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A Histochemical Study of the Apparent Deamination of Proteins by Sodium Hypochlorite*

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Summary. The possible chemical mechanisms by which neutral solutions of sodium hypochlorite containing a high concentration of sodium chloride abolish the acidophilia of proteins in sections of fixed tissue are reviewed. The most probable one is the chlorination of the protein terminal amino groups, followed by the breakdown of the N-chloramine so formed into α-ketocarboxylic acid, nitrile or aldehyde groups. Hypochlorite solutions certainly do not deaminate tissue sections as was previously thought.

Experimental evidence for the formation of relatively stable N-chloramine groups *in situ* and their limited conversion to aldehydes is reported. For example, the acidophilia of hypochlorite-treated sections was found to be restored after flooding them with hydriodic acid followed by the extraction of the liberated iodine with an alcohol. The significance of these experimental findings is discussed.

Introduction

In the presence of high concentrations of sodium chloride, sodium hypochlorite rapidly "deaminates" or somehow inactivates the free amino groups of proteins in sections of fixed tissue (Stoward and Plant, 1968; Stoward *et al.*, 1968) and is more convenient to use than nitrous acid which requires 48 hours or more to deaminate most tissue proteins. The object of the investigation described in this paper was to find out how hypochlorite reacts with proteins in this situation, as there is some *a priori* evidence that it does not deaminate proteins as had been assumed hitherto. A preliminary report has been published elsewhere (Stoward, 1970).

Previous Work

Chu *et al.* (1953) were the first to use hypochlorites for destroying protein amine groups in histochemistry. They treated acetone = fixed sections of tissue with unbuffered solutions of sodium hypochlorite and found that the cytoplasmic contents afterwards reacted with Gomori's methenamine-silver reagent. Shortly afterwards, Burstone (1955) found that sections treated with stronger solutions (10%) of hypochlorite at pH 7.5 stained strongly with Schiff's reagent, particularly in nuclei. It was claimed that this staining could be abolished by rinsing the hypochlorite-treated sections in a solution of sodium thiosulphate (to destroy the excess hypochlorite) before immersion in Schiff's reagent. Burstone concluded that

* Dedicated to Professor Dr. W. Graumann on the occasion of his 60th birthday and in honour of his contributions to histochemistry over many years both as a scientist and as an editor.

hypochlorites, unlike chloramine T, do not produce stable aldehydes in tissue proteins and attributed the Schiff-staining to loosely bound hypochlorite which oxidizes leucofuchsin to its coloured form.

In preliminary experiments I found that any solution of sodium hypochlorite whose pH is greater than about 7.5 (including the 10% solutions which Burstone used) rapidly dissolves sections of fixed tissue mounted on glass slides with an albumen adhesive. It is, therefore, difficult to understand how Chu *et al.* (1953) obtained any positive results with their unbuffered solutions whose pH must have been at least 11. Admittedly they used a starch tissue adhesive but even this does not withstand hypochlorite solutions (Stoward *et al.*, 1968).

By chance I found that if sections of Carnoy- or formalin-fixed tissue (mounted with a nitrocellulose adhesive) were treated with a very dilute solution of sodium hypochlorite to which sodium chloride had been added and whose pH had been brought down to near neutrality, they retained their integrity and lost their affinity for acid dyes (the most widely used test for the terminal amino groups of proteins).

According to Ruddell (1969), solutions of sodium hypochlorite have three effects on tissue sections. Solutions of pH less than 5 convert melanin, hair cuticle, stratum corneum and lipofuscin to a sulphated derivative as shown by a stronger basophilia. At higher pH levels, another anion (which has not been identified) is generated in all tissue components other than collagen and reticulin. In these two proteins, carbonyl groups are "unmasked" instead. Wigglesworth (1970, 1971) claims alkaline hypochlorite also unmasks bound lipid (from e.g. insect cuticle).

There are thus diverse observations which need to be explained. Unfortunately the chemical literature is not of much help: it contains no agreed account of the exact course of the reactions between hypochlorite and proteins *in vivo*. However, there are a number of clues some of which will now be explored.

Theoretical Considerations

In 1909 Langheld established that alkaline solutions of hypochlorite rapidly oxidize, decarboxylate, and deaminate primary and secondary α-amino acids. The initial reaction, he and later Aubel and Asselineau (1948) claimed, is an N-chlorination. This is followed by hydrolysis, decarboxylation and the ultimate formation of aldehydes. However, as long ago as 1916 Dakin pointed out that the course of the reactions was different if the chlorinating reagent was in excess. For example, although one equivalent of chloramine T (which is assumed to react in much the same way as sodium hypochlorite) converts α-amino acids to aldehydes as expected (Dakin *et al.*, 1916; Dakin, 1917), two equivalents leads to the corresponding nitriles being formed (Dakin, 1916). According to Wright (1936), nitriles predominate if the hypochlorite oxidation is carried out at alkaline pH levels, and N-chloramines (the precursors of aldehydes) if it is performed at lower pH levels. In contrast, hypochlorous acid itself (with a pH between 3 and 3.5) converts most α-amino acids to nitriles; very little aldehyde is formed (Pereira *et al.*, 1973). Cystine and cysteine, on the other hand, are oxidised to cysteic acid.

Some amino acids in solution react anomalously with sodium hypochlorite. Wieland *et al.* (1949) found that glutamic acid is quantitatively oxidized to

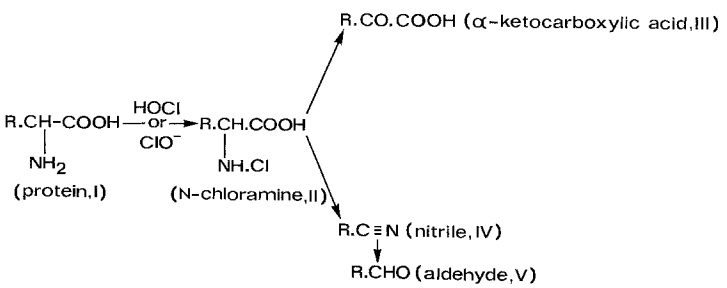

Fig. 1. Summary of the reactions that may take place between the terminal amino group of proteins (*I*) and hypochlorous acid (*HOCl*) or hypochlorite (*OCl*)

ketoglutarate, whereas aspartic acid loses only 3% of its nitrogen. They discovered that ketoglutarate and hypochlorite *together,* however, can deaminate glycine alanine, leucine, phenylalanine, tyrosine, serine, threonine, methionine, and aspartic acid. The nature of the oxidation products was not investigated but it seems likely that the corresponding ketoacids, rather than aldehydes, are formed (Ingols *et al.*, 1953).

Thus, depending on the reaction conditions, up to four products may result from the reactions of hypochlorite and amino-acids: N-chloramines, aldehydes, nitriles and α-ketocarboxylic acids (Fig. 1).

Few fundamental studies of the reactions of hypochlorites with the terminal amino acids of proteins have been carried out. This is surprising in view of the wide-spread and continued use of hypochlorites (as Chlorox and Milton's solution) as a bactericide for over 60 years. Baker (1947) suggested that most terminal amino acids are degraded in the same way as the free amino acids in solution. In addition he stated that in proteins, thiol and disulphide linkages, the tyrosine ring, the heterocyclic nuclei of histidine, tryptophan and proline, and the guanidine grouping in arginine are all liable to be attacked. However, the aromatic nucleus of phenylalanine and the hydroxyl groups of serine residues reacted, he thought, slowly.

The nitrogen function of peptide linkages probably resists hypochlorite too: although Herken and Silbersiepe (1951) and Von Arx and Neher (1963) thought hypochlorite did attack peptide bonds, Goldschmidt and Steigerwald (1929) and Pereira *et al.* (1973) on better evidence concluded the opposite, or at least hypochlorous acid (as distinct from alkaline sodium hypochlorite) had no action.

N-chloramines are the only end-groups that have been detected and sought for so far in proteins and peptides (on chromatograms) treated with chlorous acid or hypochlorite (Ryden and Smith, 1952; Von Arx and Neher, 1963). Nevertheless, in view of what can happen to free α-amino acids *in vitro*, it is possible that protein amino groups could also be converted to α-ketocarboxylic acid (formula III in Fig. 1), nitrile (IV) and aldehyde (V) groups as well. Therefore, any of the products II-V may be present in tissue sections that have been "deaminated" with hypochlorite solutions. In this paper it will be shown that N-chloramines (II) and, to a lesser extent, aldehydes are indeed formed.

Test for N-chloramines

No tests for N-chloramines have been described previously in the histochemical literature. The following two were devised for this investigation.

(a) Hydriodic Acid-Starch Test. Provided there is no unreacted hypochlorite entrapped in a hypochlorite-treated tissue section, protein N-chloramines, if present, should liberate iodine from an acidified solution of potassium iodide (after Dakin *et al.*, 1916). If starch is present in the iodide solution, an intense blue colour will be produced. If this test works, amino groups will be regenerated in the tissue proteins. Consequently if the liberated iodine is extracted (e.g. with alcohol), the tissues should take up acid dyes at acid pH levels again:

$$\text{Protein-NH-Cl} + 2\text{HI} \rightarrow \text{Protein-NH}_2 + \text{I}_2 + \text{HCl}.$$

(b) Van Urk's Test. According to Van Urk (1928), solutions of N-chloramines such as chloramine T go green turning to yellow and red when mixed with a solution of resorcinol. Solutions of sodium hypochlorites, however, turn purple. In addition, N-chloramines do *not* give a precipitate when treated with an aqueous solution of manganous chloride whereas sodium hypochlorite gives a brown one. In this study both these reagents were flooded onto hypochlorite-treated sections to test for the presence or absence of N-chloramines and unreacted hypochlorite.

Reducing Rinses for Removing Unreacted Hypochlorite

Unreacted hypochlorite adhering to tissue sections would probably interfere in all the tests used in this study for detecting any of the reaction products II-V. Ways and means of removing the excess hypochlorite were, therefore, investigated first.

Burstone (1955) used solutions of sodium thiosulphate. These solutions not only destroy excess hypochlorite but they may, under certain conditions, also react with aldehyde groups. Three other reagents were, therefore, investigated: (a) water, (b) allyl alcohol, and (c) a neutral aqueous solution of ammonia containing a trace of a copper salt.

Water would, if successful, simply wash out sodium hypochlorite from the reacted tissue section. However, there is always the possibility that hypochlorite remains in the section even after a thorough rinsing and if such sections are treated with Schiff's reagent, for example, the entrapped hypochlorite would probably oxidize the Schiff's reagent to give a coloured product and thus a false result for suspected aldehydes. To check this possibility, a reagent was sought that would give a colour reaction with free hypochlorite possibly remaining in section after rinsing. Feigl and Rossell (1957) claim that thallous hydroxide yields a brown, insoluble precipitate of thallic hydroxide with solutions of hypohalogenites. Preliminary tests on filter paper and on sections treated with solutions of sodium hypochlorite and immersed immediately in a thallous sulphate solution indicated that the thallous reagent was a specific and very sensitive test for detecting the presence of hypochlorite in tissue sections (Table 2). The Van Urk test was also used, but was found to be less sensitive than the thallous reagent test (Table 2).

Hypochlorites add across the olefinic bond of allyl alcohol to form mainly 2-chloropropane-1:3-diol (Smith, 1918). Since this addition is often quoted as being

a rapid one, this reagent seemed suitable for the rapid extraction of hypochlorite from tissue sections.

Hypochlorites also react rapidly with solutions of ammonia to form chloramines, which if cupric ions are not present (Cahn and Powell, 1954), would react with more ammonia to yield hydrazine (Raschig, 1907). Hydrazine, if formed, could block any aldehydes engendered in tissue proteins to form hydrazides.

Aldehydes, if induced in proteins by hypochlorite solutions, would be destroyed by solutions of ammonia simply because their pH is too high. Solutions of alkalis, such as barium or potassium hydroxides or even buffer solutions of pH above about 8.5, rapidly destroy aldehydes in, for example, periodate-oxidized glycogen. Therefore, since one of the purposes of this investigation was to find out whether aldehydes are introduced into tissue proteins after hypochlorite treatment, the pH of the test ammonia rinse was brought down to about 7.

Reactions of Sodium Hypochlorite with Carbohydrate-containing Substances

Theoretically sodium hypochlorite can oxidize mucosubstances (glycosaminoglycans) and polysaccharides such as glycogen in sections of fixed tissue. *In vitro* at about pH 10–11, the primary hydroxyl groups of monosaccharides are oxidized to uronic acid groups (Whistler and Schweiger, 1959; Whistler and Yagi, 1961). If the pH of the reaction mixture is lowered to about 4–5, the uronic acids are degraded further to a sugar with two fewer carbon atoms. By adjusting the pH of the hypochlorite solution to about 4–9.0 in my histochemical experiments it was hoped that little degradation of mucosaccharides would take place in tissue sections.

Methods and Materials

Methods

Paraffin-embedded sections (8 μ) of various tissues fixed in either neutral formalin or in Carnoy's fluid were stuck on grease-free, glass slides with a nitrocellulose-based adhesive (Stoward *et al.*, 1968). Their "deamination" was attempted as follows. They were:

(1) deparaffinized with four changes of xylene, taken through one change of 1:1 xylene: isopropyl alcohol, two changes of absolute isopropyl alcohol, one change of 50% aqueous isopropyl alcohol, and finally rinsed thoroughly in running water;

(2) immersed for 5–30 minutes in a freshly-prepared solution of hypochlorite obtained by mixing 3 ml stock sodium hypochlorite solution (containing about 10% w/v available chlorine) with 97 ml 8% aqueous sodium chloride. The pH of the diluted solution was adjusted just before use (normally to 4, 7, or 9) with aqueous hydrochloric acid (2N and N/10). Afterwards the sections were:

(3) rinsed thoroughly for at least 5 minutes in either (a) water, (b) allyl alcohol or (c) an ammonia solution consisting of 70 ml 95% ethanol, 10 ml distilled water, 20 ml 0.88 ammonia, one or two crystals of copper sulphate, and enough hydrochloric acid to reduce the pH to 7. After rinsing, the sections were washed thoroughly in distilled water before being subjected to any further histochemical treatment.

The absence of hypochlorite in the rinsed sections was tested by flooding them with a 2% aqueous solution of thallium sulphate (Analar grade; B.D.H. Ltd, Poole, England).

The affinities before and after hypochlorite treatment of tissue proteins for the acid dyes Fast Green FCF (Alfert and Geschwind, 1953) or Biebrich Scarlet (dissolved in 0.5M glycine; Stoward, 1968) at various pH levels (normally 3, 5, and 9.6) were used to follow the extent of the apparent deamination process.

As is indicated in Table 2, this experiment was repeated on sections immersed for 30 minutes in one of the following solutions: (a) the neutralized alcoholic ammonia rinse, (b) a

freshly-prepared 2.5% solution of phenylhydrazine hydrochloride in 5% mixed phosphate buffer (as used in the formazan reaction; Stoward, 1967*a*), and (c) a freshly-prepared 8% solution of *o*-phenylene diamine dihydrochloride in 10% aqueous acetic acid.

The detection of N-chloramines in hypochlorite-treated, water-washed sections was attempted by flooding the sections with either (a) a 2% aqueous solution of potassium iodide containing a few drops each of N hydrochloric acid and 1% starch solution; or (b) resorcinol 5% in water; or (c) a 5% aqueous solution of manganous chloride (AnalaR grade) to which a few drops of an aqueous solution of potassium hydroxide had been added until a pale-yellow flocculent precipitate just appeared in the solution; or (d) a 5% aqueous solution of ferric chloride. The flooded solution was allowed to remain on the section for at leat 5 minutes.

In another experiment, hypochlorite-treated, water-washed sections were flooded with acidified potassium iodide solution (omitting the starch solution) for 3–5 minutes, rinsed thoroughly in isopropyl alcohol to dissolve liberated iodine, washed in distilled water, covered with an acidified potassium iodide-starch solution to check that all the N-chloramines originally present in the sections had been hydrolysed, washed again in distilled water, and finally stained for 30 minutes in a 0.04% solution of Biebrich Scarlet in 0.5M glycine whose pH had been adjusted to 3.8 with dilute hydrochloric acid (after Stoward, 1968). The stained sections were rinsed in absolute isopropyl alcohol, cleared through isopropyl alcohol and xylene, and mounted in DPX.

Spot tests with the reagents used in the above experiments were performed on filter paper with (a) the sodium hypochlorite-sodium chloride solutions; (b) chloramine-T (applied as a saturated aqueous solution, concentration approximately 12.5% w/v); and (c) N^2, N^4, N^6-trichloromelamine (purchased from R. N. Emanuel Ltd., London; applied as a filtered, saturated aqueous solution, concentration approximately 0.01%)

To detect aldehyde groups, hypochlorite-treated, water-rinsed sections were treated either with Schiff's reagent for 30 minutes (followed by a rinse in running water for at least 5 minutes) or with phenylhydrazine and tetrazotized 3,3′-dimethoxybenzidine fluoroborate (TDMBF) as in steps (2)–(6) of the formazan reaction procedure described previously (Stoward, 1967*a*, p. 413). Schiff's reagent was prepared according to de Tomasi's (1936) formula. The treated sections were dehydrated through isopropyl alcohol, etc, and mounted in DPX.

The effect of hypochlorite solutions on mucosubstances was tested as follows. Sections of fixed liver (containing glycogen), stomach (whose surface epithelia contained neutral mucosubstances) and small intestine (whose goblet cells contained sialo- or sulpho-mucins or a mixture of both) were treated with sodium hypochlorite—sodium chloride solutions of pH 4.0 or 7.0 for 10–30 minutes, washed in the alcoholic ammonia—copper sulphate rinse for 5 minutes, rinsed in distilled water and finally subjected to each of the following techniques: periodic acid-salicylhydrazide-Solochrome Black AS and alum (Stoward, 1967*b*), periodic acid—Schiff (PAS) and Alcian Blue at pH 2.5 (for details, see Stoward 1967*c*).

All histochemical reactions were carried out at room temperature.

Reagents

All reagents were of AnalaR or good reagent grade quality except for the acid dyes which were used as supplied by the National Aniline Co., U.S.A. TDMBF was synthesized as described previously (Stoward, 1967*a*).

Tissues

The following tissues of male rat and female mouse (killed by carbon dioxide asphyxiation) were studied; tongue, submandibular gland, small intestine, colon, kidney, and liver. They were fixed either in neutral 4% formalin (buffered with 2% calcium acetate) for 24 hours at room temperature or in Carnoy's fluid (1:1:2 by vol. absolute ethanol: glacial acetic acid: chloroform) overnight at 5°. They were embedded in paraffin wax in the usual way. For convenience a number of organs from the same animal were embedded in a single block. It was found later that kidney and tongue exhibited all the reactions of the other tissues examined in this study. Therefore, only the results obtained with these two tissues are described here.

Table 1. Affinity of tissue section proteins for Biebrich Scarlet at pH 5 after treatment with hypochlorite followed by various other reagents. Intensity of staining estimated subjectively on scale 1 (weak) to 5 (very strong). 0 = no staining. The staining by Fast Green F is similar

Histochemical procedure	Intensity of Biebrich Scarlet staining at pH 5 Histological site:		
	Lingual skeletal muscle	Lingual serous cells	Kidney tubule cytoplasm
No treatment	5+	4+	4+
NaClO—H_2O rinse	0	0	0
NaClO—H_2O—HI	3–4+	3+	3+
NaClO—AA rinse	0	0	0
NaClO—AA—HI	2–3+	1–2+	2–3+
NaClO—NH_3 rinse	0	0	0
NaClO—NH_3—HI	2–3+	1+	1+
NaClO—PhHy	0	0	0
NaClO—PhHy—HI	3+	2+	2+
NaClO—OPD	0	0	0
NaClO—OPD—HI	2–3+[a]	2+[a]	2+[a]

[a] Orange metachromatic staining.

Abbreviations. NaClO = sodium hypochlorite-sodium chloride pH 4, 7 or 9. HI = hydriodic acid (KI + dil.HCl) followed by extraction with isopropyl alcohol. AA = allyl alcohol. PhHy = phenylhydrazine. OPD = *o*-phenylene diamine.

Results

Except where otherwise stated the results described below were obtained with sections that had been treated with sodium hypochlorite solutions containing 8% sodium chloride and afterwards washed for 5 minutes in running water.

Action of Hypochlorite Solutions

All tissues, whatever their fixation, disintegrated to some degree, in some cases completely, after less than 10 minutes immersion in solutions of sodium hypochlorite without added salt. Alkaline solutions were a little more destructive than acid or neutral ones.

In contrast, very little destruction was observed with neutral solutions containing at least 8% w/v sodium chloride. Salt in lower concentrations did not entirely repress the destruction.

All structural proteins in sections of either formalin- or Carnoy-fixed tissue lost practically all their affinities for Biebrich Scarlet or Fast Green FCF at pH 3, 5 and 9.6 after about 5 minutes treatment with sodium hypochlorite-sodium chloride solutions of pH 4, 7 or 9 followed by a rinse in water, allyl alcohol, or a neutral alcoholic ammonia solution (Table 1). After hypochlorite treatment for about 1 min, their acidophilia was reduced by roughly 50–80% and after 30 minutes treatment, was abolished completely.

Table 2. Colour reactions of hypochlorites and model substances with various test reagents on filter paper. Intensity of colour estimated subjectively on scale 1 = weak to 5 = very strong. 0 = no reaction

Test substance or solution	Reagent						
	Thallium sulphate	Manganese chloride	Resorcinol	Schiff's reagent	Hydriodic acid	Hydriodic acid + starch	o-Phenylene diamine
NaClO-NaCl solution[1]							
pH 4	2Y[2]→2Bn	3Bn	1P[3]→0	3P	5Bn	4Bn−2B[4]	3Or
pH 7	1Y[2]→1Bn	3Bn	3P[3]→0	3P	4Bn	4Bn−2B[4]	3Or
pH 9	1Y[2]→2Bn	3Bn	3P[3]→0	3P	4Bn	4Bn−2B[4]	3Or
Periodate-oxidized glycogen[5]	0	0	0	5M			0
α-Oxoglutaric acid[1,6]	0	0	0	0	0	0[7]→2B	1Y
Trichloromelamine[1,8]	0	0	0	0	4Bn→0[9]	5B	3Or
Chloramine T[1,10]	3Bn	0	2YGn	5Bn–5P[11]	5Bn[12]→3Bk →0	5Bk B[13]→2Bk →1Y	4Bn
Control[14]	0	0	0	0	0	0	0

Abbreviations. B = blue; Bn = bronze; Bk = black; M = magenta; Or = orange; P = purple; Y = yellow.

Notes. 1. Spotted on filter paper. 2. Yellow flocculent precipitate formed on testspot which went brown over a period of 10–30 minutes. 3. After about 5 seconds the purple spot became colourless. 4. Some areas of spot brown, others blue. 5. Glycogen granules in sections of Carnoy-fixed tongue and liver treated with 1% periodic acid for 10 minutes followed by a rinse in running water for 5 minutes. 6. Saturated solution, approximately 50% w/v. 7. Spot turned blue after about 10 minutes. 8. Saturated solution, about 0.01%. 9. Spot went colourless after about 10 minutes. 10. Saturated solution, approximately 12.5% w/v. 11. The brown colour was formed immediately. It turned dark purple about 5 seconds later. 12. The dark brown colour formed initially turned black approximately 20 seconds later. It became colourless after a further 5–10 minutes. 13. The times taken for the colour changes were the same as those observed for hydriodic acid alone (note 12). 14. Reagent spotted on dry, clean filter paper.

Test for Unreacted Hypochlorite

Brown amorphous granules (presumably of thallic hydroxide) were deposited over the entire tissue area of sections rinsed very briefly in water after being taken out of a hypochlorite-sodium chloride solution. Similar brown colourations appeared on filter paper spotted with hypochlorite-sodium chloride solutions of pH 4, 7 or 9 (Table 2). However, no brown colouration was observed in hypochlorite-treated sections washed for 5 minutes in allyl alcohol, or an alcoholic ammonia solution, or in running water.

Tests for N-chloramines

All sections after immersion in hypochlorite solutions of pH 4, 7 or 9 and a thorough rinse in water turned dark brown immediately when flooded with an acidified potassium iodide-starch solution. The brown colour was extractable with either ethyl or isopropyl alcohol. An intense blue colour developed slowly in

the solution immediately above the sections, but not it seemed, actually in them. The blue colour reached a maximum intensity after about 5 minutes: concomittantly the sections lost their brown colour and regained most of their affinity for Biebrich Scarlet at acid pH levels (Table 1).

Hypochlorite-oxidized sections washed in ammonia, phenylhydrazine, or o-phenylene diamine solutions did not regain quite as much acidophilia after HI treatment as those rinsed in water (Table 1).

The manganese chloride and resorcinol tests for N-chloramines did not work. Neither imparted any colour to hypochlorite-treated sections rinsed in water, but nor did they to an N-chloramine (trichloromelanine) spotted onto filter paper either (Table 2).

Tests for Aldehydes

The significant results of the Schiff and formazan tests given in Table 3 are as follows:

1. After Deamination at pH 9. Nuclei gave stronger Schiff and formazan reactions than most structural proteins. The latter in fact only gave a positive formazan reaction in Carnoy-fixed tissue; their Schiff reaction was virtually negative in both formalin- and Carnoy-fixed tissues. Lingual serous cells, on the other hand, gave positive Schiff and formazan reactions regardless of the fixation.

2. After Deamination at pH 7. Moderately strong Schiff and formazan reactions were observed in most structural proteins (e.g. in muscle) and in nuclei, those in formalin-fixed tissues giving in most cases slightly stronger reactions than those in tissues fixed in Carnoy's fluid.

The Schiff reaction of elastica was relatively much stronger but after deamination at pH 4 and 9 it was virtually negative.

It was particularly noticeable in formalin-fixed sections that some clumps of nuclei gave much stronger formazan reactions than nuclei elsewhere in the tissue. This effect was never observed in Carnoy-fixed tissues. The Schiff reactions of nuclei were uniformly much stronger in formalin-fixed tissues than in Carnoy-fixed ones.

3. After Deamination at pH 4. In contrast to the results obtained after deamination at pH 9, nuclei were, with one exception, unreactive in the Schiff and formazan reactions after deamination at acid pH levels. The exception was the strong formazan reaction of random clumps of nuclei in some tissues. This was particularly noticeable in kidney sections. The same effect was observed after deamination at pH 7.

Muscle and serous cell proteins on the whole gave stronger Schiff and formazan reactions in formalin-fixed sections than those fixed in Carnoy's fluid.

Effect of Hypochlorite on Mucosubstances (Table 3)

The results obtained with the three techniques mentioned in the methods sections for detecting mucosubstances in hypochlorite-treated, ammonia-rinsed sections appeared, in all the tissues examined, to be exactly the same as those obtained with control, untreated sections.

The reason for using the ammonia rinse instead of water was to destroy the hypochlorite-induced aldehyde groups in proteins. Otherwise it was difficult to

Table 3. Histochemical tests for aldehydes in sections treated with hypochlorite-sodium chloride followed by a 5 minute rinse in water. Reaction (colour of final reaction product) estimated subjectively on scale ± = negligible or very weak to 5+ = very strong. 0 = no reaction

Histochemical procedure	Fixation[1]	Histological site					
		Tongue				Kidney	
		Muscle[2]	Serous cell cytoplasm	Epithelium[3]	Nuclei	Tubule cytoplasm	Nuclei
Hypochlorite pH 9 followed by:							
Schiff's solution	F	±	1–2+	2	2+	0–±	2+
	C	±–1	2+[4]	1+[4]	2+	1+	1–2+
Formazan reaction	F	±	1–2+		2+	0	1–2+
	C	2+	1–2+	1+	1–2+	2+	1–2+
TDMBF control [9]	F	0	0	0	0–±	0	0
Hypochlorite pH 7 followed by:							
Schiff's solution	F	1–2+	2+	2+[5]	±–2+	2–3+	2–3+
	C	1+	1–2+	2+	±–2+	±–1+	±–2+
Formazan reaction	F	2–3+[6]	2–3+[7]	2+	2+	2+	1–2+[8]
	C	2–3+[6]	2–3+	2+	1–2+	2+	±–1+
TDMBF control [9]	F	0–±	±	0	0–±	0	0–±
Hypochlorite pH 4 followed by:							
Schiff's solution	F	1–2+	2+	1–2+	0–±	1+	0–1+
	C	±	1+	1+	0–±	0	±
Formazan reaction	F	1–2+	2+	2+	0–1+	2+	±–1+[8]
	C	2+	2+	2+	±	1+	±
TDMBF control [9]	F	±	±	±	±–0	±	0

Notes. 1. F = neutral formalin; C = Carnoy's fluid. 2. Includes both longitudinal and transversely-cut skeletal lingual muscles. 3. Squamous epithelial layer covering filliform papillae. 4. Nuclei, 3+. 5. Most nuclei, ±. 6. Mostly 2+. 7. Mostly 3+. 8. Random clumps of nuclei, 4+. 9. Treated only with TDMBF after hypochlorite, etc. Intermediate phenylhydrazine treatment omitted. Hypochlorite-treated sections reacted with phenylhydrazine but not TDMBF remained colourless.

distinguish, say, the PAS staining of glycogen granules in liver sections from the surrounding tissue.

Discussion

Assuming that proteins which have no affinity for acid dyes at any pH level do not possess free amino groups, then it appears at first sight that near neutral solutions of sodium hypochlorite containing a relatively high concentration of sodium chloride completely deaminate tissue sections very much faster than,

say, nitrous acid. Speed apart, they also have the advantage that extraction of the more soluble components from tissue sections during the apparent deamination is considerably reduced. This is illustrated by the unchanged PAS and Alcian Blue reactivities of glycogen and acid mucosubstances respectively after hypochlorite treatment. With nitrous acid, these reactivities are sometimes reduced.

Uptil now I (Stoward, 1967*a*, p.414; Stoward *et al.*, 1968) and others (e.g. Chu *et al.*, 1953; Burstone, 1955) have assumed explicitly that hypochlorite solutions deaminate proteins, that is they completely remove or destroy amine groups from or in tissue sections. This assumption is wrong; the majority of amine groups are not destroyed. Instead they are converted to relatively stable N-chloramines that apparently do not have any affinity for acid dyes. Three observations confirm this.

1. The ability of such proteins to release iodine relatively slowly from acidified solutions of potassium iodide. A similar slow release, it has been claimed, takes place with "albuminoid chloramines" *in vitro* (Robson, 1964; Dakin *et al.*, 1916).

2. The restoration of acidophilia (towards Biebrich Scarlet) to hypochlorite-treated sections after immersion in acidified solutions of potassium iodide and subsequent extraction of the liberated iodine with alcohol.

3. The negative reactions of the hypochlorite-treated, water-washed sections with manganous chloride. However, that no colour was produced, even momentarily, when such sections were flooded in this study with a solution of resorcinol could be interpreted as indicating the absence of N-chloramines.

The induced N-chloramines are surprisingly stable. This is borne out by two observations. The first is that some acidophilia is restored to hypochlorite-treated sections that have been immersed in acid or neutral solutions of *o*-phenylene diamine, phenylhydrazine or ammonia and afterwards treated successively with hydriodic acid and alcohol. Even though the two aromatic amines have probably reacted with neighbouring centres in the hypochlorite-oxidized protein (Stoward, 1970), this did not prevent the subsequent regeneration of amines and their binding with bulky acid dyes. The second observation is that acidophilia is not restored if the hydriodic acid treatment is omitted from the sequence outlined for the first observation. If the N-chloramine groups in the hypochlorite-treated sections had been as active as they are in most N-chloramine compounds *in vitro* (Robson, 1964), they would have N-chlorinated the aromatic amines phenylhydrazine and *o*-phenylene diamine used to 'rinse' the sections and would have themselves been converted back to amines in the process: if they had, they would have taken up Biebrich Scarlet.

Some of the induced N-chloramine groups seem to break down into aldehydes. Apart from their moderate staining with Schiff's solution (which is open to the objection mentioned in the Introduction), the presence of aldehydes in hypochlorite-treated, water-washed tissue proteins is confirmed by the positive formazan reaction. However, the concentration of such aldehydes never seems very high, and their distribution is curious in three respects.

(a) Nuclei appear to possess more induced aldehyde groups than structural proteins (e.g. of lingual skeletal muscle) after hypochlorite treatment at pH 9, whereas after hypochlorite treatment at pH 4, the converse holds. Hypochlorite treatment at an intermediate pH leads to roughly the same aldehyde reactivity.

(b) The cytoplasm of lingual serous cells give stronger reactions for aldehyde after hypochlorite treatment at any pH between 4 and 9 than any other proteinous site. It is possible that the protein constituent of serous cells contains more terminal amino groups than, say, the proteins making up lingual muscle. If this is so, comparatively more aldehyde groups might indeed be induced in serous cell proteins after hypochlorite treatment. However, in a previous investigation it was found that, in sections of Carnoy-fixed tissue at least, the concentrations of protein amino groups in lingual serous cells and in skeletal and smooth muscle appeared to be approximately the same (Stoward, 1968). Therefore, all that the results reported in this paper show is that proteins in serous cells differ from those of most other connective tissue components.

(c) Although most nuclei give only a weak formazan reaction in formalin-fixed sections after hypochlorite treatment at pH 7 or 4, a few isolated clumps of nuclei gave very strong reactions. These clumps have never been observed in similarly treated Carnoy-fixed sections.

Why does the concentration of aldehydes vary from site to site and depend on the pH of the hypochlorite solution? Perhaps it is because with alkaline hypochlorite solutions some of the aldehydes (such as those produced in muscle proteins) are easily converted to other products through Cannizarro condensations, while other aldehydes are relatively stable in the mildly alkaline reagent. On the other hand, the N-chloramines formed initially may break down (to aldehydes) more easily in some sites than others. *In vitro* they degenerate into aldehydes at pH levels on the alkaline side whereas most are relatively stable below about pH 5 (Robson, 1964). This may explain why more aldehydes are generated in tissue proteins on treatment with hypochlorite at pH 7 than at pH 4. Certainly at the lower pH, stable N-chloramines are formed as shown by the restoration of most of the original acidophilia to muscle proteins treated first with hypochlorite at pH 4, second with hydriodic acid, and third with alcohol.

Another explanation for the variable concentration of aldehydes may lie in the preferential breakdown of the N-chloramines at acid pH levels, although relatively small, to α-ketocaboxylic acids and nitriles. In a later paper it will be shown that α-ketocarboxylic acids are indeed formed, especially at neutral pH levels.

Sodium chloride may also have some influence on the breakdown of N-chloramines. At the very least it is difficult to understand why it is so necessary to include it in hypochlorite solutions in order to prevent sections from being dissolved. Perhaps it represses the decomposition of hypochlorite and hypochlorous acid into chlorate (Jaksic *et al.*, 1968) which in turn might oxidize tissue sections completely. However this interpretation is at present purely speculative.

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