(4) Fernandez, F. J.; Myers, S. A.; Slavin, W. *Anal. Chem.* **1980**, *52*, 741–746.
(5) Manning D. C.; Slavin, W. *Anal. Chim. Acta* **1980**, *118*, 301–306.
(6) Slavin, W.; Manning, D. C. *Spectrochim. Acta, Part B* **1980**, *35B*, 701–714.
(7) L'vov, B. V. *Spectrochim. Acta, Part B* **1978**, *33B*, 153–193.
(8) Hageman, L. R.; Nichols, J. A.; Viswanedham, P.; Woodriff, R. *Anal. Chem.* **1979**, *51*, 1406–1412.
(9) Chakrabarti, C. L.; Wan, C. C.; Hamed, H. A.; Bartels, P. C. *Anal. Chem.* **1981**, *53*, 444–450.
(10) Barnard, T. *Anal. Chem.* **1979**, *51*, 1172A–1178A.
(11) Siemer, D. D.; Baldwin, J. M. *Anal. Chem.* **1980**, *52*, 295–300.
(12) L'vov, B. V. "Atomic Absorption Spectrochemical Analysis"; Hilger: London, 1970.
(13) L'vov, B. V.; Bayunov, P. A.; Ryabchuk, G. N. *Spectrochim. Acta*, in press.
(14) Fuller, C. W. "Electrothermal Atomization for Atomic Absorption Spectroscopy"; Chemical Society: London, 1977.
(15) van den Broek, W. M. G. T.; de Galan, L. *Anal. Chem.* **1977**, *49*, 2176–2186.
(16) Manning, D. C.; Ediger, R. D. *At. Abs. Newsl.* **1976**, *15*, 42–44.
(17) Fernandez, F. J.; Iannarone, J. *At. Abs. Newsl.* **1978**, *17*, 117–120.
(18) Szydlowski, F. J.; Peck, E.; Bax, B. *Appl. Spectrosc.* **1978**, *32*, 402–404.
(19) Smith, W. H.; Leeds, D. H. *Mod. Mater.* **1970**, *7*, 139–218.
(20) Slavin, W.; Myers, S. A.; Manning, D. C. *Anal. Chim. Acta* **1980**, *117*, 267–273.
(21) Volland, G.; Kölblin, G.; Tschöpel, P.; Tölg, G. *Z. Anal. Chem.* **1977**, *284*, 1–12.
(22) Montgomery, J. R.; Peterson, G. N. *Anal. Chim. Acta* **1980**, *117*, 397–401.
(23) Julshamn, K. *At. Abs. Newsl.* **1977**, *16*, 149–151.
(24) Buzzelli, G.; Mosen, A. W. *Talanta* **1977**, *24*, 383–385.
(25) Norval, E.; Human, H. G. C.; Butler, L. R. P. *Anal. Chem.* **1979**, *51*, 2045–2048.
(26) Eames, J. C.; Matousek, J. P. *Anal. Chem.* **1980**, *52*, 1248–1251.
(27) Carrondo, M. J. T.; Lester, J. N.; Perry, R. *Anal. Chim. Acta* **1979**, *111*, 291–295.
(28) Manual for the Graphite Furnace, Analytical Procedures, 1979, Bodenseewerk, Perkin-Elmer.
(29) Zatka, V. J. *Anal. Chem.* **1978**, *50*, 538–541.
(30) Matsusaki, K.; Yoshimo, T.; Yamamoto, Y. *Talanta* **1979**, *26*, 377–380.
(31) Garmestami, K.; Blotcky, A. J.; Rack, E. P. *Anal. Chem.* **1978**, *50*, 144–147.
(32) Clyburn, S. A.; Bartschmidt, B. R.; Veillon, C. *Anal. Chem.* **1974**, *46*, 2201–2204.

RECEIVED for review February 2, 1981. Accepted May 20, 1981.

Determination of Insulin in Serum by Enzyme Immunoassay with Fluorimetric Detection

William D. Hinsberg, III, Kristin H. Milby, and Richard N. Zare*

Department of Chemistry, Stanford University, Stanford, California 94305

An enzyme immunoassay for the determination of insulin in human serum is described, based on fluorimetric detection of enzyme activity. A sandwich method using horseradish peroxidase as the label is employed; the enzyme activity is measured by fluorescence using the substrate, *p*-hydroxyphenylacetic acid. The fluorescence measurements can be carried out by liquid chromatography–laser fluorimetry or by conventional cuvette spectrofluorimetry. The procedure allows the rapid and sensitive quantitation of insulin with a 45-min total incubation period and a 7.9 pM or 46 pg/mL detection limit. There is high correlation between the insulin concentrations in serum samples determined by this method and by radioimmunoassay (correlation coefficient $r = 0.97$).

The technique of enzyme immunoassay (EIA) has undergone rapid development during the past decade (*1*). EIA has attracted considerable attention as a replacement for radioimmunoassay (RIA), since it avoids difficulties associated with the radioisotopic method (such as special handling and disposal procedures, and limited reagent shelf life). Moreover, EIA is potentially the more sensitive approach because the enzyme label may in principle be detected at the single-molecule level (*2*).

The aim of the present study is to assess the extent to which an EIA method may be improved through utilization of a more sensitive system for the measurement of enzyme activity. Earlier work in our laboratory has shown that application of laser fluorimetric techniques to the quantitation of enzyme reaction products allows more sensitive determinations than are possible with conventional methods (*3*, *4*). Recently we demonstrated that laser fluorimetry may be successfully incorporated into a practical EIA procedure (*5*) for the analysis of insulin in human serum. In this paper, we describe an extension and improvement of this approach. We also discuss the potential advantages of EIA-laser fluorimetry and its limitations in the current state of development.

Figure 1 shows a schematic representation of the "sandwich" EIA method used in this work. During step 1, the antigen, Ag (insulin), is allowed to complex with solid-phase-bound antibody (Ab). In step 2, Ab labeled with the enzyme horseradish peroxidase (HRP) is added to the sample. The labeled antibody complexes the Ag that is bound to the solid phase. The amount of solid-phase-bound enzyme is thus directly related to the amount of insulin in the original sample. After a washing step, bound enzyme activity is measured. In our case this is accomplished by using the nonfluorescent substrate, *p*-hydroxyphenylacetic acid (HPA), which is converted upon enzyme catalysis to a fluorescent product identified (*6*, *7*) as 6,6′-dihydroxy-[1,1′-biphenyl]-3,3′-diacetic acid (DBDA), as shown in Figure 2. For quantitation of DBDA, a high-pressure liquid chromatography (HPLC)–laser fluorimeter is employed (*5*).

EXPERIMENTAL SECTION

Apparatus. The equipment and chromatography conditions used in this work have been described in detail (*5*) and included a reversed-phase high-pressure liquid chromatography system. For enhancement of DBDA fluorescence, the effluent pH was adjusted by postcolumn mixing with 0.075 M NaOH in 70% CH_3OH/30% H_2O (v/v). Both this NaOH solution and the column eluent were pumped at flow rates of 1.3 mL/min. The 325-nm line of a helium–cadmium ion laser (Liconix Model 4050 UV) was focused into a 4-μL volume of the effluent in a flowing droplet detector. The fluorescence signal was isolated by liquid filters (*3*) and focused onto a photomultiplier tube. Its signal was displayed directly on a strip-chart recorder.

In those analyses carried out with conventional spectrofluorimetry, a Perkin-Elmer MPF-2A instrument was employed, using

0003-2700/81/0353-1509$01.25/0 © 1981 American Chemical Society

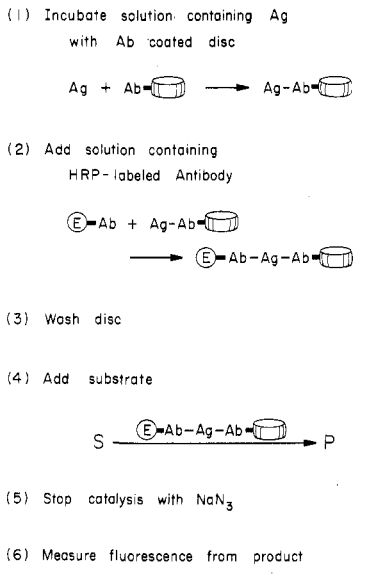

Figure 1. Experimental protocol: Ag = antigen; Ab = antibody; E = enzyme; S = substrate; P = fluorescent product.

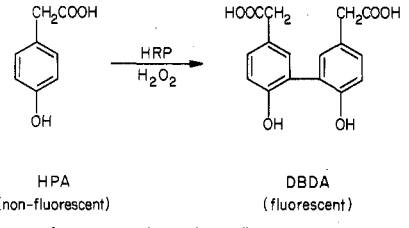

Figure 2. Enzymatic conversion of nonfluorescent substrate (HPA) to fluorescent product (DBDA).

standard 10 mm pathlength cuvettes. The excitation and emission wavelengths were 317 and 414 nm, respectively.

Reagents. The immunoassay reagents were purchased as the "Insolutec Kit" from Mochida Pharmaceutical (Tokyo) and have been described in detail elsewhere (*8*). These included plastic disks (1 cm diameter, 0.5 cm thick) coated with antiinsulin antibody, assay buffers (pH 7.4, containing animal protein), Tween 20 surfactant, 2% (w/v) sodium azide, and standard solutions of insulin. HPA was purchased from Aldrich and purified by sublimation and liquid chromatography (*5*). Human control sera were purchased as lyophilized samples from Nuclear Medical Systems (Newport Beach, CA; Lot no. R06L, R07M, R08H) and used to establish precision.

Procedures. Fresh human serum was prepared from blood samples obtained from the Stanford University Medical Center. After aliquots were taken for analysis by this method, the serum samples were frozen until RIA analysis was carried out using a modification of the method of Desbuquois and Aurbach (*9*).

In order to minimize nonspecific binding, we pretreated the antibody-coated disks by soaking at 4 °C for 24–72 h in 0.01 M phosphate buffer, pH 7.0, containing 0.01 M NaCl and bovine serum albumin (1 mg/mL). Just prior to use, the disks were rinsed with albumin-free buffer.

All incubations were carried out at room temperature (21 °C). Incubation times were controlled to within 3 s. To initiate the first reaction, disks were added to test tubes containing known (0.4 mL standards) or unknown (0.1 mL serum plus 0.3 mL assay buffer) amounts of insulin. After 15 min, 0.1 mL of HRP-labeled antibody solution was added to each tube. After an additional 15 min this second reaction was halted by adding 5 mL of a Tween 20 solution in saline. Fluid was withdrawn by aspiration. After this wash procedure was repeated three more times, the disks were transferred to clean test tubes. For the third reaction, 0.2 mL each of 0.53 mM H_2O_2 and 12 mM HPA in 0.01 M phosphate buffer, pH 7, were added to each tube. After a third 15-min interval, this reaction was halted by adding 20 μL of a 2% (w/v) sodium azide solution. A 0.2-mL aliquot of each sample was mixed with 0.2 mL of distilled methanol. Ten microliters of each resulting solution was injected onto the HPLC column. Peak heights

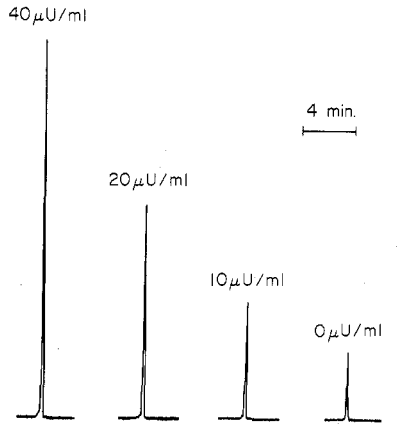

Figure 3. Chromatograms showing the DBDA peak as a function of initial insulin concentration (1 μU/mL = 42 pg/mL).

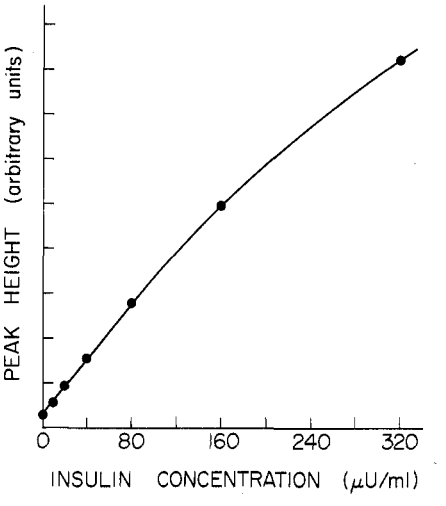

Figure 4. Standard curve showing DBDA peak height vs. insulin concentration. The solid circles are the averages of duplicate measurements.

were used to construct a standard curve and to determine insulin concentrations in the serum samples. Serum samples were analyzed in duplicate and the determinations averaged.

In some cases, 50 μL was taken from each tube after the final reaction was halted and mixed with 1.0 mL 0.075 M NaOH. The fluorescence of these samples was analyzed by using cuvettes and a conventional spectrofluorimeter.

RESULTS

Figure 3 shows typical chromatograms from four insulin standards analyzed by the above procedure. A single peak is observed to elute 2.5 min after injection. It corresponds to the fluorescent enzyme reaction product DBDA. The amount of DBDA formed is proportional to the amount of insulin in the original sample, consistent with the scheme outlined in Figure 1. It is traditional to express the concentration of insulin by its biological activity, where 1 μU/mL = 42 pg/mL = 7.2 pM. The peak for the sample containing no insulin (0 μU/mL) represents enzyme activity from nonspecifically bound HRP-labeled antibody.

Analysis of insulin standards during each run allows construction of standard curves. A typical curve is shown in Figure 4. The detection limit in this case is 1.1 μU/mL insulin (7.9 pM). This value fulfills the condition $h - h_b = 2\sigma_b$ where h and h_b are the mean peak heights for multiple samples of the lowest detectable level and the blank, respectively, and σ_b is the standard deviation in peak height measurements for multiple samples of the blank.

The precision of the present method was examined (see Table I). The intraassay (within-run) precision was estimated

Table I. Precision of the Fluorescence-EIA Method

	no. of samples	insulin concn, μU/mL	std dev	coeff of variation, %
within-run	7	17.9	1.3	7
between-run	6	13.5	2.8	21
	6	44.3	5.5	12

Table II. Comparison of Control Serum Insulin Levels[a]

	insulin concn in control serum[b]		
immunoassay method	low	medium	high
Becton-Dickinson RIA	8.7 ± 2	27 ± 5	42 ± 9
Biomed. Ref. Lab RIA	18.8 ± 4	21 ± 5	
Corning RIA	16.3 ± 4	34 ± 8	47 ± 9
Eiken RIA	6.7 ± 2	15 ± 5	33 ± 9
Immunonuclear RIA	7.0 ± 2	16 ± 4	39 ± 8
Pharmacia RIA	12.6 ± 2	24 ± 5	46 ± 9
Serono RIA	9.0 ± 2	20 ± 5	30 ± 10
present work[c]	13.5 ± 4	18 ± 2	44 ± 8

[a] RIA values from Nuclear Medical Systems RIA Control Sera Data Sheet. [b] Mean ± range in μU/mL. [c] Low and high values determined between-run; medium value determined within-run (see text).

from results of seven parallel analyses of a sample of control serum. Interassay (between-run) precisions were estimated by comparing insulin levels measured on six different days in aliquots of two control sera.

The use of control sera in this study allows a comparison of the accuracy of the present method with that of a number of RIA analyses for insulin. In Table II are listed the insulin concentrations obtained when a number of commercial RIA methods were used to analyze the control sera employed in this work. Also listed are the insulin levels obtained with the present method, which compare favorably with those from the RIA procedures.

To demonstrate the clinical applicability of our method, we made a double blind comparison to RIA using 43 samples of human serum. Figure 5 shows that our values correlate highly with RIA. The least-squares line is $y = 1.07x + 8.43$ with a correlation coefficient of 0.97.

To investigate the relationship of extended analysis time to lowered detection limit, we analyzed standard insulin samples by the above method except that the first reaction was increased from 15 to 60 min. In this case the detection limit, as defined above, was 0.5 μU/mL (3.6 pM). To assess the importance of temperature control, we carried out a series of EIA analyses, employing a water bath at 25.0 ± 0.3 °C to maintain the assay tubes at a constant temperature during incubation. No improvement in sensitivity or precision was observed.

Because our chromatographic analysis gave no indication of interfering components (only one peak was observed), we tested the possibility of using conventional cuvette spectrofluorimetry with this EIA system. Comparing peak heights from the laser fluorimetric method with the fluorescence signal using a standard instrument gave excellent correlations for both standards and serum samples (correlation coefficient $r > 0.997$). Thus at the concentrations studied, there is no need to employ the more powerful technique of HPLC laser fluorimetry.

DISCUSSION

This work demonstrates that incorporation of a sufficiently sensitive means of measuring enzyme activity into an EIA procedure can make possible substantial gains in both rapidity

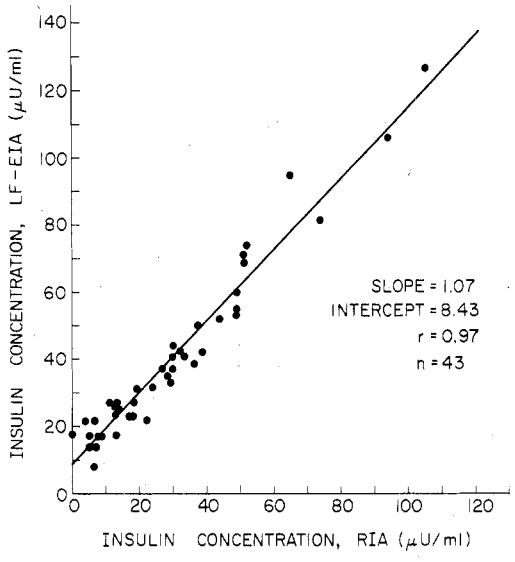

Figure 5. Correlation between insulin concentrations in serum determined by laser fluorescence enzyme immunoassay (this method) and radioimmunoassay (1 μU/mL = 42 pg/mL).

of analysis and assay sensitivity. Moreover, in the present method, measurement of enzyme activity is not a limiting factor, suggesting that further improvements are feasible, if desired.

Fluorimetric methods have been applied in both competitive binding EIA (*10*) and sandwich EIA (*11*) measurements of insulin levels in human serum. In the former, an overnight incubation was specified, and 2.5 μU/mL (18 pM) of insulin was detectable (*10*). In the latter, a total incubation period of over 5 h was called for, and the detection limit for insulin was ~2 μU/mL (14 pM) (*11*). EIA procedures for insulin in serum incorporating colorimetric enzyme determinations have been described which are similar in sensitivity and comparable or superior in rapidity to these fluorimetric EIA methods (*8, 12, 13*).

In the present work, we employ a modification of the most rapid of these colorimetric EIA methods (*5, 8, 13*). It permits application of laser fluorimetric procedures in the determination of enzyme activity. In the colorimetric EIA the reported limit of detection was 5 μU/mL (36 pM) of insulin and the total incubation period was, at a minimum, 3 h (*8, 13*). In our method, the limit of detection is almost 5 times lower, and the total incubation period is shorter by a factor of 4 than the colorimetric EIA procedure. Our detection limit may be improved an additional factor of 2 by extending the length of the first incubation.

The precision reported here is somewhat less than observed in the related colorimetric EIA (*13*), where much longer incubation periods were used. However the precision of the method is comparable to that reported for another EIA for insulin in serum (*12*) and is sufficient to allow its practical application as evidenced by the results of our double blind study (Figure 5).

Two factors prevent further optimization of the assay at this time. Improvements in sensitivity are restricted by the degree of nonspecific binding and our inability to distinguish antigen-bound label (signal) from that bound nonspecifically (blank). Further reductions in incubation period are constrained by our ability to manually process a reasonable number of samples in a rapid manner.

Detection of the fluorescent DBDA product is not a limiting element. Consequently a conventional fluorimeter can be utilized by suitable modification of the measurement protocol. Therefore, the advantages of speed and sensitivity demonstrated for this method can be realized by detecting DBDA

fluorescence in cuvettes, in flow injection analyzers, or following liquid chromatography.

The present work does indicate the potential of laser fluorescence enzyme immunoassay methods. If conventional fluorimetry is used, any further improvement in this assay will be limited by the ability to detect DBDA. However, the laser fluorimeter employed here is roughly 30 times more sensitive for detection of DBDA than is a standard spectrofluorimeter under the conditions used in this work. As a result, the following refinements of the assay should prove possible without requiring further improvement in DBDA detection: (1) the original sample size and the volumes used in the immunoassay steps may be scaled down, with an accompanying improvement in absolute sensitivity for insulin; (2) automation of the assay may allow further gains in rapidity; and (3) the limit of detection of insulin concentration in serum may be further improved by reduction of nonspecific binding.

Nevertheless, the present study based on fluorescence EIA demonstrates the most rapid and sensitive detection of insulin in human serum; moreover it is not dependent on the use of radioisotopes and it can be carried out by using conventional fluorescence equipment.

ACKNOWLEDGMENT

We thank Steven Lidofsky for assistance during the initial stages of this work and Jo Moore of the Palo Alto Veterans Administration Hospital for performing RIA analyses.

LITERATURE CITED

(1) Wisdom, G. B. *Clin. Chem (Winston-Salem, N.C.)* **1976**, *22*, 1243-1255.
(2) Rotman, B. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1981-1991.
(3) Imasaka, T.; Zare, R. N. *Anal. Chem.* **1979**, *51*, 2082-2085.
(4) Lidofsky, S. D., Dissertation, Columbia University, New York, 1980.
(5) Lidofsky, S. D.; Hinsberg, W. D., III; Zare, R. N. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1901-1905.
(6) Guilbault, G. G.; Brignac, P. J.; Juneau, M. *Anal. Chem.* **1968**, *40*, 1256-1263.
(7) Guilbault, G. G.; Sadar, M. H.; Peres, K. *Anal. Biochem.* **1969**, *31*, 91-101.
(8) Shinkai, H.; Sohma, M.; Takahashi, Y.; Kojima, R.; Hashimoto, M.; Ogawa, N. *Mol. Immunol.* **1980**, *17*, 377-381.
(9) Desbuquois, B.; Aurbach, G. *J. Clin. Endocrinol.* **1971**, *33*, 732-738.
(10) Matsuoka, K.; Maea, M.; Tsuji, A. *Chem. Pharm. Bull.* **1979**, *27*, 2345-2350.
(11) Kato, K.; Umeda, Y.; Suzuki, F.; Hayashi, D.; Kosaka, K. *Clin. Chem. (Winston-Salem, N.C.)* **1979**, *25*, 1306-1308.
(12) Albert, W. H. W.; Kleinhammer, G.; Linke, R.; Staehler, F. "Enzyme Labelled Immunoassay of Hormones and Drugs;" Pal, S. B., Ed.; de Gruyter: New York, 1978; pp 327-339.
(13) Yoshioka, M.; Tanaguchi, H.; Kawaguchi, A.; Kobayashi, T.; Murakami, K.; Seki, M.; Tsutou, A.; Tamagawa, M.; Minoda, H.; Baba, S. *Clin Chem. (Winston-Salem, N.C.)* **1979**, *25*, 35-38.

RECEIVED for review March 27, 1981. Accepted May 26, 1981. This work was supported by the National Cancer Institute of the National Institutes of Health under Grant NIH 5 R01 CA23156-04.

Inductively Coupled Plasma–BASIC Programmable Computer-Controlled Double Monochromator for Sequential Multielement Analysis

James P. McCarthy, Michael E. Jackson, Thomas H. Ridgway, and Joseph A. Caruso*

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221

An instrument is presented for the sequential determination of trace element constituents. An inductively coupled plasma source (ICP) is interfaced to a computer-controlled monochromator and is directly programmable in BASIC. The interface is simple and inexpensive to construct and utilizes a stepper motor controller chip (slave microprocessor). The reproducibility and linearity of response of the system are characterized. Accuracy and overall system performance are demonstrated for the analysis of EPA water quality control references and drinking water samples for the determination of eight elements.

The concurrent determination of both major and trace constituents in numerous applications creates the need for an analytical system capable of determinations over extended concentration ranges. The inductively coupled plasma has become increasingly popular in many diverse applications (*1–5, 7–9*). Major constituent analysis is necessary with soils (*1*) and alloys (*2*), while trace analysis is needed with food (*3*), environmental (*4*), and biological samples (*5*), as the toxicity or nutritional value of many elements is at the part-per-billion level (*6*).

Multielement detection instruments are widely utilized in ICP analysis. While the ICP polychromator system is typically capable of the analysis of 20–30 elements in 2–3 min, it is expensive and wavelength selection is inflexible, as the analytical wavelengths are fixed upon initial system alignment and are not conveniently modified by the user. Several commercially available polychromator systems employ auxiliary monochromators to add versatility in wavelength selection of at least one channel. The major advantage of these polychromator systems is that they provide simultaneous analysis and high sample throughput.

Recently, sequential slew scanning monochromator systems that provide rapid wavelength change between analytical lines and acquire data only at preselected wavelengths have become available with computer controlled/stepper motor operated gratings. Several commercial ICP sequential slew scanning spectrometer systems (ICP-SSS) are available with programmable monochromators (*11–16*). Floyd et al. (*11*) discuss ICP-SSS and suggest development criteria for a sequential system. Many of these design features, including a double monochromator for high stray light rejection, have been incorporated in the system described here.

Drawbacks of many of the sequential systems (*11–16*) and indeed of any computer-controlled hardware, include the

0003-2700/81/0353-1512$01.25/0 © 1981 American Chemical Society