Studies on the Reaction between Sodium Hypochlorite and Proteins

1. PHYSICO-CHEMICAL STUDY OF THE COURSE OF THE REACTION

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In connexion with their application in surgery, a comprehensive investigation of the action of solutions of sodium hypochlorite on proteins is being undertaken. The reactions have been studied first from physico-chemical standpoints, using egg albumin.

Earlier work on this subject is reviewed by Engfeldt (1922). More recently, Price (1931) has dealt with the nature of the reaction between sodium hypochlorite and gelatin. Wright (1926, 1936) emphasized the relative parts played by chlorination and by oxidation. Lieben & Bauminger (1933) also found, with casein, that the protein was both oxidized and chlorinated in alkaline solution by sodium hypochlorite. Commercial interest has led to work on the reaction between sodium hypochlorite and proteins (e.g. Cross, Bevan & Briggs, 1908; Briggs, 1918; Trotman, Trotman & Brown, 1928).

Reactions between sodium hypobromite and proteins, peptones, amino-acids and 'model-compounds' have received extensive attention in Germany by Goldschmidt *et al.* (e.g. Goldschmidt & Strauss, 1929) and by Brigl, Held & Hartung (1928).

This earlier work indicates that: (1) amino-acids and the amino-acid residues in proteins are attacked by sodium hypochlorite at speeds which differ greatly from one such acid or residue to another; (2) groupings other than the 'peptide linkage' in proteins are attacked; (3) both chlorination and oxidation occur; (4) groupings of different types comprising 'active chlorine' are formed. These groups show widely divergent degrees of stability; (5) the nature of the reaction does not vary greatly from one protein to another, and changes in concentrations and temperature bring about only changes of degree; (6) in distinctly acid solution, chlorination predominates over oxidation, and the nature of the reaction varies little over the range pH 1-6. In alkaline solution oxidation is more marked, but the reaction is profoundly influenced by the concentration of hydroxyl ions.

Preliminary unpublished work in the present investigation showed that albumin reacted rapidly with sodium hypochlorite with evolution of heat, and that as the reaction proceeded the pH of the

reaction mixture rose sharply at first before exhibiting the fall reported by other authors (e.g. Norman, 1936).

It was found, also, that casein and solid heat denatured egg albumin gave protein- and peptone-like bodies of comparatively high solubility, together with products of much lower molecular weight. In all fractions of the reaction mixture containing 'Kjeldahl nitrogen', combined electropositive chlorine was detected. The protein-fraction resembled somewhat the chloro-protein described by Salkowski (1923), and the bromo-protein precipitated by acetic acid from alkaline solution (Vandervelde, 1924). The absence of chlorate from the final reaction mixture was established, after precipitation of peptone, etc., by the ferrous sulphate—permanganate method.

The work now described falls into three parts: (A) A curve was constructed to show titres of electropositive chlorine, determined iodometrically, at different stages during the reaction of sodium hypochlorite with egg albumin in alkaline solution. Corresponding figures were obtained for the amount of nitrogenous (Kjeldahl) material that could be precipitated by phosphotungstic acid, thus giving a curve representing the progressive degradation of protein as a function of time. (B) Curves for temperature against time were constructed for the reaction. (C) Curves for pH value against time were constructed.

EXPERIMENTAL

A. Degradation of protein

Methods. The hypochlorite solution ('Milton') of which 101 ml. were taken, contained 1.12 g. NaOCl and 18.65 g. NaCl/100 ml. It was stirred mechanically in a thermostat at 36° and two samples of 0.5 ml. each were withdrawn for analysis, using KI solution and acetic acid, followed by titration with 0.02 N- $\text{Na}_2\text{S}_2\text{O}_3$. The reaction with protein was started at zero time by running in 25 ml. of solution containing 1 g. of granular egg albumin (moisture + ash = 14.5%) in 0.5% NaCl solution. Samples of convenient volume (1-2 ml.) were withdrawn successively over the first hour and analyzed as above. A final sample was titrated after 19 hr.

Degradation was followed by treating reaction-mixtures as above after different times to recover material of the

protein-to-peptone class. At the predetermined time, 20 ml. of KH_2PO_4 buffer (0.154 M, pH 4.6) were added and SO_2 was bubbled through, avoiding excess, until the reaction of starch-KI paper was negative. Phosphotungstic acid (2% in 1.5 N- H_2SO_4 , 25 ml.) was stirred in and after 15 min. the precipitate was separated by decanting and centrifuging. It was washed once with 20 ml. of a liquid of the composition: 130 ml. 11.3% NaCl solution + 19 ml. phosphotungstic precipitant + 20 ml. phosphate buffer (as above) and again centrifuged. Original supernatant liquids and washings showed no biuret reaction. The solid was taken up with 14 ml. of 2 N- Na_2CO_3 and made up with water to 25 ml., from which duplicate samples of 0.64 ml. were taken (microburette) for Kjeldahl analysis in which the ashing and the indicator were as described by Miller & Houghton (1945); NH_3 was distilled into 0.143 N-HCl from 40% NaOH containing $\text{Na}_2\text{S}_2\text{O}_3$. Excess acid was titrated with 0.143 N-NaOH. Results for total N of the precipitate were recorded as percentages of the value found in a control experiment in which NaCl (16.5%) was used in place of the hypochlorite. Duplicate results were reproducible within 1.2% (overall) of the mean. Duplicate determinations of N differed by less than 0.3% from the mean. The control experiment showed that the recovery of N was 88% of the calculated value.

Results. These (Fig. 1) show a close correspondence between the rapid reduction of hypochlorite and the degradation of protein. It is calculated that after

Fig. 1. Exothermic, oxidative degradation of albumin by NaOCl at 36°. 1.12 g. NaOCl + 1.0 g. albumin (84.5%) in 125 ml.

10 min., when an almost steady state has been reached, for each mol. of NaOCl reduced, 33.7 g. of albumin are rendered incapable of coprecipitation with phosphotungstic acid, while the remainder of the original protein (22.5 g.) was found by qualitative examination to be changed to polypeptide precipitable by phosphotungstic acid but not precipitated by trichloroacetic acid or saturation with $(\text{NH}_4)_2\text{SO}_4$. The reduction of hypochlorite is closely coupled also with the exothermic effect as seen from the temperature-time curve (Fig. 1).

B. The exothermic reaction

Methods. Reactions with albumin and NaOCl (as above) were carried out in a silvered vacuum vessel with mechanical stirring. Temperatures were measured with a

thermometer reading to 0.1°. Before an experiment, water (250 ml.) at 34° was stirred in the vessel until thermal equilibrium was reached. The water was removed by suction and the hypochlorite solution, with or without buffer, in a total volume of 200 ml., and at the equilibrium temperature, was introduced. A cooling curve was constructed for the course of 5 min. The albumin solution (25 or 50 ml.), adjusted to the zero-time temperature, was added, and temperatures were recorded at suitable intervals over the course of about 12 min. Trials with water alone showed that the error due to incorrect matching of the temperature of the albumin solution was less than 0.03°, and that fluctuation was smoothed out within 15 sec.

With the total volume and the quantity of the second reagent fixed, experiments were carried out using different quantities of protein and of hypochlorite. A hypochlorite solution containing 1.3% of NaCl was then used to detect any effect due to the high salt concentration generally employed. No such effect was observed.

For reactions in the presence of buffer, phosphate (0.068 M in the reaction mixture) was used in the pH range 6.8–10.0. In view of the statement (Mauger & Soper, 1946) that phosphate takes part in *N*-chlorination, temperature curves were repeated with more dilute phosphate (0.0171 M) and with 0.4 M-carbonate-bicarbonate buffers of approximately the same pH values.

Comparable experiments were carried out at pH 9.0 and 5.5 with *N*-methylacetamide solution (2.78%) of the same N content as the albumin solution used.

Fig. 2. Exothermic reaction of albumin with NaOCl. 2.24 g. NaOCl + x g. albumin in 250 ml., initially at 32°. $x = (1), 2.0; (2), 1.4; (3), 0.8; (4), 0.4; (5), 0.2$.

Table 1. The effect of varying hypochlorite concentrations on the rise of temperature in the reaction between egg albumin and sodium hypochlorite

(Reaction system: 50 ml. 4% albumin in 0.5% NaCl solution + x ml. NaOCl solution (1.12 g. NaOCl + 18.5 g. NaCl/100 ml.) + (200 - x) ml. NaCl solution (18.5 g./100 ml.). Initial temperature: 34°.)

x	200*	100	70	50	30	10
Maximum rise (°)	5.63	2.54	1.87	1.32	0.63	0.06
Initial rate of heating (°/min.)	10.0	6.3	6.0	5.4	3.4	0.9

* Secondary rise apparent: cf. Fig. 2.

Results. In the absence of buffer, the reaction appears to proceed in two distinct stages when relative excess of oxidizing agent is used (Fig. 2). With excess of protein, however, smooth curves with the characteristics given in Table 1 were

obtained. Time-pH curves for such reactions are discussed in the next section.

The rate of evolution of heat was directly proportional to the concentration of (a) protein, and (b) hypochlorite, when one of the reagents was present in excess (Fig. 6). The linear relationship with respect to protein was observed when the weight of NaOCl was not less than 2.8/unit wt. of protein taken. The linear relationship with respect to hypochlorite was maintained, while the same weight ratio did not exceed 0.3:1.0.

Fig. 3. Exothermic reaction of albumin with NaOCl at different pH. NaOCl (1.12 g.) + 1.0 g. albumin in 250 ml. solution. 0.0616 M-phosphate; 32°; initial pH: (1), 9.6; (2), 9.0; (3), 8.5; (4), 7.7; (5), 7.3; (6), 6.9.

With the buffered system, the exothermic reaction varies greatly with pH (Fig. 3); and it also appears that two distinct reactions occur, since in all cases where the initial pH is less than 8.9 an instantaneous rise in temperature is found, followed by the slower and more strongly exothermic reaction. The rapid heating effect appears to be due to a reaction catalyzed by hydrogen ions (Table 2) since it increases almost linearly with decreasing pH value.

Table 2. *The catalysis of the reaction by hydrogen ions*

Initial pH	9.60	8.95	8.50	7.60	7.25	6.85
Instantaneous rise in temp. (°)	0.00	0.00	0.10	0.24	0.29	0.33

Results of both experiments with *N*-methylacetamide (pH 9.0 and 5.5) are different from those with protein. In alkaline medium, a rise of not more than 0.05° was found, with no further change after 1 min. In acid solution the rise during the first minute, after which no further change was observed, was 0.18°.

C. Changes in pH value during the reaction

Methods. Hypochlorite and 4% albumin solutions were the same as above. The former (100 ml.) was stirred mechanically in a wide-necked bottle in a thermostat at 36°. A glass electrode, an immersion-type calomel half-cell and a thermometer were dipped into the liquid. pH values were determined (Cambridge pH meter) for 5 min.; the reaction was then started by introducing 25 ml. of the protein solution, and pH values were measured at intervals for 10–15 min. A glass electrode adapted for use in high

ranges of pH values (Cambridge 'Alki') was used, after early trials with the ordinary type of glass electrode had given consistent but erroneous results with both reaction mixtures and alkaline buffer solutions. Tests with buffer solutions showed that the 'Alki' electrode gave a true reading within a few seconds and that the instantaneous value differed by only 0.4 pH unit from the equilibrium figure. Adequate earthing of metal apparatus was found to be essential. Erratic readings found initially were due to a calomel element whose appearance and behaviour when used in conjunction with an ordinary glass electrode, did not reveal any fault. Immediately before each experiment the electrode system was standardized against borate buffer of pH 9.10 at 36°; checks after experiments showed drifts never exceeding 0.03 pH. A series of curves were constructed for reaction mixtures of different ratios and concentrations of reagents. A second series was constructed with phosphate buffers in the mixture.

Fig. 4. Variation of pH with time for reactions of albumin with NaOCl in buffered solutions. 1.12 g. NaOCl + 1.0 g. albumin in 250 ml. at 36°. (1), no buffer; (2)–(5), 0.0616 M-phosphate.

Fig. 5. Variation of pH with time for reactions of albumin with NaOCl in buffered solutions. 1.12 g. NaOCl + 1.0 g. albumin in 250 ml. at 36°. (1), no buffer (pH adjusted with 2N-H₂SO₄); (2), 0.0616 M-phosphate; (3), 0.246 M-phosphate.

Results. (Figs. 4 and 5.) When the initial pH of the reaction mixture is 8.5–9.0, a considerable increase in alkalinity is found, followed by an

approximately smooth and relatively large fall. The magnitudes of rise and fall decrease as the initial pH is made to approach 7.5. On the acid side of this point the initial effect is inverted. Results for buffered and for adjusted reaction mixtures are shown in Fig. 5. Five experiments with the reaction mixture x ml. hypochlorite solution + $(100 - x)$ ml. water + 25 ml. albumin solution gave curves whose slopes, immediately following the initial rise in pH, are directly proportional to x (Fig. 6).

Fig. 6. Reaction between NaOCl and albumin (varied amounts) in 250 ml. at 36°. Rates of exothermic reaction and of falls in pH.

- (1) x = ml. NaOCl (0.16 M) to 2.0 g. albumin (84.5%).
 y = initial rate of rise in temperature ($^{\circ}$ C/min.).
- (2) x = ml. 4% albumin solution to 2.24 g. NaOCl.
 y = initial rate as in (1).
- (3) x = ml. NaOCl as in (1).
 y = initial rate of fall in pH value from maximum attained.

To estimate the amount of added base represented by the increase in pH value shown in Fig. 5, curve 2, suitably diluted buffered hypochlorite was titrated electrometrically with 0.1N-NaOH. The increase in pH value in the reaction with protein was found to be equivalent to the addition of 17.5 ml. 0.1N-NaOH to the reaction mixture.

DISCUSSION

The results of the thermal study emphasize the complexity of the reaction. Three reactions appear to have been distinguished; two in alkaline solution, and a third which takes place only in acid or weakly alkaline conditions. The second alkaline reaction is found only when the weight ratio of NaOCl to albumin exceeds 1.3:1, i.e. with relative excess of hypochlorite, and when the pH value is initially greater than 9.6. That such a rapid reaction should set in after at least a minute, and only with sufficient hypochlorite, suggests that a product of the first

alkaline reaction is involved. From measurements of slopes, both alkaline reactions are found to proceed at rates which are approximately proportional to the initial concentration of protein. The variation of the rate with pH (Fig. 7) supports the

Fig. 7. Rate of exothermic reaction as function of initial pH. (1), 0.068 M-phosphate. (2), 0.0171 M-phosphate or 0.4 M-carbonate-bicarbonate.

conclusion of previous workers (Price, 1931; Wright, 1936; Norman, 1936). The work of Mauger & Soper (1946) is also confirmed qualitatively within the limits of the present data. The third reaction mentioned above is very rapid and its effect decreases as the pH is increased (Table 2) to an extinction point at pH 9.0. All three reactions are considered to be oxidations in view of the close connexion with the reduction of hypochlorite and their strongly exothermic nature.

The evolved heat as measured in part (B) is made up of various components. In chlorination, hydroxyl ions are produced. In the oxidation of protein, new carboxyl groups are formed and ammonia and other basic substances may appear. Thus, in addition to the heat evolved in the oxidation proper there is the heat ascribable to ionic reactions consisting of mutual neutralization of products and to the reaction of excess acid with buffer. Such secondary effects should be corrected for, if the oxidation is to be followed by thermal measurements.

Since the cleavage of protein in the conditions used is oxidative, while hydrolytic action, as shown by Engfeldt (1922), is negligibly small, it is assumed that basic groups are produced in very small amounts only. Thus, as a first approximation, correction of the total heat evolved is concerned only with the heat due to reaction of the new acidic groups with the buffer:

and with neutralization of the base formed in chlorination:

The heat of formation of water is 13,700 cal./equiv. Since, as found above, the amount of alkali produced by chlorination is equivalent to approximately 17.5 ml. 0.1N in the reaction of 1.0 g. protein with 1.12 g. sodium hypochlorite, the neutralization of this amount of alkali contributes 24 cal., equivalent in the experimental conditions to a rise in temperature of not more than 0.1°, i.e. approximately 3% of the total rise in temperature recorded. If it is assumed that one carboxyl group is produced for each peptide link broken, i.e. for each 2.9 mol. of sodium hypochlorite reduced (see below), the maximum error is not greater than 0.3°, i.e. 10%. This is considered to be an outside figure since the value of 2.9 taken above includes hypochlorite reduced in side reactions. The total error in the thermal measurements is therefore not greater than 15%, and will be little in excess of 10% in reactions at pH values nearer 7.0 since chlorination as shown by the initial rise in pH is now diminished.

Results with *N*-methylacetamide indicate that the behaviour of the —CONH— grouping in this compound is not in any way comparable with its behaviour in proteins. Thus, the same linkage in protein would appear not to suffer oxidation (Goldschmidt & Steigerwald, 1925). Peptide links, however, differ from the —CONH— grouping in *N*-methylacetamide since in the structure —CONH.CHR.CONH— the CHR— and —NH— groups are under the influence of two electro-negative groups.

There are important groups of minor abundance in the albumin molecule which may be oxidized by sodium hypochlorite. The sulphur linkages in cystine (and cysteine) and perhaps even in methionine, the cyclic nitrogenous systems in histidine, tryptophan and proline, free amino-groups in, e.g. lysine, the guanidino grouping in arginine, and the aromatic group of tyrosine are all liable to rapid attack. The aromatic ring of phenylalanine, and hydroxyl groups as in serine probably react but slowly with sodium hypochlorite, and carboxyl groups as in glutamic acid are probably fairly inert towards this reagent.

The initial increase in pH value in the reactions can only be due to decarboxylation of free acidic groups or to *N*-chlorination:

In view of the relatively inert nature of aliphatic acids towards sodium hypochlorite (Engfeldt, 1922), the first supposition is excluded. Further, the maximum pH value is reached before any significant reduction of hypochlorite has occurred. Difficulty in preventing the early rise in alkalinity with buffer shows that a strong base is liberated. The chlorination is therefore put forward as an explanation of the effect observed.

Complete reaction with excess of chlorine-acceptor would, with the amount of hypochlorite taken, lead to the formation of sodium hydroxide equivalent to approximately 150 ml. 0.1N. The electrometric titration shows that alkali appears in an amount which is 12% of this value, and it is inferred that active chlorine represented by at least 12% of the original quantity taken is present within a short time in combined form. Subsequent oxidation would depend on progressive hydrolysis of the 'bound active chlorine', leading to production of acid:

The overall effect is therefore:

Since the ultimate pH value is lower than the initial figure either organic acid is produced by the oxidation or basic groups are destroyed, or both. It is well known that amines and ammonia are oxidized by hypochlorites in alkaline solution, whereas aliphatic acids are not in general attacked (Engfeldt, 1922). If both acids and nitrogenous bases are produced by oxidative fission of the protein the bases will be destroyed by subsequent action of the hypochlorite and the acids remaining will effect a lowering in the pH of the reaction mixture. Close correspondence between pH- and temperature-time curves suggests that the exothermic oxidation results in a lowering of the pH of the reaction mixture.

It is noted (Cohn & Edsall, 1943) that a well-defined break in the titration curve of egg albumin is found at pH 8.5 and this is attributed to free amino-groups. Such groups are probably oxidized readily by hypochlorite and much further work is necessary to allow of a full explanation of the secondary breaks in the present experimental curves.

From quantitative data on the amino-acids obtained by hydrolysis of egg albumin (Block, 1945) the average mol. wt. $\bar{m}$ of an amino-acid is 116.4. In the protein molecule each acid is represented by its residue. If terminal groups are neglected the average residue weight is that of the acid less that of water, i.e. 98.4. If bonds such as —S.S— are disregarded and albumin is considered as a peptide chain of molecular weight 34,000, and if it is assumed that the protein is degraded either to a peptone of molecular weight 2000 or to substances of average original molecular weight 98, it is calculated from the results of (A) that for each peptide link broken, 2.9 molecules of sodium hypochlorite are reduced. The less accurate average value for this quantity given by the limits of concentrations for 'linear' exothermic reaction (break-points in curves 1 and 2, Fig. 6) is 2.5, if 98 is taken as the average residue weight.

SUMMARY

1. Reaction between egg albumin and sodium hypochlorite results in degradation of protein and reduction of hypochlorite accompanied by evolution of heat, all of which follow a similar course. There is no significant change after ten minutes.

2. From thermal data it is concluded that three oxidations occur. Oxidation is most rapid at pH 9.8.

3. The pH falls as the reaction proceeds: in alkaline solution there is first a rise, and this rise increases with greater initial alkalinity.

4. Approximately 2.9 molecules of NaOCl are reduced for each amino-acid residue attacked.

5. *N*-Chloro-groups form rapidly and hydrolyze as the oxidation proceeds.

6. *N*-Methylacetamide is not oxidized significantly by sodium hypochlorite under the conditions used.

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Antidotal Activity of British Anti-Lewisite against Compounds of Antimony, Gold and Mercury

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Ever since the discovery of 2:3-dimercaptopropanol (British Anti-Lewisite, or BAL) as an arsenical detoxicant (Stocken & Thompson, 1940, 1941; Peters, Stocken & Thompson, 1945), its application as a possible antidote for compounds of other metalloids and metals had been envisaged in this laboratory, and a few experiments were tried at an early date. For various reasons it was not possible to report this work till 1945 when Whittaker (1945*a*) submitted an account of various aspects of the problem to the Ministry of Supply.

Meanwhile, reports had appeared from America on the effects of BAL on the toxic actions of Bi, Hg, Cd, V and Se (Barron & Kalnitsky, 1944), Cd (Sulzberger & Baer, 1944) and Sb (Calvery, Braun & Lusky, 1944), and in this country on effects obtained with Zn (Bruner, 1945).

From the earlier work with arsenic, theoretical considerations alone rendered it likely that antidotal

effects should be obtained with antimony and also with divalent toxic metals such as copper or mercury. As evidence had been obtained, however, showing that a considerable proportion of cases of post-arsphenamine dermatitis responded clinically to a course of injections of BAL (Carleton, Peters, Stocken, Thompson, Williams, Storey, Levvy & Chance, 1946) it was decided to study first those metals whose compounds are used therapeutically. The work described here therefore deals only with antimony, gold and mercury, and chiefly with the organic compounds of these metals in use in medicine at the present time.

Extension of this work to the treatment of the dermatitis that develops occasionally in patients receiving therapy with compounds of gold has already begun.

As in the earlier work with arsenic, the initial experiments were designed to test the effect of BAL