Nil.
2*nd horn* (see Fig. 1).	(1) 2 mg. Duck. Nil.
	(2) 0·1 mg. Hen. Maximal contraction.
	Change Ringer.
	(3) 0·1 mg. Hen. Nil.
	(4) 1 mg. Hen. Nil.

This is an example of very perfect specificity.

Expt. 3. Sensitised with 1 mg. hen. 30th day.

1*st horn.*	(1) 0·1 mg. Duck. Maximal contraction.
	Change Ringer.
	(2) 0·01 mg. Hen. Weak contraction.
	(3) 0·01 mg. Hen. Nil.
	(4) 0·1 mg. Hen. Moderate contraction.
	(5) 1 mg. Hen. Very weak contraction.

2nd horn.

(1) 0·01 mg. Duck. Moderate contraction.
(2) 0·1 mg. Duck. Nil.
(3) 1 mg. Duck. Weak contraction.
Change.
(4) 1 mg. Duck. Nil. (Desensitised to duck.)
(5) 0·01 mg. Hen. Weak contraction.
(6) 0·1 mg. Hen. Moderate contraction.
Change Ringer.
(7) 1 mg. Duck. Nil.
Change Ringer.
(8) 1 mg. Hen. Moderate contraction.

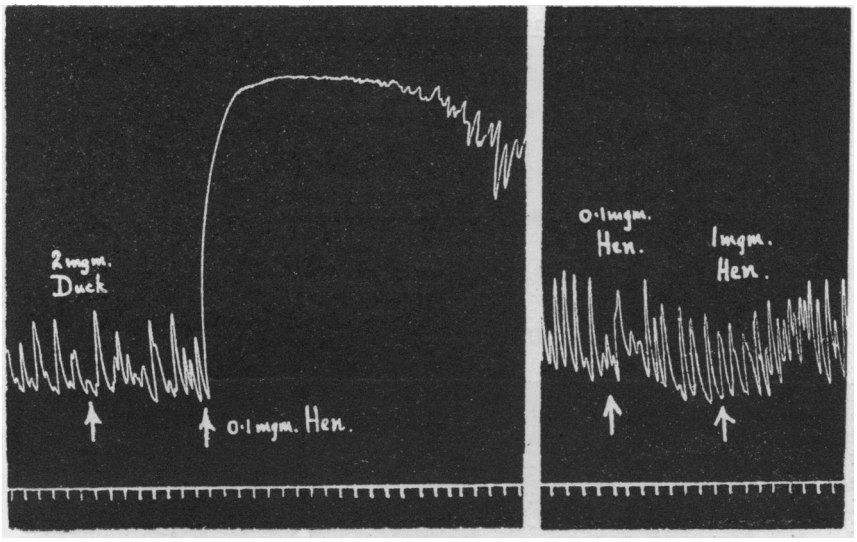

Fig. 1. See Expt. 2, 2nd horn. Sensitised with 1 mg. of Hen albumin, 21st day.

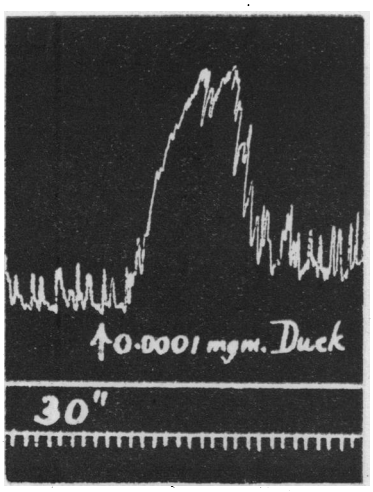

Fig. 2. See Expt. 4, 2nd horn. Effect of 1 part of specific antigen (Duck albumin) in 500 millions of Ringer's solution.

The maximal response given to the first dose might easily give the impression that the sensitisation to duck albumin was as intense as to hen. The record of the second horn, however, shows clearly that complete desensitisation to duck leaves a power of responding even to 0·01 mg. of hen albumin. The same distinction is shown in the next record, in which the non-specific sensitisation is of a high order, but that to the specific antigen is much greater. It may be noted that the bath containing the organ held 50 cc., so that a dose of 0·0001 mg. produces a concentration of only 1 in 500 millions.

Expt. 4. Sensitised with 1 mg. duck. 31st day.

1*st horn.*	(1)	0·01 mg. Hen. Maximal contraction. Change Ringer.
	(2)	0·01 mg. Hen. Very small contraction. Change Ringer.
	(3)	0·01 mg. Hen. Nil.
	(4)	0·01 mg. Duck. Moderate contraction.
2*nd horn.*	(1)	0·0001 mg. Duck. Moderate contraction. (See Fig. 2.) Change Ringer.
	(2)	0·001 mg. Duck. Nil.
	(3)	0·01 mg. Duck. Maximal contraction. Change.
	(4)	0·1 mg. Hen. Nil.

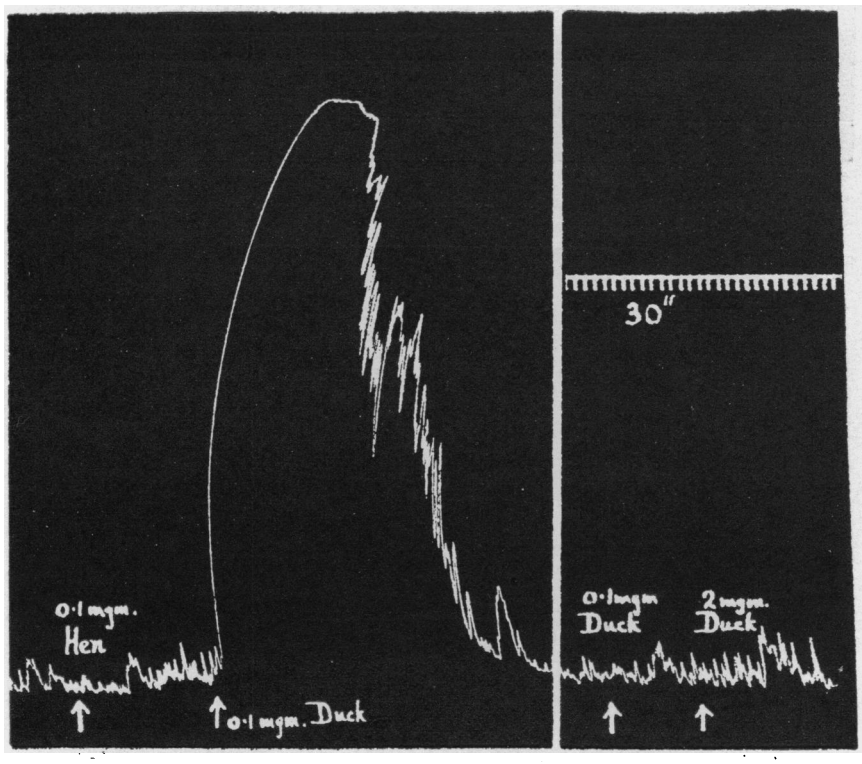

Fig. 3. See Exp. 6, 1st horn. Sensitised with 1 mg. of Duck albumin, 28th day.

Expt. 5. Sensitised with 1 mg. duck. 30th day.

1*st horn.*	(1)	0·001 mg. Hen. Nil.
	(2)	0·001 mg. Duck. Moderate contraction.
		Change Ringer.
	(3)	0·1 mg. Hen. Nil.
	(4)	0·1 mg. Duck. Larger contraction than at (2). Not maximal.
2*nd horn.*	Not tested.	

Expt. 6. Sensitised with 1 mg. duck. 28th day.

1*st horn.* (See Fig. 3.)	(1)	0·1 mg. Hen. Nil.
	(2)	0·1 mg. Duck. Maximal contraction.
		Change Ringer.
	(3)	0·1 mg. Duck. Nil.
	(4)	2 mg. Duck. Nil.
2*nd horn* (see Fig. 4).	(1)	2 mg. Hen. Moderate contraction.
		Change Ringer.
	(2)	0·1 mg. Duck. Maximal contraction.
		Change Ringer.
	(3)	0·1 mg. Duck.
	(4)	2 mg. Duck.

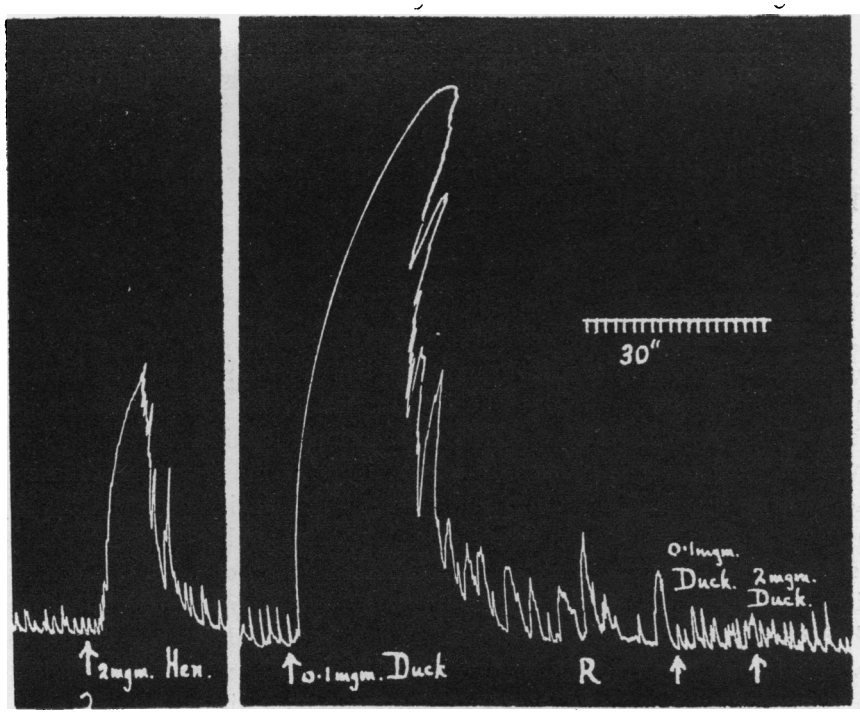

Fig. 4. Continuation of Expt. 6. 2nd horn.

Experiments on Intact Guinea-pigs.

Sensitising injections hypodermic; test injections intravenous.

1. Sensitisation—1 mg. hen albumin.

Weight in g.	Days after first injection	Second dose		Result
300	32	0·1 mg.	Hen	† in 4′
290	30	0·05	Hen	† in 4′
200	30	0·01	Hen	Slight symptoms
180	33	0·01	Hen	Slight symptoms
270	32	0·1	Duck	Moderate symptoms
245	30	0·5	Duck	Slight symptoms

The lethal dose for hen albumin apparently lies between 0·01 and 0·05 mg. For duck albumin it is not reached even at 0·5 mg. though there are symptoms of reaction even with 0·1 mg.

2. Sensitisation—1 mg. duck albumin.

Weight in g.	Days after first injection	Second dose		Result
290	32	0·1 mg.	Duck	† in 3′
250	30	0·05	Duck	† in 3′
225	30	0·01	Duck	Slight symptoms
200	33	0·01	Duck	Slight symptoms
290	32	0·1	Hen	† in 3′
240	30	0·05	Hen	Nil

Again the distinction is clear, but a guinea-pig dies, in this series, after 0·1 mg. of the non-specific albumin.

Discussion.

So far as they go, the results support the conception that the stereochemical structure of the protein molecule is at least an important factor in antigenic specificity. Without the information given by the racemisation method these two proteins would have been indistinguishable, except by an immunological test. The racemisation having shown a structural difference, the presumption that structure and antigenic specificity are related seems to be warranted. This conclusion, however, provides only a first step towards a conception of the relation between antigen and antibody. In due course it will be of interest to discover whether that protein of an immune serum, which carries the specific "antibody" action, shows any difference of molecular pattern from the corresponding protein of a normal serum. Meanwhile the only kind of relation which seems to provide even a distant analogy is that of enzyme and substrate; but the discrimination of the antibody is far more delicately specific.

SUMMARY.

The crystalline albumins from the eggs of the domestic fowl and duck behave as distinct antigens for the anaphylactic reaction. This difference corresponds with a difference in structure, as revealed by the fact that, when the proteins are racemised, the amino-acids escaping racemisation are not identical in the two cases.

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