CXLII. THE IMMUNOLOGICAL PROPERTIES OF ALKALI-TREATED PROTEINS.

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THE influence of acid and alkali on proteins in relation to the antigenic properties of these proteins has been studied by many authors. Wells [1909] noted the marked loss of antigenic power when crystalline egg-albumin was treated with sodium hydroxide, whereas little loss occurred following similar treatment with hydrochloric acid. Ten Broeck [1914] found that racemised egg-albumin (prepared by Dakin's [1912] method) was non-antigenic, and similar results with other proteins were obtained by Landsteiner and Barron [1917] and by Kahn and McNeal [1918].

Evidence of restoration of antigenic properties to alkali-treated proteins was furnished by Landsteiner and Barron [1917], who showed that when the non-antigenic alkali-treated serum-proteins were treated with nitric acid, antigenic xanthoproteins were formed; these xanthoproteins prepared from alkali-treated proteins gave precipitin and complement-fixation reactions with antisera to xanthoproteins from untreated serum-proteins and also acted as true antigens when injected into rabbits. These results, indicating some reversal of the process or processes which originally caused loss of antigenic power, are very significant, and it is possible that a further study of this phenomenon would add considerably to our knowledge of protein antigens.

In view of the importance of this relationship between the action of alkali and the loss of antigenic properties, it was decided to attempt to define more precisely the relationship between p_H and loss of antigenicity, and also to try to obtain, by some method other than that used by Landsteiner and Barron [1917], confirmatory evidence of the restoration of antigenic power to alkali-treated proteins. For the latter purpose, it was considered that iodination of the alkali-treated proteins would be most suitable, since iodination can be effected in neutral or faintly alkaline solution, and, although it replaces the original species specificity of serum proteins by a specificity determined principally by the 3 : 5-diiodotyrosine groupings [Wormall, 1930], iodination does not lead to any appreciable loss of antigenic properties.

In the investigation described here, the proteins of horse-serum were rendered non-antigenic by treatment with sodium hydroxide for approximately 24 hours at 18° in one series and 30° in another series. Fractions of these "alkali-proteins" were nitrated and others iodinated, and precipitin

and complement-fixation tests were then carried out with these preparations to determine whether the power to react with various antisera had been restored by nitration and iodination. These protein preparations, the alkali-treated proteins and the corresponding iodinated and nitrated alkali-treated proteins, were also tested for antigenic power by injection into rabbits, precipitin and complement-fixation tests being made at intervals.

EXPERIMENTAL.

Antigens used for injection.

The following antigens were prepared for immunisation purposes. NaCl to give 0.9 %, and phenol to give 0.25 % were added to all the antigen solutions, except those containing iodoprotein, which received NaCl only. All the solutions were kept in the ice-chest.

NaOH-treated serum-proteins. (a) At 18°. A mixture of 1500 cc. of horse-serum, 235 cc. of 7.4 *N* NaOH and 15 cc. of toluene was kept at 18° for 22 hrs. Conc. HCl was added to give maximum precipitation, and the precipitated proteins were filtered off and washed well with water; they were then dissolved in water with the addition of a small amount of *N*/2 NaOH and sufficient 10 % Na_2CO_3 solution to give a clear solution with p_H about 7.8. NaCl and phenol were then added in the amounts given above and the solution kept in the ice-chest. The final volume was 1000 cc. and the solution contained 5.75 % of protein (solution A).

(b) At 30°. A mixture of 2500 cc. of horse-serum, 225 cc. of 12.15 *N* NaOH and 25 cc. of toluene was kept in an incubator at 30° for 24 hrs. The proteins were precipitated by HCl as above, and finally obtained in solution (800 cc.) at about p_H 7.8. The solution contained 5.6 % of protein (solution B).

Iodinated NaOH-treated proteins. (a) 250 cc. of the solution of NaOH-treated proteins prepared as above (solution A) were mixed with 250 cc. of distilled water and 250 cc. of 7 % NH_4OH solution, and treated with *N*/10 iodine solution until there was a considerable excess of iodine [cf. Wormall, 1930]. The iodinated proteins were precipitated by the addition of 10 % acetic acid to give maximum precipitation, and the precipitated protein was removed by centrifuging. This precipitate was washed with water and dissolved in water with the addition of sufficient 10 % Na_2CO_3 solution to give a clear solution, the reaction of the solution being adjusted to p_H 7.6. 250 cc. of a solution containing 5.2 % of protein were obtained in this manner.

(b) 250 cc. of the solution obtained by treating horse-serum with NaOH at 30° (solution B) were iodinated as above.

Nitrated NaOH-treated proteins (xanthoproteins). The method used for nitration was that involving the action of conc. HNO_3 at 18° in the presence of urea [cf. Landsteiner and Prašek, 1913]. The products of this reaction react immunologically like proteins nitrated by a method which does not involve treatment with strong acid, viz. the tetranitromethane method [Wormall, 1930], and in this paper the terms xanthoprotein and nitrated protein are used synonymously.

(a) To 300 cc. of a solution of NaOH-treated proteins (solution A) a few grams of urea were added followed by 600 cc. of conc. HNO_3 slowly with constant stirring and cooling. The mixture was kept for 45 mins. at 18° and then poured into a large volume of distilled water. The precipitate was then filtered off, washed with water and dissolved in water with the addition of a little *N*/5 NaOH and sufficient 10 % Na_2CO_3 solution to give a clear solution. The p_H was adjusted to 7.8 and 300 cc. of a solution containing 3.5 % of protein were obtained.

(b) 300 cc. of the protein solution obtained by NaOH-treatment at 30° (solution B) were nitrated in a similar manner.

Immunisation.

Each antigen solution was tested on four rabbits, the injections being made weekly. Serum from each rabbit was tested 7 days after the third and each subsequent injection, i.e. just before the fourth, fifth, sixth and seventh, and, in certain instances, the eighth injection, and 7 days after the final injection.

Series (a). For the first three injections, an amount of the solution (solution A or the corresponding iodinated or nitrated protein solution) which contained approximately 0.25 g. of protein (4.3 cc. to 7 cc.) was injected intravenously into each rabbit. Five further injections of a volume of the solution which contained approximately 0.35 g. of protein were made intraperitoneally.

Series (b). With solution B and the corresponding iodinated and nitrated protein solutions each rabbit received at each injection a volume of the solution which contained 0.25 g. of protein; each animal had seven injections, the first three or four being intravenous and the rest intraperitoneal.

Precipitin tests.

Two, and, in a few tests, three drops of the immune serum were added to 0.25 cc. of the diluted antigen solution in a small test-tube ($2'' \times \frac{1}{4}''$). The mixture was shaken and observed after it had stood 5 mins., 30 mins., 1 hr. and 3 hrs. at room temp. ($16-20^{\circ}$) and after a further 18 hrs. in the ice-chest. The amount of protein in each antigen solution had previously been determined, and the various antigen dilutions were made to correspond to the following dilutions of a 5 % protein solution; 1 : 20, 1 : 100, 1 : 500 and 1 : 2500. Several immune sera were used for each series of the tests, but since similar antisera gave essentially the same results, the reactions in each instance with one typical antiserum only are recorded in the Tables of this paper. The readings, unless otherwise stated, are those obtained after 1 hour.

The extent of precipitin formation is recorded as follows: — (no reaction), f.tr. (faint trace), tr. (trace), $\pm$, +, $+\pm$, ++ (increasing degrees of precipitation).

Complement-fixation tests.

A range of the antigen solution was made in a series of small test-tubes, each tube containing 0.5 cc. of the diluted antigen; 0.06 cc. of 1 : 5 guinea-pig-serum and 0.10 cc. of the inactivated rabbit-“immune” serum were added to each tube, the tubes shaken and allowed to stand for 45 mins. to 1 hr. 0.5 cc. of a 4 % suspension of sensitised ox-red blood cells was then added to each tube, the tubes shaken and incubated at 37° . The amount of haemolysis after various intervals, usually 15 mins., 30 mins. and 1 hr., was recorded. On some occasions similar series of tests were carried out with 0.05 and 0.15 cc. of immune serum and varying concentrations of antigen. Control tests with no antigen solution, and others with varying amounts of antigen and no immune serum, were always carried out.

The influence of p_H on the power of horse-serum proteins to react with antibodies to unchanged horse-serum.

In these experiments samples of normal horse-serum were kept at various hydrogen ion concentrations for 24 hrs. at 19° , and the power of such treated sera to react with antisera to untreated horse-serum was tested at intervals.

The adjustment of the horse-serum to a definite p_H was made by the addition of N HCl or N NaOH, and to the solution obtained a buffer solution (the universal buffer mixture of Prideaux and Ward [1924] (B.D.H. preparation) of the same p_H was added, the presence of this buffer mixture preventing any appreciable alteration in the p_H of each solution during the course of the experiment. For the solutions to be kept at p_H 1, N HCl was added to the diluted serum to give the desired p_H , and the solution then diluted with $N/10$ HCl; N and $N/10$ NaOH were used in the same way for the tests at p_H 13.

In the experiment recorded in Table I, 6 cc. of normal horse-serum were diluted with about 20 cc. of 0.9 % NaCl, and N HCl or N NaOH was added until the desired p_H was obtained; 20 cc. of universal buffer mixture of the corresponding p_H were added, and the mixture diluted to 60 cc. with 0.9 % NaCl. These mixtures were then kept at 19°, and samples withdrawn from each solution at the start, after 45 mins., 3½ hrs. and 24 hrs. Each sample, except those from the solution at p_H 7.5, was neutralised by the addition of acid or alkali and the reaction adjusted to p_H 7.5, after which the required dilutions were made and precipitin tests carried out. In Table I the results with p_H 4, 5, 6, 7, 8, 9 and 10 are omitted, since these did not differ appreciably from those given for p_H 7.5.

Table I. *The influence of p_H on the power of horse-serum-proteins to react with antibodies to untreated horse-serum-proteins.*

Tests carried out with anti-horse-serum immune serum No. 107 (readings after 1 hr.).

Duration of treatment at different p_H values	Precipitin reactions with horse-serum-proteins kept for various periods at the following p_H and subsequently neutralised							Dilution* of antigen after neutralisation
	1	2	3	7.5	11	12	13	
Start	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	1 : 20
	++	++	++	++	++	++	++	1 : 100
	+	+	+	+	+	+	+	1 : 500
	tr.	tr.	tr.	tr.	tr.	tr.	tr.	1 : 2500
45 mins.	+ ±	+ ±	+ ±	+ ±	+ ±	+	±	1 : 20
	+ ±	++	++	++	++	++	+	1 : 100
	±	±	+	+	+	±	tr.	1 : 500
	f.tr.	f.tr.	tr.	tr.	tr.	tr.	f.tr.	1 : 2500
3½ hrs.	+	+ ±	+ ±	+ ±	+	+	tr.	1 : 20
	+ ±	++	++	++	+ ±	+	±	1 : 100
	±	±	+	+	+	±	tr.	1 : 500
	tr.	tr.	tr.	tr.	f.tr.	—	—	1 : 2500
24 hrs.	+	+ ±	+ ±	+ ±	±	±	—	1 : 20
	+ ±	++	++	++	++	±	—	1 : 100
	±	±	+	+	+	tr.	—	1 : 500
	f.tr.	f.tr.	f.tr.	tr.	tr.	—	—	1 : 2500

* All antigen dilutions in this and in the following Tables are in terms of dilution of a 5 % protein solution.

The results of these experiments confirm the findings of the earlier workers in that they show marked destruction of antigenicity in alkaline solution and a less pronounced loss by treatment with acid. This investigation of the exact influence of p_H , rather than the study of the action of excess of strong acid

or alkali, leads to the conclusion, however, that in moderately alkaline solutions the loss of the power to give precipitation with antibodies to the unchanged serum is not as rapid as some authors have suggested. Thus, horse-serum kept at p_H 11 for 24 hrs. at 19° shows a slight loss only of this antigenic power. At p_H 12, however, marked loss occurs in $3\frac{1}{2}$ hrs. and at p_H 13 a similar destruction occurs in less than 1 hr.; after 24 hrs. at p_H 13, the serum no longer possesses the power to react with antisera to the native proteins of horse-serum.

In some of the above, and in later experiments, antisera obtained by the injection into rabbits of fat- and lipin-free proteins of normal horse-serum, obtained by the method used by Hartley [1925], were used. In all these experiments, antisera obtained in this way gave results identical with those given by antisera obtained by the injection of untreated horse-serum.

The action of strong alkali on the power of serum-proteins to react with antibodies to the unchanged serum-proteins and the immunological properties of these alkali-treated proteins after iodination and nitration.

Preliminary tests were carried out to determine the amount of NaOH necessary for the destruction, in 20–24 hrs. and at various temperatures (18° , 30° and 37°), of the power of horse-serum-proteins to react with antisera to untreated horse-serum-proteins. After the mixtures had stood for this length of time at the required temperature, conc. HCl was added to give maximum precipitation and the precipitated proteins were centrifuged and dissolved in 0.9 % NaCl solution with addition of the minimum amount of 10 % sodium carbonate solution to give a clear solution. Portions of these solutions of alkali-treated proteins were iodinated or nitrated by the methods indicated above, and the products obtained tested with various antisera. In the majority of these experiments it was found that, although sufficient strong alkali had been added to render the proteins incapable of reacting (in precipitin and complement-fixation tests) with antibodies to untreated horse-serum-proteins, the proteins formed could still take part in immunological reactions after iodination or nitration; thus, after iodination they gave reactions with antisera to iodinated horse-serum-proteins, and after nitration they reacted with antisera to nitrated horse-serum-proteins, the latter observation confirming those of Landsteiner and Barron [1917]. The iodinated alkali-treated proteins were not, however, always as strongly antigenic *in vitro* as were the corresponding nitrated proteins, but usually, after iodination or nitration, these “alkali-proteins” gave good precipitin reactions. In other experiments, where the amount of NaOH was much greater than in the experiments described here, no regeneration of antigenic properties by iodination or nitration could be effected, but this is not surprising in view of the pronounced hydrolytic and other destructive changes which occur when serum-proteins are treated with strong alkali. Table II records an experiment which illustrates some of these features. This experiment, involving the action of NaOH at 37° , shows that

on occasions the antigenic powers *in vitro* can best be restored with HNO_3 , for here the nitrated proteins of solution Y (a solution of alkali-treated horse-serum-proteins) gave good precipitin reactions with suitable antisera, whereas little precipitation was obtained with iodinated proteins of solution Y and antisera to iodinated horse-serum-proteins. In the case of solution Z, where much more alkali was used, no appreciable regeneration of the power to give precipitin reactions could be effected by iodination or nitration.

Table II. *Precipitin reactions.*

Antigen	Antigen dilution	Immune serum		
		Anti-horse-serum (No. 107)	Anti-iodo-horse-serum (No. 1657)	Anti-nitro-horse-serum (No. 1881)
Horse-serum (X)	1: 100	++	—	f.tr.
	1: 500	+ ±	tr.	tr.
	1: 2500	+	tr.	f.tr.
NaOH-treated horse-serum (Y)	1: 100	—	—	—
	1: 500	—	—	—
	1: 2500	—	—	—
NaOH-treated horse-serum (Z)	1: 100	—	—	—
	1: 500	—	—	—
	1: 2500	—	—	—
Iodinated X	1: 100	tr.	++	tr.
	1: 500	tr.	±	±
	1: 2500	—	tr.	tr.
Iodinated Y	1: 100	—	±	—
	1: 500	—	tr.	—
	1: 2500	—	—	—
Iodinated Z	1: 100	—	f.tr.	—
	1: 500	—	—	—
	1: 2500	—	—	—
Nitrated X	1: 100	—	—	f.tr.
	1: 500	—	—	+
	1: 2500	—	—	±
Nitrated Y	1: 100	—	—	+
	1: 500	—	—	±
	1: 2500	—	—	f.tr.
Nitrated Z	1: 100	—	—	f.tr.
	1: 500	—	—	tr.
	1: 2500	—	—	—

Experimental details.

X.	30 cc. of horse-serum +	1 cc. of toluene.
Y.	„ „	+ 6.9 cc. of 5.35 N NaOH + 1 cc. of toluene.
Z.	„ „	+ 10.5 cc. „ + „

These mixtures were kept at 37° for 20 hrs. The “alkali-proteins” of solutions Y and Z were precipitated by HCl and dissolved as described for the antigens used for injection. The amount of protein in each solution was determined and the antigen dilutions were calculated to refer to 5 % protein solutions.

Table III gives the results of precipitin tests with some of the antigens used for the injection experiments (series (a)), and here the restoration of *in vitro* antigenic power to alkali-treated proteins appears to be effected just as readily by iodination as by nitration. Results of a similar nature were obtained in complement fixation tests. The iodinated alkali-treated proteins also reacted with antisera to brominated horse-serum-proteins.

Table III. *Precipitin reactions of the antigens used for the injections (series (a)).*

Antigen	Antigen dilution	Immune serum			
		Anti-horse-serum (No. 107)	Anti-iodo-horse-serum (No. 1657)	Anti-bromo-horse-serum (No. 1822)	Anti-nitro-horse-serum (No. 1881)
NaOH-treated horse-serum-proteins (a)	1 : 20	-	-	-	-
	1 : 100	-	-	-	-
	1 : 500	-	-	-	-
	1 : 2500	-	-	-	-
Iodinated NaOH-treated horse-serum-proteins (a)	1 : 20	-	-	tr.	-
	1 : 100	-	+ ±	+	tr.
	1 : 500	-	+	±	f.tr.
	1 : 2500	-	f.tr.	f.tr.	-
Nitrated NaOH-treated horse-serum-proteins (a)	1 : 20	-	-	tr.	±
	1 : 100	-	-	tr.	+
	1 : 500	-	-	-	±
	1 : 2500	-	-	-	tr.
Horse-serum	1 : 20	++	-	-	-
	1 : 100	++	tr.	-	f.tr.
	1 : 500	+	tr.	-	tr.
	1 : 2500	±	f.tr.	-	f.tr.

The in vivo antigenic properties of alkali-treated proteins and of alkali-treated proteins which have been iodinated or nitrated.

The results of the experiments described above show that proteins which have been treated with sufficient alkali to destroy the power to react with antibodies to the original protein have not necessarily lost their antigenic capabilities, and that they can function as *in vitro* antigens after iodination or nitration. From these test-tube experiments it would not be justifiable to conclude, however, without further evidence, that restoration of antigenic properties had occurred, for several other possible explanations could be presented. The action of the alkali might, for example, result in the formation of denatured proteins with a new specificity, though such denatured proteins would be expected to give some reaction with antisera to the original protein. The most conclusive evidence would undoubtedly be furnished by immunisation experiments if they showed that, whereas no antibodies are produced in the animal body in response to the alkali-treated proteins, antibodies are readily formed after the injection of the corresponding iodinated or nitrated alkali-treated proteins.

The experiments to be referred to under this heading can be divided into two groups: series (a), in which were used horse-serum-proteins which had been treated with sufficient sodium hydroxide solution to give a final concentration approximately *N* and the mixture kept for 22 hrs. at 18°, and series (b) with a somewhat similar mixture kept at 30° for 24 hrs. Portions of each solution of alkali-treated proteins were iodinated and others converted into xanthoproteins, and immunisation experiments carried out with the "alkali-protein" and the corresponding iodinated and nitrated proteins. Four rabbits

were used for each antigen solution, and the serum of each animal was tested frequently, in precipitin and complement fixation tests, against the antigen solution used for the injections and against certain other antigens. Each rabbit received seven or eight weekly injections, and the serum tests were made 7 days after the third and subsequent injections. The results of these experiments supported, in general, the conclusion drawn from the results of the experiments already described above, the experiments with the xanthoproteins prepared from the "alkali-proteins" confirming the findings of Landsteiner and Barron [1917]. The antibody response to the iodinated and nitrated compounds, particularly to the former, was perhaps not as marked as was expected, but possibly this can be accounted for by the more drastic treatment with alkali. In these experiments the concentration of sodium hydroxide was approximately the same, but the incubation period was longer than in the experiments of Landsteiner and Barron (22 and 24 hrs. compared with 16 hrs.) and in series (b), at least, the incubation temperature was higher. The mixtures prepared by the latter authors were kept at room temperature, whereas those used in the experiments described here were kept at 18° (series (a)) and 30° (series (b)).

Table IV. *Precipitin reactions between the antigens used for the injections (series (a)) and the sera of the rabbits injected.*

Rabbit No.	Rabbit injected with	Antigen used in test	A Antigen dilution				B Antigen dilution			
			1:20	1:100	1:500	1:2500	1:20	1:100	1:500	1:2500
120	NaOH-treated	NaOH-treated	—	tr.	tr.	—	tr.	tr.	f.tr.	—
121	—	—	—	—	—	—	—	—	—	—
122	serum-proteins (a)	serum-proteins (a)	—	tr.	tr.	—	—	tr.	f.tr.	—
123	—	—	—	—	—	—	—	—	—	—
124	Iodinated	Iodinated	—	—	—	—	f.tr.	tr.	tr.	—
125	NaOH-treated	NaOH-treated	f.tr.	±	±	f.tr.	tr.	+	±	—
126	—	—	—	—	—	—	—	—	—	—
127	serum-proteins	serum-proteins	—	tr.	f.tr.	—	tr.	±	f.tr.	—
128	Nitrated	Nitrated	tr.	±	±	f.tr.	±	±	tr.	f.tr.
129	NaOH-treated	NaOH-treated	f.tr.	±	±	f.tr.	tr.	±	tr.	—
130	—	—	tr.	+	±	f.tr.	+	+	±	f.tr.
131	serum-proteins	serum-proteins	tr.	tr.	f.tr.	—	f.tr.	tr.	—	—

In the above table, A refers to tests made with sera of the rabbits after these animals had received three injections of the antigen, and under B are given the results with the sera obtained after five injections.

Table IV gives some typical results of the precipitin tests with the sera of the rabbits of series (a), and Table V some similar complement-fixation tests. The most pronounced differences are found when the NaOH-treated proteins and the corresponding nitrated compounds are compared. The former engender practically no antibody response, whereas the injection of the latter causes the production of antibodies for the antigen used. The iodinated alkali-treated proteins give some antibody response, and, although it is not as marked as that with the corresponding nitrated compounds, it is not insignificant.

Table V. *Complement-fixation tests with each antigen used for the injections (series (a)) and the sera of the rabbits injected with this antigen.*

Rabbit No.	Proteins used as antigen in test and for immunisation	Antigen dilution used					Control (NaCl)
		1:20	1:80	1:320	1:1280	1:2560	
120	NaOH-treated serum-proteins	4	4	3	3	4	4
121		4	4	4	4	4	4
122		4	2	1	—	1	4
123		4	4	4	4	4	4
124	Iodinated NaOH-treated serum-proteins	4	4	4	—	1	4
125		4	3	—	—	—	4
126		3	2	1	—	—	4
127		2	1	—	—	—	4
128	Nitrated NaOH-treated serum-proteins	4	2	—	—	—	4
129		2	—	—	—	—	4
30		2	—	—	—	—	4
131		3	2	—	—	1	4

Note. Control tests indicated that the antigen solutions, in the concentrations used here, had neither anticomplementary nor haemolytic powers.

In the above Table, 4 indicates complete haemolysis, — indicates no haemolysis, and 3, 2 and 1 give intermediate degrees of haemolysis.

The results with the proteins of series (b) (alkali-treatment at 30°) were similar to those in Tables IV and V, with somewhat less marked precipitin and complement-fixation reactions. Treatment of serum-proteins with strong alkali at this higher temperature probably results in such pronounced hydrolytic and other changes that regeneration, even in part, of antigenic properties is no longer possible. The nitrated proteins of this series, however, did give some antibody response, and marked complement-fixation reactions were obtained. The sera of the rabbits injected with iodinated proteins of this series gave no significant precipitin reactions, but did give some complement-fixation when tested against the corresponding antigen. Thus, in this series, the only satisfactory positive results were obtained in complement-fixation tests.

The sera of all these animals were tested on certain occasions with similar proteins which had not been subjected to the action of strong alkali, *viz.* with horse-serum-proteins, and also with iodinated and nitrated horse-serum-proteins. The results were similar to those described above and recorded in Tables IV and V, with, as was expected, more pronounced precipitation or complement fixation. Thus, the sera of rabbits injected with iodinated alkali-treated proteins reacted with iodinated horse-serum-proteins, but not with nitrated horse-serum-proteins or with untreated horse-serum-proteins, and the sera of rabbits injected with nitrated alkali-treated proteins reacted with the nitrated serum-proteins only.

DISCUSSION.

The destructive action of alkali on protein antigens has long been recognised [Wells, 1909, *etc.*] and several explanations have been put forward to account for this loss of antigenicity. Some loss of this function does occur during similar treatment with acid of approximately the same strength, but this action of acid is very mild compared with that of alkali. Alkalis appear

to have, therefore, a somewhat specific destructive action on protein antigens, and this action does not seem to be related to hydrolytic changes effected in the protein molecule.

The immunological properties of horse-serum-proteins which have been kept in buffered solutions of known p_H for varying lengths of time were first investigated in this work, with results which are of some value in relation to the general study of the immunology of chemically altered protein antigens. As was expected, destruction of the power of such proteins to react with antibodies to the untreated proteins occurred most readily in alkaline solution, but it was rather remarkable that there was no appreciable loss of this function after exposure for 24 hrs. (at 19°) to alkaline solutions at or below p_H 11. At p_H 12 there was moderate, and at p_H 13 marked, loss of this power in less than 1 hr. In acid solutions of p_H 1 and 2 there was only a very slight diminution in 24 hrs. at 19° of the power to give a precipitate with antisera to untreated horse-serum-proteins, and no detectable loss occurred in the solutions kept for this period at p_H 3–10 inclusive. The serum-proteins are certainly not the least sensitive of proteins to treatment with acid or alkali, and thus it appears rather remarkable that such changes in the reaction of the medium can occur without appreciable loss of these immunological properties. Any prolonged exposure of proteins to relatively strongly acid or alkaline solutions will undoubtedly cause serious alteration of the immunological behaviour of these proteins, but from the experiments recorded here it appears justifiable to conclude that exposure for short periods and at the ordinary temperatures to reactions between p_H 2 and p_H 11 will not appreciably alter the protein antigens of serum. In studying the immunological properties of chemically altered protein antigens therefore, although it is always advisable to avoid, where possible, the use of strong acid or alkali, especially the latter, the use of reagents of this type must not necessarily be condemned.

The loss of antigenic properties as a result of the action of alkali might be attributed to any one, or to a combination of two or more, of the changes which occur in the protein molecule. Hydrolysis, racemisation and changes in the sulphur-containing constituents of the protein take place in the alkaline medium, and in all probability there are other ways in which the protein molecule is modified by such treatment. Most authors consider that the loss of antigenicity is related to racemisation. For a discussion of the evidence see Wells [1929] and Hartley [1931]. Hartley suggests, however, that antigenicity is lost before racemisation is complete. No attempt has been made in the work described here to study this relationship or indeed to attempt to relate loss of antigenicity with any definite change or changes in the protein molecule, and the main problem was concerned with the possibility of reversing this destructive action of sodium hydroxide. Regeneration of antigenic power in alkali-treated serum-proteins can be effected by treatment with conc. nitric acid [Landsteiner and Barron, 1917], and the experiments described here have fully confirmed this observation.

The other method used in these attempts to obtain antigenic preparations from non-antigenic alkali-treated serum-proteins involved iodination. Iodination of proteins leads to no loss of antigenic power, but the original species specificity is lost and a new specificity acquired [Obermayer and Pick, 1906], this new specificity being characteristic for the 3 : 5-dihalogenated tyrosine grouping [Wormall, 1930]. Iodination of the alkali-treated proteins used in this work led to some regeneration of antigenic function (*cf.* Tables II, III, IV and V); thus the iodinated alkali-treated proteins reacted *in vitro* with antisera to iodoproteins from horse-serum, and after injection into rabbits they produced characteristic iodoprotein antibodies. The antigenic power regenerated was, however, not very high and certainly was not as pronounced as that released by nitration of the same alkali-treated proteins. It is not to be expected, however, that iodination or nitration will lead to complete regeneration of antigenic power, and the margin between temporary loss (which perhaps is reversed in part by nitration or iodination) and irreversible destruction of antigenicity will probably be small. This loss of antigenicity is probably due to several changes in the protein molecule, and nitric acid (or iodine in ammoniacal solution) may be able to reverse certain changes only. Whatever the reason for the regeneration of antigenic properties, it will probably be concerned with the tyrosine groupings of the protein molecule, groupings which are affected by nitration and iodination, and these findings emphasise still further the significance, from the immunological viewpoint, of the tyrosine molecules which are present in the protein. The species specificity of serum and other proteins appears to be linked in some way with these groupings, although it must not be overlooked that antigenic specificity is not always due to aromatic groupings. The experiments of Landsteiner and Barron and those described here furnish some additional support for the view that the tyrosine molecules play an important part also in determining antigenicity, a view which still rests mainly on the experiments of Wells [1908] and later authors, who have shown that gelatin, a protein deficient in aromatic amino-acids, is non-antigenic.

SUMMARY.

The action of acid and alkali on the power of horse-serum-proteins to react with antibodies to the untreated serum-proteins has been investigated with special reference to the precise influence of solutions of definite p_H values. The results agree with the earlier observations of other authors (Wells, *etc.*) that alkalis have a more marked destructive action than have acids. With moderate degrees of alkalinity, however (up to p_H 11 or so) there is very little loss of these antigenic properties in 24 hrs. at 19°.

In view of the findings of Landsteiner and Barron [1917] that the conversion of non-antigenic alkali-treated-proteins into xanthoproteins results in a regeneration of antigenic properties, attempts have been made to effect the same change by iodination. Some success was obtained, but iodination is not

as efficient as is nitration in regenerating antigenic properties in these alkali-treated proteins. The significance of these findings in relation to the chemistry of protein antigens is discussed.

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