square noise in the average wave form from four distilled-water blanks. The detection limits for the magnetron arc–graphite furnace system are very good when compared to most reported dc arc values. Note that the Fe, the detection limits for the ion line in Ar are more than a factor of 2 lower than for the neutral-atom line. With He as the cooling gas, the Fe ion line detection limit is more than an order of magnitude lower than the value for the neutral-atom line with the Ar cooling gas.

This preliminary investigation suggests that the combination of the magnetron rotating arc and an electrothermal atomizer may be very attractive for the determination of some trace metallic elements in microsolution samples. The significant improvements in the powers of detection and precision observed with the graphite tube furnace relative to previous studies with a glass-frit nebulizer (*11*) suggest that the magnetron arc may be an excellent atomization and excitation source, but it may be less useful as a means of vaporizing the sample particles produced by nebulization techniques. This is probably the result of the relatively small vertical dimensions of the plasma and the correspondingly short sample residence times.

The magnetron rotating arc is very simple and easy to construct and operate. The gas flow requirements are relatively modest. A single anode and cathode typically can be used for about 100 shots before erosion by the plasma requires electrode replacement. A single, compromise set of operating conditions can be used for several elements with detection limits in the 0.005–0.5 μg/mL range and with relative standard deviations typically in the 1–3% range.

Since the heart of the atomizer and the heart of the rotating arc are both graphite cylinders of comparable dimensions and properties, it should be possible to combine the two functions in a single device. A prototype system is under construction. This should nearly eliminate any sample loss during transport. In addition, narrower, taller peaks should be produced. This may reduce the detection limits. In addition, the elimination of the transport tube may allow the use of pure He as the transport and cooling gas. This may result in improved detection limits for some elements.

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Direct Monitoring of Enzyme-Catalyzed Reactions via Laser-Based Polarimetry

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A new method is described in which a laser-based polarimetric detection system is used to monitor enzyme-catalyzed reactions. This method provides direct detection of the reaction progress based on the inherent chirality of either the substrate or product. The technique is tested on the glucose oxidase catalyzed conversion of β-D-glucose to D-gluconic acid. The ability of this system to negate the large background signals produced by optically active substrates is examined, and a mass limit-of-detection for glucose oxidase of 34 fmol is demonstrated. Results obtained with the polarimetric technique are compared to those from an accepted spectrophotometric method, and the technique is shown to be as accurate with an LOD 15 times lower. The theoretical relationship between the polarimetric response and various experimental parameters will be developed and verified.

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INTRODUCTION

The use of kinetic methods of analysis has expanded greatly in the past quarter century, drawing upon recent advances in computer technology for data acquisition and manipulation. Techniques such as continuous-flow monitoring (*1*), electrode kinetics (*2*), and elution chromatography (*3*) are a few of the many analytical areas where kinetic methods play a major role.

One important application of kinetic methods involves the monitoring of enzyme-catalyzed reactions. The use of enzyme-based determinations is widespread, particularly in medical applications, and because of the routine use of automated clinical techniques, kinetic methods of analysis now outnumber both equilibrium-based and direct instrumental measurements (*4*).

The inherent selectivity of enzyme-catalyzed reactions makes these techniques an important area of study in the field of chemical analysis. However, in many enzymatic reactions the properties of the substrate or product are such that de-

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tection of these species is difficult or impossible. Thus the sensitivity of the technique can be severely degraded. One approach to circumvent this limitation is to couple one or more additional chemical reactions to the primary enzymatic reaction such that a final product is produced that can be monitored by using UV–vis spectroscopy (*5*) or some other detection method such as fluorescence (*6*). The concentration determined for the monitored species is then used to determine the rate or concentration of interest. A key assumption in this approach is that the ancillary reactions proceed at a rate far in excess of the enzymatic reaction. To ensure that this assumption is valid, the reaction conditions will frequently have to be adjusted. This change in conditions may prevent the enzymatic reaction from occurring at its optimum rate.

Another method of monitoring enzyme-catalyzed reactions entails the manufacture of an enzyme-bonded electrode that can be used in combination with potentiometric (*7*) or amperometric detection (*8*). A drawback to this method is the lack of a universal response in that a separate electrode will be required for each reaction of interest. For reactions where the exchange of a proton with a background electrolyte is involved, direct pH sensing is possible without the need for a coupled reaction. However, the large changes in pH that often occur in these types of systems can inhibit the activity of the enzyme (*9*).

There are many important problems that could benefit by the development of more sensitive techniques for monitoring enzyme levels. For example, in prematurely born infants, blood levels of specific enzymes are measured to assess the development and performance of a variety of physiological systems (*10*). Obviously, the quantity of sample available for testing is severely limited in such situations, and the levels of the enzyme of interest are exceptionally low.

Most enzyme-catalyzed reactions involve a compound with a chiral center in either the substrate, the product, or both. This suggests that optical activity detection would be a good possibility for monitoring the progress of these reactions. Further, since the detected property would be due to the substrate or product, the reaction could be followed directly, thereby eliminating the need for coupled reactions. Recent reports have described a laser-based polarimeter with rotational detectabilities at the 5-μdeg level (*11*). The excellent sensitivity of this polarimetric system makes it well suited for monitoring enzyme reactions. The near universality of this approach is a significant advantage compared to the methods described previously. However, because many enzyme-catalyzed reactions involve an optically active substrate, the large background signal generated by these substrates presents a potential problem for the polarimetric system. As an example, for the glucose oxidase catalyzed reaction of β-D-glucose to D-gluconic acid, a 20 mg/mL solution of glucose would possess a rotation of 1°/dm. To maintain the 5-μdeg detectability of the polarimetric system, an instrumental dynamic reserve in excess of $1:10^5$ would be required. As has been shown previously (*11*, *12*), with proper experimental control, polarimetric systems do have the capability to achieve dynamic reserves at the required level. To achieve this, the rotational signal due to an optically active substrate can be nulled out by rotation of the polarizing prism (analyzer) an amount exactly equal to the signal created by the substrate. Thus a new null position would be obtained for the analyzer; however, the amount of the light reaching the photodetector would be unchanged from that observed with a non-optically active substrate. This would ensure that the detectability of the polarimeter would be constant, independent of the optical activity of the substrate.

In this report a new technique will be described for studying enzyme reactions that can provide significant advantages over other approaches. A laser-based polarimeter is used to monitor the glucose oxidase catalyzed conversion of β-D-glucose to D-gluconic acid. This reaction is of medicinal importance as it is used in the diagnosis of diabetes. The relationship between the measured signal and the kinetic parameters will be established, and the influence of several key experimental parameters on the technique will be examined.

EXPERIMENTAL SECTION

Apparatus. The laser-based polarimeter used in this report is based on a 5-mW He–Ne laser and has been described in detail earlier (*13*, *14*). Briefly, the polarizer and analyzer are both select, Glan-Thompson polarizing prisms (Model MGT-E8; Karl Lambrecht, Chicago, IL) chosen by using the same procedure as described earlier (*14*). The detection cell was constructed in-house and had a volume of 238 μL with a path length of 0.46 dm. Modulation of the laser light at 2 kHz was accomplished using an acetonitrile-filled Faraday cell. The light passing through the analyzer was detected with a red-sensitive PMT (Model R928, Hamamatsu, Middlesex, NJ). The photomultiplier tube (PMT) output was demodulated, amplified, and digitized with a Stanford Research Systems (Palo Alto, CA) lock-in amplifier (Model SR510). Data were sampled at a 2-Hz rate and transferred to a personal computer via an IEEE interface (Model PC-2A; National Instruments, Austin, TX). A base-line noise level corresponding to 3×10^{-6}° was demonstrated with this system.

Sample introduction was accomplished using a syringe pump (Model LC-5000; ISCO, Lincoln, NE) coupled to the detection cell with a Model 7010 injector (Rheodyne, Cotati, CA). A 400-μL injection loop was used for the stopped-flow work, while a 2.25-mL injection loop was used for the continuous-flow studies. The 400-μL loop was large enough to ensure that the detection cell would be adequately flushed before filling. The system was not thermostated during this study, although the temperature was recorded for each kinetic run.

Reagents. The glucose and glucose oxidase (Sigma) were used as received. Glucose concentrations used for this study were all in excess of 100 times the enzyme concentration to ensure pseudo-first-order kinetics. The water was deionized and distilled. All solutions were buffered at pH 5.5 with 0.05 M phosphate buffer, which is within the optimum pH range for glucose oxidase (*15*).

Procedures. The detection cell was filled with a solution of β-D-glucose by using the syringe pump, and the resulting rotational signal manually cancelled by rotation of the analyzer. Enzyme solutions were prepared by volumetric dilution of a 0.15 mg/mL solution of glucose oxidase. Aliquots of this solution were mixed with the glucose solution, and the mixture was introduced into the detection cell within 45 s of mixing via the injection valve. For the stopped-flow study, the pump was shut off after a period of time equal to the system dead volume and the cell volume, and data collection initiated at that point. For the continuous-flow study the pump was left on, and the mixture under study was pumped through the cell during the entire time of data collection.

RESULTS AND DISCUSSION

The overall reaction for the glucose oxidase catalyzed conversion of β-D-glucose to D-gluconic acid is

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

The specific rotation $[\alpha]$ of β-D-glucose is 51.8° $(g/mL)^{-1}$ dm^{-1} at the He–Ne laser line (633 mn), while D-gluconic acid possesses a specific rotation of 13.5° $(g/mL)^{-1}$ dm^{-1}. A net change of approximately 38° $(g/mL)^{-1}$ dm^{-1} in $[\alpha]$ is observed for this reaction. Thus, the substrate and product properties are such that this reaction would be advantageously probed by polarimetric means. Further, by keeping the glucose concentration several orders of magnitude greater than the enzyme concentration, this reaction will follow pseudo-first-order kinetics (*16*), thereby simplifying the data analysis. The inherent rotational sensitivity of the laser-based polarimeter detection system and the ability to maintain this detectability

independent of the glucose concentration ensures that this assumption will always be valid.

To detect glucose oxidase via its reaction chemistry, the detection cell is first filled with an appropriate solution of glucose, and the rotational signal observed is minimized by manipulation of the analyzer. The enzyme is mixed with the glucose solution, and the detection cell is filled with this mixture. The monitored signal is then indicative of the rotation due to the β-D-glucose (reactant) and the D-gluconic acid (product). Since these have different specific rotations, the net optical activity in the detection volume will change as the reaction proceeds.

Mathematical Interpretation of Signal. The polarimetric system will register a change in rotation as the reaction proceeds which is given as $d\alpha/dt$. The relationship between this signal and the properties of the enzyme, substrate, and product can be determined by expanding the polarimetric response as follows:

$$\frac{d\alpha}{dt} = \frac{d\alpha}{dC}\frac{dC}{dt} \tag{2}$$

Thus the measured signal will be dependent on both the properties of the products and reactants ($d\alpha/dC$) and the rate that the reaction proceeds (dC/dt). The latter term is a function of the specific enzyme and the reaction conditions, and for pseudo-first-order kinetics at constant substrate concentration, it is given by

$$dC/dt = k[E]^\circ \tag{3}$$

In eq 3, k is the pseudo-first-order rate constant for the enzyme (s^{-1}) and $[E]^\circ$ is the concentration of the enzyme (molarity).

The total rotation observed initially in the detection cell (α_T) will be dependent on the rotational properties of the substrate, product, and enzyme and their respective concentrations:

$$\alpha_T = [\alpha]_S l C_S + [\alpha]_P l C_P + [\alpha]_E l C_E \tag{4}$$

In eq 4 $[\alpha]$ is the specific rotation (deg $(g/mL)^{-1}$ dm^{-1}), l is the interaction path length (dm), and C is the concentration (by convention, g/mL). The subscripts S, P, and E refer to substrate, product, and enzyme, respectively. Because the enzyme concentration will be several orders of magnitude lower than the concentration of either the substrate or product, the enzyme contribution to the overall rotational signal can be ignored.

From eq 1, it is clear that the conversion of β-D-glucose to D-gluconic acid occurs with 1:1 stoichiometry. Thus, the concentration of D-gluconic acid at time t, $C_P(t)$ is given by

$$C_P(t) = C^\circ_S - RC_S(t) \tag{5}$$

where C°_S is the initial concentration of glucose, $C_S(t)$ is the concentration of glucose at time t, and R is the ratio of the formula weights for β-D-glucose and D-gluconic acid.

Substituting eq 5 into eq 4 and taking the derivative with respect to the concentration of glucose yields

$$d\alpha/dC_S = [\alpha]_S - R[\alpha]_P \tag{6}$$

Substitution of eqs 3 and 6 into (2) gives

$$d\alpha/dt = ([\alpha]_S - R[\alpha]_P)k[E]^\circ \tag{7}$$

Equation 7 predicts a linear response between the signal observed with the polarimetric system, $d\alpha/dt$, and the enzyme concentration. Further, for many enzyme-catalyzed reactions the structural differences between the substrate and product are small. For these reactions, the value of R is close to 1 and the magnitude of the measured signal will be directly proportional to the difference between the specific rotations of the substrate and the product.

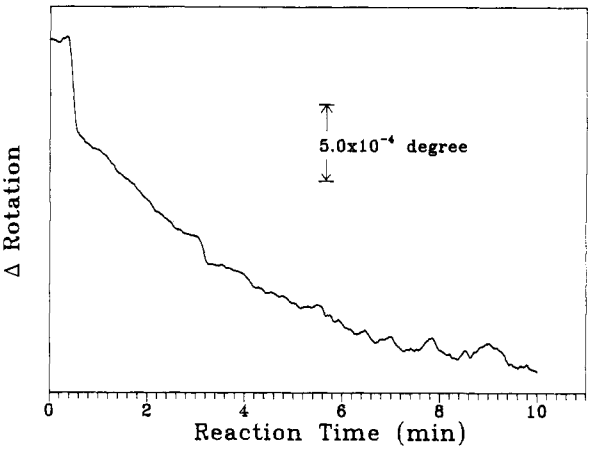

Figure 1. Rotational response versus time for the glucose oxidase catalyzed conversion of β-D-glucose into D-gluconic acid as measured by laser-based polarimetry. The decreased slope of the response curve at times greater than 7–8 min is due to oxygen depletion in the detection cell limiting the reaction rate.

Figure 1 shows a typical response versus time curve obtained with the polarimetric system. The large drop in signal that takes place within the first minute after injection of the enzyme–substrate mixture is due to refractive index effects occurring within the detection cell as the sample solution enters. The increase in noise occurring at times greater than 7 min is due to the decomposition of H_2O_2 (reaction product) in the cell. The specific rotations for β-D-glucose and D-gluconic acid were measured at the He–Ne laser wavelength (633 nm) and have been given earlier. Using these specific rotation values and assuming that the rate constant for glucose oxidase is 3400 s^{-1} (*17*), under these conditions $d\alpha/dt$ is predicted to be 3.3 μdeg/s. Experimentally, with the data from Figure 1, $d\alpha/dt$ is determined to be 3.1 μdeg/s. This underscores the validity of eq 7 within the limits of the assumptions used to derive it.

Limitations Due to Oxygen Content. The large excess of glucose present ensures oxygen will become the limiting reagent for the glucose–glucose oxidase reaction under the conditions used for this study. Henry's law (*18*) can be used to estimate the concentration of oxygen present in water at 20 °C. With this value, it is calculated that it would take approximately 7.2 min before the oxygen concentration would limit the reaction. Adequate data were collected in approximately 3–4 min, so the oxygen content did not inhibit the technique. It is possible to calculate the maximum rotational signal that can be obtained before oxygen depletion occurs, stopping the reaction. This value should be constant and independent of enzyme concentration. On the basis of the amount of β-D-glucose that will be converted into D-gluconic acid for the available oxygen and with eq 7, a maximum rotation of 1.2 mdeg is predicted. Experimentally, an average maximum signal of 1.3 mdeg has been measured for a variety of enzyme concentrations. This value has been determined by using an air-based Faraday cell as a rotational standard corresponding to a rotation of 0.286 mdeg (*19*).

Quantitative Analysis. From the standard error of the slope of a blank, the mass limit of detection (LOD) is determined to be 5.2 ng of glucose oxidase in the detection cell (S/N = 2). With the molecular weight of glucose oxidase (154 kDa), this mass LOD corresponds to 3.4×10^{-14} mol of the protein. The system response was found to be linear with enzyme concentration from effectively the detection limit to 38 μg of enzyme in the cell (r = 0.998). For comparison, a conventional spectrophotometric method (*20*) for glucose oxidase analysis was used under similar conditions. The LOD for this method was larger than that obtained with the po-

Table I. Comparison of the Calculated Enzyme Activities for Two Detection Methods

enzyme concn, μg/mL	temp, °C	method	calcd activity,[c] units/g	corr[d,e] to 25 °C
6.0	22.3	P[a]	1490	1663
40.9	22.1	P	1450	1633
136.4	22.5	P	1530	1693
60	24	S[b]	1660	1727
120	24	S	1570	1633
150	24	S	1690	1727

[a] Polarimetric detection. [b] Spectrophotometric detection. [c] 1 unit = amount of enzyme needed to convert 1 μmol of substrate in 1 min. [d] From ref 21. [e] Manufacturer's assay 2700 units/gm (35 °C), 1658 units/gm (25 °C).

larimetric technique by a factor of 15. Recent work (*12*) has shown that the polarimetric system can be used with a 1.0-μL volume, 1.0-cm path detection cell. Incorporation of this cell would result in a loss of a factor of 5 in the path, but the LOD would benefit by a 240-fold reduction in the detection volume. This would lower the mass LOD for the technique by a factor of approximately 50. Even as configured, the polarimetric system can provide significant improvement in the ability to measure enzyme levels directly.

Equation 7 predicts that the polarimetric response should be independent of the substrate concentration as long as the pseudo-first-order approximation is not invalidated. This assumption was tested for four glucose concentrations spanning the range from 1 to 20 mg/mL. Each glucose solution was tested with four different enzyme concentrations. As predicted, the response was found to be independent of glucose concentration. Further, the system noise, that is, the standard error of the polarimetric response ($d\alpha/dt$), did not increase with increasing substrate concentration. This can be attributed to the ability of the polarimeter to effectively null out the substantial background signal from the glucose substrate. In effect, the number of photons arriving at the photomultiplier tube is the same, independent of the chirality or concentration of the substrate. The linear response coupled with the ability to tolerate a variety of substrate concentrations allows for a broad range of experimental conditions to be utilized with the polarimetric detection system.

The activity of the glucose oxidase sample was determined via the polarimetric technique for several different concentrations of the enzyme. The enzyme activity was also determined by using a conventional method (*20*) in which the H_2O_2 produced in the glucose oxidase catalyzed reaction is coupled to a starch/I_3^- reaction (under Mo(VI) catalysis) to produce an absorbing product. The product of this reaction was measured by using a photodiode array spectrophotometer. The results for the two techniques, as well as the manufacturer's assayed value are listed in Table I. The results are not immediately comparable since the three measurements were done at different temperatures. To facilitate comparison, an empirically determined temperature coefficient for the glucose oxidase system (*21*) was used to correct all values to 25 °C. These corrected values are listed in the last column of Table I.

From the data in Table I it is clear that the polarimetric system provides results that agree well with those available from either the manufacturer or an accepted analytical method. The polarimetric results have a relative standard deviation of only 1.8% in the measured enzyme activity over a 20-fold variation in enzyme concentration. Clearly, polarimetric measurement of enzyme activity demonstrates both reasonable accuracy and precision when compared to other techniques. Further improvements could be anticipated if more precise control of temperature were available. Alternately, recent work has shown the potential for temperature compensation in kinetic methods using an extended Kalman filter (*22*, *23*). Application of techniques such as this in combination with point-by-point temperature monitoring could produce effective buffering from the effect of temperature variations on the substrate signal. Since the polarimetric technique is limited by the temperature drift induced by the substrate signal, this approach warrants further investigation.

Finally, since many biomedically important enzymes act to either alter or produce chirality, polarimetric measurement of enzyme activity should have wide application. Table II lists several biomedically and industrially important enzymes as well as the reactant(s) and product(s) whose conversion they catalyze. Excellent limits of detection would be expected for most of the listed enzymes. In addition, the polarimetric technique eliminates the need to consider extraneous reactions to facilitate detection.

CONCLUSIONS

A new technique has been demonstrated for the kinetic measurement of enzyme activity. The technique is fast, easy to implement, and does not require the use of coupled reactions to follow the reaction progress. The presence of optical activity in either the reactant or the product is the only re-

Table II. Partial Listing of Medicinal and Industrial Enzyme Systems Suitable for Polarimetric Detection

enzyme	substrate(s)	$[\alpha]_D^{25}$, deg	product(s)	$[\alpha]_D^{25}$, deg
serum glutamate oxaloacetate transmutase	L-aspartate	−25.5	L-glutamate	+32.5
	α-ketoglutarate	0	oxaloacetate	0
serum glutamate pyruvate transmutase	L-alanine	−13.6	L-glutamate	+32.5
	α-ketoglutarate	0	pyruvate	0
lactate dehydrogenase	pyruvate	0	L-lactate	−2.3
α-hydroxybutyrate dehydrogenase	α-ketobutyrate	0	α-hydroxybutyrate	−24.5
aspartase	fumerate	0	L-aspartate	−25.5
fructose diphosphate aldolase	D-fructose 1,6-diphosphate	+1.2	D-glyceraldehyde 3-phosphate	+14.5
			dihydroxyacetone phosphate	0
ornithine carbamyltransferase	carbamyl phosphate	0	citrolline	+3.7
	ornithine	+11.5	phosphate ion	0
β-fructofuranosidase	β-sucrose	+66.4	D-fructose	−13.3
			D-glucose	+57.2
α-glucosidase	α-maltose	+140.7	D-glucose	+57.2
β-galactosidase	β-lactose	+34.2	D-glucose	+57.2
			D-galactose	+83.3

quirement for application of the technique. However, considering the biological function of enzymes this requirement is not limiting, and the technique should be applicable to a wide range of enzyme systems. Theoretical analysis of the relationship between the measured signal and the kinetic parameters has shown that the primary experimental parameter to consider is the net difference in specific rotation for the reactant(s) and product(s). In support of this, it was demonstrated that the glucose concentration did not affect either the magnitude of the signal or the noise. Polarimetric measurement of glucose oxidase activity provided values that were in good agreement with those obtained with accepted analytical procedures. A mass limit of detection of 34 fmol of glucose oxidase was demonstrated by using the polarimetric system, and the system response has been shown to be linear with enzyme concentration over 4 orders of magnitude. Although this LOD is impressive, significant improvements can be expected when procedures are implemented to buffer the system from the effect of temperature variations.

Registry No. Glucose oxidase, 9001-37-0.

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Bulbed Capillary External Referencing Method for Proton Nuclear Magnetic Resonance Spectroscopy

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Through fundamental reanalyses of the external referencing system and the chemical shift equation, an entirely new referencing method using a new bulbed capillary reference tube device is derived. The device was initially for a direct diamagnetic correction needing no susceptibility and geometry factor values to be theoretically performed on the spectrum. Then, the same device method was straightforwardly developed to eventually give the first bias-free or true chemical shifts δ°_{cor} unequivocal to samples in NMR with accuracies estimated as high as ±0.004-0.009 ppm in addition to high precisions obtained as 0.004-0.009 ppm. Thus, we could reasonably introduce "accuracy", by analogy to instrumental analysis cases, for the first time into NMR that has been governed only by experimental precisions. Therefore, by comparison, the usual external referencing shifts relative to the TMS cylinder reference are reasonably found as showing precisions as high as 0.004-0.005 ppm but accuracies as poor as a mere -0.2-+0.8 ppm. Similarly, the usual internal referencing shifts are suggested to have as unexpectedly poor accuracies as -0.3-+0.5 ppm by solute-solvent effects, reasonable for the sample solutions. The new method can be carried out with simple procedures under practically moderate experimental conditions. Unique theoretical and technical features of the method are discussed in detail.

Several years ago when we were restudying the characteristic ^{1}H NMR spectra of ion-exchange resins immersed in water (*1-4*), a question was raised concerning the diamagnetic correction (DC) for external referencing (ER). Although DC had been theoretically required as important (*5, 6*), only one of nearly 30 papers (*3*) studying ion-exchange resin spectra with ER did not perform DC. Even that one (*3*) in a brief note could not show the practicality or effectiveness of DC in their ER measurements.

A literature study of this point also revealed that no practical DC technique had yet been established for any other sample systems probably because of technical difficulties against its theoretical importance. Instead, most ^{1}H chemical shifts had been measured with internal referencing (*7*), which needed no DC (*6*) to be used more easily. Thus, the DC problem in ER seemed to have been neglected and left practically unsolved since the advent of NMR.

However, we had actual ion-exchange resin samples that could not be measured without ER, where a practical DC technique was urgently needed. Therefore, to search for an usable method, we began to restudy the DC problem from theoretically considering why observed ER shifts had to be actually corrected. In this course, we noticed that DC had usually been called a "susceptibility correction (SC)" (*6, 8*), meaning that the correction was to be done only by evaluating

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