(18) Burdo, T. G.; Seitz, W. R. *Anal. Chem.* **1975**, *47*, 1639.
(19) Meites, L.; Zuman, P. "Electrochemical Data", Part I, Vol. A.; John Wiley: New York, 1974; p 454.
(20) Higuchi, T. *U.S. Dep. Commer., Off. Tech. Serv.*, 1962, AD 412,343; *Chem. Abstr.* **1964**, *60*, 9115g.
(21) Edsall, J. T.; Felsenfeld, G.; Goodman, D. S.; Gurd, F. R. N. *J. Am. Chem. Soc.* **1954**, *76*, 3054.
(22) Martin, R. B.; Edsall, J. T. *J. Am. Chem. Soc.* **1958**, *80*, 5033.
(23) Delumyea, R. Ph.D. Thesis, Wayne State University, Detroit, Mich., 1974.
(24) Nau, V.; Nieman, T. A. *Anal. Chem.* **1979**, *51*, 424.
(25) Stieg, S.; Nieman, T. A. *Anal. Chem.* submitted for publication.
(26) Veazey, R.; Nieman, T. A. *Anal. Chem.* **1979**, *51*, in press.

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Enzyme Amplification Laser Fluorimetry

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Laser fluorimetry has been applied to the detection of enzyme reaction products at ultra-low concentrations using the 325-nm line of a He–Cd laser as an excitation source and liquid filters to isolate the fluorescence. In one direct enzyme reaction, glucose-6-phosphate is converted to 6-phosphogluconolactone as NADP is reduced to NADPH. Measurement of the fluorescence from NADPH permits quantitation of glucose-6-phosphate with a detection limit of 2 nM. In another direct enzyme reaction, α-ketoglutaric acid is converted to L-glutamic acid as NADPH is oxidized to NADP. Fluorescence from the alkaline-treated NADP is used to quantitate the α-ketoglutaric acid with a detection limit of 4 × 10^{-12} mol. By combining these two enzyme reactions, an enzyme cycle results in which both enzyme reaction products increase in concentration. After a fixed period of time, the enzyme cycle is stopped and the initial concentration of NADP is determined by measuring the final concentration of 6-phosphogluconolactone, using yet another direct enzyme reaction. This enzyme amplification method allows determination of 1 × 10^{-14} mol of NADP, which is about 30 times more sensitive than previously reported results.

Most enzyme reactions are monitored by measuring the coenzyme concentration of reduced nicotinamide adenine dinucleotide, NADH, or reduced nicotinamide adenine dinucleotide phosphate, NADPH (*1–3*). The low oxidation–reduction potential of these compounds (0.32 V) allows the enzyme reaction to proceed under moderate conditions. The most interesting property of NADPH or NADH might be its amplification capacity through enzyme cycles, as shown in Figure 1. In the presence of enzymes 1 and 2, NADP is reduced to NADPH and then oxidized to NADP repeatedly (cycle) as substrates 1 and 2 are transformed to products 1 and 2 (Figure 1a). After some fixed period of time, the enzyme cycle is stopped by destroying the enzymes. Then the concentration of product 1 or product 2 is measured by another enzyme reaction (Figure 1b) which converts NADP to NADPH. In our experiment, product 1 or 2 is quantitated by measuring the fluorescence from NADPH. Each enzyme cycle transforms one substrate molecule into a product molecule. By letting many cycles occur, the original NADP concentration is amplified.

Absorption measurements at 340 nm (ϵ = 6270) provide the most common means for determining the concentration of NADPH or NADH (*2, 3*). However, fluorescence measurements may be used to advantage especially for low concentration samples where more than a hundred-fold improvement in detection sensitivity is realized (*4, 5*). Since NADPH or NADH does not fluoresce strongly, use of a conventional fluorescence spectrophotometer suffers from low sensitivity at reasonable spectral resolution. At the lowest concentrations it is recommended that the fluorescence spectrophotometer should be replaced by a tungsten lamp and filters. In this case, the detection limit is determined by the background signal, which is composed of the scattered light from the excitation source, slight fluorescence from the filters and impurities in the sample, and the Raman signal from water (*2, 3*).

The technique of laser fluorimetry can provide high sensitivity in the analysis of trace species (*6–9*). The detection of 0.02 parts-per-trillion of fluorescein dye is readily demonstrated using dye laser excitation and pulse-gated photon counting (*10*). Laser fluorimetry is so sensitive that the detectable concentration is limited by the background signals from the Raman spectrum of water and contaminant fluorescence in the solvent, even under good spectral resolution. For the detection of still lower concentrations, some "amplification procedure" becomes attractive, in which the concentration of the trace species is amplified without changing the background level (*11*). An example is the detection of 1 × 10^{-18} mol of ornithine δ-aminotransferase by enzyme amplification (*12*).

Our strategy for the measurement of NADP (NADPH) is the use of a He–Cd laser (325-nm line) to induce fluorescence, nonfluorescent inorganic liquid filters to reduce the background signal, and enzyme amplification to increase the signal intensity from the sample under study. We show here that laser fluorimetry is able to detect NADP (NADPH) at lower concentrations than previously reported, and that this technique may be applied to the sensitive detection of glucose-6-phosphate and α-ketoglutaric acid. Through the use of an enzyme cycle, the concentration of NADP (NADPH) is measured at levels of 1 × 10^{-10} M.

EXPERIMENTAL

Fluorescence Detection System. Figure 2 presents a schematic drawing of the experimental apparatus. The 325-nm output of a helium–cadmium laser (Liconix model 405 UV) passes

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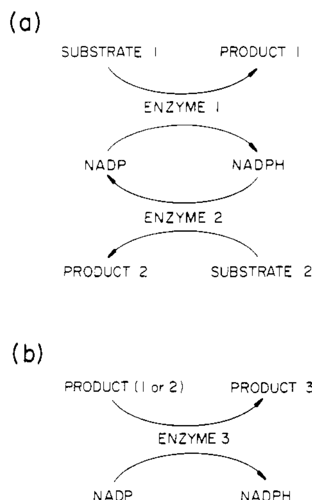


Figure 1. Enzyme amplification. In (a) enzymes 1 and 2 convert substrates 1 and 2 into products 1 and 2 while the coenzyme NADP is repeatedly reduced and reoxidized. After a set incubation time, enzymes 1 and 2 are destroyed stopping the reaction cycle. Then enzyme 3 is added which converts product 1 or 2 into product 3 while NADP is reduced to NADPH. After completion of reaction (b) the fluorescence from NADPH is measured

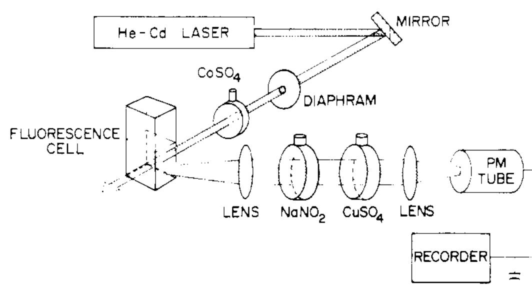


Figure 2. Laser fluorimeter

through a diaphragm and a $CoSO_4$ (300 g/L, 1.0-cm path length) liquid filter before entering a quartz cell (1 cm^2 in cross section) that contains the sample under study. The diaphragm and liquid filter combination reduces to a negligible level the visible background light from the laser discharge tube, particularly the 442-nm Cd emission line. Fluorescence from the sample is collected by an optical system that focuses an image of the fluorescent line onto the face of a photomuliplier (Centronic model Q4249 BA). The optical train consists of an $f/1$ lens, a $NaNO_2$ filter (133 g/L, 1.0-cm path length), a $CuSO_4$ filter (saturated solution, 1.8-cm path length), and an $f/3$ lens. The $NaNO_2$ filter removes the scattered light from the He–Cd laser and the Raman bands of water; the $CuSO_4$ filter reduces contaminant fluorescence at wavelengths longer than 530 nm. The filter combination is quite effective in isolating the NADPH fluorescence since the latter has a 7800 cm^{-1} Stokes shift. The output power (4 mW) of the He–Cd laser is well regulated (~0.5% rms noise). The experiment is limited by the background signal from the sample when no NADPH is present. The dark current from the photomultiplier is less than 1% of this signal. The dc output of the photomultiplier is displayed on a stripchart recorder using a 1-s time constant.

Figure 3a illustrates the transmission curves of the excitation filter (Corning 5840) and the fluorescence filter (Corning 4303 and 3387) typically used in conventional detection systems for NADPH. Also shown in this figure is the spectral profile of the Raman bands of water excited by light passing through the broadband excitation filter. There are three major problems with this excitation–detection system: (1) the Raman signal is only poorly suppressed; (2) the maximum transmission of the emission filter does not coincide with the fluorescence maximum of NADPH (460 nm); and (3) the Corning 3387 filter is fluorescent when irradiated by light passing through the excitation filter. Figure 3b illustrates the location of the laser line, the transmission curve

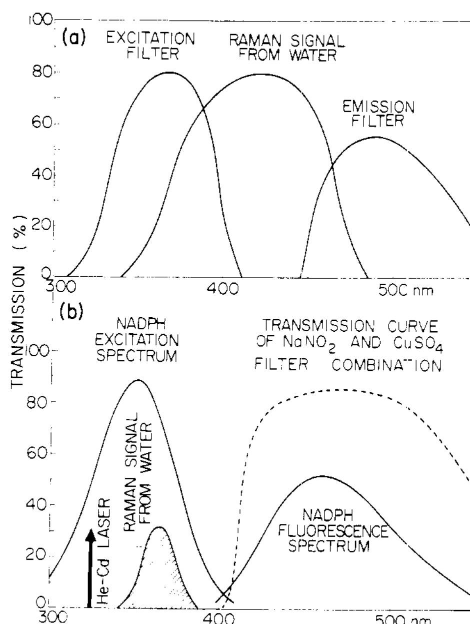


Figure 3. Transmission curves: (a) for a conventional fluorimeter; and (b) for the laser fluorimeter. The excitation and fluorescence spectra of NADPH are shown in (b). The Raman signal for each setup is also presented

of the detection filters used in this study, and the excitation and fluorescence spectra of NADPH. Note that the nonfluorescent liquid filter combination completely blocks the scattered light of the laser and the Raman signal from water. Moreover, this filter system has high transmission at the fluorescence maximum of NADPH.

Reagents. The enzymes (glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, and 6-phosphogluconate dehydrogenase), coenzymes (NADP and NADPH), and substrates (glucose-6-phosphate and α-ketoglutaric acid) are obtained from Boehringer and are used without further purification. The imidazole is nonfluorescent grade (Sigma). Doubly-distilled water and 6 M sodium hydroxide are used after exposure to sunlight (1 day). This photobleaching procedure reduces the background fluorescence significantly (by a factor of about five).

Procedure. The experimental procedures described in ref. 2 and 3 are followed closely. Because the coenzyme and the buffer solution have fluorescent contaminants, care is taken to use as little of either as is needed. The sample volume is adjusted to 1 mL for comparison with conventional fluorescence measurements. Because of photobleaching effects caused by the 325-nm output of the He–Cd laser, the fluorescence intensity from the sample is recorded only for several seconds. The sample recovers in the dark after about 30 s. Typically the fluorescence intensity is measured twice.

The detection limit is calculated from the results of several (usually four) measurements of samples having identical concentrations. The signal-to-noise ratio, S/N, is calculated from the expression:

$$S/N = \frac{n_S - n_N}{\sqrt{\sigma_S^2 + \sigma_N^2}}$$

where n_S and n_N are signal and background intensities, σ_S and σ_N are their standard deviations. The detection limit is defined as $S/N = 2$ in this study.

RESULTS

Background Signal. The performance of the present instrument was determined by measuring the fluorescence from NADPH solutions at different concentrations (Figure 4). The high sensitivity of the instrument and the excellent rejection of the Raman bands from water and unwanted fluorescence and scattered light in this laser fluorescence system allow the detection of NADPH at concentrations in

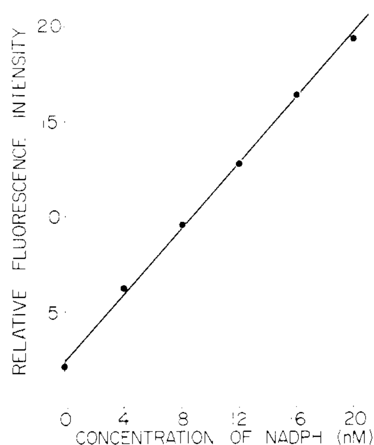
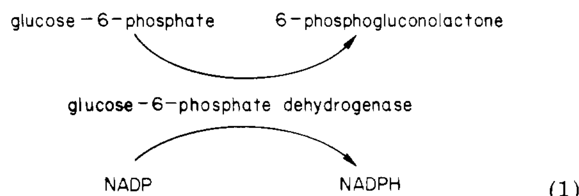


Figure 4. Analytical curve for the quantitation of NADPH

the nanomolar range. Most of the background signal comes from distilled water. The background signal, corresponding to 2 nM of NADPH, slightly varies from day to day and depends on the water container and the extent of photobleaching. This fact shows that the contribution from Raman bands of the water is much less important than that from impurities in the distilled waters. The low background signal of the laser fluorimeter implies that laser fluorescence analysis may be quite promising for the measurement of enzyme reactions at low concentrations.

Glucose-6-phosphate. The concentration of glucose-6-phosphate is determined using the enzyme reaction



(1)

the fluorescence intensity from NADPH is used to quantitate the glucose-6-phosphate. The analytical curve is linear over the range 0–80 nM. The detection limit is 2 nM, corresponding to 2×10^{-12} mol of glucose-6-phosphate in the 1-mL sample volume. The detection limit is set by fluctuations in the background signal from distilled water and NADP. Reactions are quite reproducible; the scatter in the data is about 5%.

Alkaline Development of NADP. The NADP is itself nonfluorescent, but it may be converted to a strongly fluorescent molecule by adding concentrated alkaline solution (*2, 3, 5*). The spectral properties of alkaline-treated NADP are similar to NADPH, and the same detection system is used in conventional fluorimetry. The analytical curve using our detection system is linear over the range 0–25 nM with a detection limit of 4 nM, corresponding to 4×10^{-12} mol. The long incubation time (10 min) at elevated temperature (60 °C) with concentrated alkaline solution (6 M NaOH) damaged the quartz cell through repeated use. Consequently, disposable Pyrex test tubes were used instead for incubation. However, the background fluorescence from contaminants then could not be subtracted for the individual samples. The background signal corresponds to 1×10^{-11} mol of NADP. Although alkaline-treated NADP can be detected with high sensitivity by this method, the detection limit is not so low as expected. The reason appears to be that maximum in the excitation spectrum shifts from 350 nm for NADPH to 375 nm for alkaline-treated NADP. Consequently, the 325-nm line of the He–Cd laser is more than ten times less effective in exciting fluorescence.

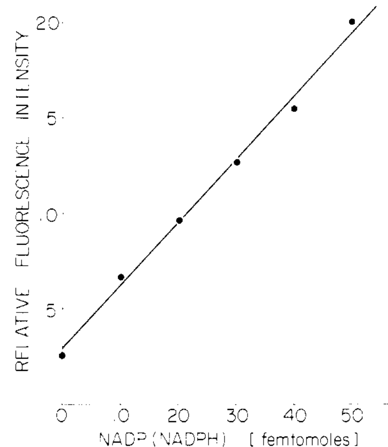
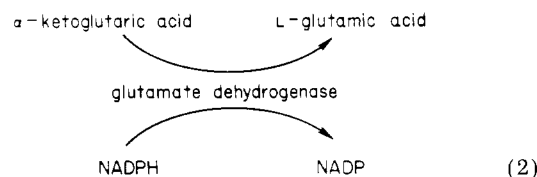


Figure 5. Analytical curve for the quantitation of NADP (NADPH) by enzyme amplification laser fluorimetry. The cycling volume is 100 μL. The detection limit is 10 fmol (0.1 nM)

α-Ketoglutaric Acid. The application of the alkaline development of NADP is demonstrated by the quantitation of α-ketoglutaric acid using the enzyme reaction



(2)

After the enzyme reaction proceeds at room temperature for 20 min, 0.3 M HCl is added to decompose the fluorescent NADPH that remains. Following alkaline development, a linear analytical curve is obtained over the range 0–20 $\times 10^{-12}$ mol with a detection limit of 4×10^{-12} mol. One third of the background signal originates from the distilled water, while the rest comes from the alkaline development.

Detection of NADP by Enzyme Amplification. By combining reactions 1 and 2, an enzyme cycle is obtained (see Figure 1a, where substrate 1 = glucose-6-phosphate, enzyme 1 = glucose-6-phosphate dehydrogenase, product 1 = 6-phosphogluconolactone, substrate 2 = α-ketoglutaric acid, enzyme 2 = glutamate dehydrogenase, and product 2 = L-glutamic acid). The sample is incubated for 2 h at 38 °C. During this period several thousand cycles are expected to occur if the enzymes have full activity (*2, 3*). The sample is then heated to 85–90 °C for 3 min to destroy the enzymes. Next 6-phosphogluconate dehydrogenase is added which reduces NADP to NADPH at the same time that 6-phosphogluconolactone is converted to ribulose-5-phosphate (see Figure 1b).

In this enzyme amplification procedure, the concentration of NADP and NADPH is determined unspecifically, and thus both are measured to the same sensitivity. If it is desired to measure only one of them, then the other must be decomposed before measurement by the addition of acid or alkaline solution (*2, 3*). Figure 5 shows the analytical curve for the detection of NADP (NADPH) by enzyme amplification laser fluorimetry. The detection limit of 1×10^{-14} mol is set by the fluctuations in the enzyme reaction rates occurring in the individual incubation tubes. This fluctuation is found to be very sensitive to the cleanliness of the glassware.

DISCUSSION

The use of enzyme reactions combined with laser fluorimetry offers many advantages for trace analysis of biomedical species. Table I summarizes the detection limits obtained in this study and compares them to previous literature values.

Table I. Comparison of Detection Limits

substance	present work	literature value	ref.
glucose-6-phosphate	2 nM	200 nM	*2, 3*
α-ketoglutaric acid	4 pmol	20 pmol	*2, 3*
NADP	0.1 nM	1 nM	*2*
	10 fmol	300 fmol	*3*

The background signal of our laser fluorimeter corresponds to a 2 nM concentration of NADPH. This value is 50 times lower than that obtained using a conventional fluorescence detection system (*2, 3*). Thus the laser excitation source and nonfluorescent filter system provides a very effective means of reducing the background signal. The present excitation–detection system allows the quantitation of glucose-6-phosphate by a direct enzyme reaction to concentrations of 2 nM. This detection limit is 100 times better than that measured by the usual fluorescence detection system. It is noteworthy that the sensitivity of the direct measurement of NADPH in this study almost equals that of the enzyme amplification technique in a conventional system (1 nM). The present method using direct enzyme reaction is quite simple and requires only 10 min incubation time while the use of enzyme amplification is not so straightforward and requires long incubation times. However, when enzyme amplification is combined with laser fluorimetry, it is possible to measure as little as 10 femtomoles of NADP. This amount is 30 times smaller than previously reported results.

Only a small improvement is obtained by using the alkaline treatment of NADP for the quantitation of the latter. The detection limit in the present study is equal to or slightly better than the literature values. The use of the 364-nm line of the argon ion laser, for example, might enhance the fluorescence intensity of the alkaline-treated NADP by more than an order of magnitude compared to that of the 325-nm line of the helium–cadmium laser, since the 364-nm line nearly coincides with the absorption maximum of the alkaline-treated NADP.

The present detection system is versatile because it may be applied not only to the quantitation of NADPH but also to NADH, since the spectral properties of NADH are almost identical to those of NADPH. The detection of NADPH and NADH is quite general for monitoring enzyme reactions. Even if the enzyme reaction is not a redox reaction, the reaction products may undergo a subsequent redox reaction so that NADPH or NADH can be used (*13*). The quantitation of NADPH (NADH) may also be used in the analysis for inorganic substances, such as for phosphate (*2, 3*) or nitrate (*14*) by using appropriate enzyme reaction systems.

In this study the sample volume is adjusted to 1 mL so that the results can be readily compared to other studies. However, because laser light can be focused easily to a small spot size without loss of intensity, microanalysis may be possible. This would permit the measurement of microliter samples, for example, without the need for dilution. Care should be taken to avoid or minimize photodecomposition of the sample. With the use of a quartz cell, this problem can become severe. However, it may be possible to use flow injection and immobilized enzymes, in which the sample is continuously passed over the enzymes which are bound to some solid support structure (*15*). For the detection part, the use of a flowing droplet might provide high sensitivity since cell wall fluorescence is avoided (*16*).

When fluorescence from contaminants in the sample becomes too strong, some separation procedure may be necessary, such as centrifugation or chromatography. High-pressure liquid chromatography (HPLC) is increasingly being used in biomedical applications. For example, a reverse phase μBondapak C_{18} column separates NADH from contaminants on the basis of polarity (*17, 18*). The application of laser fluorimetry as a detection system for HPLC (*19*) may offer advantage in this case. However, while alkaline development is useful for conventional fluorescence analysis, in HPLC this procedure may not be practical because the proper pH level is difficult to maintain. For example, the fluorescence from alkaline treated NADP decreases to 50% of its value at pH 9.6 and falls to zero at pH 6 (*5, 20*).

An alternative for overcoming the fluorescence interference from contaminants is to use enzyme amplification since the latter technique has the potential for dramatically increasing the signal-to-noise because typically several thousand enzyme cycles occur per hour. Recently many researchers have reported the use of enzyme-linked immunoassay as a replacement for radioimmunoassay in biomedical studies (*21, 22*). In enzyme-linked immunoassay, each enzyme produces typically several thousand NADPH (NADH) coenzyme molecules. The concentration of NADPH (NADH) can then be amplified by use of an enzyme cycle, as described in this study. Thus by using enzyme-linked immunoassay together with enzyme amplification laser fluorimetry, it may be possible to detect and quantitate hormones at very low concentrations.

LITERATURE CITED

(1) Fishman, M. M. *Anal. Chem.* **1978**, *50*, 261R–73R.
(2) Lowry, O. H.; Passonneau, J. V., "Flexible System of Enzymatic Analysis"; Academic Press: New York, 1972.
(3) Anan, K.; Konno, K.; Tamura, Z.; Matsuhashi, M.; Matsumoto, J. "Kiso-Seikagaku-Jikkenho"; Maruzen, 1976.
(4) Greengard, P. *Nature (London)* **1959**, *178*, 632–4.
(5) Lowry, O. H.; Roberts, N. R.; Kapphahn, J. I. *J. Biol. Chem.* **1957**, *224*, 1047–64.
(6) Smith, B. W.; Plankey, F. W.; Omenetto, N.; Hart, L. P.; Winefordner, J. D. *Spectrochim. Acta, Part A* **1974**, *30*, 1459–63.
(7) Bradley, A. B.; Zare, R. N. *J. Am. Chem. Soc.* **1976**, *98*, 620–21.
(8) Richardson, J. H.; Wallin, B. W.; Johnson, D. C.; Hrubesh, L. W. *Anal. Chim. Acta* **1976**, *86*, 263–7.
(9) Imasaka, T.; Kadone, H.; Ogawa, T.; Ishibashi, N. *Anal. Chem.* **1977**, *49*, 667–8.
(10) Ishibashi, N.; Ogawa, T.; Imasaka, T.; Kunitake, M., *Anal. Chem.*, in press.
(11) Blaedel, W. J.; Bagusloski, R. C. *Anal. Chem.* **1978**, *50*, 1026–32.
(12) Kato, K.; Hamaguchi, Y.; Okawa, S.; Ishikawa, E.; Kobayashi, K.; Katunuma, N. *Lancet* **1977**, (1), 40.
(13) Lloyd, B.; Burrin, J.; Smythe, P.; Alberti, K. G. M. M., *Clin. Chem.* **1978**, *34*, 1724–9.
(14) Kiang, C. H.; Kuan, S. S.; Guilbault, G. G. *Anal. Chem.* **1978**, *50*, 1323–5.
(15) Betteridge, D. *Anal. Chem.* **1978**, *50*, 832A–46A.
(16) Diebold, G. J.; Zare, R. N. *Science* **1977**, *196*, 1439–41.
(17) Loshom, C. A.; McComb, R. B.; Bond, L. W.; Bowers, G. N., Jr.; Coleman, W. H.; Gwynn, R. H., *Clin. Chem.* **1977**, *23*, 1576–80.
(18) Margolis, S. A.; Howell, B. F.; Schaffer, R., *Clin. Chem.* **1977**, *23*, 1581–84.
(19) Lidofsky, S. D.; Imasaka, T.; Zare, R. N. *Anal. Chem.* in press.
(20) Kaplan, N. O.; Colowick, S. P.; Barnes, C. C. *J. Biol. Chem.* **1951**, *191*, 461–72.
(21) Ishikawa, E. *J. Biochem.* **1973**, *73*, 1319–21.
(22) Yoshioka, M.; Taniguchi, H.; Kawaguchi, A.; Kobayashi, T.; Murakami, K.; Seki, M.; Tsutou, A.; Tamagawa, M.; Minoda, H.; Baba, S. *Clin. Chem.* **1979**, *25*, 35–8.

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