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The Use of Radioactive Isotopes in Immunological Investigations

9. THE REACTIONS OF ANTISERA TO ANTIGENS CONTAINING MULTIPLE DETERMINANT GROUPS

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Although it is generally accepted that the specific combining centres of antibody molecules are in some way complementary to certain polar groupings in the antigen molecule which determine its serological specificity, very little is known as to the exact nature of either the immunologically determinant groups of natural protein antigens or the specific combining centres (receptor groups) of antibodies.

The immunological specificity of natural protein antigens can readily be altered, by treatment with suitable chemical reagents, so that antisera to these altered proteins will no longer precipitate the original untreated protein, but will precipitate many other proteins treated with the same chemical reagent. There is evidence, however, that in these antisera the antibodies still retain some specificity for the untreated protein, since the amount of antibody precipitable by a heterologous protein treated with the chemical reagent is usually distinctly less than the amount precipitable by the fully homologous antigen. Thus the introduction of new determinant groups into a protein antigen does not usually completely mask all the serologically determinant groups of the original protein.

Antibodies, unlike antigens, cannot have a different specificity conferred on them by chemical treatment, but their specific serological activity can be reduced or abolished by denaturation or by treatment with iodine, formaldehyde, keten or a diazonium salt (see the reviews by Landsteiner (1945), Boyd (1947) and Haurowitz (1950)). The receptor groups of the antibody may contain tyrosine, histidine and possibly cysteine residues

(Pressman & Sternberger, 1951), but from the fact that treatment of precipitating antibodies with appreciable amounts of di-2-chloroethyl sulphone (mustard gas sulphone; HO_2) does not lead to a loss of their precipitating power, it is concluded that free amino groups are not essential components of the receptor groups of these antibodies (Francis, Mulligan & Wormall, 1955b). There is also much supporting evidence for the latter conclusion, for Coons and his colleagues (for the references, see Coons, 1951) have found that antibodies can be conjugated with fluorescein isocyanate without apparent loss of specificity, and Porter (1950) has obtained evidence that specific combination of ovalbumin and rabbit antiovalbumin does not result in the blocking of the N^1 -amino groups or any significant quantity of lysine N^5 -amino groups of the antibody.

Haurowitz & Schwerin (1942, 1943), using antisera to antigens containing multiple determinant groups, have obtained further information about the nature of the antibody receptor groups, and from their results have also deduced that the antibodies they studied were univalent, i.e. contained only one receptor group per molecule. These authors used as antigens 'arsanil proteins' (proteins coupled with diazotized arsanilic acid), and iodinated 'arsanil proteins', and thus had three distinct types of determinant group in their antigens, viz. arsanil groups, 3:5-diiodotyrosine groups and the unknown group (or groups) responsible for the immunological specificity of the original protein.

In the series of investigations described here, we have also used as antigens proteins treated with two different chemical reagents, iodine and mustard gas sulphone. These doubly labelled protein anti-

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gens differ structurally, as well as with respect to the nature of the introduced groups, from those used by Haurowitz & Schwerin, for whereas iodine and diazotized arsanilic acid both react with the tyrosine residues in the protein molecule, di-2-chloroethyl sulphone reacts mainly with free amino groups. Since iodine and the diazonium salt cannot both react with the same tyrosine residue, the two introduced determinant groups in Haurowitz's antigens must have been in different parts of the protein molecule, and probably well separated. In our 'iodinated sulphone-proteins', on the other hand, at least some of the two introduced groupings are probably close together, since steric hindrance seems to occur between them during the preparation of these antigens (Francis, Mulligan & Wormall, 1955*a*). Although the exact nature of the determinant groupings in natural protein antigens is unknown, it seems probable that their distribution in the molecule is more likely to resemble that of the diiodotyrosine and sulphonazan groupings in 'iodinated sulphone-proteins' than that of the diiodotyrosine and arsanil groupings in iodinated arsanil proteins. Haurowitz & Schwerin (1942, 1943) have, in fact, found that the reactions of antisera to arsanil globulins differed considerably from those of antisera to iodinated arsanil proteins, and these differences may well be attributable to differences in the relative positions within the antigen molecules of the two determinant groupings concerned in each case (arsanil and globulin factor in the former case, and arsanil and diiodotyrosine in the latter).

Nomenclature. For convenience in describing antigens containing multiple determinant groupings, and their antisera, the following abbreviations are adopted here. The artificially introduced determinant groups in the antigens are denoted by letters (*A* = arsanil groups, *I* = 'iodo' groups (e.g. 3:5-diiodotyrosine residues), *S* = sulphonazan groups). Ovalbumin is denoted by *Ov* and globulins by *Gl* (mixed serum globulins, except where otherwise stated). Thus *IOv* denotes iodinated ovalbumin and *ISGl* iodinated sulphone-globulin. The presence of a radioactive label (^{131}I or ^{35}S) is indicated by an asterisk before the letter denoting the group containing the label.

Whole antisera to *ISGl* and *ISOv* are designated 'anti-serum-*ISGl*' and 'anti-serum-*ISOv*' respectively. The presence in an antibody molecule of a receptor group or groups directed specifically and exclusively towards a single type of determinant group (or towards the 'carrier protein') is indicated by designating the antibody as 'type anti-*A*', 'type anti-*Gl*', etc. ('Anti-*A*' antibodies will give specific reactions with *AOv*, *AGl*, etc., and 'anti-*Gl*' with *Gl*, *AGl*, *IGl*, etc.) The presence of a receptor group (or groups) having a complex specificity, such that each single group could react with an antigen containing one or more, but not necessarily all of the types of determinant group involved, is indicated by 'type anti-*IS*', 'type anti-*ISGl*', etc. Thus type anti-*IS* antibody molecules could react with antigens containing either *I* groups or *S* groups or both, but

a single type anti-*IS* receptor group could not, normally, react with more than one molecule of such an antigen. The presence within a single antibody molecule of more than one different receptor group will be indicated as 'anti-*I*-anti-*S*', etc. An 'anti-*I*-anti-*S*' molecule should be capable of combining simultaneously with two molecules of antigen, one of which must contain *I* groups (e.g. *IOv*, *IGl*, *ISOv* or *ISGl*) and the other *S* groups (*SOv*, *SGL*, *ISOv* or *ISGl*).

The investigations described in this paper are divided into three sections. In the first, haptens corresponding in their structure to the *I*-, *S*- and *IS*-antigens have been used to investigate the inhibition of the reaction between anti-serum-*ISOv* (or anti-serum-*ISGl*) and various 'partial antigens'. (The term 'partial antigen' is used in this paper to indicate an antigen which contains only some of the types of determinant group present in the immunizing or fully homologous antigen.) In the second section, the antisera have been exhausted with the partial antigens in turn, in a manner similar to that employed by Haurowitz & Schwerin (1942, 1943), but, in addition, the effect of varying the order in which the partial antigens were added has been studied. In the third section, an attempt has been made to differentiate between antibodies of the types anti-*IS* and anti-*I*-anti-*S*, by studying the capacity of precipitates formed by the reaction between an **S*-antigen and *IS*-antiserum to combine with an **I*-antigen.

MATERIALS AND METHODS

Antigens. Thrice-recrystallized hen ovalbumin and mixed horse serum globulins were treated with iodine, di-2-chloroethyl sulphone, or with both these reagents, as described by Francis *et al.* (1955*a*). Solutions of the products in 0.9% (w/v) NaCl, adjusted to pH 7.5 by the cautious addition of Na_2CO_3 solution and centrifuged (and filtered where necessary) to remove particulate matter, were used for the precipitin reactions.

Antisera. Rabbit antisera to *ISGl* and *ISOv* were prepared by courses of intramuscular injection of the antigen after it had been precipitated by alum as described by Proom (1943). At each injection 5 ml. of an approximately 2% suspension of the antigen was injected into each of the hind legs of rabbits weighing approximately 3 kg. Injections were made at intervals of 10–20 days, good antisera usually being obtained after the second or third injection. Pooled sera were used for all the experiments described.

Inhibitors. 'Sulphone-tyrosine' (β -*p*-hydroxyphenyl- α -thiazan-4-ylpropionic acid 1:1-dioxide) was prepared as described by Boursnell, Francis & Wormall (1946); 3:5-diiodotyrosine was prepared from tyrosine as described by Harington (1928), and 'sulphonediiodotyrosine' was prepared in a similar manner from 'sulphone-tyrosine'. The *sulphone-diiodotyrosine* (β -4'-hydroxy-3':5'-diiodophenyl- α -thiazan-4-ylpropionic acid 1:1-dioxide) had m.p. 135–140° (decomp.). (Found: N, 2.77%; I, 43%. $\text{C}_{13}\text{H}_{15}\text{O}_4\text{NI}_2\text{S}$ requires N, 2.55%; I, 46%.) Solutions of these inhibitors (0.02 M in 0.9% NaCl adjusted to pH 7.5 by adding Na_2CO_3) were used in the precipitin inhibition tests.

Quantitative precipitin reactions. These were carried out as described by Francis *et al.* (1955*b*).

Determinations of radioactivity, total iodine and total nitrogen. These were made as described by Francis *et al.* (1955*a*).

Serial precipitations of antibody by 'partial antigens'. A small measured volume of one of the partial antigens (i.e. sulphone-protein or iodinated protein in the experiments with antisera-*ISOv* or *ISGL*), in suitable concentration (usually about 25 μ g. N/ml.), was added to a measured volume (1-4 ml.) of antiserum-*ISGL* (or antiserum-*ISOv*) in a 15 ml. centrifuge tube. The mixture was kept at 37° for 30 min. and then in the refrigerator for 30 min., and centrifuged. A further small volume of the same antigen was then added to the mixture, and the procedure repeated until the final addition of antigen caused no further precipitation. The total precipitate was then centrifuged down and washed with 0.9% NaCl as described above. The second partial antigen was then added in the same way to the combined supernatant solution and washings. In these serial precipitations the amount of each antigen added at the

three washings with ice-cold 0.9% NaCl. Since, in some of our experiments on serial precipitation of antibodies, a considerable number of washings would be required *in toto*, it was decided to investigate whether repeated washing involved any serious loss of precipitate.

The separated γ -globulins from an antiserum-*ISOv* were labelled with ^{131}I as described previously (Francis *et al.* 1955*b*) and samples were treated with antigen **ISOv* to give precipitates in the equivalence zone and the zones of slight antigen and antibody excess. Each precipitate, after incubation, refrigeration and centrifuging as described above, was washed 5 times with ice-cold 0.9% NaCl. The ^{131}I and ^{35}S contents of each washing and of the fully washed precipitates were determined, and from the results the corresponding antibody and antigen N values were calculated.

Table 1. *The effect of washing serological precipitates with cold saline*

Antibody: γ -globulins separated from antiserum to sulphone-ovalbumin, and labelled with ^{131}I (0.33% total iodine).
Antigen: ^{35}S -labelled sulphone-ovalbumin.

Precipitates: samples (0.5 ml. containing 0.5 mg. N) of antibody solution were mixed with the antigen solution and the separated precipitates were washed repeatedly with 0.25 ml. portions of ice-cold 0.9% NaCl.

N-contents of the washings and the antibody N in the precipitate were calculated from ^{131}I measurements. The antigen N in the precipitate was calculated from ^{35}S measurements. All the results are averages of duplicate experiments.

Zone	Antigen N added (μ g.)	Total N (μ g.) in washings					Composition of washed precipitates		
		1	2	3	4	5	Antibody N (A) (μ g.)	Antigen N (B) (μ g.)	Ratio (A/B)
Slight antibody excess	1.42	10.5	1.1	0.4	0.2	0.3	21.7	1.2	18
Equivalence	2.85	13.3	1.6	0.7	0.5	0.7	38.4	2.5	15
Slight antigen excess	5.69	8.9	1.4	0.8	0.5	0.7	56.3	5.0	11

first addition was selected as that corresponding to about one-third to one-half of the total quantity of that antigen required. After the total amount of antigen required had been determined, a duplicate set of precipitations was carried out in which this quantity of antigen was added at one step. With antiserum-*ISOv* this second method usually gave more precipitate than the first, presumably owing to the inclusion in it of incomplete antibodies, but with antiserum-*ISGL*, the precipitate produced by multiple small additions of antigen was always the larger. In serial precipitation experiments with these antisera the method of antigen addition which gave the greater amount of precipitate was used where possible.

Quantitative inhibition reactions. These were carried out like the other quantitative precipitin reactions, the inhibiting agent being added to the antiserum, and the mixture allowed to stand at room temperature for 5 min. before the test antigen was added.

RESULTS

The effect of washing serological precipitates with 0.9% NaCl

Heidelberger & Kendall (1932) have shown that complete removal of non-specific serum proteins from serological precipitates is achieved by two or

The results (Table 1) show that under the conditions of these experiments practically the whole of the loss of nitrogen from the precipitate occurs in the first two washings. There is, however, a small fairly constant additional loss with each further washing, possibly due to further removal of non-specific protein or to a slight but definite solubility of the precipitate in the cold saline. Thus it is inadvisable that small precipitates should be washed more than 3 times. No ^{35}S could be detected in any of the washings, though a ^{35}S -content corresponding to 0.05 μ g. antigen N could have been detected.

Inhibition of the precipitin reaction between antiserum-ISGL and iodinated and sulphone-treated proteins, by 3:5-diiodotyrosine, sulphone-tyrosine and sulphone-diiodotyrosine

The precipitation of antibody by an antigen can be inhibited by any hapten which is capable of combining sufficiently strongly with the receptor groups of the antibody molecule which are concerned in the reaction with the antigen studied. In order to gain information about the receptor groups of antibodies in an antiserum-*ISGL* we have com-

pared the inhibitory actions of sulphone-tyrosine, 3:5-diiodotyrosine and sulphone-3:5-diiodotyrosine (haptens-*S*, hapten-*I* and hapten-*IS*) on the precipitation of these antibodies by antigens containing *I*, *S* and *IS* groupings.

In preliminary qualitative experiments it was found that the reactions with antigens prepared from horse serum globulins were only slightly inhibited by the haptens used, the globulin factor being too strongly determinant. When ovalbumin was used as the carrier protein for the determinant groups in the test antigens, however, the reaction of the antiserum with all three antigens (*IOv*, *SOv* and *ISOv*) was inhibited by hapten-*I*, hapten-*IS* and a mixture of hapten-*I* and hapten-*S*; with antigen-*SOv* there was inhibition by hapten-*S* also. For quantitative investigations, therefore, it was decided to use the system antigen **SOv*-antiserum-*ISGL*.

Fig. 1. Precipitation of antibodies to iodinated sulphone-horse serum globulins by ^{35}S -containing sulphone-ovalbumin in presence of inhibiting haptens. All mixtures contained the requisite amount of antigen ($9.38\text{ }\mu\text{g. N}$) to give maximal precipitation in absence of inhibitors. Inhibitors: A, 3:5-diiodotyrosine; B, sulphone-tyrosine; C, mixture of 3:5-diiodotyrosine and sulphone-tyrosine; D, sulphone-diiodotyrosine.

Samples (2 ml.) of antiserum-*ISGL* were mixed with varying amounts of solutions 0.02 M with respect to (a) hapten-*I*, or (b) hapten-*S*, or (c) both hapten-*I* and hapten-*S*, or (d) hapten-*IS*. The same amount ($18.75\text{ }\mu\text{g. N}$) of antigen-**SOv* was added to each tube, preliminary tests having shown that this was the amount required to give maximal precipitation. The mixtures were then treated as described above, and the precipitates analysed for ^{35}S and total N.

The results of this experiment (Fig. 1) show that the reaction between antigen-**SOv* and antiserum *ISGL* is strongly inhibited by hapten-*S* and also significantly by hapten-*I*. A mixture of these two

haptens proved more effective than either separately, but less effective than the compound (hapten-*IS*) containing both a sulphonazan and a diiodotyrosine structure.

Serial precipitation of antibodies to iodinated sulphone-proteins by 'partial antigens'

In the first experiment described below 'partial antigens' were prepared from the same carrier protein as was used for the preparation of the immunizing antigen, but in order to exclude the protein factor as far as possible from the reaction, the antiserum (antiserum-*ISOv*) was first absorbed with denatured homologous carrier protein (ovalbumin). In the second experiment both homologous and heterologous carrier proteins were used for the preparation of the test antigens, and the antiserum (antiserum-*ISGL*) was absorbed with denatured horse serum globulins. Since a certain amount of complement nitrogen may be present in antigen-antibody precipitates prepared from whole antisera, complement was also removed from both antisera before use by incubation with the washed precipitate from an unrelated precipitin system.

Expt. 1. Antiserum-*ISOv*, mixed with washed heat-denatured ovalbumin (approximately $0.25\text{ mg. ovalbumin/ml. serum}$) was kept at 37° for 2 hr. with occasional shaking, and at $0-4^\circ$ overnight. The mixture was then centrifuged and the clear serum was treated similarly with the washed precipitate from a horse globulin anti-horse globulin system (approximately 0.6 mg./ml. serum). The resulting absorbed serum gave no visible precipitate when tested with antigen *Ov*. Separate portions (3 ml.) of the absorbed, complement-free serum were treated with antigens **SOv*, **IOv* and **ISOv* in the orders and amounts shown in the columns of Table 2, each mixture being treated and analysed for antigen and antibody as described under 'Methods'. All precipitations and analyses were carried out in duplicate.

The results (Table 2) show that: (a) the total amount of antibody precipitated by successive addition of the two partial antigens is practically the same, irrespective of the order in which they are added, but that the amount precipitated by either antigen is considerably diminished if the other is added first (i.e. some antibody is precipitable by either antigen); (b) there is some antibody which is not precipitated by either antigen *SOv* or *IOv*, but is precipitable by antigen *ISOv*; and (c) the total amount of antibody precipitated by the three antigens added successively is less than that precipitated by antigen *ISOv* added alone.

Expt. 2. Antiserum-*ISGL*, mixed with washed heat-denatured horse serum globulins (approximately $0.4\text{ mg. protein/ml. antiserum}$) was kept at 37° for 2 hr. and at $0-4^\circ$ overnight and then centrifuged. The clear serum was then treated similarly with the washed precipitate from a horse globulin anti-horse globulin system (approximately 0.5 mg./ml. serum). The final product gave no visible precipitate when treated with antigen *GL*. Separate portions (4 ml.) of

the absorbed, complement-free serum were then titrated with antigens **SOv*, **IOv*, **ISOv*, **SGL*, **IGl* and **ISGL* in the orders and amounts shown in the columns of Table 3, as described above.

The results of this experiment (Table 3) showed again that a certain amount of antibody was precipitable by either an *I*- or an *S*-antigen, but there is no evidence of a similar behaviour with antigens *IGl* and *SGL* added after the removal of all antibody precipitable by antigen *ISOv*, in that the amounts of antibody then precipitated by each of these two antigens is essentially independent of the order in which they are added. In this system there was much less, or, with the *Gl*-series of antigens, no significant difference between the amounts of antibody precipitated by an *IS*-antigen on the one hand, and an *I*-antigen, *S*-antigen and *IS*-antigen added

serially on the other. It is remarkable also that, although the serum gave no precipitate with antigen *Gl*, complete absorption with antigen *ISOv* (or serially with antigens *SOv*, *IOv* and *ISOv*) removed only about two-thirds of the antibody, the remainder being precipitable only by antigens containing *Gl* groupings, together with *I*, *S* or *IS* groups.

A further point of importance arises when the amounts of radioactive antigen added and precipitated at each stage are being considered. In both the systems, with antiserum-*ISOv* and antiserum-*ISGL*, the precipitation of added antigens which are not fully homologous with the antiserum is often incomplete, and since the second and subsequent precipitations are therefore carried out in the presence of more than one antigen, it is important to verify that the radioactivity of these

Table 2. *Precipitation of antibodies to iodinated sulphone-ovalbumin by partial antigens*

Antiserum: rabbit antiserum-*ISOv*, absorbed with denatured ovalbumin and freed from complement; 4 ml. used for each experiment.

Antigens: ³⁵S-containing sulphone-ovalbumin (**SOv*); ¹³¹I-containing iodinated ovalbumin (**IOv*) and iodinated sulphone-ovalbumin (**ISOv*).

Precipitates: the antigens were added in the orders and amounts shown in the columns of the Table, these amounts being those required to give maximal precipitation. The precipitates were centrifuged down and washed before the next antigen was added to the supernatant solution plus washings. The antigen in each precipitate was determined by radioactivity measurements, and the antibody by subtracting the corresponding weight of antigen N from the total N.

(All figures are given as μ g. N/ml. antiserum.)

Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate	
	Antigen	Antibody		Antigen	Antibody		Antigen	Antibody
<i>*SOv</i> (5.85)	4.9	54.1	<i>*IOv</i> (7.68)	6.7	59.1			
<i>*IOv</i> (2.55)	2.5	23.2	<i>*SOv</i> (1.58)	1.6	17.6			
<i>*ISOv</i> (6.0)	4.8	62.6	<i>*ISOv</i> (6.0)	5.1	62.9	<i>*ISOv</i> (18.6)	16.3	172.0
Total antibody		139.9			139.6			172.0

Table 3. *Precipitation of antibodies to iodinated sulphone-horse globulins by partial antigens*

Antiserum: absorbed rabbit antiserum-*ISGL*.

Antigens: ovalbumin derivatives containing ³⁵S or ¹³¹I or both isotopes (cf. Table 2) and the corresponding horse serum globulin derivatives.

Precipitates: details as in Table 2, each antigen being added a little at a time, until no further precipitate was obtained.

(All figures are given as μ g. N/ml. antiserum.)

Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate	
	Antigen	Antibody		Antigen	Antibody		Antigen	Antibody
<i>*IOv</i> (4.38)	1.75	24.3	<i>*SOv</i> (10.0)	7.33	81.3			
<i>*SOv</i> (3.75)	3.25	44.8	<i>*IOv</i> (1.25)	0.06	10.9			
<i>*ISOv</i> (2.81)	2.56	33.9	<i>*ISOv</i> (2.19)	1.45	22.7	<i>*ISOv</i> (11.56)	7.0	123.8
Total antibody		103.0			114.9			123.8
<i>*IGl</i> (6.25)	4.65	63.6	<i>*SGL</i> (10.62)	8.98	113.5			
<i>*SGL</i> (3.75)	3.75	61.8	<i>*IGl</i> (1.25)	1.15	17.6			
<i>*ISGL</i> (5.31)	4.10	49.3	<i>*ISGL</i> (5.31)	4.03	42.0			
Total antibody		174.7			173.1			
<i>*ISOv</i> (11.56)	6.9	125.5	<i>*ISOv</i> (11.56)	7.1	122.0			
<i>*IGl</i> (1.25)	0.88	17.4	<i>*SGL</i> (1.88)	1.63	25.8			
<i>*SGL</i> (1.88)	1.35	23.8	<i>*IGl</i> (1.88)	1.03	18.7			
<i>*ISGL</i> (2.19)	2.1	19.0	<i>*ISGL</i> (2.20)	2.20	22.8	<i>*ISGL</i> (18.8)	14.6	186.5
Total antibody		185.7			189.3			186.5

precipitates is derived from one antigen only. It was not possible to do this in every case, but in each case where a precipitate was obtained on adding an antigen containing ^{35}S subsequent to one containing ^{131}I , radioactivity measurements made with an aluminium filter (capable of absorbing all the ^{35}S β -particles) superimposed on the sample showed that the precipitates contained no ^{131}I .

*Treatment of washed precipitate from the system
SOv-antiserum-ISGL with an iodoprotein antigen

The previous two experiments have shown that some of the antibodies in antiserum-ISOv or antiserum-ISGL can be precipitated by either an *I*-antigen or an *S*-antigen, i.e. they contain receptor groups directed towards both *I* and *S* determinant groups in the antigen molecules (type anti-*IS* or anti-*I*-anti-*S*). The following experiment was planned to establish whether those antibodies precipitated by one of the partial antigens still contained free receptor groups capable of combining with the other.

globulin, tested and shown to give a precipitate with the original antiserum) were added, and each mixture was diluted to 25 ml. with 0.9% NaCl. After they had been kept at 37° for 2.5 hr., with occasional shaking, the mixtures were recentrifuged and the precipitates washed with 1.0 ml. portions of ice-cold 0.9% NaCl until the washings showed only a negligible radioactivity (four washings in all). Each precipitate was then dissolved in alkali and analysed for ^{131}I , ^{35}S and total N.

Control experiments were also set up, in which serological precipitates quite different from the experimental precipitates, but containing about the same amount of protein, were incubated with the *I*-antigen and analysed in the same way. These control precipitates were chosen from widely variant homologous and heterologous systems, including as many points of similarity with the test system as possible; e.g. antisera to sulphone-proteins and to horse serum globulins, and serum γ -globulins as antigens.

From the results (Table 4) it will be seen that the uptake of *I*-antigen by the **SOv*-anti-*ISGL* precipitates was small and invariably less than that taken up by the control precipitates, the latter being in some cases surprisingly high.

Table 4. Addition of iodinated rabbit γ -globulins to serological precipitates from the system
sulphone-ovalbumin plus anti-iodinated sulphone-horse serum globulins

The precipitates, prepared in the antibody excess zone from ^{35}S -containing sulphone-ovalbumin and an antiserum to iodinated sulphone-horse serum globulin, were suspended in ^{131}I -containing iodinated rabbit serum γ -globulin solution. Control serological precipitates of approximately the same size were treated similarly. All washed precipitates were analysed for ^{35}S , ^{131}I and total N. All results are expressed/100 μg . of total N in the treated precipitate.

Iodinated protein antigen added (μg . N)	Composition of washed precipitate			
	Sulphone antigen (μg . N)	Iodoprotein antigen (μg . N)	Antibody (μg . N)	Ratio (antibody N/antigen N)
				For sulphone antigen For iodoprotein antigen
0.0	9.3	0.0	90.7	9.8 —
2.11	10.74	0.49	88.8	8.3 181
6.07	9.70	0.74	89.6	9.2 121
8.00	10.60	0.50	88.9	8.4 178
21.32	10.34	0.96	88.7	8.6 92
Control systems				
1.50	—	1.20	Sulphone-ovalbumin plus anti-sulphone- ovalbumin (antibody N/antigen N ratio in precipitates approximately 9)	
5.65	—	3.73		
19.6	—	8.03		
50.6	—	4.30	Iodinated sulphone-horse serum globulins plus anti-sulphone-ovalbumin	
39.1	—	16.50	Horse serum γ -globulin plus anti-horse γ -globulins (separated γ -globulins)	

Antiserum-ISGL (30 ml.) was treated with antigen **SOv* in quantity (12.64 μg . N/ml. serum) slightly more than half that required to give maximal precipitation. The mixture was kept at 37° for 2 hr., well mixed, and separate portions (each corresponding to 5.5 ml. serum) were centrifuged and the precipitates (each containing about 300 μg . N) washed 3 times with 0.2 ml. portions of ice-cold saline. Each precipitate was then resuspended in 0.9% NaCl, varying quantities of an *I*-antigen (iodinated rabbit serum γ -

DISCUSSION

In order to explain the high degree of immunological specificity shown by a natural antigenic protein it is necessary to assume that the determinant groupings in this protein occupy a significantly large area in the surface of the molecules, since it is inconceivable that a configuration containing, for example, a

particular grouping of two or three amino acid residues, should not also occur in many other immunologically different proteins. It is logical to conclude that the corresponding receptor groups in the antibody molecules also occupy a fairly large area.

In antigen-antibody mixtures where the antigen is not in excess one might reasonably expect combination between antigen and antibody to occur over the greater part or the whole of the area of a receptor group. Furthermore, if each antibody molecule contains only one receptor group, or even if there are several receptor groups, provided these are well separated, the antigen molecule attached to each receptor group should be capable of aligning itself so that an appreciable part of its surface is sufficiently close to the antibody molecule for non-specific or van der Waal's intermolecular attractive forces to take effect, or for the polar groupings on these surfaces to 'interfere' with the water-binding action of the polar groups on non-specific parts of the antibody molecule, as postulated by Boyd (1942).

With excess of antigen in the mixture, on the other hand, there will be competition for the limited total receptor group area of the antibody molecules. Under these circumstances, more than one antigen molecule might combine with each receptor area, each antigen molecule being only attached to a part of this area by a part of one of its own determinant areas. A similar view has been put forward by Haurowitz (1952), and this conception would reconcile the observation that a molecule of antibody to iodinated proteins apparently contains only one receptor group capable of specific combination with the diiodotyrosine groups of the antigen (Banks, Francis, Mulligan & Wormall, 1951) with the evidence, obtained by a number of workers from a study of the soluble complexes formed in the antigen excess zone, that in this zone some antibody molecules can combine with more than one molecule of antigen (see, for example, Marrack, Hoch & Johns (1951) and Becker (1953)).

A combination of this type would not only lead to a smaller neutralization of charge on the antigen (and possibly antibody) molecules during the specific combination, but would also tend to prevent the non-specific parts of the antigen and antibody molecules from coming sufficiently close for any extensive interaction between their polar groupings. Both these factors would cause less bound water to be lost from the complex, and hence less decrease in its solubility, than would occur with complexes formed in zones where antigen was not present in excess.

Many of the investigations which have helped to elucidate the nature of the specific reaction between antigen and antibody have been made with protein

antigens containing artificially introduced determinant groupings, in which the original protein specificity has been largely or entirely lost. It does not necessarily follow that these chemically altered (or conjugated) proteins behave immunologically and serologically exactly like natural proteins, and in particular their principal determinant groupings probably correspond to relatively much smaller areas of the protein molecule. If they also contain large determinant areas, however, the corresponding antibodies should also contain large receptor groups, and to demonstrate the existence of these it would be necessary to show that the receptor groups in, for example, antibodies to iodinated globulins, were of the type designated here anti-*I**Gl* rather than anti-*I* and anti-*Gl* or anti-*I*-anti-*Gl*, and antibodies to iodinated sulphone-proteins were of the type anti-*IS* rather than anti-*I*-anti-*S*. Incidentally, if the *I* and *S* groupings are well separated in the antigen molecule (and some of them most probably are), and if the antibodies to these proteins were bivalent, it seems almost certain that some of the antibody molecules in antisera to antigen *ISGl*, for example, should be of the type anti-*I*-anti-*S* (anti-*I**Gl*-anti-*S**Gl*, etc.) and any demonstration of the absence of this type of antibody would constitute supporting evidence for the view that each molecule of antibody contains only one receptor group.

In the experiments of Haurowitz & Schwerin (1942) an antiserum-*AGl* (i.e. anti-arsanil-globulin) was exhausted with antigen *AOv* and subsequently treated with a mixture of antigens *AOv* and *Gl*, or with the single antigen *AGl*. Whereas no further precipitate was obtained with the mixture, precipitates were obtained with *AGl*. (This result is paralleled by the finding, reported above, that during serial precipitation of antiserum-*ISOv* or antiserum-*ISGl* by partial antigens, no precipitates containing two different antigens were obtained.) This was taken to indicate the absence of anti-*A*-anti-*Gl* types, and the presence of anti-*AGl* types of antibody molecule. In these experiments, however, the nature of the *Gl*-determinant groups of the antigen was unknown, and there was no evidence as to whether the antibodies precipitated in the first stage were of the anti-*A* or anti-*AGl* types. In later experiments (Haurowitz & Schwerin, 1943), an antiserum-*IAGl* was successively exhausted with antigens, *AOv*, *IOv*, *IAOv*, *Gl* and *IAGl*. Precipitates were obtained at every stage except with the antigen *IAOv*, which would appear to indicate in this case the absence of any antibodies with a double specificity for *I* and *A* groupings. However, if any anti-*IA* types had been present, these might have been precipitated by the addition of antigens *AOv* and *IOv*, but no information concerning this point is available from these experiments. In our own experiments on serial precipitation, precipitates

were always obtained with the 'double-determinant' antigens added after the two 'single antigens' (Tables 2 and 3), but this difference may well be attributable to the distribution of the *A* and *I* groups in Haurowitz's antigen making the production of anti-*AI* types of antibody impossible. The presence of complex types of receptor groups in antiserum-*IAGl* is indicated, however, since Haurowitz & Schwerin obtained further precipitates with antigen *IAGl*. The antibodies precipitated at this stage must therefore have had combining groups directed towards the *Gl*-determinant as well as towards the *I*- or *A*-determinants; these may, therefore, have had complex combining sites of the types anti-*IGl* or anti-*AGl* (or even anti-*IAGl*, though this point cannot be settled since only the one antigen was used). The possibility that they may have been of the type anti-*I* (or *A*)-anti-*Gl* may be excluded in the light of the earlier results of Haurowitz & Schwerin (1942).

In our inhibition experiments (Fig. 1) the precipitin reaction between antiserum-*ISGl* and antigen **SOv* was inhibited by hapten-*I*. This shows that at least some of the molecules of antibody involved in precipitation with the antigen **SOv* have an *I*-type of specificity as well; i.e. they are either of the type anti-*I*-anti-*S* or anti-*IS*. If these antibodies are multivalent it is difficult to see how anti-*I*-anti-*S* types could fail to be present, though if they are univalent these types could not be present, and the double specificity must be attributed to anti-*IS* types. On either theory, the inhibition of the reaction by hapten-*I* is explicable. On the 'lattice theory' of precipitate formation, anti-*I*-anti-*S* types would be rendered effectively univalent by combination with hapten-*I*, and 'zero-valent' by combination with haptens containing both *I* and *S* groups. It seems doubtful, however, if this could account for the great difference between the inhibition effected by hapten-*I* alone and that by the mixed or complex haptens, unless the antiserum contains a considerable proportion of the anti-*S* type of antibody molecule, an assumption which is not supported by the results of later experiments.

The experimental results are readily explicable if the antibodies are largely of the type anti-*IS*. These would not be expected to have their precipitating power for the antigen *SOv* seriously diminished by combination with hapten-*I*, which would leave a considerable part of their receptor areas unaffected. Combination with both hapten-*I* and hapten-*S*, or even more so with hapten-*IS*, would, however, render them effectively zero-valent. The results of these experiments, therefore, are more suggestive of the presence of anti-*IS* types than of anti-*I*-anti-*S*, which should be present if the antibodies were multivalent.

The results of the inhibition experiments de-

scribed in this paper do not preclude the possibility of the presence of the separate anti-*I* and anti-*S* types of antibody in addition to the compound anti-*IS* type, but in the successive precipitation experiments with antiserum-*ISOv* and with antiserum-*ISGl* (Tables 2 and 3) the amounts of antibody precipitated by the *I*-antigens or the *S*-antigens were greatly diminished if the other type of antigen was added first. This behaviour was not observed, however (Table 3), when antigens *IGl* and *Sgl* were added to antiserum-*ISGl* after antigen *ISOv*. This indicates that much of the antibody in these antisera must contain anti-*I*-anti-*S* or anti-*IS* types of receptor group.

Since, in these serial precipitation experiments, the partial antigens are not always completely precipitated, there is the possibility that, if anti-*I*-anti-*S* types of antibody are present, the addition of a second partial antigen might cause the formation of a precipitate containing both antigens. However, Haurowitz & Schwerin did not obtain precipitates of this type with the mixed antigens they used (*AOv* and *Gl*), and, as mentioned above, in our serial precipitation experiments no signs of anti-*I*-anti-*S* types of antibody were detectable either. The formation of a further precipitate with the antigen **ISOv* after the addition of antigens **IOv* and **SOv* cannot therefore be attributed to the presence of anti-*I*-anti-*S* types of antibody. A more probable explanation is that although *I*-antigens and *S*-antigens are both capable of precipitating anti-*IS* (or anti-*ISGl*) types of antibody, some of these antibody molecules, though they may combine with partial antigens containing only *I* or *S* groups, are insufficiently well adapted to the *IS* structure to precipitate unless a larger part of their receptor areas are 'covered' by antigen than can be achieved by the partial antigens.

It should also be noted, in the experiment with antiserum-*ISOv* (Table 2) that the difference between the amount of antibody precipitated by antigen-*ISOv* added in one stage, and the total amount precipitated by the three antigens added serially, indicates the presence of some antibody analogous to the incomplete antibody observable in anti-ovalbumin sera. We believe that the non-precipitability of this antibody is not due to univalence (cf. Francis *et al.* 1955*b*) but is due to its being insufficiently well-adapted to the antigen to precipitate unless there is, in addition, a sufficiency of more complete (i.e. better adapted) antibody present in the system. Under these conditions it will be precipitated (along with the more complete antibody) in complexes having a molecular ratio of antibody to antigen in excess of unity. In the serial precipitations with partial antigens reported in this paper, and in the experiments of Haurowitz & Schwerin, the more fully adapted antibody

molecules will naturally be the most readily precipitable by partial antigens, and will thus not be available in the later stages to assist in the precipitation of the incomplete or less well-adapted antibody.

In the experiment with antiserum-*ISGL* exhausted with antigen *ISOv* (Table 3) precipitates were obtained on the addition of antigens *IGl* and *ISGL*, but there was no evidence of the presence of anti-*IS* types of antibody molecule in these precipitates. These antibodies, although incapable of precipitating antigen *Gl*, must therefore have contained receptor groups adapted mainly towards *Gl* determinant groups, but partially towards *I* or *S* groups also, and requiring the presence of these latter groups in the antigen for combination with it to be sufficiently intimate to cause precipitation.

The experiments discussed so far have provided strong evidence that the antisera studied contained relatively large amounts of antibody containing receptor areas directed towards some considerable portion of the surface area of the antigen molecule, i.e. types anti-*IS*, anti-*ISGL*, etc., and indicate the absence of the multivalent types such as anti-*I*-anti-*S*. Further evidence on this point may be deduced from the results of the experiment recorded in Table 4. When the antigen **SOv* was added to antiserum-*ISGL* in the antibody excess zone it should have precipitated well over half the total antibodies precipitable by *SOv*, including antibodies of types anti-*S* and anti-*IS* (and anti-*I*-anti-*S* if present), but not the simple type anti-*I*. Since the antibody/antigen molecular ratio in such a precipitate would be fairly high, any free anti-*I* receptor groups in the precipitated antibodies should still be capable of further combination with an *I*-antigen without rendering the precipitate soluble. This should only occur if anti-*I*-anti-*S* types of antibody are present, as distinct from anti-*IS* types, since the combining sites of this latter type will already be 'covered' by the molecules of the antigen **SOv*. These possibilities are represented diagrammatically in Fig. 2.

The results of this experiment (Table 4) showed that although the **I*-antigen was precipitable by the whole serum, the amounts taken up by the precipitate used in the experiment were extremely small, and in every case appreciably less than the amounts taken up by completely unrelated serological precipitates. This uptake of **I*-antigen by the control precipitates is almost certainly due to non-specific adsorption, and it seems highly probable that the small uptake of **I*-antigen by the **SOv*-anti-*ISGL* precipitates was also non-specific. Even if it is assumed that the whole of the **I*-antigen taken up in the latter case was specifically combined, the ratio of antibody to iodoprotein-antigen in the treated precipitates was so high that it indicates that only a very small fraction of the

antibody precipitated could have been of the anti-*I*-anti-*S* type.

We now suggest that the following views on the nature of the receptor areas of antibody molecules are most in accord with experimental findings. First, the antibody receptor areas are complementary to a relatively large area of the surface of the immunizing antigen molecule, corresponding to a fairly large number of adjacent amino acid residues. Secondly, the antibody molecules within a single serum may vary considerably in their adaptation to the fully homologous antigen. The degree of adaptation towards one part of the large determinant area of the antigen may exceed that towards another, and individual antibody molecules

Fig. 2. Diagrammatic representation of the possible reactions between a sulphone-ovalbumin antigen and antibodies to iodinated-sulphone-globulins, and between the resulting precipitate and an iodinated protein antigen.

may vary in the part of the antigen determinant area towards which they are most fully adapted, producing, for example, simple types anti-*I*, anti-*S* or anti-*Gl* at one extreme, and the complex type anti-*ISGL* at the other. Those antibody molecules showing the most complete adaptation ('complementariness') towards part or all of the antigen determinant groupings will precipitate antigens containing only a relatively small part of the structure of the determinant area of the fully homologous antigen, since the degree of 'closeness of fit' between antigen and antibody over this small

area is sufficient to cause enough loss of bound water, due to mutual charge neutralization, to render the complex insoluble. With decreasing degrees of adaptation between the precipitable antibody and the immunizing antigen, increasingly large parts of the structure of this antigen must be present in any test antigen for it to be specifically precipitable. In extreme cases, only the fully homologous antigen will be precipitated, and some antibody (incomplete antibody) may not be sufficiently well-adapted to precipitate even the homologous antigen unless some better adapted antibody is also present. In some cases, suitable chemical treatment (e.g. with di-2-chloroethyl sulphone), by rendering these least well-adapted antibody molecules less soluble, may render them in part precipitable by antigens with which they were incapable of precipitating before treatment. Thirdly, many antibody molecules may each contain only one large receptor area, but in presence of an excess of antigen this group may be capable of combining with more than one molecule of antigen, each molecule of the latter combining with part of the receptor area by virtue of part of its own determinant area. In such complexes much of the polar determinant area of the antigen molecule, and possibly some of the receptor area of the antibody molecule, will not be closely 'fitted' to a complementary group, and since van der Waals forces will be operative to a lesser extent between the non-specific parts of the molecules, the complexes will therefore be soluble.

SUMMARY

1. The effect of washing serological precipitates with ice-cold saline has been investigated. Adequate removal of non-specific protein can be achieved by two or three washings with relatively small amounts of saline, and further washing removes traces of non-specific protein, or possibly antibody, only.

2. Antisera to iodinated sulphone-treated ovalbumin (and horse serum globulins) have been prepared, and their reactions investigated. (Sulphone=di-2-chloroethyl sulphone; mustard gas sulphone.)

3. The precipitin reaction between these antisera and protein antigens containing diiodotyrosine groups or sulphone groups is inhibited by 3:5-diiodotyrosine, by a tyrosine-sulphone compound, better by a mixture of these two, and best of all by a diiodotyrosine-sulphone compound.

4. The antisera have been 'exhausted' successively with antigens containing either the iodine groups or the sulphone groups, and with antigens containing both types of determinant groups, and the amounts of antibody precipitated at each stage

have been determined. Both homologous and heterologous carrier proteins have been used for the preparation of the antigens used in these experiments.

5. Precipitates obtained by treating one of these antisera with an antigen containing the sulphone determinant group only showed no significant capacity to combine with a second antigen containing iodine determinant groups only.

6. The experimental results are discussed from the point of view of the nature of the specific combining sites (receptor areas) on the antibody molecules. It is concluded that these are relatively large areas, specifically adapted towards equally large areas of the surfaces of the antigen molecules, but that the degrees of adaptation of different parts of the receptor areas may vary considerably from molecule to molecule. The evidence further suggests that it is unlikely that there is more than one such receptor area on each molecule of antibody.

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