

THE IMMUNOLOGICAL SPECIFICITY OF CHEMICALLY ALTERED PROTEINS

HALOGENATED AND NITRATED PROTEINS

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The immunological specificity of protein antigens has interesting aspects from the chemical viewpoint. Protein specificity appears to be closely associated with, and dependent on, chemical differences in the protein molecules. In most instances these differences are such that it is not possible to distinguish between similar proteins of different species by the usual methods of chemical investigation, but there are instances in which such a differentiation is possible by chemical or physical as well as by immunological tests. The differences which are responsible for serological specificity may not necessarily involve variations in the amount of certain special amino acids or even of free groups such as amino or carboxyl groups, but may be due merely to differences in the structural or spatial arrangement of certain groupings. A fundamental difference between antigenicity and specificity is indicated by the more recent work on complex antigens and haptens.

There are several methods applicable to the study of the relationship between immunological specificity and the chemical properties of antigens; (a) alterations in the chemical constitution of the protein can be effected by various chemical processes such as oxidation, iodination, nitration, esterification, etc. (Obermayer and Pick (1), Landsteiner (2)), (b) a comparison can be made of the immunological properties of closely related vegetable proteins (Wells and Osborne (3)) or animal proteins (cf. review by Wells (4) pp. 68-74), and (c) compounds of well-defined chemical nature can be attached to the protein molecule (Landsteiner (5)). Each of these methods has a special

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value in furnishing evidence about the nature of the groups which are concerned with specificity. The third method permits a study to be made of the effect produced by the introduction into the protein molecule of substances of related constitution and configuration, and many variations can be made in the constitution of the substituent group. The first method, the study of the specificity of chemically altered proteins, does not offer the same widespread opportunities since the methods of producing chemical changes in the protein molecule are rather limited; in addition there are few, if any, instances which cannot be objected to on the ground that the treatment to which the protein is subjected may produce other alterations besides that desired, for it is hardly possible to state with absolute certainty that the protein molecule can be attacked in such a way that only one significant change in the molecule occurs.

The first observations on the specificity of chemically altered proteins were made by Obermayer and Pick (1). The most significant changes were observed when serum was subjected to the action of iodine, nitric acid and nitrous acid and the so-called iodo-, nitro (xantho-) and diazo-proteins were obtained. These protein compounds still possessed antigenic properties but the chemical treatment had resulted in a loss of species specificity and the acquisition of a new specificity. Thus an iodoprotein from horse serum produced in a rabbit antibodies which reacted with iodized horse serum and with an iodoprotein derived from the serum of any species but not with native horse serum. Nitrated horse serum produced antibodies for any nitrated protein but not for horse serum or for iodized serum proteins. From these experiments the conclusion was reached by Obermayer and Pick (cf. also Pick (6)) that the species specificity of serum proteins is fundamentally related to the aromatic radicals present in the protein molecule and that any change in the aromatic nucleus destroys the original specificity and endows the molecule with a structural specificity characteristic for the new structure. Little evidence was available at that time however, that the action of iodine, nitrous acid or nitric acid was directed solely towards the aromatic amino acids, in particular the tyrosine molecules, and whilst these reagents are known to effect changes in the benzene ring, there may be other processes such as oxidation and hydrolysis which occur simultaneously. The methods of iodination used by Obermayer and Pick, iodination in hot acid solution (Hofmeister (7)) and iodination by the method of Blum and Vaubel (8) which involves treatment of the serum with iodine in the presence of sodium bicarbonate and heating at 50° for some time, will produce changes in the protein molecule other than simple iodination in the benzene ring. Also, in the preparation of nitrated and diazotized proteins, the treatment with acid may effect changes such as denaturation or hydrolysis.

Although in these three processes (iodination, nitration and diazotization) a distinct change occurs in the aromatic radicals of the protein, notably the tyrosine, the rest of the protein molecule is undoubtedly affected by the treatment.

One of the most interesting of these chemically altered proteins is the iodoprotein and several authors have obtained general confirmation of the observations made by Obermayer and Pick that treatment with iodine destroys, more or less completely, the original species specificity and occasions a new specificity.

Wells (9) obtained inconstant results with iodized serum in anaphylactic experiments with guinea pigs but with one preparation found that the iodized serum possessed the property of sensitizing guinea pigs to either the iodized serum or normal homologous serum. In general, however, no loss of species specificity occurred on iodination. Freund (10) carried out experiments similar to those of Obermayer and Pick and obtained confirmatory evidence that the specific complex of the protein is bound to the aromatic groupings of the molecule. Anaphylactic experiments have been carried out by various authors (11, 12, 13, and 14). Bauer and Murschhauser (15) described immunization experiments with iodized serum and iodized casein and conclude that only the labile proteins, such as serum proteins, lose their specificity on iodination and that the less labile proteins (cf. caseinogen) do not behave in this way.

The serological properties of nitrated proteins have recently been discussed by Ottensooser and Strauss (16).

There appears to be satisfactory evidence that the process of iodination leads to a more or less complete loss of species specificity and to the acquirement of a new specificity for iodinated proteins. It appeared desirable however, that more evidence should be available as to the nature of the changes involved in the iodination process before the loss of specificity is attributed solely to the introduction of iodine into the benzene ring of the tyrosine molecules present in the original protein. The view that the aromatic radicals largely determine the species specificity appeared to receive support from the fact that gelatin, which is deficient in aromatic amino acids, possesses no antigenic properties (Wells (9)). It is quite possible however, that the whole of the particular grouping or complex which is necessary for antigenicity may play little or no part in determining specificity, and indeed, the more recent observations on the haptens of pneumococci (Avery and Heidelberger) and the Forssman antigen (Landsteiner and Levene) indicate that the grouping which determines specificity may be entirely devoid of aromatic radicals. From the studies of the immunological properties of the iodoproteins and those of the nitrated proteins, it appeared probable, however, that the tyrosine groupings are concerned in some way with protein specificity, but the evidence upon which this view was based is not entirely convincing.

Several authors (Hofmeister (7), Hopkins (17), Blum and Vaubel (8) etc.) had observed that iodized proteins react negatively to Millon's test, a finding which appeared to justify the conclusion that substitution in the tyrosine nucleus occurs; but it was not until afterwards that Oswald isolated 3:5 diiodotyrosine from naturally occurring iodoproteins (18) and from artificially iodized proteins (19).

The methods of iodination used for the immunological experiments above mentioned—those of Hofmeister (7), and Blum and Vaubel (8)—may, as discussed above and as pointed out by Hofmeister, produce changes in the protein molecule in addition to simple substitution in the benzene ring of the tyrosine molecules. Iodization by the method of Blum and Vaubel leads, according to Blum and Strauss (20), to iodination in the tyrosine grouping, some oxidation processes and a change involving a destruction of one of the groups responsible for the biuret reaction. The latter authors also discuss the possibility of iodination in parts of the protein molecule which are unrelated to the tyrosine groupings. Very recently an investigation was made by Bauer and Strauss (20-a) on the chemical changes which occur in iodination and nitration of proteins. According to these authors nitration of proteins may involve a substitution in the tyrosine and tryptophane groups.

It seems desirable that a full investigation should be made of the serological properties of the halogenated and nitrated proteins with a view to gaining more information as to the rôle played by the aromatic groupings of the protein molecule in determining specificity. Consequently, halogenation or nitration has been effected by methods which have been modified in order to limit, as far as possible, the other changes such as hydrolysis and denaturation which readily occur in acid and alkaline solutions. For halogenation, bromine has been used in addition to iodine, and a study has been made of the serological properties of the brominated proteins.

EXPERIMENTAL

Preparation of Antigens

Iodinated Serum Proteins.

(a) This method was a modification of that of Blum and Strauss (21). Five hundred cubic centimeters of horse serum, 500 cc. of water and 500 cc. of 7% NH_4OH were mixed well and $\text{N}/10$ iodine solution added, with shaking, in quantities of about 50 cc. at each time. The iodine solution was added until free iodine was detectable by starch-iodide paper 5 minutes after the last addition of iodine, and then acetic acid was added to give maximum precipitation. The solution was centrifuged and the precipitate was dissolved in about 1500 cc. of distilled water by the aid of a little dilute Na_2CO_3 solution, the pH of the solution being kept below 8.5. The iodoprotein was reprecipitated twice more from a very faintly alkaline solution by acetic acid and then dissolved in 0.9% NaCl solution by the aid of a little dilute Na_2CO_3 solution. The reaction of the solution was adjusted to pH 7.5 and sufficient phenol to give 0.25% was added to that part of the solution required for injection. The horse serum iodoprotein, reprecipitated several times

from slightly alkaline solution by acetic acid and washed well with alcohol, contained 9.0% of iodine. Similar preparations were made with beef, chicken and rabbit serum. All the iodoproteins prepared by this method reacted negatively to Millon's test.

(b) *Iodination in Hot Acid Solution.* ((7.1) cf. Landsteiner and Prášek (22).) The brown product was dissolved as far as possible in dilute sodium carbonate solution and reprecipitated with dilute acetic acid. The product was then re-dissolved in 0.9% NaCl by the addition of a small amount of dilute Na_2CO_3 . The reaction of the solution was adjusted to pH 7.5. This preparation and the corresponding antisera were used for the experiments recorded in table IX only.

Brominated Serum Proteins. A modification of the method of Hopkins and Pinkus (23) was found most satisfactory for the purpose. A mixture of 250 cc. of horse serum and 500 cc. of distilled water was treated with bromine water until free bromine could be detected after the solution had been well shaken and allowed to stand for a few minutes. One per cent sodium carbonate solution was then carefully added to give maximum precipitation and the solution centrifuged. The precipitate was dissolved in about 1 liter of water with the aid of a small amount of dilute Na_2CO_3 , the pH being kept below 8.5 and the bromoproteins precipitated by the addition of a small amount of 10% acetic acid. This precipitate was dissolved in about 300 cc. of 0.9% NaCl by the addition of a little dilute Na_2CO_3 , the reaction of the solution adjusted to pH 7.5, and sufficient phenol to give 0.25% was added to the solution used for animal injections. The brominated horse serum proteins, purified by several precipitations with acetic acid, washed with alcohol and dried, contained 4.7% of bromine. The brominated sera reacted negatively to Millon's test. Attempts were made to modify this process by brominating in approximately neutral solution by adding Na_2CO_3 or NaOH after the addition of bromine to the horse serum but the products obtained differed from that obtained by the method described above in their insolubility in neutral or faintly alkaline solution (pH 7.5 to 8.0).

Chlorinated Serum Proteins. A method analogous to the bromination method was used and a product obtained which had properties similar to those of the brominated proteins. A fairly considerable amount of chlorine was absorbed during the chlorination process but when the crude chloroproteins had been purified by several reprecipitations from slightly alkaline solutions with acetic acid and had been washed well with alcohol and then dried, they contained no combined chlorine. The results of the immunization experiments with the chlorinated proteins are, therefore, not included here since a comparison with the other halogenated proteins is not possible. The results showed that some slight loss of the original species specificity had occurred.

Nitrated Serum Proteins.

(a) With nitric acid ("xantho" proteins)—cf. Landsteiner and Prášek (22).

(b) With tetranitromethane ("nitro" proteins). One hundred cubic centimeters of serum, 100 cc. of pyridin, 200 cc. of distilled water and 30 cc. of tetranitromethane were mixed and shaken at intervals at room temperature. The mix-

ture assumed a reddish orange color which finally became orange brown, sometimes with the intermediate formation of a deep green colour. Further additions of pyridine were made to keep the solution neutral or faintly alkaline. The solution was centrifuged, after the addition in some cases of a small amount of dilute acetic acid to complete the precipitation, and the precipitate washed with about 200 cc. of acetone, centrifuged and washed twice more with about 200 cc. of acetone. The yellow precipitate was then suspended in water and dilute sodium carbonate added to render slightly alkaline. NaCl was added to give 1%, and the pH adjusted to 7.5. Sufficient phenol to give 0.25% in the solution was added to the suspension of nitrated horse serum used for the injections. The amount of protein in the filtered solutions was 0.5–1.5%.

Metaprotein. Twenty cubic centimeters of serum were mixed with 200 cc. of distilled water and 2 cc. of concentrated HCl (spec. grav. 1.18). The mixture was allowed to stand at 37°C. for 24 hours and the metaprotein precipitated by the addition of dilute sodium carbonate. The precipitate was washed with a little 0.9% NaCl solution and dissolved in water with the aid of a little dilute Na₂CO₃ or very dilute NaOH. The solution had to be kept slightly alkaline to prevent precipitation of the metaprotein. Thymol was added as a preservative.

Diazotized Serum Proteins. (cf. Landsteiner and Prášek (22).)

Immunization

Five rabbits received intravenous, or in some cases intraperitoneal, injections of the antigen at weekly intervals and the sera were tested 7 days after the third and, where necessary, the subsequent injections. The antigens used for injection were prepared from horse serum in every case.

Iodo-horse Protein. Five cubic centimeters of a solution containing 4.5% of iodoprotein were injected intravenously on the first occasion. Subsequent injections were made intraperitoneally since several fatalities occurred on the second intravenous injection. Two rabbits gave good antisera after three injections and two more after four injections.

Acid Iodinated Protein. (Hofmeister's preparation.) Two intravenous injections were made of 5 cc. of a solution containing 1.5% of iodoprotein, followed by intraperitoneal injections of 10–15 cc. of the solution at the subsequent injections. Four to six injections were necessary.

Brominated Protein. Intravenous injections were made of a solution containing 4% of bromoprotein. Two animals were bled after 3, one after 4 and one after 5 injections.

Nitrated Protein ("Xanthoprotein"). Intravenous injections were given of 5 cc. of a solution containing 2% of xanthoprotein. One animal bled after 3, one after 4, and one after 5 injections.

Nitrated Protein ("Nitroprotein") Intraperitoneal injections were made of 10 cc. of a suspension containing 2.5% of nitroprotein. Four animals bled 7 days after the third injection.

Precipitin Tests

For these tests the antigen solutions were diluted to 1:100, 1:500, 1:2500 and in certain cases 1:12500 in terms of a 5% stock solution, and 0.2 cc. used for each test. Two drops of the immune serum were added to each tube, the solutions shaken and readings made after 1 hour at room temperature (25 to 30°C.). The intensity of the reaction was indicated as follows:—(no reaction), f. tr. (faint trace), tr. (trace), \pm , +, $+\pm$, ++, etc.

The first readings in the following tables are those recorded after 1 hour at room temperature and the second readings after 2 hours at room temperature and overnight in the ice box. In most of the tables the results of tests with two antisera only, and in certain instances with only one serum, are recorded. Other antisera gave similar results and in each case at least three antisera were tested.

The iodoprotein preparation and the iodoprotein antisera used for the following experiments, except those recorded in table IX, were those corresponding to the first method of iodination, namely, iodination in the presence of ammonium hydroxide.

The serological properties of the iodinated and brominated proteins were investigated by experiments the results of which are given in tables I, II, and III. From table I it will be seen that the iodoprotein antiserum reacts to the maximum extent with iodinated serum and somewhat less with brominated sera. The bromoprotein antisera react with brominated or iodinated proteins, to a slightly greater extent with the former. Iodination and bromination lead therefore to a more or less complete loss of the original species specificity (tables II and III), but the reaction between an iodo-horse antiserum and the homologous antigen is always more marked than that with any other iodinated proteins. The same holds true for the brominated proteins, and in all cases there seems to be a retention of some species characteristics. The effect is more pronounced when the readings are made after a shorter interval of time, e. g., 5 minutes after mixing.

The results recorded in table IV and V show that the proteins nitrated by the two methods used have similar serological properties and that nitration by either method leads to the acquirement of a new characteristic specificity.

Cross tests made between nitrated and halogenated proteins (table VI) indicate that some interaction occurs between certain halogenated proteins (in the main the brominated products) and antisera to nitrated proteins. In these cases there is no question of a species reaction since

TABLE I

Antigen	Antigen dilution	Iodo-horse antiserum			Bromo-horse antiserum		
		1635	1643	1643	1822	1822	1823
Iodo-horse	1:100	++±	+++	+++±	++±	++±	++±
	1:500	+±	+±	+±±	+±	+±	+±
	1:2500	±	±	+	+	+	+
Iodo-beef	1:100	+±	+±	+±±	+±	+±±	+±
	1:500	+±	+	+±	+±	+±	+±
	1:2500	tr	tr	±	±	+	+
Iodo-chicken	1:100	+	+	+	+±	+±	+±
	1:500	+±	+±	+±	+	+	+±
	1:2500	tr	tr	±	±	±	+
Iodo-rabbit	1:100	+±	+	+±	+±	+±±	+±
	1:500	+±	+±	+±	+±	+±	+±
	1:2500	±	±	+	±	±	+
Bromo-horse	1:100	+±	+±	+±±	+±	+±±	+±
	1:500	+	±	+	+±	+±	+±
	1:2500	tr	-	±	±	+	+
Bromo-beef	1:100	+±	+	+±	+±	+±	+±
	1:500	±	±	±	+±	+±	+±
	1:2500	tr	-	±	±	±	+
Bromo-chicken	1:100	+	+±	+±	+±	+±	+±
	1:500	+	±	±	+	+	+±
	1:2500	f. tr	-	-	tr	±	±
Bromo-rabbit	1:100	+±	+	+±	+±	+±	+±
	1:500	+	±	±	+±	+±	+±
	1:2500	tr	-	-	±	±	+

the antigens tested were prepared from proteins different from that which gave rise to the antiserum. A similar cross reaction occurs between bromo-horse antibodies and nitrated proteins, antigens (cf. table VII).

Table VIII gives the results of experiments with diazotized proteins and shows that diazoproteins and nitrated proteins are very closely related serologically. Thus diazo-beef and nitro-beef proteins, for

TABLE II

Antigen	Antigen dilution	Iodo-antiserum						Bromo-antiserum		
		1657		1635		1643		1822	1823	1821
Horse serum	1:100	±	+	—	f.tr.	—	—	—	—	—
	1:500	±	+	—	f.tr.	tr.	tr.	—	—	—
	1:2500	tr.	±	—	f.tr.	f.tr.	tr.	—	—	—
	1:12500	—	—	—	—	—	—	—	—	—

TABLE III

Immune serum	Antigen dilution	Antigen					
		Iodo-horse		Bromo-horse		Horse serum	
Anti-horse No. 55	1:100	f.tr.	f.tr.	—	—	+++	+++±
	1:500	tr.	tr.	—	—	++	++
	1:2500	—	—	—	—	±	+
Anti-horse No. 1807	1:100	—	±	—	—	++	+++±
	1:500	—	tr.	—	—	+	+±
	1:2500	—	—	—	—	±	+

example, react equally well with antisera to any type of nitrated protein investigated. Diazotized proteins, like nitrated proteins, also give some cross reactions with brominated proteins.

In table IX are given the results of experiments with the proteins iodinated in acid solution (Hofmeister's preparation). These antigens do not appear to be very active serologically but give antisera which react quite well with iodinated proteins prepared by the more gentle treatment (method (a)) or with brominated proteins. The antigens obtained by iodination in acid solution gave, however, remarkably

weak reactions with antisera to iodoproteins prepared by the action of iodine in ammoniacal solution.

Since it has been shown (Landsteiner (24), Landsteiner and van der Scheer (25)) that the precipitin reaction of azo-proteins can be inhibited specifically by simple substances containing the specific reactive grouping, it was thought possible that similar tests would give some

TABLE IV

Immune serum	Antigen dilution	Antigen							
		Xantho-horse		Xantho-beef		Xantho-chicken		Xantho-rabbit	
Xantho-horse No. 1770	1:100	+	+±	±	±	tr.	±	tr.	±
	1:500	+±	++	+	+±	+	+±	+	+±
	1:2500	+	+	±	+	tr.	+	±	+
Xantho-horse No. 1766	1:100	+±	++	±	+	+	+	±	+
	1:500	+	++	+	+±	+	+±	+	+±
	1:2500	±	+	±	+	±	+	±	+
Nitro-horse No. 1879	1:100	++	++±	±	+	tr.	±	tr.	tr.
	1:500	++	++±	+	+±	+	+±	+	+±
	1:2500	+	+±	±	+	±	+	+	+
Nitro-horse No. 1881	1:100	++	++±	+	+±	+	+±	+	+±
	1:500	+±	++	+	+±	+	+	+	+
	1:2500	±	+	tr.	+	tr.	+	±	+
Anti-horse No. 1807	1:100	-	-	-	-	-	-	-	-
	1:500	-	-	-	-	-	-	-	-
	1:2500	-	-	-	-	-	-	-	-

The term "xanthoprotein" is used here to refer to the nitrated proteins obtained by treatment with nitric acid and "nitroprotein" for the products obtained by the tetranitromethane method.

indication as to the nature of the reacting groups of the halogenated proteins.

Inhibition tests with equimolecular quantities of various substances (table X) show that whereas tyrosine and several other amino acids have no inhibitory effect on the reaction between halogenated proteins and the corresponding antisera, 3:5 diiodo-, 3:5 dibromo- and 3:5 dichloro-tyrosine have a very marked inhibitory influence on the

TABLE V

Immune serum	Antigen dilution	Antigen				Horse serum
		Nitro-horse	Nitro-beef	Nitro-chicken	Nitro-rabbit	
Xantho-horse No. 1770	1:100	+	+	+	+	-
	1:500	+	+	+	+	-
	1:2500	f. tr	±	±	±	-
Xantho-horse No. 1764	1:100	+	+	+	+	-
	1:500	+	+	+	+	-
	1:2500	tr	±	tr	+	-
Nitro-horse No. 1879	1:100	+	+	+	+	+
	1:500	+	+	+	+	±
	1:2500	+	+	+	+	±
Nitro-horse No. 1881	1:100	+	+	+	+	±
	1:500	+	+	+	+	±
	1:2500	+	+	+	+	±
Anti-horse serum No. 1807	1:100	-	-	-	-	+
	1:500	-	-	-	-	+
	1:2500	-	-	-	-	±

See note at the bottom of table IV for an explanation of the terms xanthoprotein and nitroprotein.

iodoprotein or bromoprotein precipitin reaction. It is interesting to note that the diiodo-compound is most active in this respect and the dichloro-compound least active. Stronger solutions of all three dihalo-

TABLE VI

Immune serum	Antigen dilution	Antigens							
		Iodinated serum			Brominated serum				
		Beef		Chicken		Beef		Chicken	
Xantho-horse No. 1770	1:100	—	—	—	—	±	+	tr	±
	1:500	—	—	—	—	±	±	tr	tr
	1:2500	—	—	—	—	—	tr	—	—
Xantho-horse No. 1766	1:100	—	±	—	tr	±	+	±	+
	1:500	—	tr	—	tr	±	±	tr	±
	1:2500	—	f.tr	—	f.tr	tr	tr	—	f.tr
Nitro-horse No. 1879	1:100	tr	±	—	tr	±	+	±	+
	1:500	tr	+	—	tr	tr	±	tr	±
	1:2500	f.tr	tr	—	—	f.tr	tr	—	f.tr
Nitro-horse No. 1883	1:100	—	tr	—	—	±	+	tr	±
	1:500	—	tr	—	—	±	±	f.tr	f.tr
	1:2500	—	—	—	—	—	tr	—	—

TABLE VII

Antigen	Antigen dilution	Antigens					
		Xantho-proteins		Nitrated proteins			
		Beef		Chicken			
Iodo-horse No. 1635	1:100	—	—	—	—	—	—
	1:500	—	—	—	—	—	—
	1:2500	—	—	—	—	—	—
Bromo-horse No. 1823	1:100	—	—	—	—	tr	±
	1:500	—	—	—	—	f.tr	±
	1:2500	—	—	—	—	—	f.tr
Anti-horse serum No. 1807	1:100	—	—	—	—	—	—
	1:500	—	—	—	—	—	—
	1:2500	—	—	—	—	—	—

generated tyrosine compounds inhibit completely or almost completely the reaction between iodoprotein antigen and iodoprotein antiserum, and that between the brominated protein antigen and bromo-protein

TABLE X

Inhibition Tests. 0.2 cc. of a solution of the substance tested for inhibiting action (containing 0.5 millimol per 100 cc. of 0.9% NaCl in each case) + 0.05 cc. of the antigen solution + 2 drops of immune serum. The strength of the antigen solution for the first four horizontal columns was 1:100 and for the others 1:20, of a 5% stock solution.

Antigen	Immune serum	Control	Substances tested for inhibitory action													
			Tyrosine	3.5 diiodo-tyrosine	3.5 dibromo-tyrosine	3.5 dichloro-tyrosine	Alanin	Phenylalanin	Histidin	Phenol	o-iodophenol	Potassium iodide				
Horse serum	Horse serum No. 1807	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±
Xantho-chicken	Xantho-horse No. 1770	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±	+ +±
Iodo-chicken	Iodo-horse No. 1635	+ +±	-	tr	±	±	±	±	±	±	±	±	±	±	±	±
Bromo-chicken	Bromo-horse No. 1822	+ +±	-	f. tr	tr	±	±	±	±	±	±	±	±	±	±	±
Bromo-chicken	Iodo-horse No. 1635	+ +±	-	tr	tr	±	±	±	±	±	±	±	±	±	±	±
Iodo-chicken	Bromo-horse No. 1822	+ +±	-	-	f. tr	tr	f. tr	tr	tr	tr	tr	tr	tr	tr	tr	tr

antiserum or the cross reactions between iodoproteins and bromoproteins. None of these inhibiting substances have any influence on the reaction between nitrated proteins and the corresponding antisera or on the reaction between horse serum antiserum and the homologous antigen, indicating that the inhibiting effect of 3:5 dihalogenated tyrosine on the iodoprotein and bromoprotein precipitin reactions is specific.

All the antisera were tested with metaprotein antigens (beef and chicken) but in no case was any positive reaction noted.

DISCUSSION

The results of the experiments with halogenated and nitrated proteins undoubtedly offer support for the view that the aromatic radicals of the protein molecule may be concerned with the determination of specificity; but there are changes in the protein molecule which do not appear to be concerned with the aromatic nuclei but which lead to a loss of species specificity. Such an effect is produced by esterification with acid alcohol, acetylation, or methylation (Landsteiner and Prášek (26), cf. also (27, 28)).

Whilst the methods previously used were rather drastic in nature, the methods of halogenation employed here were such that hydrolytic and oxidative changes were reduced as far as possible.

Iodination and bromination result in a more or less complete loss of the original species specificity and a new specificity characteristic of halogenated proteins is produced. The injection of iodinated horse serum into a rabbit gives rise to the formation of antibodies which react with iodinated heterologous sera or with the brominated sera. Similar antibodies are produced by the injection of brominated serum and in these antigens there appears to be some common active grouping which is responsible for the new specificity. This new grouping seems to be bound up with the tyrosine molecules and for several reasons it appears probable that the serological properties of the halogenated protein antigens are due to the disubstitution of halogen in the 3:5 position of the tyrosine molecules. Ample evidence exists for the view that iodoproteins contain the 3:5 diiodotyrosine structure (Oswald (18, 19), Blum and Vaubel (8)), but in addition the possibility that substitution occurs in other places, (e. g. in the histidine groups), must not

be overlooked. According to Blum and Strauss (21), however, gentle iodination in the presence of ammonium hydroxide does not result in any appreciable attachment of iodine to the histidine nitrogen atoms.

Iodination or bromination leads to a loss of the power to give a positive Millon's reaction and this loss is attributed to disubstitution of halogen in the tyrosine ring in the ortho positions relative to the hydroxyl group (Blum and Vaubel (8)). This disappearance of a positive Millon's reaction runs parallel in the experiments described here, with the loss of the original species specificity, and incomplete iodination results in the formation of a product which still gives Millon's reaction and retains some of the species specificity. More convincing proof of the view that the new specificity of halogenated proteins is dependent upon the presence in the protein of 3:5 dihalogenated tyrosine groupings is derived however from the inhibition tests, since 3:5 diiodotyrosine has a marked inhibitory effect on the reaction between iodoproteins and the iodoprotein antiserum whereas tyrosine and other amino acids have none. Thus the iodoprotein antibodies have some active grouping which has a special affinity for the 3:5 diiodotyrosine grouping and indeed for any 3:5 dihalogenated tyrosine structure, since the corresponding bromo- and chloro-derivatives of tyrosine have a similar combining capacity for iodoprotein, or bromoprotein antibodies. These inhibitory reactions appear to be specific, and from other evidence (Landsteiner (24)) it seems possible that reactions of this type will be useful for the determination of the structure or configuration of chemically altered antigens.

The results of the experiments with nitrated (or xantho) proteins confirm the findings of Obermayer and Pick (1), that after nitration with concentrated nitric acid there is a more or less complete loss of species specificity with the production of a new specificity characteristic for nitrated proteins. This method of nitration undoubtedly results in fairly drastic hydrolytic, and possibly oxidative, changes, and hence it was considered desirable that some other method of nitration of proteins, preferably one in which strong acids are not used, should be devised. The new method adopted, a method based on that of Schmidt and Fischer (29) and involving nitration with tetranitromethane in neutral or slightly alkaline solution, yields nitroproteins which resemble, in general chemical properties and immunological

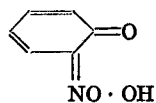
reactions, those obtained by the nitric acid method. Thus the antiserum to nitrated horse serum (tetranitromethane method) reacts with any nitrated serum or with the xanthoproteins (nitric acid method) from any serum, and the nitrated proteins react with the xanthoprotein antiserum.

A reinvestigation of some of the immunological properties of diazotised proteins has confirmed the observations of Landsteiner and Prášek (22) rather than those of Obermayer and Pick (1). The latter authors imply that xanthoprotein and diazoprotein are serologically well differentiated on account of the different nature of the substituents, and they conclude that substitution of a diazo group, presumably in the benzene ring, leads to a new structural specificity. Landsteiner and Prášek, however, found that the xanthoproteins and diazotised proteins show close serological relationship; thus the diazo antigens and the xantho antigens reacted almost equally well with diazotised protein antiserum or with xanthoprotein antiserum. In the experiments described here, diazotised serum proteins react like the xanthoproteins or the nitrated proteins and can hardly be differentiated in precipitation tests with xanthoprotein and nitrated protein immune sera. These facts seem to indicate that the diazotized protein and the nitrated protein have constitutions which are closely related.

Diazotization of proteins by the action of nitrous acid has proved difficult to account for chemically, since, according to all the evidence available, proteins contain no aromatic amino groups. Richard (30), who first described the reaction, reached the conclusion that proteins must contain aromatic amino groups, but another interpretation was advanced by Landsteiner (31) who showed that treatment of aromatic oxyacids, such as salicylic acid or tyrosine, with nitrous acid leads to the formation of diazo compounds (cf. also Weselsky (32), Morel and Sisley (33)). One may conclude that a similar process occurs when proteins are treated with nitrous acid, and that a diazo group is introduced into the tyrosine nucleus, probably in the ortho position to the hydroxyl group.

It is rather remarkable that diazotized salicylic acid and the diazotized proteins have a distinct yellow colour, comparable with that of the nitrated proteins. The marked yellow colour of the ortho-

nitrophenols has been attributed by Armstrong (34) to a quinoid structure (cf. also Hantzsch (35)).



This explanation might reasonably be extended to the case of the nitrated proteins and it is not improbable that a similar structure exists in the diazotized phenolic compounds or diazotized proteins. Such a change in the aromatic nucleus of the tyrosine molecule which leads to the formation of a quinoid structure might account for the serological alteration effected by nitration or diazotization of proteins. This view would offer a reasonable explanation for the close serological similarity between nitrated and diazotized proteins. At any rate it appears that a considerable difference in the nature of the substituent grouping has, in this case, no influence on the serological specificity.

The results of the work of Obermayer and Pick are generally accepted as a proof that the serological differences observed by the authors are merely due to chemical differences in the radicals introduced into the protein molecule. In the present investigation, however, an intense cross reaction between nitrated proteins and diazotized proteins and a strong interaction between iodo- and bromoproteins, were observed. Apart from slight cross reactions there was a sharp serological distinction between the nitrated and diazotized protein on the one hand and the halogenated protein on the other but this difference is not necessarily dependent upon the nature of the substituents only. It may depend on the place of substitution and on the number of entering groups which is probably different in halogenated and nitrated proteins (cf. 19, 36, 20-a).

It is not possible from the evidence available at the present time,—and to acquire more will be difficult,—to decide how far the introduction of different radicals into the same position of the aromatic nucleus would lead to serological differentiation. Thus it would be of interest to know whether the mere replacement of halogen atoms of halogenated proteins by, for example, hydroxyl, nitro, or methyl groups or by more complex radicals, would lead to the formation of antigens which would no longer react with antibodies to the halogenated pro-

tein. From other evidence, especially in regard to the azo-proteins (Landsteiner and van der Scheer (37)) it has been found that various substituents situated in the same position in the benzene ring do not necessarily occasion pronounced differences in serological behaviour.

In all the experiments with halogenated and nitrated proteins it has been observed that the antisera to a chemically altered horse serum react to the maximum extent with the homologous antigen. These differences, although distinct, are not so pronounced as some of those previously noted in the case of the xanthoproteins by Landsteiner and Prášek (22). These observations tend to indicate that the species characteristics are not entirely destroyed by nitration or halogenation, although the power to react with an immune serum to the native homologous serum may be entirely or practically entirely lost.

One possible explanation of these facts would involve the assumption that more than one group in the protein molecule is responsible for the species characteristics and that chemical treatment such as halogenation or nitration does not change or destroy the whole of these groupings. It is also possible that several active groupings of the antigen molecule combine with a similar number of groupings in the corresponding antibody and thus an antigen would give rise to the formation of an antibody which has its combining groups orientated in a manner similar to the orientation of the specificity groupings of the antigen. Thus the maximum reaction will be obtained only when the spatial distribution of the combining group or groups of the antigen and antibody are closely related, i.e., when the antiserum reacts with the homologous antigen.

SUMMARY

The serological properties of iodoproteins prepared by a method which involves less drastic treatment of the protein than the methods previously used for this purpose confirm the findings of Obermayer and Pick (1) and later authors, that iodination of proteins results in a more or less complete loss of species specificity and that a new specificity characteristic for iodoproteins is produced.

A serological investigation of brominated proteins has been made for the first time. These preparations are only slightly different from iodized proteins in their serological properties.

Evidence is submitted which indicates that the radical in iodoproteins which is responsible for the specificity is not iodine but the 3:5 dihalogenated tyrosine grouping. Thus marked inhibition of the iodoprotein (or bromoprotein) precipitin reactions is effected by 3:5 dihalogenated tyrosine, not by iodophenol or potassium iodide.

A reinvestigation has been made of the serological properties of nitrated and diazotized proteins. Proteins nitrated by nitric acid, or by a method which does not appear to have been used for proteins hitherto, namely nitration with tetranitromethane in neutral or slightly alkaline solution, acquire a new common serological specificity. The nitrated proteins and diazotized proteins show, in confirmation of the results of Landsteiner and Prášek (22) and in contrast to the findings of Obermayer and Pick, very little difference in their reactions. Thus diazotized proteins and proteins nitrated by either of the two methods above mentioned react equally well with any nitroprotein antiserum. This interaction exists in spite of the difference in the substituents, either because the substitution with the nitro- or diazo-group occurs in the same position in the aromatic nucleus, possibly in the ortho position to the hydroxyl group, or because of some other structural similarity. In the last connection it is suggested that both compounds may have a quinoid structure as has been assumed for ortho-nitrophenols.

Whilst this assumption could account for the marked serological difference of nitrated and halogenated proteins it should also be mentioned that iodination (and bromination) lead to a disubstitution of halogen in the two ortho positions relative to the hydroxyl group of the tyrosine whereas nitration of proteins probably results in the formation of mononitrotyrosine and substitution in the tryptophane group as well (19, 36, 20-a). It is probably impossible therefore, to draw a strict analogy between nitration (or diazotization) and halogenation of proteins since a comparison of their immunological properties is not exactly a comparison of the effect of substituting a different group in the same position. Accordingly it would appear that as yet no definite conclusions can be drawn as to the serological effect of differences in the chemical nature of various substituents in the aromatic nucleus although some influence is likely for general reasons.

All of the chemically altered proteins still retain a small amount of

the original species specificity, and the antisera always react to a slightly greater extent with the homologous antigen than with similarly treated antigens prepared from heterologous sera. This difference occurs even when the possibility of some unaltered protein being present in the antigen can be practically excluded.

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