

tert-Butyl Hypochlorite for Detection of Nitrogenous Compounds on Chromatograms

DANIEL P. SCHWARTZ and MICHAEL J. PALLANSCH

Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

A large number of nitrogen-containing compounds of biological interest may be readily detected on paper chromatograms by forming their *N*-chloro derivatives with *tert*-butyl hypochlorite and subsequently spraying with starch-potassium iodide.

The procedure is less tedious and hazardous than other chlorinating techniques.

SEVERAL METHODS have been reported for forming and detecting on filter paper the *N*-chloro derivatives

of organic nitrogen compounds. Chlorine gas and a starch-potassium iodide solution were used in the original procedure of Rydon and Smith (?), whereas Reindel and Hoppe (5, 6) used chlorine dioxide and chlorine as *N*-chlorinating reagents and benzidine or *o*-toluidine as

indicators. However, these methods are somewhat inconvenient and may leave a high background. Sodium hypochlorite has been used as an *N*-chlorinating agent, but this procedure is admittedly almost entirely specific for mono-substituted amides (4). For these reasons the authors attempted to develop a rapid, simple, and convenient method for forming *N*-chloro derivatives of a wide variety of nitrogen-containing compounds of biological interest. It has been found that *tert*-butyl hypochlorite is an ideal reagent for this purpose. Of 98 nitrogen-containing compounds tested, only five were unreactive.

EXPERIMENTAL

Reagents. *tert*-Butyl hypochlorite was synthesized by the method of Teeter and Bell (8) and purified by distillation in an all-glass apparatus. The fraction distilling at 78° at atmospheric pressure was collected and stored at 5° in the dark in a glass-stoppered bottle.

The spray reagents used to detect the spots formed on the chromatograms by nitrogen-containing compounds were prepared as follows:

SOLUTION I. 1 part of *tert*-butyl hypochlorite to 99 parts of 10 volume % glacial acetic acid in 1,2-dichloroethane (Eastman Kodak White Label, or equal). This solution was stable for at least 1 month at room temperature in ordinary laboratory light.

SOLUTION II. 1% potassium iodide-1% soluble starch containing 5 mg. % mercuric iodide as a preservative.

Paper Chromatography. All chromatograms were run on 8-inch-square sheets of Whatman No. 1 paper prewashed three times in water by ascending technique. Unwashed paper gave rise to a blue-black band about 1/4 inch wide and about 2 inches from the upper edge of the paper after chromatography. Solutions of the compounds studied were made in water, 0.5*N* hydrochloric acid, or 1*N* ammonium hydroxide. Samples were spotted 2 cm. apart at the origin, using a microliter pipet. Hydrochlorides were neutralized on the sheet with 1 μ l. of 2*N* ammonium hydroxide. Where dilution was necessary, microliter volumes were pipetted onto a polyethylene strip and diluted to the desired concentration with water.

The chromatograms were developed by the ascending technique in 9 \times 10 \times 3 inch museum jars, with the solvent system methanol-water-pyridine (80-20-4). When the solvent reached the upper edge of the paper (about 2.5 hours), the papers were removed, dried overnight at 40° to 50°, and sprayed with Solution I. The sheets were allowed to stand from 5 to 10 minutes in a hood with a good draft, then sprayed lightly and evenly with Solution II. Under these conditions, compounds forming *N*-chloro derivatives, when in the proper concentration, were immediately evident as blue or purple-blue spots on a colorless background. The circumference of the spots was

traced immediately and the enclosed areas were measured with a planimeter.

It was undesirable to aerate the papers for more than 10 minutes after spraying with Solution I, as many of the compounds tested (especially most of the common amino acids) apparently form unstable *N*-chloro derivatives. Under no conditions should the chromatograms be heated after chlorination.

Paper Electrophoresis. The protein zones on ionograms produced by conventional apparatus can be speedily located in a qualitative fashion by the technique described.

After electrophoresis the paper strips were heated in a circulating air oven at 130° C. for 20 minutes. Chlorination and spraying were carried out as for conventional paper chromatograms.

The response to *tert*-butyl hypochlorite of a number of naturally occurring protein mixtures and purified proteins was studied with a commercial paper electrophoresis apparatus of the Durrum type (2). Ten-microliter samples of the protein to be studied were placed on the Whatman 3 MM paper strips in the apparatus and subjected to electrophoresis for 16 hours at a current of approximately 0.625 ma. per strip. Human blood serum, concentrated milk serum, and solutions of crystalline β -lactoglobulin, α -lactalbumin, catalase, urease, and lipase were studied in an acetate buffer of pH 5.4, a phosphate buffer of pH 7.5, and a borate buffer of pH 8.6.

RESULTS AND DISCUSSION

Results of the study are presented in Table I. Concentrations are given in micrograms for approximately the lower limits of detection per square centimeter under the conditions of this investigation.

The extremely low specificity of the reagent renders it practically useless for the detection of any one class of nitrogen-containing compounds. However, the reagent can be applied successfully after ninhydrin, and, theoretically, can be used after any reagent which will not react with it, oxidize potassium iodide to free iodine, or interfere with the formation of the starch-iodine complex. Despite its low specificity the reagent is, in most instances, relatively sensitive toward the majority of compounds within a given class, except the nitrogen-containing vitamins.

When compared to ninhydrin as a reagent for amino acids on paper, the *tert*-butyl hypochlorite-starch-potassium iodide spray is noticeably superior for detecting the basic amino acids and tryptophan. According to Auclair and Dubreuil (1), the lowest detectable quantities of arginine, lysine, histidine, and tryptophan with ninhydrin on two-dimensional chromatograms are 2.0, 1.5, 7.5, 2.0 γ , respectively. Thus, as shown in Table I, the hypochlorite

reagent is approximately four times as sensitive. This increased sensitivity may be used advantageously on amino acid chromatograms by applying Solutions I and II prior to treatment with ninhydrin. The spots due to the starch-iodine complex fade with time (depending on concentration of the amino acid and the paper may then be sprayed with ninhydrin to obtain the purple spots from the regenerated amino acids. Duplicate chromatograms may be run and sprayed separately for comparison.

Although the sulfur-containing amino acids cysteine, cystine, and methionine failed to give spots at high concentrations, lanthionine, cystathione, homocystine, cysteine acid, methionine sulfone, and methionine sulfoxide were readily detectable.

For the monoamino, monocarboxylic amino acids, the chain length, branching, and position of the amino group apparently influence the reactivity of the amino group to *N*-chlorination, in some instances. Thus, much higher concentrations of leucine and isoleucine were needed for detection than with valine. Norvaline and norleucine were relatively less reactive than leucine and isoleucine, and γ -amino-*n*-butyric acid was approximately 15 times as reactive as the corresponding α amino acid. No spot was given by α -aminoisobutyric acid even in concentration as high as 55 γ , whereas the beta isomer reacted at 1.1 γ . The relative insensitivity of the alpha form of these pairs to *N*-chlorination might be used as a means of differentiating the isomers in either pair, as they are sometimes difficult to separate on paper.

Tyrosine has been reported to be unreactive when chlorinated with chlorine gas (7) or chlorine dioxide (6). In the present procedure, however, it was easily detected.

The present method failed to detect hippuric acid, even in very high concentrations, whereas Rydon and Smith (7) reported a positive response with their reagent. This may be due to the relatively large size of *tert*-butyl hypochlorite, and the close proximity of the benzene ring to the reactive group in hippuric acid.

All protein mixtures analyzed produced patterns similar to those obtained with the conventional bromophenol blue dye (3) as a protein stain. Because of the transient nature of the color produced by *tert*-butyl hypochlorite-starch-potassium iodide, it is of little value for quantitative work. As barbituric acid reacts with the reagent, veronal buffers cannot be used.

The reagent is more sensitive than bromophenol blue. This extreme sensitivity was a serious handicap in analyzing mixtures in which the component with highest electrophoretic mobility was strongly adsorbed on the

