Trace determination of biological thiols by liquid chromatography and precolumn fluorometric labeling with o-Phthalaldehyde

ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 1984

Impact Factor: 5.64 · DOI: 10.1021/ac00277a064 · Source: PubMed

2 AUTHORS:

Kenneth Mopper
Old Dominion University
147 PUBLICATIONS **9,368** CITATIONS
SEE PROFILE

Delmas Daniel
Institut Français de Recherche pour l'Exploit…
47 PUBLICATIONS **922** CITATIONS
SEE PROFILE

Reprinted from Analytical Chemistry, 1984, 56, 2557.

Trace Determination of Biological Thiols by Liquid Chromatography and Precolumn Fluorometric Labeling with *o*-Phthalaldehyde

Kenneth Mopper*

University of Miami, Rosenstiel School of Marine and Atmospheric Science, Division of Marine and Atmospheric Chemistry, 4600 Rickenbacker Causeway, Miami, Florida 33149-1098

Daniel Delmas

CREMA—L'Houmeau, Case 5, 17137 Nieul-sur-Mer, France

Organic thiols were converted to highly fluorescent isoindole derivatives by reaction with *o*-phthalaldehyde and 2-aminoethanol in aqueous solution and at a mildly basic pH. The reaction was complete within 1 min. The derivatives were then separated by reversed-phase high-performance liquid chromatography. Optimal reaction and chromatographic parameters were determined and were similar to the analogous reaction of primary amines with *o*-phthalaldehyde and 2-mercaptoethanol. Unlike earlier precolumn derivatization techniques for thiols, there are no interfering reagent or reagent byproduct peaks, which is advantageous for ultratrace analysis of thiols in aqueous samples. The detection limit is about 25 fmol per injected thiol and the precision is about ±7% at the 2–3 pmol level. The linearity of response was examined over 3 orders of magnitude (nM–μM) and was linear in that range. Examples of the use of the method are given for the analysis of thiols in urine and reducing marine sediment porewaters.

Low molecular weight, hydrophilic thiols (such as, glutathione, mercaptoethanesulfonic acid, cysteine, thioglycolic acid, and methanethiol) have been the subject of intense research in numerous biochemical and environmental studies (*1*, *2*). They are key components of some coenzymes and of metallothioneins which, through selective complexation, are involved in the intracellular availability, or detoxification of trace metals, such as copper, zinc, and mercury (*3*). In addition, these compounds are widely dispersed in the environment as they are important intermediates in the anaerobic decomposition of organic matter in soils, sediments, and sewage (*4–6*).

Despite the importance of these compounds, biochemical and environmental studies have been limited because of shortcomings in existing analytical techniques. Published precolumn derivatization techniques for liquid chromatographic analysis of thiols (*7–11*) generally function well for most biological samples but are not particularly suited for ultratrace (low or subnanomolar) analysis of thiols. For example in some cases, the reagent itself and reaction byproducts interfere with the chromatographic separation; in other cases, multiple derivatives are formed for each thiol or lengthy reaction times are required. To circumvent these problems Nakamura and Tamura (*12*) used postcolumn fluorometric derivatization based on the reaction of thiols with *o*-phthalaldehyde and primary amines (*13*, *14*). However, this technique requires the assembly of a dedicated postcolumn reaction system and the sensitivity is ultimately limited by the fluorescent background of the reagent stream.

It is well known that the reaction of primary amines with excess *o*-phthalaldehyde (OPA) and an excess thiol yields unique, isoindole derivatives (*14*) that are readily separated by reversed-phase HPLC (*15*). Likewise, the reaction of thiols with excess *o*-phthalaldehyde and an excess primary amine should also yield unique derivatives suitable for chromatography. In addition, like the amine method, the reaction for thiols should be rapid, mild, and proceed in aqueous media; also, no interferences from the reagent or reaction byproducts are expected. We present here our findings regarding use of this reaction for precolumn derivatization and reversed-phase liquid chromatographic separation of thiols. Emphasis is placed on optimization of reaction parameters and on applications to natural samples.

EXPERIMENTAL SECTION

Apparatus. The pumping system consisted of an Eldex Laboratories (Menlo Park, CA) gradient controller, Model Chomat-A-Trol, and an Eldex Model AA dual piston pump. Gradients were formed by low pressure ratioing valves programmed by the controller. To ensure complete mixing of solvents, a high-pressure mixer was placed between the pump and injection valve (Valco Model CK6UHPa-N60, 20-μL loop). Detection was accomplished with a Gilson Medical Electronics (Middleton, WI) Model 121 fluorometer equipped with a 9-μL flow cell and 340 ± 6 nm (excitation) and 450 ± 20 nm (emission) filters. Results were recorded on a Hewlett-Packard (Avondale, PA) Model 3390A reporting integrator.

Chemicals. Double-distilled water (from alkaline permanganate) and HPLC-grade salts and solvents were used in the mobile phases as well as in the preparation of reagent solutions and standards. The mobile phases consisted of 0.05 M sodium acetate (pH 5.7) ("A" phase) and acetonitrile or methanol ("B" phase). Mobile phases were filtered (0.45 μm) prior to use. The reagent consisted of two parts: the first solution was 20 mg of *o*-phthalaldehyde (Fluka Chemical, Hauppauge, NY) in 1 mL of methanol; the second was 20 μL of 2-aminoethanol (Sigma Chemical, St. Louis, MO) in 1 mL of 0.8 M sodium borate, pH 7.4. The reagent solutions are stable at least 1 week, but for trace analysis they were prepared fresh daily. Stock solutions of thiol standards (Sigma Chemical; Aldrich Chemical, Milwaukee, WI) were prepared: 0.5 mM *N*-acetylcysteine; 1 mM each glutathione, 2-mercaptoethanesulfonic acid, 3-mercaptopropionic acid, monothioglycerol, 2-mercaptoethanol, methanethiol, ethanethiol, allyl mercaptan, 1-propanethiol, 2-propanethiol; 20 mM thioglycolic acid; 0.1 M mercaptosuccinic acid; and 10 mM sodium sulfite. The stock solutions were prepared in 5 mM sodium acetate buffer (pH 4.0) containing 0.1 mM EDTA. We found that these solutions were stable at least 1 week, which is in agreement with Nakamura and Tamura (*12*). Mixed standards were prepared from stock solutions and diluted with water to appropriate concentrations (either 1:1000 or 1:10000) just prior to derivatization.

Derivatization. The reaction solution was prepared by adding 20 μL of each of the two part reagent to 1 mL of mixed, diluted

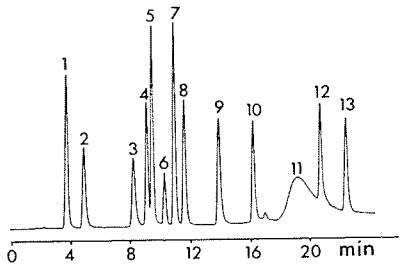

Figure 1. Gradient separation of OPA derivatives of 12 thiols and sodium sulfite, derivatization and chromatographic parameters as in Experimental Section: (1) mercaptosuccinic acid (0.1 nmol); (2) sodium sulfite (0.1 nmol); (3) glutathione (10 pmol); (4) thioglycolic acid (0.2 nmol); (5) *N*-acetylcysteine (7 pmol); (6) thiolactic acid (0.5 nmol); (7) 2-mercaptoethanesulfonic acid (10 pmol); (8) 3-mercaptopropionic acid (10 pmol); (9) monothioglycerol (10 pmol); (10) 2-mercaptoethanol (10 pmol); (11) cysteine (2 nmol); (12) methanethiol (10 pmol); (13) ethanethiol (10 pmol). Sodium sulfide elutes between peaks 9 and 10.

standard or sample. After precisely 1 min at room temperature, 20 μL was injected. If necessary, deproteination and/or filtration of biological samples and sediments were performed prior to derivatization. For physiological fluids, samples were diluted by a factor of 100–200 with water prior to derivatization. Due to the instability of thiols, sample manipulations and derivatization should be performed in an oxygen-free environment (glovebox).

HPLC Conditions. Chromatographic separations were carried out with a 100 × 4.6 mm stainless-steel Adsorbosphere C18 reversed-phase column containing 3-μm packing (Applied Science, State College, PA). A guard column containing 40 μm, C_{18} reversed-phase packing (Applied Science) was attached directly to the analytical column. Separations were performed at ambient temperature and at a flow rate of 1 mL min^{-1}. The gradient typically used with acetonitrile was as follows: isocratic at 10% B for 1 min; 10% to 25% B in 1 min; isocratic at 25% B for 4 min; 25% to 50% B in 7 min; isocratic at 50% B for 11 min; isocratic at 100% B for 2 min; 100% to 10% B in 2 min; 10 min conditioning at 10% B.

RESULTS AND DISCUSSION

Mobile Phases. Methanol and acetonitrile were tested for their effect on resolution and selectivity of OPA–thiol derivatives in reversed-phase HPLC. Both solvents worked well, although the overall selectivity was somewhat better with acetonitrile than with methanol. A typical chromatogram of a standard mixture of OPA-derivatized thiols is depicted in Figure 1. When 10 mM tetrabutylammonium chloride (pH 6.0) was substituted for the buffer, the derivatives eluted late (in a high percentage of B phase) and as groups of poorly resolved peaks. This increased retention was undoubtably due to ion pair formation between the tetrabutylammonium ion and the ionized carboxyl and sulfonic acid groups on the derivatives. Better control over the selectivity of these compounds could be achieved by slight pH adjustment of the acetate buffer ("A" phase).

Fluorescence Wavelengths and Intensities. Results for a number of organic and inorganic thiols were in good agreement with those reported by Nakamura and Tamura (12). Uncorrected excitation and emission maxima of some compounds not given by those investigators are as follows: sulfite 346/390 nm; hydrosulfite 335/410 nm; thiosulfate 338/394 nm; and thioglycolate 352/460 nm. These wavelengths were measured in 50% aqueous methanol. We found that through use of narrow band-pass filters it was possible to selectively enhance the detection of either organic thiols (emission maximum 420–430 nm) or inorganic sulfur compounds (emission maximum 390–410 nm).

pH of the Reaction. Nakamura and Tamura (*12*) reported the optimal pH range for the reaction of thiols with OPA and taurine to be 8.5–10.5. Figure 2 shows our results for pH 4.0–12.5. Evidently substituting 2-aminoethanol for taurine shifts the optimal pH range downward to about 6.5–9.5, which

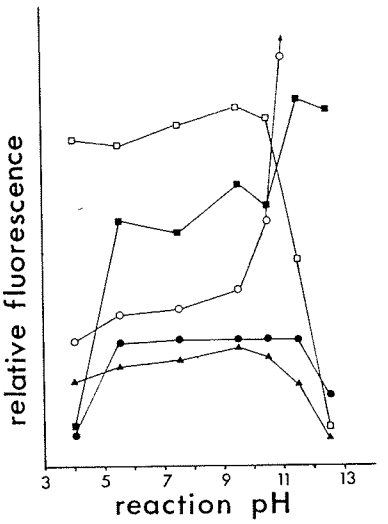

Figure 2. Effect of pH of the reaction mixture on the reactivity of OPA (8.6 mM) and 2-aminoethanol (23.6 mM) toward thiols and sodium sulfite: reaction time 1 min at room temperature; Chromatographic conditions as in the text; (●) sodium sulfite, (□) glutathione, (▲) 2-mercaptoethanesulfonic acid, (○) 2-mercaptoethanol, (■) ethanethiol.

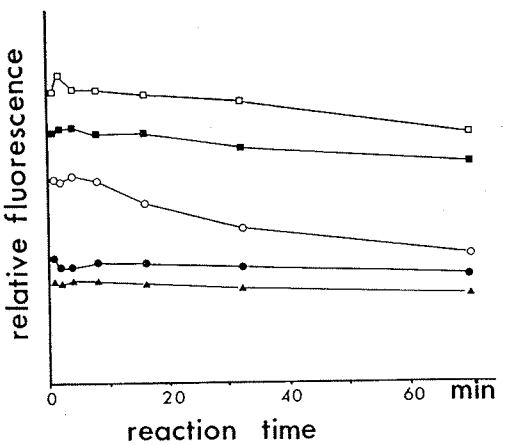

Figure 3. Effect of reaction time on the derivatization of thiols and sodium sulfite with OPA (8.6 mM) and 2-aminoethanol (23.6 mM); reaction pH was 8.4. Notation is the same as that give in the in caption to Figure 2.

is close to that of most natural waters and physiological fluids. The increase in fluorescence of 2-mercaptoethanol and ethanethiol above pH 9.5 is due to the formation and coelution of fluorescent degradation products. These products formed much more slowly at the lower end of the optimal pH range (see below).

Reaction Time. The reaction time prior to injection was varied from 1 min to 70 min, Figure 3. The reaction temperature was ambient and the reaction pH was 8.4. In agreement with Nakamura and Tamura (*12*), the reaction appeared to be nearly instantaneous. At pH 8.4, fluorescent degradation products were not observed, except in the 70 min run where minor peaks due to these products were encountered. A reaction time of 1 min or less and a low reaction pH (<9) are recommended.

Concentration of OPA. The concentration of OPA in the reaction mixture was varied from 1 to 21 mM, Figure 4. The concentrations of 2-aminoethanol and total thiols in the reaction mixture were maintained at 24 mM and 0.067 mM, respectively. From Figure 4, the optimal concentration range for OPA is 2–11 mM. At least a 50-fold excess of OPA over total thiols is recommended for quantitative analysis when thiol concentrations are in the μM–mM range. For samples containing submicromolar concentrations of thiols, at least

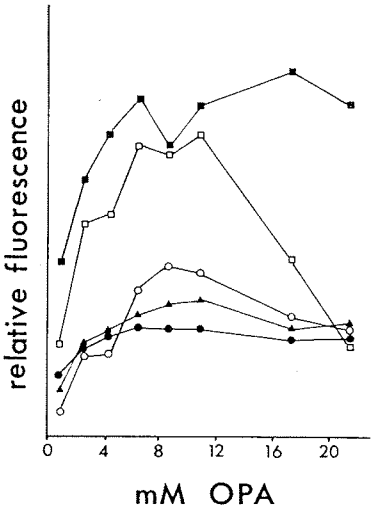

Figure 4. Effect of OPA concentration on the derivatization of thiols and sodium sulfite. The reaction time was 1 min at room temperature; the reaction pH was 9.5. Notation is the same as that given in the caption to Figure 2.

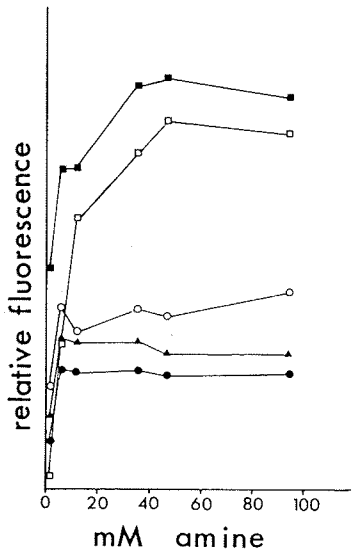

Figure 5. Effect of 2-aminoethanol concentration on the derivatization of thiols and sodium sulfite. The reaction time was 1 min at room temperature. Notation is the same as that given in the caption to Figure 2.

a 100-fold molar excess of OPA should be present.

Concentration of Amine. The concentration of 2-aminoethanol in the reaction solution was varied from 2 to 94 mM, while the concentrations of OPA and total thiols were held at 8.6 mM and 0.067 mM, Figure 5. The optimal concentration for 2-aminoethanol is >4 mM. Taurine (*12*) displayed a similar reactivity. At high amine concentrations (>12 mM) we observed a rather poor fluorescence response for cysteine (about 100-fold lower than that of other thiols). When the amine concentration in the reaction solution was lowered to <1 mM, the reactivity of cysteine with OPA improved dramatically, but the derivative eluted at the system dead-volume. We have not explored the reasons for this anomalous behavior, but we think it may be caused by involvement of cysteine's amino group in the reaction at low amine reagent concentrations. An alternative method for the determination of cysteine (as well as cystine) involves oxidation of the thiol group followed by derivatization of the amino group with OPA (*16*).

Reproducibility, Linearity, Blank, and Detection Limit. A series of five gradient runs were performed with a

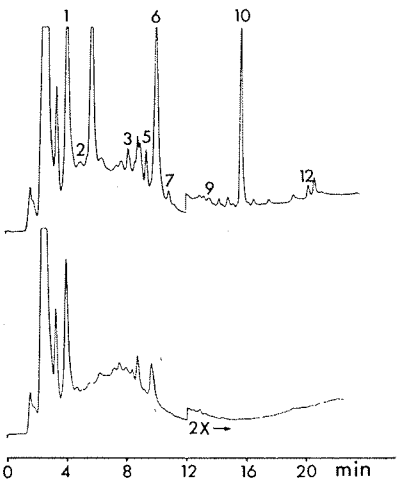

Figure 6. (Upper) Deproteinated urine sample diluted by a factor 150 prior to derivatization with OPA and 2-aminoethanol. Derivatization procedure and chromatographic conditions are given in the text. (Lower) As above, but underivatized. Notation is given in the caption to Figure 1; peak 6 can also be β-mercaptopyruvic acid; 2X, increase in detector sensitivity by a factor of 2.

seven-component thiol standard mixture containing sodium sulfite, glutathione, *N*-acetyl-L-cysteine, 3-mercaptopropionic acid, monothioglycerol, 2-mercaptoethanol, and ethanethiol. The derivatization was performed prior to each injection and the reaction time was 1 min. Using peak areas, the average coefficient of variation was 7.5% at the 2.5 pmol level for the thiols, except glutathione which was 5.5% at the 25 pmol level and sodium sulfite which was 9.1% at the 0.1 nmol level. The average coefficient of variation for retention times was 1.2%.

The linearity of response was examined over a concentration range of about 3 orders magnitude (0.1–64 pmol injected thiols) using the above seven-component standard. Responses were linear ($r > 0.999$) in this range.

The reagent blank was evaluated by derivatizing freshly distilled water followed by analysis at a high detector sensitivity setting. A few minor peaks were observed, Figure 6 (lower), corresponding to about 0.1–0.5 pmol thiols in a 100-μL injection (i.e., 1–5 nM). The identity and source of these compounds were not investigated since they do not interfere with the separation of known thiols. We suspect that these compounds may be trace contaminants in the reagents or fluorescent products formed from the direct condensation of the amine with OPA.

The detection limit was found to be about 25 fmol per injected thiol (200 fmol for sodium sulfite) with a signal-to-noise ratio of about 3. This corresponds to an injection of 100 μL of sample with 0.25 nM levels of thiols and 2 nM level of sodium sulfite.

Stability and Extraction of Fluorescent Products. The fluorescent isoindole derivatives of the thiols were found to be relatively unstable in the aqueous reaction mixture, Figure 3, which is in agreement with Simons and Johnson (*14*). For field sampling and for analysis of large numbers of samples using automatic injectors, it is desirable to have a method for stabilizing the products. Therefore, we attempted to extract the derivatives onto small cartridges packed with C_{18} bonded material (Sep-PAK, Waters Associates, Milford, MA). The following procedure was tested: Cartridges were conditioned with 5 mL of methanol and 10 mL of water and then loaded with 5 mL of derivatized standard solution (1 μM thiols, 10 μM sodium sulfite). The cartridges were rinsed with 1 mL of water and followed by 5 mL of methanol to elute the derivatives. Recoveries for the nine thiols tested average 76 ± 8% (52 ± 12% for sodium sulfite). The derivatives were stable in methanol at least 3 h. Unfortunately this procedure was

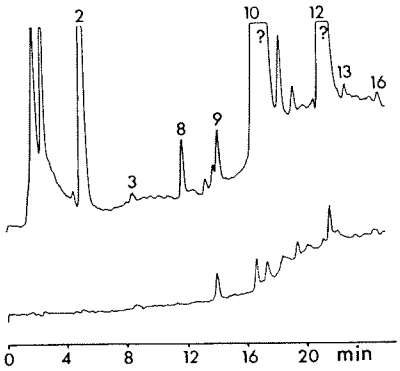

Figure 7. (Upper) Thiols in porewater of reducing sediment. Porewater was displaced from the sediment with degassed seawater. Derivatization procedure and chromatographic conditions are given in the text. Notation is the same as that given in the caption to Figure 1: peak 16, 1-propanethiol; the two large unknown peaks coeluting with 2-mercaptoethanol and methanethiol, 10 and 12, may be inorganic sulfides. (Lower) Reagent blank run under the same conditions as the sediment porewater sample.

not found useful for samples containing high concentrations of sulfide (>0.1 mM) due to the formation of fluorescent byproducts which interfere with the detection of some thiols.

An alternative to the above approach is to use an automated precolumn derivatization and injection method. This method has been successfully applied to the chromatographic determination of amino acids with OPA (*17*, *18*) and therefore should be easily adapted to the present thiol method.

Applications. Like the analogous method for amino acids (*15*), one of the major advantages of the present technique is its extremely high sensitivity and selectivity compared to other LC and GC techniques for thiols. The method is particularly well suited for the analysis of aqueous samples such as physiological fluids, food extracts (e.g., onion and garlic extracts), and sewage. By use of reversed-phase chromatography these samples may be injected without cleanup steps prior to derivatization, if they are sufficiently diluted, as shown in Figure 6. It should be pointed out that peak identifications on Figures 6 and 7 were made only by coinjection with standards; therefore, these identifications must be considered tentitive at this stage.

Because of the lack of interfering reagent peaks and the low blanks, the method can also be used for ultratrace determination of thiols. For example, subnanomolar to nanomolar levels of thiols, as found in natural waters, can be readily determined with this technique, Figure 7. We are currently using this method to study the nature and distribution of thiols in reducing marine environments. Preliminary results of this study will be presented elsewhere (*19*).

Registry No. *o*-Phthalaldehyde, 643-79-8; 2-aminoethanol, 141-43-5; mercaptosuccinic acid, 70-49-5; sodium sulfide, 1313-82-2; glutathione, 70-18-8; thioglycolic acid, 68-11-1; *N*-acetylcysteine, 616-91-1; thiolactic acid, 79-42-5; 2-mercaptoethanesulfonic acid, 3375-50-6; 3-mercaptopropionic acid, 107-96-0; monothioglycerol, 38098-46-3; 2-mercaptoethanol, 60-24-2; cysteine, 52-90-4; methanethiol, 74-93-1; ethanethiol, 75-08-1; 1-propanethiol, 107-03-9.

LITERATURE CITED

(1) Jocelyn, P. C. "Biochemistry of the SH Group"; Academic: New York, 1972.
(2) Balzer, W. In "Marine Organic Chemistry"; Duursma, E. K., Dawson, R., Eds.; Elsevier Scientific: Amsterdam, 1981; Chapter 13.
(3) Sanders, B. M.; Jenkins, K. D.; Sunda, W. G.; Costlow, J. D. *Science* **1983**, *222*, 53–55.
(4) Freney, J. R. In "Soil Biochemistry"; McLaren, A. D., Peterson, G. H., Eds.; Marcel Dekker: New York, 1967; Chapter 10.
(5) Zinder, S. H.; Brock, T. D. *Appl. Environ. Microbiol.* **1978**, *35*, 344–352.
(6) Adams, D. D.; Richards, F. A. *Deep-Sea Res.* **1968**, *15*, 471–481.
(7) Takahashi, H.; Nara, Y.; Meguro, H.; Tuzimura, K. *Agric. Biol. Chem.* **1979**, *43*, 1439–1445.
(8) Fahey, R. C.; Newton, G. L. In "Functions of Glutathione"; Larsson, A., et al., Eds., Raven: New York, 1983; pp 251–260.
(9) Lankmayr, E. P.; Budna, K. W.; Müller, K.; Nachtman, F.; Rainer, F. *J. Chromatogr.* **1981**, *222*, 249–255.
(10) Kuwata, K.; Uebori, M.; Yamada, K.; Yamazaki, Y. *Anal. Chem.* **1982**, *54*, 1082–1087.
(11) Toyo'oka, T.; Imai, K. *J. Chromatogr.* **1983**, *282*, 495–500.
(12) Nakamura, H.; Tamura, Z. *Anal. Chem.* **1981**, *53*, 2190–2193.
(13) Jocelyn, P. C.; Kamminga, A. *Anal. Biochem.* **1970**, *37*, 417–421.
(14) Simons, S. S., Jr.; Johnson, D. F. *Anal. Biochem.* **1978**, *90*, 705–725.
(15) Lindroth, P.; Mopper, K. *Anal. Chem.* **1979**, *51*, 1667–1674.
(16) Turnell, D. C.; Cooper, J. D. H. *Clin. Chem.* (*Winston-Salem, N.C.*) **1982**, *28*, 527–531.
(17) Winspear, M. J.; Oaks, A. *J. Chromatogr.* **1983**, *270*, 378–382.
(18) Turnell, D. C.; Cooper, J. D. H. *J. Autom. Chem.* **1983**, *5*, 36–39.
(19) Mopper, K.; Delmas, D. *EOS*, in press.

RECEIVED for review May 21, 1984. Accepted July 5, 1984. This work was supported by grants from the National Institutes of Health (Biomedical Research Support Grant to K.M.) and from C.N.E.X.O., P.I.R.O, and C.N.R.S. (to D.D.).