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# A Spectrophotometric Method for the Direct Detection and Quantitation of Nitric Oxide, Nitrite, and Nitrate in Cell Culture Media

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**A method for the spectrophotometric determination of nitric oxide, nitrite, and nitrate in tissue culture media is presented. The method is based on the nitric oxide-mediated nitrosative modification of sulfanilic acid that reacts with *N*-(1-naphthyl)ethylenediamine dihydrochloride forming an orange-colored product absorbing at 496 nm. Nitric oxide levels were determined in culture media from this absorbance measurement using chemiluminescence standardization. Extinction coefficients of 5400 and 6600 $M^{-1}$ $cm^{-1}$ were determined for the nitric oxide product in assay solutions containing 0.1 or 100 mM $KPO_4$ buffer (pH 7.4), respectively, with a limit of detection of 1 $\mu$M. Acidification of these reactions (pH 2.4) generated a pink-colored product absorbing at 540 nm allowing for quantitation of total nitric oxide/nitrite levels using extinction coefficients of 38,000 and 36,900 $M^{-1}$ $cm^{-1}$, for the assay solutions described. The limit of detection of this assay was approximately 300 nM. Using the 100 mM $KPO_4$ buffer system, nitrate levels were determined following reduction to nitrite using a copper-coated cadmium reagent with an extinction coefficient of 29,500 $M^{-1}$ $cm^{-1}$ and a detection limit of 0.5 $\mu$M. The utility of these assays was demonstrated in the standardization of nitric oxide-saturated cell culture media, and the release of nitric oxide by the NONOate compound DEA/NO.** © 2000 Academic Press

***Key Words:*** **nitric oxide; quantitation; chemiluminescence; spectrophotometric assay; nitrite; nitrate; cell culture.**

The growing interest in the effects of nitric oxide ($^{\bullet}NO$) as both a cytotoxic and a signal transduction molecule has warranted the need for rapid and accurate evaluation of $^{\bullet}NO$ concentrations in carrier solutions used in cell culture experiments. To date, several methods for measurement of $^{\bullet}NO$ concentrations have been routinely employed. Spectrophotometrically, $^{\bullet}NO$ levels have been indirectly evaluated via analysis of nitrite ($NO_2^-$) concentrations using the acidic Griess reaction, or by quantifying the formation of methemoglobin or metmyoglobin following the $^{\bullet}NO$-induced oxidation of oxyhemoglobin or oxymyoglobin (1, 2). Beyond these methods, chemiluminescence, electrochemical, and mass spectral detection have also provided a sensitive means for the measurement of $^{\bullet}NO$ concentrations in carrier solutions (3–5). Though frequently used, these methods have some disadvantages. Although the acidic Griess reaction provides a rapid and easy assay, its utility is hindered by the fact that it provides an indirect estimation of $^{\bullet}NO$ via the measurement of $NO_2^-$. The spectrophotometric detection of $^{\bullet}NO$ via oxidation of oxyhemoglobin is limited by the fact that oxyhemoglobin is relatively unstable and not commercially available and therefore must be prepared prior to each assay. Although chemiluminescence provides the most sensitive and specific method for the direct detection of $^{\bullet}NO$, the detector is expensive and requires significant maintenance, making it unaffordable in many laboratory settings. Likewise, electro-

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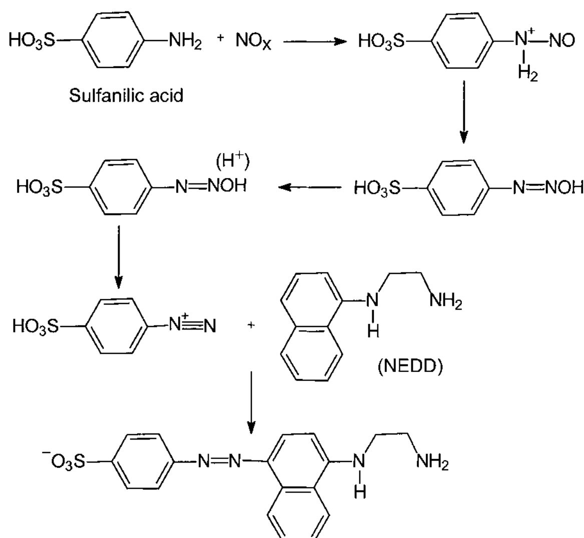


**FIG. 1.** Chemistry associated with the $NO_x$-mediated modification of sulfanilic acid and its reaction with NEDD leading to the formation of orange product.

chemical and mass spectral methods require significant instrumentation and maintenance support.

Recently, Nims *et al.* have described a method for the direct measurement of ˙NO in 100 mM phosphate buffer, pH 7.4, containing 17 mM sulfanilamide and 0.4 mM NEDD (6). The mechanism of this reaction relies on the production of $NO_x$ species generated by the reaction of ˙NO with $O_2$. Once formed, the $NO_x$ intermediates react in a nitrosative reaction with sulfanilic acid (SA)[3] which in the presence of *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) results in the formation of an orange azo dye that absorbs at 496 nm as shown in Fig. 1 (7). The difference between this product and the acidic Griess product is believed to be the protonation state of the sulfoxide.

The current work extends the method of Nims *et al.* by: (1) adapting it to cell culture conditions commonly used in biological research; (2) rigorously standardizing the methodology for direct evaluation of ˙NO as well as $NO_2^-$ and $NO_3^-$; and (3) demonstrating the utility of the assay under experimental conditions using ˙NO-saturated cell culture media, as well as ˙NO released from a NONOate compound (DEA/NO).

## MATERIALS AND METHODS

Serum-free Eagle's MEM without glutamine or phenol red was from Washington University Tissue Culture Facility. Nitrogen gas (oxygen-free) was obtained from Genex (St. Louis, MO). Nitric oxide gas (2.5 grade 99%) was obtained from Praxair, Inc. (Danbury, CT). The delivery of $N_2$ gas to media was monitored via an FM-1050 series flow meter from Matheson Instruments (Montgomeryville, PA). Gas-tight syringes were from SGE Inc. (Austin, TX). Sulfanilic acid and NEDD were purchased from Sigma Chemical Co. (St. Louis, MO). Copper acetate and 100 mesh cadmium were purchased from Aldrich, Milwaukee, WI. Spectrophotometric measurements were performed on a DU640 spectrophotometer from Beckman Instruments, Inc. (Fullerton CA). Gas-phase chemiluminescence detection of ˙NO was performed using a Model 280 nitric oxide analyzer (NOA) from Sievers (Boulder, CO).

*Preparation of ˙NO saturated media.* All ˙NO stock solutions were prepared in Eagle's MEM without serum, phenol red, or glutamine. Initially, 100 mL media was aliquoted into sterile 250-mL glass bottles. The bottles were sealed with a rubber septum and then covered with tape to minimize $O_2$ leakage. A Lurlock valve was then inserted to release pressure during the gassing process. The media were purged of $O_2$ by saturation with ultrapure $N_2$ gas. This was accomplished with the aid of a FM-1050 series flow meter adjusted to a setting of 10. The media were gassed for 1 min per 2-mL volume. At the same time, a 1 N NaOH liquid solution was being saturated with $N_2$ gas followed by a 15 min exposure to ˙NO gas adjusted to a flow of 2–5 psi. At the end of the $N_2$ gassing process, the media were linked to the output of the NaOH liquid solution and saturated with ˙NO for 30 min per 100-mL volume.

*Chemiluminescence measurement of ˙NO.* A Sievers Model 280 NOA was used for the gas-phase chemiluminescence detection of ˙NO. All data were analyzed using the Sievers' 280 NOA software. For the generation of standard curves $NO_2^{\cdot-}$ was reduced to ˙NO using KI in glacial acetic acid; the ˙NO gas was swept into the NOA reaction chamber where it reacted with ozone to form electronically excited nitrogen dioxide. Emission from the excited nitrogen dioxide was detected by the NOA, and peak areas were used to construct standard curves. Nitric oxide levels were determined directly from these standard curves. Standards were prepared from a 1.0 mM stock solution of $NO_2^-$ in Eagle's MEM. Samples and standards were injected into the NOA using a 50-$\mu$L gas-tight syringe. The syringe was rapidly rinsed with 3 vol of standard or ˙NO sample; after wiping the needle with a Kimwipe, the fourth volume was injected into the NOA. Each solution to be quantified was sampled in triplicate by injecting 20- to 30-$\mu$L volumes.

*Spectrophotometric measurement of ˙NO.* Nitric oxide concentrations in cell culture media samples were directly measured using a modification of the method of Nims *et al.* (6). Aliquots (10–40 $\mu$L) of ˙NO-saturated media were withdrawn from each bottle using a gas-tight syringe. The media were added to 900 $\mu$L of 0.1 or

[3] Abbreviations used: SA, sulfanilic acid; NEDD, *N*-(1-naphthyl) ethylenediamine dihydrochloride; NOA, nitric oxide analyzer.

100 mM potassium phosphate buffer, pH 7.4, containing 17 mM SA and 0.4 mM NEDD, that was previously volume adjusted with media for a final volume of 1 mL. The solution was immediately mixed by inversion incubated at room temperature for 3 min and then $A_{496nm}$ was measured. The ˙NO concentration of the solution was calculated according to Beer's law using an extinction coefficient of 5400 $M^{-1}$ $cm^{-1}$ (0.1 mM $KPO_4$ assay buffer) or 6600 $M^{-1}$ $cm^{-1}$ (100 mM $KPO_4$ assay buffer) as determined from experiments using chemiluminescence standardization. Multiple samples of increasing volume from ˙NO-saturated media preparations were employed for the calculation of these extinction coefficients.

*Spectrophotometric detection of $NO_2^-$.* Nitrite levels were determined in the same cuvettes used for ˙NO determination. This was accomplished by the addition of 2.5 μL 85% phosphoric acid (0.1 mM $KPO_4$ buffer assays) or 10.0 μL phosphoric acid (100 mM $KPO_4$ buffer assays) to the neutral Griess reagent, to yield an acidic Griess reaction (pH 2.4). The solution was immediately mixed by inversion and incubated at room temperature for 15 min; then $A_{540nm}$ was measured. These absorbance readings were used to determine sample $NO_2^-$ levels from a standard curve of genuine $NO_2^-$ dissolved in the same media volumes. Extinction coefficients of 38,000 $M^{-1}$ $cm^{-1}$ (0.1 mM $KPO_4$) or 36,900 $M^{-1}$ $cm^{-1}$ (100 mM $KPO_4$) may be employed for the calculation of total nitrite/nitric oxide levels only when <20-μl aliquots of ˙NO-saturated media were assayed. Higher volumes resulted in absorbance measurements exceeding Beer's law.

*Spectrophotometric detection of nitrate.* Nitrate ($NO_3^-$) levels were evaluated using a copper-coated cadmium reagent (1). The cadmium reagent was prepared by washing 2–3 g of 100-mesh cadmium with 50 mL of nanopure water in a 250-mL Erlenmeyer flask, discarding the supernatant and then washing twice with 50 mL of 0.5 N HCl. At this point the cadmium texture resembled stones resting on the bottom of the flask. Next the cadmium was washed twice with 50 mL of $H_2O$ and then washed with 50 mL of 5% copper acetate to coat the cadmium with copper. The wash with copper acetate was repeated two to three times until the solution above the cadmium remained blue for 60 s to ensure that the cadmium was saturated with copper. If at any time the cadmium turned red or black, the cadmium reagent was unusable and discarded. Once the cadmium was saturated with copper, the excess copper was removed quickly by rinsing with four washes of 25 mL of $H_2O$. The copper-coated cadmium was then washed twice with 30 mL of 0.1 N HCl and stored in this HCl solution. The copper-coated cadmium appeared gray and slightly more buoyant than the original metal grains and was usually stable for 6 months at room temperature.

Immediately prior to assay an aliquot (approximately 5 mL from the reagent layer at the bottom of the flask) of the copper-coated cadmium reagent was washed twice in media, pH 9.0, and centrifuged; the supernatant discarded and resuspended in 3.0 mL of pH 9.0 media. The media samples and standards were all adjusted to pH 9.0 (using NaOH) to ensure efficient conversion of nitrate to nitrite. To accomplish the conversion of nitrate to nitrite, 0.1 mL of cadmium reagent was mixed with 0.1 mL of sample or standard in Eppendorf microfuge tubes and vortexed intermittently for 30 min at room temperature. Following the conversion, reaction tubes were placed in a microfuge and spun at 14,000 rpm for 2 min. Then 0.1 mL of supernatant was mixed with 0.9 mL of neutral assay reagent (17 mM SA and 0.4 mM NEDD in 100 mM $KPO_4$ buffer, pH 7.4) in a 1-mL cuvette and incubated at room temperature for 5 min. Ten microliters of 85% phosphoric acid was then added; solutions were mixed by inversion and then incubated at room temperature for 15 min. Absorbance values were read at 540 nm and sample $NO_3^-$ levels were determined from $NO_3^-$ standard curves. Using these conditions the extinction coefficients for nitrate and nitrite were nearly identical (see Fig. 5). Therefore the samples could be measured in the absence of cadmium reagent and the nitrite concentration could be determined. The measurement could then be repeated in the presence of cadmium reagent and the total nitrate and nitrite concentration could be determined and subtracted from the nitrite concentration to obtain the nitrate concentration.

*Calculation of ˙NO extinction coefficient.* Using the ˙NO concentrations as determined by chemiluminescence, the molarity of ˙NO corresponding to each absorbance measurement was calculated using the volume ratio of the ˙NO aliquot to the total reaction volume. Absorbance values were plotted vs molarity ˙NO, and linear regression analysis was performed. The slope obtained from the regression line was used to calculate the ˙NO extinction coefficient. This value was then employed to calculate ˙NO concentrations from absorbance data using Beer's law.

## RESULTS

The objective of this work was the development of a method providing rapid, inexpensive, and accurate spectrophotometric quantitation of ˙NO, $NO_2^-$, and $NO_3^-$ concentrations in cell culture media using the chemistry described in Nims *et al.* (6). Initially the optimal time of incubation for the development of colored product for the neutral (˙NO, Fig. 2A) and acidic (total ˙NO/$NO_2^-$, Fig. 2B) reactions was carried out in 0.1 and 100 mM $KPO_4$-buffered neutral Griess reagents (NGR). Figure 2A shows that the ˙NO-mediated development of orange color at 496 nm was maximal by 3 to 5 min in either buffer system. Similarly,

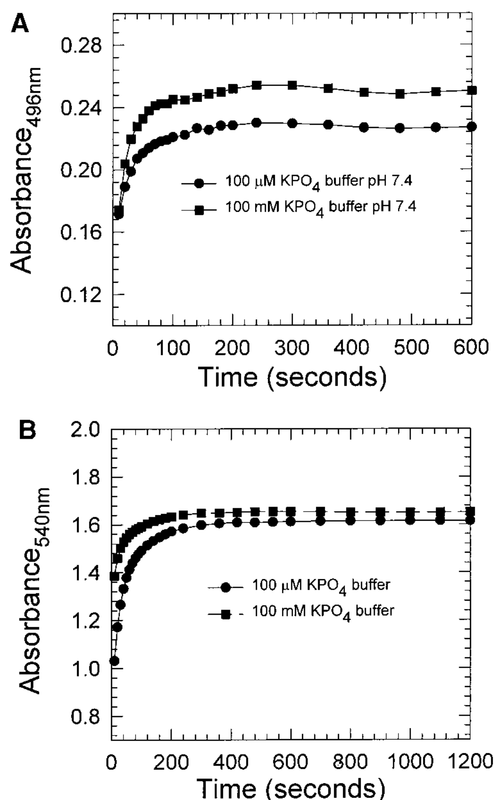


**FIG. 2.** Time course and stability of the absorbance changes seen in the neutral and acidic reactions. (A) 30 μL of ˙NO-saturated media was added to 900 μL neutral Griess reagent containing 70 μL media, for a final volume of 1 mL. The solution was mixed by inversion and the change in absorbance at 496 nm was monitored over time. (B) NGR reactions were acidified (pH 2.4) and absorbance at 540 nm was determined.

Fig. 2B demonstrates a plateau in color change that occurred between 10 and 15 min at 540 nm following acidification of either reaction. Therefore, the neutral reactions were analyzed following 3- to 5-min room temperature incubation for the direct detection of ˙NO; the solutions were then acidified and read after 15-min incubation at room temperature for the determination of total ˙NO/$NO_2^-$.

The extinction coefficients for the measurement of ˙NO and $NO_2^-$ in tissue culture media were determined. This was accomplished by directly measuring ˙NO concentrations in three independent preparations of ˙NO stock solution in media via chemiluminescence, then measuring the absorbance change of the neutral reactions as described for Fig. 2A. The ˙NO concentration of each stock solution in media, as determined by chemiluminescence, was then used to calculate moles of ˙NO in each sample volume employed to obtain absorbance measurements. Absorbance values were then plotted vs concentration of ˙NO for the neutral reactions carried out in 0.1 mM (Fig. 3A) and 100 mM (Fig. 4A) $KPO_4$-buffered NGR. The slope of the lines as determined by linear regression yields the ˙NO extinction coefficients representative for these assay conditions (Figs. 3A and 4A). To determine the extinction coefficients for the acidic reactions, stock solutions of $NO_2^-$ prepared in media were diluted and mixed with NGR as described. The solutions were incubated at room temperature for 3 min then acidified, and incubated 15 min. The absorbance was measured at 540 nm. The measurements were obtained in triplicate for the 0.1

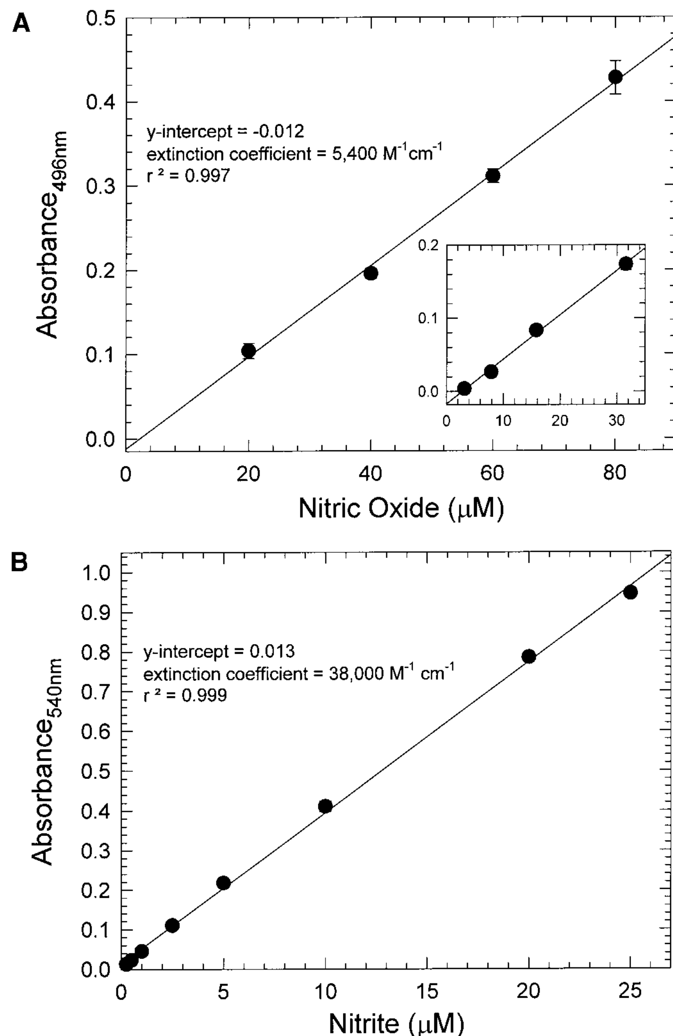


**FIG. 3.** The standardization of the neutral and acidic reactions buffered in 100 μM $KPO_4$. (A) Three separate bottles with 100 mL Earle's MEM without phenol red, glutamine, or serum were saturated with genuine ˙NO gas as described under Materials and Methods. Nitric oxide concentrations were then determined using chemiluminescence detection. Absorbance measurements were also obtained, and the data were plotted as $A_{496}$ vs ˙NO (μM) final concentration. Linear regression was performed, and the extinction coefficient for ˙NO was 5400 $M^{-1}$ $cm^{-1}$. A limit of detection of 3 μM is shown in the inset to A. (B) Sodium nitrite was dissolved in media and analyzed using the acidic reaction. Each standard was prepared in triplicate and analyzed with linear regression to determine the extinction coefficient. Errors represent ± 1 standard deviation. If no error bar appears, then the error is within the size of the symbol used.

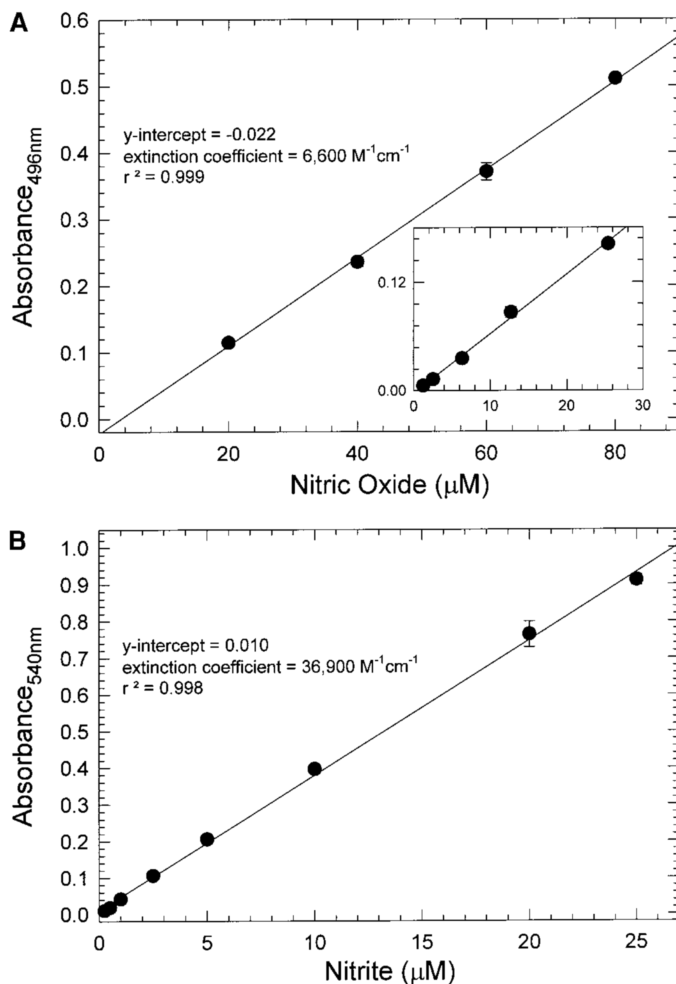


**FIG. 4.** The standardization of the neutral and acidic reactions buffered in 100 mM $KPO_4$. (A) Three bottles with 100 mL Earle's MEM without phenol red, glutamine, or serum were saturated with genuine ˙NO gas as described under Materials and Methods. Nitric oxide final concentrations were determined using chemiluminescence detection. Absorbance measurements were then obtained using the 100 mM buffered assay system. Linear regression was performed, and the extinction coefficient for ˙NO was calculated to be 6600 $M^{-1}$ $cm^{-1}$. A limit of detection of 1 μM is shown in the inset to A. (B) Genuine $NO_2^-$ was dissolved in media and analyzed using the acidic reaction. Each standard was prepared in triplicate and analyzed with linear regression to determine the extinction coefficient. Errors represent ± 1 standard deviation.

mM (Fig. 3B) and 100 mM (Fig. 4B) $KPO_4$-buffered NGR. The extinction coefficients were calculated as described for the ˙NO reaction.

In some experimental settings it is desirable to measure $NO_3^-$ under cell culture conditions in the presence of $NO_2^-$. This usually requires reducing $NO_3^-$ to $NO_2^-$ and measuring $NO_2^-$ spectrophotometrically (1). A similar system was developed based on the reduction of $NO_3^-$ to $NO_2^-$ using a copper-coated cadmium reagent. Standards of $NO_3^-$ and $NO_2^-$ were prepared in media, exposed to the copper-coated cadmium reagent, and assayed in the 100 mM NGR reagent as described under Materials and Methods. These reaction conditions yielded essentially complete conversion of $NO_3^-$ to $NO_2^-$ as evidenced by the results in Fig. 5 showing that the extinction coefficients obtained with genuine nitrate (Fig. 5A) and nitrite (Fig. 5B) were nearly identical (29,500 vs 29,800 $M^{-1}$ $cm^{-1}$ for $NO_3^-$ and $NO_2^-$, respectively). Using this methodology complete conversion of $NO_3^-$ to $NO_2^-$ should be verified in each assay to ensure the quality of the copper-coated cadmium reagent. To determine sample $NO_3^-$ concentrations in the presence of $NO_2^-$, the concentration of $NO_2$- should be determined in the absence of cadmium and the concentration of $NO_2^-$ + $NO_3^-$ should be determined in the presence of cadmium. The concentration of $NO_2^-$ + $NO_3^-$ should then be subtracted from the $NO_2^-$ concentration to obtain the concentration of $NO_3^-$. Using this method we were able to detect 0.5 μM $NO_3^-$.

Following characterization of these assays, the neutral and acidic Griess reactions were used to standardize ˙NO-saturated cell culture media routinely used in the lab. To determine the length of time required for ˙NO saturation of Eagle's MEM (serum-, glutamine-, and phenol red-free), a time-course experiment was performed (Fig. 6A) using 100 mM $KPO_4$-buffered

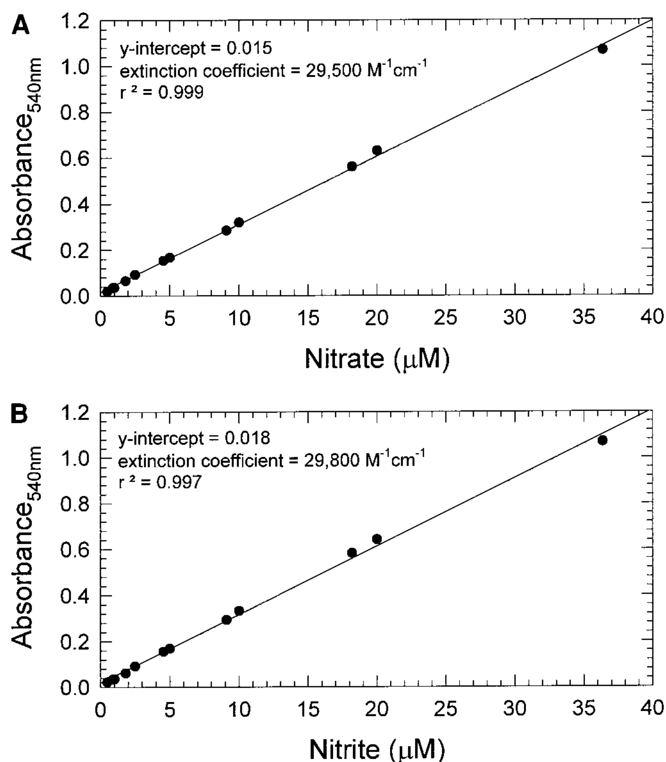


**FIG. 5.** Standardization of the assay system for the measurement of $NO_3^-$ using the copper-coated cadmium reagent. $NO_3^-$ (A) and $NO_2^-$ (B) dissolved in tissue culture media were analyzed in the presence of the cadmium reagent using the assay conditions described under Materials and Methods. $NO_3^-$ gave no color change in the absence of cadmium (data not shown). All data were obtained using the 100 mM $KPO_4$ buffered NGR.

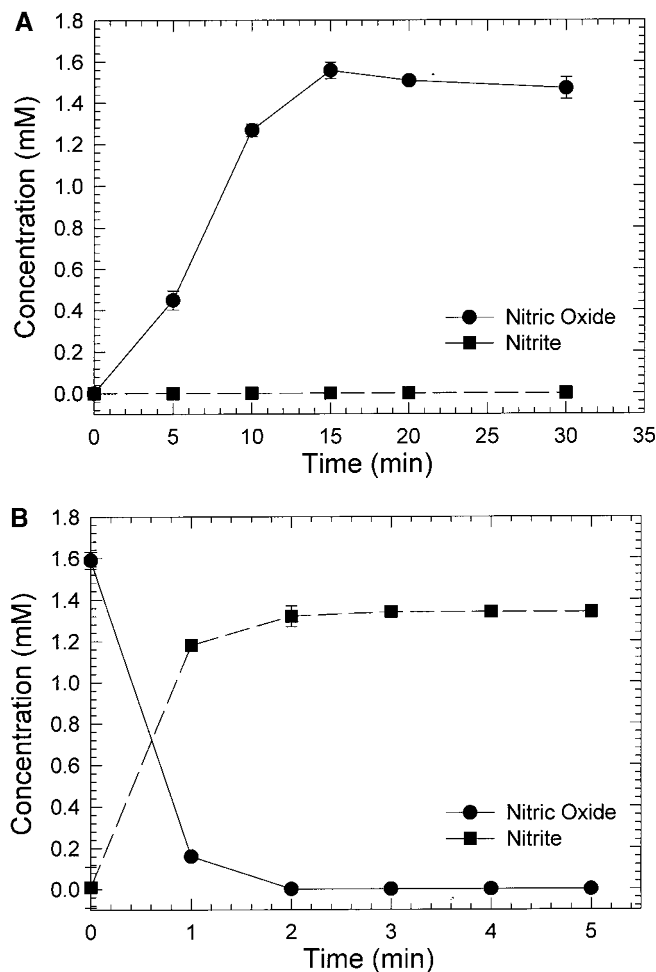


**FIG. 6.** The time course for saturation of media with genuine ˙NO and the disappearance of ˙NO from media in a culture plate. (A) The time required for saturation of media with ˙NO gas was determined by gassing 100-mL volumes of media for increasing time intervals and determining ˙NO and $NO_2^-$ concentrations using the 100 mM $KPO_4$-buffered NGR. Nitric oxide and $NO_2^-$ concentrations were plotted vs time of gassing. (B) Using the same assay system the disappearance of ˙NO and the appearance of $NO_2^-$ were determined by the addition of 4 mL of ˙NO-saturated media to a 60-mm cell culture plate at room temperature in room air. Errors represent ± 1 standard deviation.

NGR. Following saturation with $N_2$ gas, 100-mL volumes of media in 250-mL bottles were exposed to ˙NO gas for increasing time intervals. Thirty-microliter aliquots were added to NGR as described, and absorbance values at 496 nm were obtained to determine the ˙NO concentration of the media. The solutions were then acidified and absorbances at 540 nm were determined and employed for the calculation of total ˙NO/$NO_2^-$. Nitrite levels were calculated by subtracting the ˙NO concentration from total ˙NO/$NO_2^-$. If the total ˙NO/$NO_2^-$ concentration was less than or equal to the ˙NO concentration, $NO_2^-$ contamination within the bottle was assumed to be insignificant. Using this system, both ˙NO and $NO_2^-$ concentrations were plotted vs time and a typical set of results are shown in Fig. 6A. These data demonstrate a rapid increase in ˙NO levels with time, followed by plateau at approximately 1.5 to 1.6 mM ˙NO, which occurred between 15 and 30 min. Nitrite contamination was insignificant if the ˙NO-saturated gas was bubbled through the liquid NaOH solution, and there were no $O_2$ leaks in the system. These data are in agreement with literature values determined by chemiluminescence detection at room temperature. Varying the volume of media in the bottles prior to gassing or storing the bottles in the dark at room temperature for up to 3 days had little effect on ˙NO concentration (data not shown).

The disappearance of ˙NO and the appearance of $NO_2^-$ in ˙NO-saturated media following exposure to air was also determined. These experimental conditions were designed to mimic the exposure conditions employed during a typical cell culture experiment. This was accomplished by removing 4 mL of ˙NO saturated media using a gas-tight syringe and placing it in a 60-mm culture plate (following sterile filtration). Media aliquots (30-$\mu$L) were withdrawn from the culture plate during increasing time intervals, then assayed for ˙NO and total ˙NO/$NO_2^-$ concentrations. Figure 6B shows the rapid drop in [˙NO] through 3 min at which point ˙NO concentrations were undetectable. Similarly, [$NO_2^-$] increased rapidly over the first 3 min then reached a plateau. From these experiments we were unable to show significant concentrations of $NO_3^-$ under the specified conditions; therefore, the majority of ˙NO appeared to be converted to $NO_2^-$. A slight difference in ˙NO vs total ˙NO/$NO_2^-$ was consistently observed (Fig. 6B), which may be explained by a portion of the ˙NO coming out of the saturated media solution and escaping into the gas phase, as a function of time in the culture dish.

Figure 7 shows the release of ˙NO from the NONOate drug (DEA/NO) dissolved in media and analyzed using the 100 mM $KPO_4$-buffered NGR. One hundred microliters of media containing 980 $\mu$M DEA/NO was added to 900 $\mu$L of NGR mixed by inversion and immediately placed in the spectrophotometer. Figure 7A shows the absorbance change as a function of time while Fig. 7B shows the rate at which ˙NO was released during that time. Peak rates of ˙NO release occurred in the first 2 to 5 min; however, the release of ˙NO continued for 30 to 35 min. These results demonstrate the utility of this assay for following the release of the ˙NO from a drug that is commonly used as a source of ˙NO in culture experiments.

## DISCUSSION

The results presented here indicate that the nitrosative modification of SA under neutral conditions coupled with the color change of NEDD is a quick and inexpensive means of detecting ˙NO in media prepara-

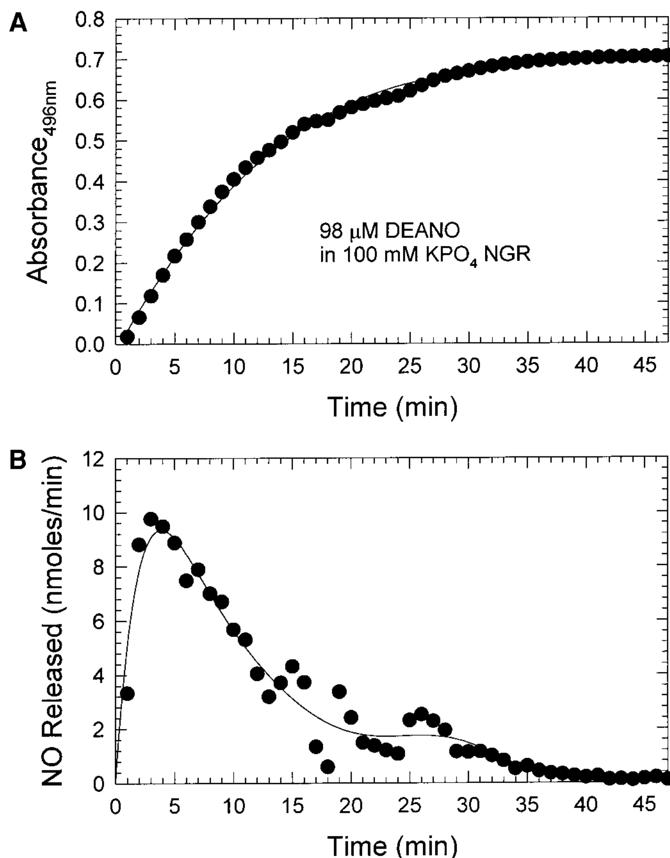


**FIG. 7.** Release of ·NO from DEA/NO determined in the 100 mM $KPO_4$-buffered assay system. DEA/NO was dissolved in NaOH and the concentration was determined as described (8). (A) DEA/NO in media was then added to the assay system at a final concentration of 98 $\mu$M and the absorbance at 496 nm was followed as a function of time. (B) The data for each minute of decomposition were then utilized to calculate the rate of ·NO release.

tions commonly used in cell culture experiments. The standardization of this method to results obtained with chemiluminescence detection provides accuracy in the use of absorbance data for determination of ·NO concentrations. This reaction can be accomplished in a plastic disposable cuvette. Following ·NO evaluation, the reaction can be acidified to provide quantitative information pertaining to $NO_2^-$ levels in the same cuvette. In addition, this method demonstrates the utility of the copper-coated cadmium reagent for determining $NO_3^-$ concentrations in a given media solution. The simplicity, accuracy, and versatility of this system provide a quantitative means for the assessment of nitrogen oxide species in tissue culture experiments.

To utilize this assay system accurately, several technical considerations must be addressed. First, the tissue culture media should be made without serum or phenol red to obtain the most sensitive and accurate results. If serum is required, deproteination is suggested prior to obtaining absorbance measurements, and the percentage yield for given circumstances should be determined using known standards. Also, the preparation and sampling of the ·NO-saturated media should be done carefully to avoid any contamination with $O_2$. Finally, the ·NO-containing solutions must be added and mixed quickly in the cuvette to obtain reproducible data. If these technical considerations are addressed, this assay provides a rapid and reproducible method for the detection of ·NO in cell culture media.

In summary, this method was used to measure ·NO in several situations commonly encountered in cell culture experiments. The assay system was capable of accurately detecting ·NO in media with a limit of detection of 1 $\mu$M. Also, the release of ·NO from a NONOate drug was easily detected. Nitrite levels could be detected in the same reactions with a limit of detection of 300 nM. In addition, $NO_3^-$ could be measured with a limit of detection of approximately 0.5 $\mu$M. These data demonstrate the utility of this assay system for the spectrophotometric determination of ·NO concentrations commonly employed in cell culture models.

## ACKNOWLEDGMENTS

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