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Pitfalls in Using Sodium Hypochlorite as a Seed Disinfectant in ^{14}C Incorporation Studies

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ABSTRACT

Seeds sterilized with sodium hypochlorite (NaOCl) retained sufficient amounts to interfere with studies of amino acid metabolism of the sterilized seeds during germination. Repeated washing in water did not remove NaOCl completely. However, soaking the seeds for 10 min in 0.01 N HCl removed NaOCl completely, without reducing germinability.

Residual NaOCl reacted with the amino acids and reduced their concentrations in the incubation media. This reaction resulted in high production of CO_2 and low uptake of amino acids by the seeds. Decarboxylation of the amino acids occurred in the incubation medium outside the seed, was independent of the presence of seeds in the reaction, and therefore was not related to amino acid metabolism by the seeds. Effects of NaOCl on uptake, incorporation, and CO_2 production from indoleacetic acid were similar to those of the amino acids studied.

Sodium hypochlorite (NaOCl) is effective as a disinfecting and sterilizing agent against a broad range of bacteria, viruses, and fungi (4, 10, 18, 19). Disinfecting seeds with strong solutions of NaOCl such as 5.25% (equivalent to NaOCl concentration in commercial Clorox) for 1 to several min does not reduce germinability. However, this compound has a strong oxidizing property which makes it highly reactive with amino acids (2, 7), nucleic acids (6), amines, and amides (16, 17). The general reaction between amino acids and NaOCl produces the respective aldehyde, NH_4Cl and CO_2 (7, 14). These reports led me to investigate the effects of small amounts of NaOCl on metabolism of amino and organic acids by the seed.

I report on reactions between NaOCl and several amino and organic acids and the effects of these reactions on uptake, and incorporation of these acids into protein. I also describe an acid treatment that removes the NaOCl which remains adsorbed to the seed after washing, without affecting germinability.

MATERIALS AND METHODS

Material. Seeds of tomato (*Lycopersicon esculentum* L. cv. Potomac), barley (*Hordeum vulgare* L. cv. Himalaya), and lettuce (*Lactuca sativa* L. cv. Grand Rapids) of high viability were used. Clorox[1] (commercial product of the Clorox Co., Oakland, Calif.) was the source of NaOCl, and all concentrations used were dilutions of the original commercial preparation which contained 5.25% NaOCl. The following amino and organic acids (New England Nuclear) were used: L-leucine-U-^{14}C (220 mc/mmole), L-leucine-1-^{14}C (15 mc/mmole), L-tyrosine-U-^{14}C (300 mc/mmole), L-lysine-U-^{14}C (220 mc/mmole), L-aspartate-U-^{14}C (150 mc/mmole), L-histidine-U-^{14}C (155 mc/mmole), acetate-1-^{14}C (50 mc/mmole), and indoleacetic acid-1-^{14}C (10 mc/mmole).

Application of NaOCl. Sodium hypochlorite was introduced into the incubation media that contained amino or organic acid in one of two ways. The first was in minute quantities (4 μmoles or less per 100 tomato seeds as determined by titration with HCl) that remained adsorbed on the seed surface as a result of soaking the seeds for 5 min in 1% (v/v) solution of NaOCl, then draining the solution and washing the seeds several times, each time in 30 ml of sterile water (Tables I and IV). This is the most common practice for seed sterilization in numerous laboratories (3, 8, 11, 15). In a set of experiments (Table IV), the seeds were disinfected with 1% NaOCl, transferred into a solution of 0.1 N HCl for 10 min, then washed eight times with water. The second way was to add specific amounts of NaOCl directly to the reaction mixture at the initial time of the reaction and to determine its reactivity with amino or organic acids during a specific time (Tables II, III, and Fig. 1). Seeds that were used in these reactions were not previously sterilized with NaOCl. Unless otherwise specified, NaOCl was added to a final concentration of 0.52% (v/v). This concentration produced maximum reactivity between NaOCl and solutions of 0.5 mM leucine when the reactions were carried out in air (Fig. 1).

Incubation. Details of the contents of the incubation mixtures and durations of incubations appear under individual tables. All incubations were carried out in respiration flasks at 25 C (23 C for lettuce seeds to avoid high temperature dormancy). The reaction was started by adding seeds, NaOCl, or both to the incubation medium which already contained the labeled acid and antibiotics. The flask was stoppered, placed in a water bath at 25 C, and shaken constantly. Carbon dioxide, produced during the incubation period, was collected on a filter-paper wick that was secured to the rubber stopper with a stainless steel pin. One drop of 14 N KOH was placed on the filter-paper wick for trapping the CO_2. The reaction was terminated by transferring the flask into ice, washing the seeds several times in cold water, and holding at −20 C for extracting proteins.

Decarboxylation, Uptake, and Incorporation of Acids. Decarboxylation of amino and organic acids was determined by measuring the radioactivity that was trapped in KOH. The filter-paper wick and stainless steel pin were immersed in 1 ml of hyamine hydroxide to which scintillation solution was added and radioactivity was determined. The procedure for extracting

[1] Mention of tradenames is made for identification purposes and does not imply endorsement by the United States Department of Agriculture.

protein (acid-insoluble) in 10% cold trichloroacetic acid was reported elsewhere (1).

Seed Germination. Samples of 300 seeds for each treatment (H_2O, NaOCl, or NaOCl then HCl) were germinated for 5 days in a growth chamber at 22 C and 16-hr light period/day. Seeds that showed normal roots and shoots were considered germinated. Lettuce and tomato seeds were germinated in 10-cm Petri dishes using 100 seeds/dish. Barley seeds were germinated in 30 × 40 cm trays using 300 seeds per tray for each treatment. The bottom of the Petri dishes and trays contained two layers of germination paper, moistened with water.

RESULT AND DISCUSSION

Decarboxylation of Leucine by NaOCl. Decarboxylation of leucine (0.5 mM) by NaOCl was established in the concentration curve shown in Figure 1. Maximum decarboxylation was reached at 1.2 mM of NaOCl in air and 6 mM in N_2. Final pH values of the leucine solutions at NaOCl concentrations of 0, 0.24, 1.2, 6 and 30 mM were 5.9, 6.3, 6.6, 8.3 and 8.9, respectively. The decreased decarboxylation of leucine at NaOCl concentrations above 6 mM was due to the alkalinity of the reaction mixture. At pH 7 and above, reactivity of NaOCl fell rapidly because most of the hypochlorous acid in the solutions remained undissociated (10).

No attempt was made to characterize all the products of the reaction between leucine and NaOCl. However, in preliminary experiments the reactions were terminated by adding 2,4-dinitrophenylhydrazine followed by extraction with methylene chloride. Chromatography of the methylene chloride-soluble fractions on microcel-coated plates developed with hexane saturated with polyethylene glycol, showed two major labeled products which co-chromatographed with isovaleraldehyde and isovaleric acid.

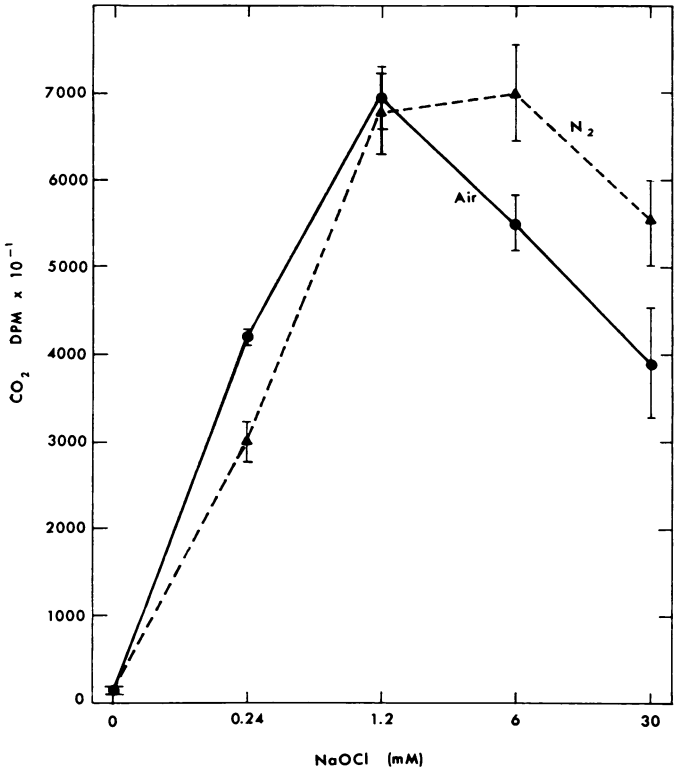

FIG. 1. Effect of NaOCl on decarboxylation of leucine. The reaction mixtures contained 2 ml of 0.5 mM leucine, 40 μg of penicillin-G and streptomycin sulfate, and 0.66 μc of leucine-U-^{14}C. The reactions were started by adding specific amounts of NaOCl and maintained under air or N_2 for 2 hr at 25 C.

Table I. *Removal of NaOCl from Surface of Tomato Seeds by Successive Washing in Water and Its Effect on Decarboxylation, Uptake, and Incorporation of ^{14}C-1-Leucine into Protein*

Samples of hundred seeds were soaked for 5 min in water (control) or 1% solution of NaOCl (v/v), washed up to eight times each in 30 ml of water, drained, and incubated for 2 hr at 25 C in 2 ml of 0.5 mM leucine, 40 μg of penicillin-G and streptomycin sulfate, and 0.6 μc of ^{14}C-1-leucine. Extraction of protein is described in reference 1.

Treatment	No. of Washes	$^{14}CO_2$	Acid-insoluble	Acid-soluble	Total Uptake	Incorporated into Protein
		dpm × 10^{-1}/100 seeds				%
H_2O	0	18	258	2,310	2,568	10.05
NaOCl	0	51,927	232	665	897	25.86
NaOCl	2	27,910	359	1,301	1,660	21.63
NaOCl	4	19,423	369	1,384	1,753	21.05
NaOCl	8	16,443	385	1,827	2,212	17.41

Table II. *Effect of NaOCl on Decarboxylation of ^{14}C-1-leucine*

Groups of 50 tomato seeds or equal number of ground seeds were incubated for 1 hr at 25 C in 2 ml of 0.5 mM leucine containing 0.6 μc of ^{14}C-1-leucine, 40 μg of penicillin-G and streptomycin sulfate ± 0.52% NaOCl (v/v) in a final concentration. Released CO_2 was collected on a filter-paper wick that contained 1 drop of 14 N KOH.

Seed	Radioactivity Recovered in CO_2	
	−NaOCl	+NaOCl
	dpm × 10^{-1}/reaction	
None	14	4,588
Whole seed	7	4,278
Ground seed	18	20,211

Effects of NaOCl on Decarboxylation, Uptake, and Incorporation of Leucine. Data in Table I are typical of experiments in which seeds are sterilized with NaOCl, washed several times in water, then used to follow uptake and incorporation of amino acids into protein. All reaction mixtures that contained NaOCl, either as traces that washing with water failed to remove (Tables I and IV), or in 0.52% concentration added to incubation media (Tables II and III), exhibited two common features: high decarboxylation and high percentage of incorporation of the labeled amino acid into protein.

Decarboxylation of leucine (expressed as CO_2) was high in all reactions that contained NaOCl (Tables I–IV), and the reactions had no requirement for O_2 (Fig. 1). Decarboxylation proceeded equally well with and without the seeds (Table II). This observation suggests that most of the CO_2 arose from decarboxylation of leucine by NaOCl in the incubation medium outside the seed and was not part of the metabolism of leucine by the seed. In the reactions which contained ground seeds, it is likely that the fine particles provided a large surface area for adsorption of NaOCl which later reacted with leucine.

Measurements of CO_2 provide a highly sensitive and simple method for determining the effectiveness of washing the seeds to remove NaOCl. In general, in the absence of NaOCl, the percentage of CO_2 produced from most amino acids comprised a small part of total uptake by the tissue (Table III). In many published reports on metabolism of amino or organic acids in

seeds and tissue slices that had been disinfected with NaOCl, no data on CO_2 were reported (3, 12). In these cases, it is difficult to determine the extent to which the presence of NaOCl might have affected the data and their interpretation. In the few reports, where CO_2 was measured, high decarboxylation of amino and organic acids was reported (5, 8). In one report (13), almost all the alanine-1-^{14}C fed to NaOCl-treated pea seeds appeared as CO_2, and no explanation was offered for this observation.

The increase in percentage of incorporation of leucine into protein by seeds in reaction mixtures that contained NaOCl appeared to result from two factors. One was the reduction in uptake of the amino acid (Table I). The other was an actual, although slight, stimulation of incorporation into protein by NaOCl. This stimulation became evident only when NaOCl was washed away completely before incubation (acid-insoluble in Table I). Of the two factors, reduced uptake was the major cause of increased percentage of incorporation. Apparently, uptake was reduced by decarboxylation of leucine on the surface of the seed making it less available for incorporation into protein. Through either excessive washing (Table I) or acid treatment (Table IV) more NaOCl was removed and decarboxylation, uptake, and incorporation of leucine into seed protein, in reactions that contained NaOCl approached those exhibited by the control.

Effect of NaOCl on Other Amino and Organic Acids. The effects of NaOCl on five other amino acids and two organic acids (acetate and IAA) were similar to those on leucine (Table III). All amino acids and IAA were strongly decarboxylated in reactions that contained NaOCl, and responses were similar to those of leucine. However, NaOCl increased the uptake of the two basic amino acids (lysine and arginine) and reduced the uptake of the others. Those data confirm published information on the strong reactivity of NaOCl with amino acids (7, 14). They also point to the strong decarboxylation of IAA by NaOCl (Table III).

Table III. *Effect of NaOCl on Decarboxylation, Uptake, and Incorporation of Amino Acids, IAA, and Acetate into Protein*

The 2-ml reactions contained 0.66 μc of substrate, carrier-free, 40 μg of penicillin-G, and streptomycin sulfate ± 50 tomato seeds ± 0.52% NaOCl (v/v) in a final concentration. Incubation period was 4 hr at 25 C.

Reaction Contents			CO_2	Acid-insoluble	Acid-soluble	Total Uptake	Incorporated into Protein
Acid	Seed	NaOCl	*dpm × 10^{-1} /reaction*				%
His-^{14}C(UL)	+	−	51	1,325	11,525	12,850	10.31
	+	+	17,003	2,413	2,218	4,631	52.11
	−	+	6,190				
Asp-^{14}C(UL)	+	−	34	83	649	732	11.34
	+	+	24,716	267	572	839	31.82
	−	+	5,117				
Lys-^{14}C(UL)	+	−	16	1,400	6,613	8,013	17.47
	+	+	12,212	33,549	15,581	49,130	68.29
	−	+	8,819				
Tyr-^{14}C(UL)	+	−	101	851	4,342	5,193	16.39
	+	+	10,160	1,426	2,257	3,683	38.72
	−	+	14,139				
Arg-^{14}C(UL)	+	−	17	150	5,242	5,392	2.78
	+	+	5,383	6,188	3,223	9,411	65.75
	−	+	3,065				
Leu-^{14}C(UL)	+	−	148	474	1,011	1,485	31.92
	+	+	10,837	599	614	1,213	49.38
	−	+	14,693				
IAA-1-^{14}C	+	−	442	878	4,616	5,494	15.98
	+	+	9,849	410	373	783	52.36
	−	+	2,947				
Acetate-1-^{14}C	+	−	625	47	1,046	1,093	4.30
	+	+	353	48	761	809	5.93
	−	+	142				

Table IV. *Removal of NaOCl from Surface of Seeds by Washing with Water or HCl Followed by Water*

Sterilization was for 5 min in 1% NaOCl (v/v). The HCl treatment was for 10 min in 0.01 N. Each washing was accomplished by soaking seeds in 30 ml of water. Incubation medium contained 0.3 μc of ^{14}C-1-leucine in 3 ml of 0.5 mM leucine. Incubation period was 2 hr at 25 C (23 C for lettuce). Each replicate utilized 100 seeds of tomato and lettuce and 20 seeds of barley.

Seed	Treatment	Germination	CO_2	Acid-insoluble	Acid-soluble	Total Uptake
		%	*dpm × 10^{-1}/sample*			
Tomato	H_2O → 8 washes	92	49	91	373	464
	NaOCl → 8 washes	94	1230	177	343	520
	NaOCl → HCl → 8 washes	95	55	127	443	570
Lettuce	H_2O → 8 washes	98	49	31	582	613
	NaOCl → 8 washes	99	110	51	688	739
	NaOCl → HCl → 8 washes	99	47	35	632	667
Barley	H_2O → 8 washes	99	48	142	1009	1252
	NaOCl → 8 washes	100	471	135	904	1039
	NaOCl → HCl → 8 washes	100	57	124	952	1076

Removal of NaOCl from Seed Surface after Use. Because amino acids were reactive with NaOCl, it was important to wash away all NaOCl from the seed surface after sterilization. The present data indicate that even after several washes with water there was enough NaOCl on the seeds to alter the pattern of amino acid metabolism (Table I). On the other hand, 0.01 N HCl for 10 min removed NaOCl completely, as evidenced by CO_2 production equal to the control, without any effect on seed germinability (Table IV). Lower concentrations were less effective and 0.1 N HCl for 10 min reduced germinability by about 10%.

The high reactivity of amino acids with trace amounts of NaOCl, and the difficulty of washing it away from seed surface with water may yield misleading data when amino acids are used in metabolic studies of NaOCl-treated seeds. Special efforts should be made to remove NaOCl completely from seed surface before incubating in amino acids, especially when long incubation periods and high specific radioactivity, carrier-free, amino acids are used. Under such conditions the label would be depleted by reacting with NaOCl in the incubation medium before it has the chance to be taken in and metabolized by the tissue.

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