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o-Phthalaldehyde: Fluorogenic Detection of Primary Amines in the Picomole Range. Comparison with Fluorescamine and Ninhydrin

(amino-acid analysis/peptide analysis)

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ABSTRACT ***o*-Phthalaldehyde, in the presence of 2-mercaptoethanol, reacts with primary amines to form highly fluorescent products. Picomole quantities of amino acids, peptides, and proteins can be detected easily. *o*-Phthalaldehyde is five to ten times more sensitive than fluorescamine and is soluble and stable in aqueous buffers.**

o-Phthalaldehyde, in the presence of 2-mercaptoethanol, has been described by Roth (1, 2) as a reagent for fluorometric detection of α-amino acids. However, Roth found that lysine and cystine showed only about 5% of the fluorescence of other natural amino acids, and that peptides also showed greatly reduced fluorescence values. Roth's work was confirmed by Taylor and Tappel (3), who also found that peptides yielded greatly reduced fluorescence with *o*-phthalaldehyde. Probably because of these shortcomings, the reagent has not been widely used for routine detection of primary amines.

In this report we demonstrate that a modified formulation of Roth's reagent can be used to detect α-amino acids, peptides, and proteins in the picomole range. By increasing the concentration of 2-mercaptoethanol 10-fold and adding Brij to the reagent mixture, we have overcome most of the shortcomings described. In comparison with ninhydrin and fluorescamine, *o*-phthalaldehyde exhibits greater sensitivity.

Udenfriend *et al.* (4) first described fluorescamine, a fluorogenic reagent that also allows high sensitivity detection of primary amines. They compared fluorescamine with ninhydrin, attempting to show the greater sensitivity of the fluorogenic reagent. Georgiadis and Coffey (5) later published their own comparison of ninhydrin and fluorescamine, and their results also imply much greater sensitivity with the fluorometric method. As has been observed by Hare (6), these data are misleading because the amino-acid analyses displayed were not performed on the same instrument. The analysis utilizing ninhydrin was performed using 9-mm diameter resin beds, whereas the analysis utilizing fluorescamine was performed with 2.8-mm diameter resin beds. The reduction in resin bed cross-sectional area alone would account for a 10-fold increase in sensitivity; consequently, the increase in sensitivity attributed to fluorescamine is exaggerated.

We compared ninhydrin with fluorescamine and *o*-phthalaldehyde using a single analytical system. The 0.20 × 25-cm stainless steel column was filled with DC-4A cation exchange resin (Durrum Chemical Corp., Palo Alto, Calif.). A single-column procedure was used with sodium formate and sodium borate buffer solutions in stepwise addition. Thick-walled polystyrene reservoirs, pressurized with nitrogen gas, were used to drive the buffers through the resin bed (7). The effluent passed into a Kel-F mixing manifold into which ninhydrin or fluorogenic reagents could be introduced.

For ninhydrin detection, the formulation of Liao *et al.* (8) was used. The ninhydrin reagent and column effluent were mixed in an equal volume ratio and allowed to react at 115° for 3 min in a coil of Teflon tubing of 0.3-mm inner diameter. The mixture then passed into a 60-mm flow cell, where absorbance was monitored at 570 nm. One nanomole of an amino-acid calibration mixture was applied to the resin bed; the resulting chromatogram is depicted in Fig. 1A. Full-scale deflection on the recorder corresponded to an absorbance of 1.0.

For fluorometric detection, the ninhydrin system was replaced by an Aminco filter fluorometer (American Instrument Co., Silver Spring, Md.) with a 70-μl flow cell. A Corning 7-51 filter was used for excitation, and a Wratten 2A filter was used for emission‡. Because fluorescamine and *o*-phthalaldehyde fluorophors fluoresce maximally from pH 9 to 11, buffering of column effluents is usually necessary. *o*-Phthalaldehyde is stable in water and can be conveniently dissolved in an appropriate buffer solution. Fluorescamine, however, rapidly hydrolyzes in water to form nonfluorescent products; consequently, it is usually dissolved in acetone. The necessary buffering is accomplished by separately adding a buffer solution to the column effluent.

Fluorescamine (Roche Diagnostics, Nutley, N.J.) was dissolved (0.30 mg·ml^{-1}) in acetone (Fisher Scientific Co., spectroscopic grade). The borate buffer solution was made from 0.40 M boric acid titrated to pH 9.7 with potassium hydroxide. The column effluent, borate buffer, and fluorescamine–acetone reagent were simultaneously mixed in equal volume ratios in the Kel-F mixing manifold. Teflon tubing (0.3-mm inner diameter) connected the manifold to the fluorometer flow cell. Excess tubing was used to allow sufficient reaction time for fluorophor formation by aspartic and glutamic acids, which react more slowly than other amines. One nanomole of the calibration mixture was applied as before; the resulting chromatogram is shown in Fig. 1B. To achieve this result, the fluorometer was set at its least sensitive scale and the recorder was adjusted to 50 mV full-scale deflection.

o-Phthalaldehyde (Eastman) was dissolved in 95% ethanol

‡ Optimum excitation wavelengths are 390 nm for fluorescamine and 340 nm for *o*-phthalaldehyde; optimum emission wavelengths are 475 nm for fluorescamine and 455 nm for *o*-phthalaldehyde.

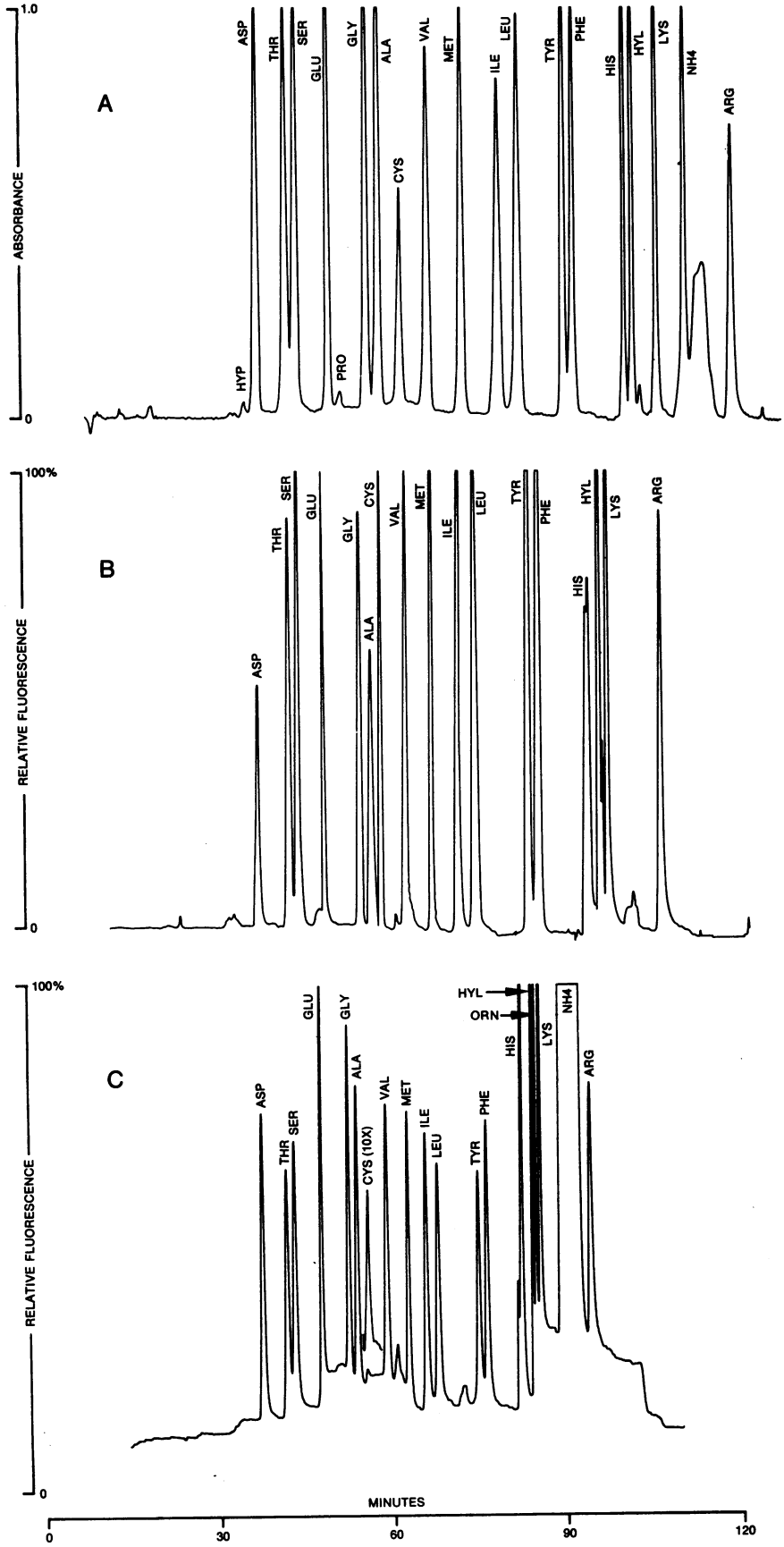

FIG. 1. Amino-acid analysis performed on a single resin bed 0.20 × 25 cm. (A) Sample contained 1.0 nmol of each amino acid detected with ninhydrin using a flow cell with a 60-mm pathlength. Full-scale absorbance = 1.0. (B) Sample contained 1.0 nmol of each amino acid detected with fluorescamine and an Aminco fluorometer. Full scale deflection = 53 mV. (C) Sample contained 100 pmol of each amino acid

(*Figure 1 legend continued on next page*)

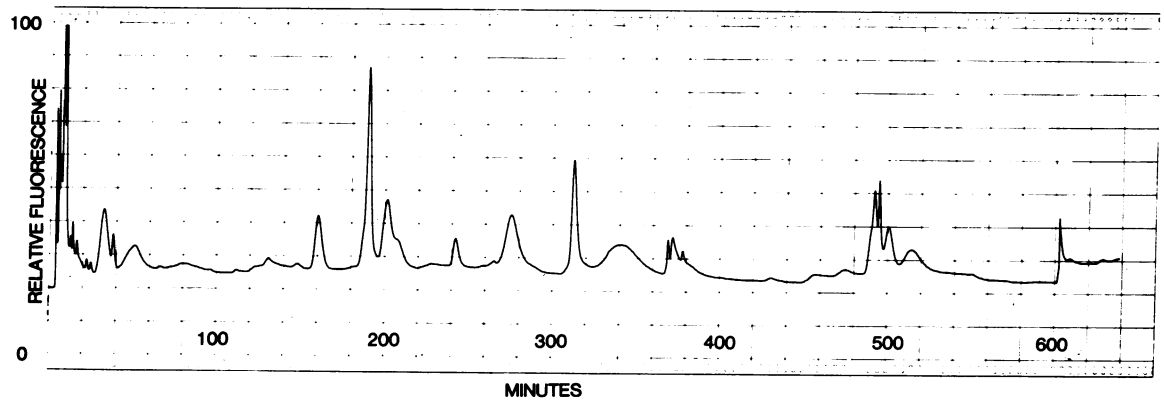

FIG. 2. Tryptic peptides of *S*-aminoethylated human globin detected by *o*-phthalaldehyde. Only 10 μg (300 pmol) of globin were used for this analysis. Six stepwise buffers were used to elute the peptides; their pH values were 4.6, 5.5, 6.3, 7.2, 8.3, and 9.2. The 0.20 × 25-cm column contained Durrum DC-4A resin. Column temperature was constant at 57°, and buffer flow rate was 7 ml·hr^{-1}. Recorder full-scale deflection was 10 mV.

(800 mg in 10 ml); this was added to 1.0 liter of 0.40 M boric acid solution titrated to pH 9.7 with KOH and containing 2.0 ml of 2-mercaptoethanol. Lysine, for an unknown reason, yields relatively low fluorescence unless Brij is present, as noted by Schwabe and Catlin (9). Consequently, 1.0 g·liter^{-1} Brij (Pierce Chemical Co., Rockford, Ill.) was added to the previously filtered reagent. The *o*-phthalaldehyde reagent was added in an equal volume ratio to the column effluent, and the mixture was passed directly to the fluorometer flow cell via a short length of a Teflon tubing of 0.3-mm inner diameter. Contrary to Roth's observation that fluorophor formation with *o*-phthalaldehyde required 2–5 min of reaction time, we observed maximum fluorescence when the mixture was immediately passed into the fluorometer. This is in contrast with the fluorescamine reaction, which achieves maximum fluorescence after a slight delay. One hundred picomoles of the calibration standard were applied to the resin bed; the results are shown in Fig. 1C. This analysis was achieved with the fluorometer and recorder adjusted as before.

As can be seen from Fig. 1A and B, fluorescamine shows very little improvement in sensitivity over ninhydrin when the latter is monitored with a 60-mm flow cell. Although Roth reported little difference in sensitivities, our results show that *o*-phthalaldehyde is between five and ten times more sensitive than fluorescamine. The magnitude of these differences, which vary for individual amino acids, is indicated in Fig. 1C, which depicts an analysis of one-tenth the sample shown in Fig. 1B.

Although Roth was unable to obtain significant fluorescence with peptides, we succeeded in detecting tryptic peptides from hemoglobin. The α and β chains of purified normal human globin were treated with ethylenimine to aminoethylate the sulfhydryl groups. The *S*-aminoethylated globin was then reacted with TPCK trypsin (Worthington Biochemicals, Freehold, N.J.) at pH 8–9 and the resultant peptide mixture, adjusted to pH 3.0, was stored in a freezer. This formed the stock solution of peptides for subsequent experiments.

The analytical system used for separating the peptides was similar to that described above. Six stepwise buffer solutions were used as eluants for the analysis. They were prepared from sodium citrate, phosphate, and borate, all 0.20 M in sodium. A manual sample injection valve (Durrum Chemical Corp.) was used to inject 20-μl sample volumes. The concentration of the peptide solution was adjusted so that 20 μl corresponded to 10 μg of the original globin. The *o*-phthalaldehyde reagent was prepared as before, except the Brij was omitted and the buffer solution was made 0.80 M in boric acid and titrated to pH 9.1 with KOH.

Fig. 2 illustrates the elution pattern of the tryptic peptides from 10 μg (300 pmol) of human globin. Note that the very acidic peptides co-elute early in the chromatogram and that only neutral and basic peptides are resolved with this protocol. Acidic peptides could be separated with an anion-exchange resin and decreasing pH gradient.

The high sensitivity of this reagent for peptides provides a valuable tool for protein chemists who have limited amounts of material for structural analysis. Membrane proteins and viral proteins can be partially characterized with picomole amounts of purified material. Weidekamm *et al.* (10) reported the use of *o*-phthalaldehyde and 2-mercaptoethanol for the detection of proteins in gel electrophoresis experiments and were able to detect as little as 10 ng of protein. We examined both *o*-phthalaldehyde and fluorescamine in relation to their reactivity to proteins. A stock solution of bovine serum albumin was prepared in 1.0 M NaCl. This was then serially diluted to provide a solution containing 1.5 pmol of bovine serum albumin per 10 μl. The solution was injected into the column effluent stream such that it passed directly into the fluorometer flow cell after reacting with *o*-phthalaldehyde or fluorescamine. The results showed *o*-phthalaldehyde to be nearly five times more sensitive than fluorescamine. Using bovine serum albumin, the linearity of *o*-phthalaldehyde was established over the range of 1.5–12 pmol.

Neither *o*-phthalaldehyde nor fluorescamine forms fluorophors with secondary amines. Weigele *et al.* (11) described a method of oxidizing secondary amines with *N*-chlorosuccinimide to form primary amines that can then react with fluorescamine. Felix and Terkelsen (12, 13) have applied Weigele's

(Figure 1 legend continued from previous page.)
detected with *o*-phthalaldehyde and the same fluorometer. Full-scale deflection = 50 mV. Cystine was detected by use of 10X scale expansion. All analyses were performed with five stepwise buffers with pH values 2.90, 3.45, 4.00, 5.50, and 10.0. Column temperature was constant at 50°. Total analysis times vary since flow rates were not exactly matched for each analysis. These varied from 5.5 to 6.5 ml·hr^{-1}.

method to an automatic amino-acid analyzer. We have not succeeded in using Weigele's method with *o*-phthalaldehyde. *N*-Chlorosuccinimide apparently oxidizes *o*-phthalaldehyde, rendering it useless for fluorophor formation.

In spite of this yet unresolved problem, *o*-phthalaldehyde shows several advantages over fluorescamine. (*a*) *o*-Phthalaldehyde exhibits greater fluorescent quantum yields (nearly one order of magnitude). (*b*) *o*-Phthalaldehyde is soluble and stable in aqueous buffers. We have allowed it to remain at room temperature for several days and have not observed loss of sensitivity or increased fluorescent background. Chromatographs utilizing *o*-phthalaldehyde are thereby simplified, because only one reagent needs to be added to column effluents. (*c*) Quieter baselines are obtained, since mixing of two aqueous solutions is not impaired by the simultaneous addition of organic solvents. The latter can cause Schlieren effects, precipitation problems and gas formation in the flow cell, all of which create baseline artifacts. (*d*) *o*-Phthalaldehyde is considerably less expensive than fluorescamine. As such, it could be used economically in standard amino-acid analyzers using higher eluent flow rates. Because of the high cost of fluorescamine, its use is usually limited to microbore systems.

It is interesting to note that the limits in sensitivity of analyses of primary amines using any of the methods described above are determined by impurities in the buffer solutions used as eluents. Ninhydrin-positive components and fluorogenic contaminants deposited on the resin bed by the buffer solutions are eluted later by the high pH borate buffer. The effect of these contaminants is seen in the region of the chromatogram in which the basic amino acids elute. We estimate that, were it not for these contaminants, femtomole (10^{-15}) quantities of amino acids and peptides could be detected using *o*-phthalaldehyde.

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