Superoxide-dependent consumption of nitric oxide in biological media may confound *in vitro* experiments

Robert G. KEYNES, Charmaine GRIFFITHS and John GARTHWAITE[1]
Wolfson Institute for Biomedical Research, University College London, Cruciform Building, Gower Street, London WC1E 6BT, U.K.

NO functions ubiquitously as a biological messenger but has also been implicated in various pathologies, a role supported by many reports that exogenous or endogenous NO can kill cells in tissue culture. In the course of experiments aimed at examining the toxicity of exogenous NO towards cultured cells, we found that most of the NO delivered using a NONOate (diazeniumdiolate) donor was removed by reaction with the tissue-culture medium. Two NO-consuming ingredients were identified: Hepes buffer and, under laboratory lighting, the vitamin riboflavin. In each case, the loss of NO was reversed by the addition of superoxide dismutase. The effect of Hepes was observed over a range of NONOate concentrations (producing up to 1 μM NO). Furthermore, from measurements of soluble guanylate cyclase activity, Hepes-dependent NO consumption remained significant at the low nanomolar NO concentrations relevant to physiological NO

signalling. The combination of Hepes and riboflavin (in the light) acted synergistically to the extent that, instead of a steady-state concentration of about 1 μM being generated, NO was undetectable (< 10 nM). Again, the consumption could be inhibited by superoxide dismutase. A scheme is proposed whereby a 'vicious cycle' of superoxide radical ($O_2^{\bullet-}$) formation occurs as a result of oxidation of Hepes to its radical species, fuelled by the subsequent reaction of $O_2^{\bullet-}$ with NO to form peroxynitrite ($ONOO^-$). The inadvertent production of $ONOO^-$ and other reactive species in biological media, or the associated loss of NO, may contribute to the adverse effects, or otherwise, of NO *in vitro*.

Key words: cytotoxicity, Hepes, peroxynitrite, riboflavin, soluble guanylate cyclase.

INTRODUCTION

NO functions as a physiological cell-to-cell messenger throughout the body but can also be toxic to cells. As such, NO has been implicated in the pathogenesis of many different disorders that affect humans, such as atherosclerosis, septic shock, renal failure and neurodegenerative disease [1]. The best-understood pathway for physiological NO signal transduction is binding to the NO receptor, soluble guanylate cyclase (sGC), and the subsequent generation of cGMP [2,3]. NO toxicity is usually attributed to other mechanisms, including direct inhibition of mitochondrial respiration and reaction with superoxide radicals ($O_2^{\bullet-}$) to give peroxynitrite ($ONOO^-$), which can result in covalent modifications to proteins and the generation of reactive radicals, such as nitrogen dioxide, carbonate and hydroxyl radicals [4].

Much of the impetus for considering NO as a toxic substance derives from *in vitro* experiments performed using tissue-culture techniques in which it can readily be demonstrated that expression of the inducible isoform of NO synthase or addition of NO-releasing chemicals can cause the death of many cell types over periods of hours or days. In the case of brain neurons, the Ca^{2+}/calmodulin-dependent neuronal NO synthase can apparently also become sufficiently active for periods long enough to contribute to neurodegeneration [5]. However, some authors find no link between glutamate-mediated neuronal death and NO [6–8]. It has been postulated that variable expression of NO synthase in different tissue-culture models, timing and intensity of the insult may all play a role in generating these conflicting results [9]. Similar conflicts exist in other areas of biology.

One of the key criteria to be met in order to sustain the hypothesis that NO is an endogenous toxin is that exogenous NO, at concentrations relevant to those produced endogenously,

should be able to elicit cell death. Unfortunately, there is little knowledge of what constitutes a pathological concentration of NO when it is generated endogenously *in vivo*. Nevertheless, various NO-releasing chemicals have been used in various concentrations to investigate the toxic potential of NO [10–12]. Historically, the most commonly used ones include sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine ('SNAP') and 3-morpholinosydnonimine ('SIN-1'). However, the primary species produced by these donors may not be NO. For example, 3-morpholinosydnonimine decomposition is associated with $O_2^{\bullet-}$ release and $ONOO^-$ generation [13]. Furthermore, reactive nitrogen species that act as nitrosonium ion (NO^+) donors have been implicated in the actions of *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside [14,15]. The advent of the NONOates (diazeniumdiolates), which release authentic NO with predictable kinetics, has simplified matters. The rate of NO release from the NONOates is a function of pH, temperature and the identity of the nucleophile carrier. In aqueous aerobic solutions NO is inactivated by reaction with O_2 in a process termed autoxidation [16]. Knowing the kinetics of NO release and inactivation, the resulting NO concentrations in biological buffers can be predicted [17].

In the course of some studies originally aimed at determining the toxicity of exogenous NO towards brain neurons maintained in primary culture, we decided to use the NONOate (*z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO), which has a long half-life (20 h). In simple buffer solutions, the NO generated by DETA/NO accumulates to a steady-state concentration when the rate of autoxidation is equal to the rate of NO release, making it potentially useful for studies of the cytotoxicity of NO at defined concentrations and over long time periods in tissue culture. On measuring the

concentrations attained in the tissue-culture media itself, how-ever, we found a large and unexpected difference compared with predictions. Here we report on the origin of the discrepancy and the potentially serious implications this has for working with NO in tissue-culture media, or even simple buffer solutions.

MATERIALS AND METHODS

Measurement of NO

NO concentrations were measured in 1 ml samples of tissue-culture medium, Tris (25 mM) or Hepes (25 mM), all at pH 7.4, using an electrochemical probe (ISO-NO; World Precision Instruments, Stevenage, Herts., U.K.) placed in a sealed vessel maintained at 37 °C. The tissue-culture media tested (all from Gibco Life Technologies, Paisley, U.K.) were: minimal essential medium (MEM) buffered with Hepes (catalogue number MEM 10012) or $NaHCO_3$ (MEM 31095), Neurobasal medium (21103), Dulbecco's modified Eagle's medium (DMEM 41965) and RPMI-1640 (31870). The NONOate DETA/NO (Alexis Bio-chemicals, Nottingham, U.K.) was made freshly each day in 10 mM NaOH and kept on ice until use. Where indicated, superoxide dismutase (SOD; Sigma-Aldrich, Poole, U.K.) was added to give an activity of 1000 units/ml [4]. The metal chelators and radical scavengers diethylenetriaminepenta-acetate (DTPA), neocuproine, cuprizone, uric acid and deferoxamine (all from Sigma-Aldrich) were added from at least 100-fold-concentrated stock solutions to Tris or Hepes buffer and, where appropriate, the pH was re-adjusted to 7.4. Vitamins (100 × MEM vitamin mix; Gibco Life Technologies) or riboflavin (0.1–0.2 mg/l; Sigma-Aldrich) were added to Tris or Hepes buffer. In ex-periments designed to examine the effect of laboratory lighting, a lightproof cardboard box covering the entire apparatus, whose lid could be opened and closed as desired, was used. Upon opening the lid the apparatus was exposed, at a distance of approx. 2 m, to standard laboratory lighting conditions (four fluorescent strip bulbs). NO traces were collected using DUO.18 software version 1.1 (World Precision Instruments) and sub-sequently analysed using Microcal™ Origin™ software. All experiments were undertaken at least three times and statistical differences analysed using one-way or multiple-comparison ANOVA with Dunnett's *post-hoc* test; P values of < 0.05 were regarded as significant.

Determination of sGC activity

Enzyme activity was measured at 37 °C in 1.5 ml Eppendorf tubes. Purified sGC from bovine lung (Alexis) was diluted in buffer (pH 7.4) containing Tris (10 mM), dithiothreitol (1 mM) and BSA (0.05 %) to give a stock concentration of 5 μg/ml and stored on ice. DETA/NO (3 μM) was pre-equilibrated at 37 °C for 3 h in Tris or Hepes (25 mM), $MgCl_2$ (3 mM) and BSA (0.05 %) with or without the addition of SOD (1000 units/ml). Substrate (GTP, 1 mM) was added to the reaction 10 s before the addition of sGC to give a final concentration of 0.05 μg/ml. Aliquots of the reaction mix were removed after 10 min, inacti-vated by addition to boiling buffer (50 mM Tris/3 mM EDTA) and cGMP levels measured by radioimmunoassay. Results are given as means ± S.E.M. and were analysed by one-way ANOVA with Dunnett's *post-hoc* test.

RESULTS

Inactivation of NO in tissue-culture medium

Addition of the slow-releasing NO donor DETA/NO (300 μM) to simple Tris buffer (25 mM, pH 7.4) resulted in a build up of
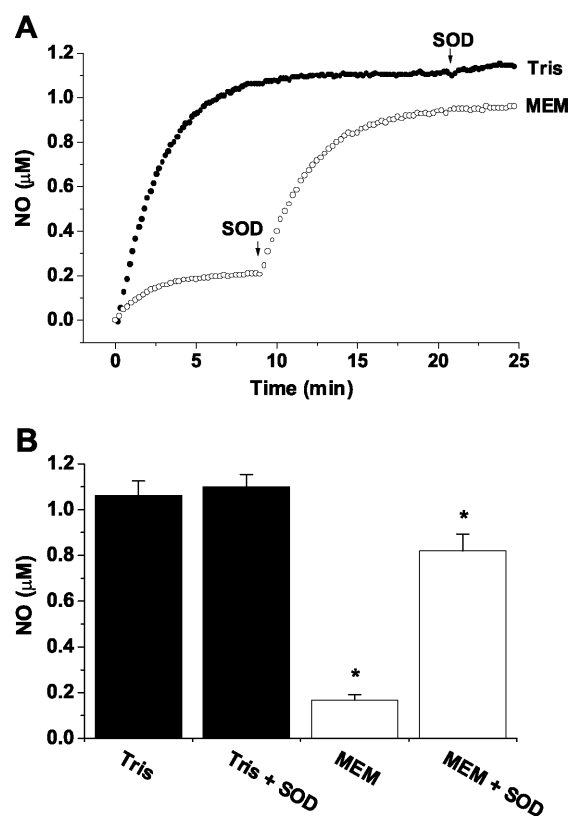
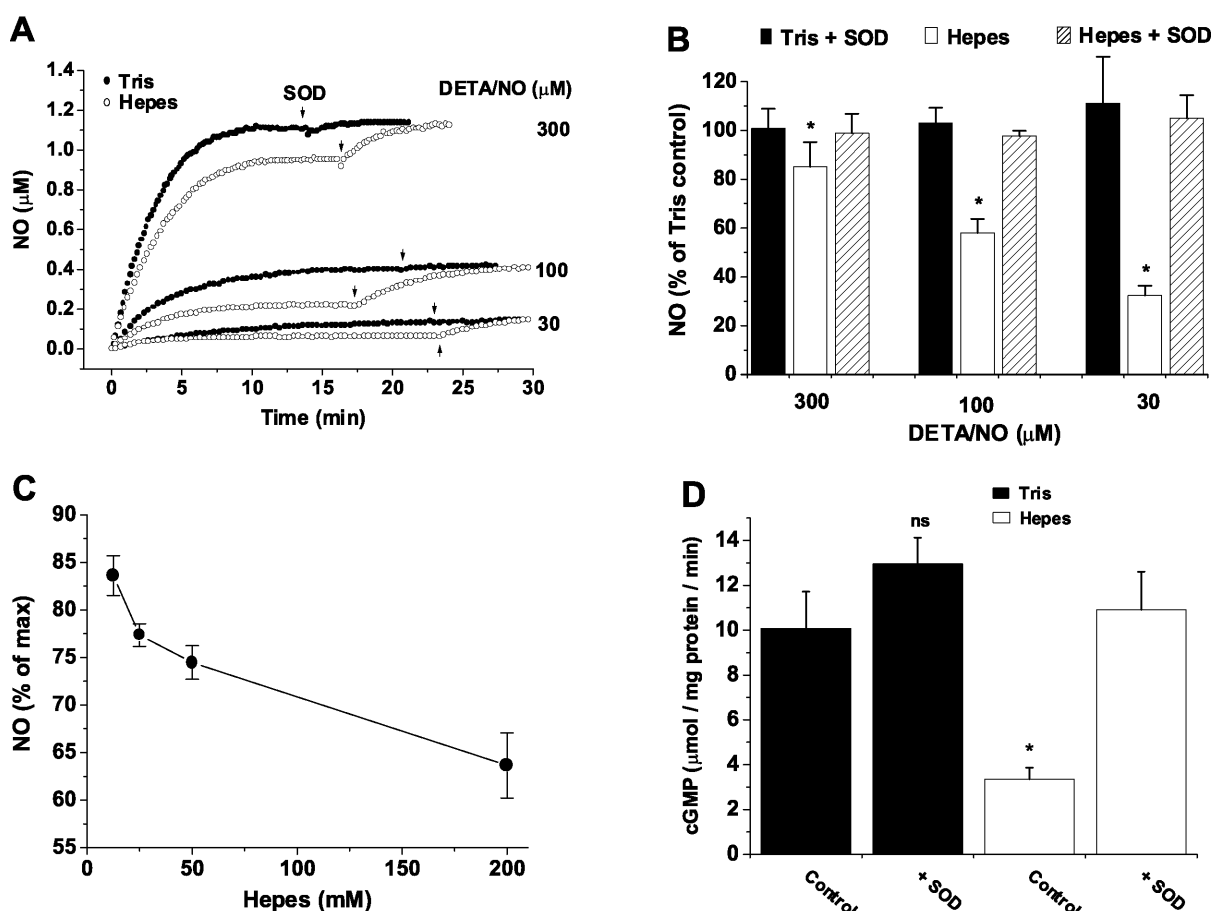**Figure 1   Inactivation of NO by MEM**

(**A**) Representative traces of NO accumulation upon addition of DETA/NO (300 μM) to Tris buffer (●) or MEM (○); subsequent additions of SOD (1000 units/ml) are marked by arrows. (**B**) Summary data from three independent experiments; *$P < 0.05$ versus the control NO concentration in Tris buffer.

NO, which reached a plateau of around 1 μM after about 10 min (Figure 1) as predicted by the kinetics of autoxidation [17]. Subsequent addition of SOD (1000 units/ml) had no significant effect. When a similar test was performed in MEM (Gibco Life Technologies catalogue number 10012), which forms the basis of many different cell-culture media, the NO plateau was much lower, about 0.2 μM. Addition of SOD then resulted in an increase in the NO concentration to a value (about 0.8 μM) close to, but still significantly less than, that found in Tris buffer. These findings suggest that, in Tris, NO is being consumed largely by autoxidation whereas, in MEM, reaction with $O_2^{\cdot-}$ is mainly responsible.

Importance of the buffer

Broadly, MEM contains four main ingredients: inorganic salts, amino acids, vitamins and other compounds (D-glucose, Hepes, Phenol Red and sodium succinate).

Initially, Hepes and Tris buffers (25 mM) were compared (Figures 2A and 2B). In response to 300 μM DETA/NO, the mean steady-state NO concentration attained in Hepes was consistently less than in Tris (1.0 ± 0.12 versus 1.19 ± 0.09 μM; $n = 3$). Addition of SOD to Hepes restored NO to the concen-tration found in Tris (1.17 ± 0.09 μM). As the DETA/NO concentration was reduced to 100 μM and then to 30 μM, a greater proportion of the NO was consumed in the Hepes buffer

A

B

C

D

Figure 2   Inactivation of NO by Hepes buffer

(**A**) Representative traces of NO accumulation upon addition of DETA/NO (30–300 μM) to Tris (●) or Hepes (○); subsequent additions of SOD (1000 units/ml) are marked by arrows. (**B**) Summary data from three independent experiments; *$P < 0.05$ versus the control NO concentration found in Tris buffer. Key: solid bars, Tris+SOD; open bars, Hepes; hatched bars, Hepes+SOD. (**C**) Summary data of steady-state NO concentrations generated from DETA/NO (300 μM) in different Hepes concentrations (12.5–200 mM). Data are expressed as a percentage of the maximum NO signal (approx. 1 μM in all cases) generated following further addition of SOD (1000 units/ml). (**D**) sCG was stimulated by addition to a reaction mix pre-equilibrated with DETA/NO (3 μM) and buffered with either Tris or Hepes in the presence or absence of SOD as indicated. Data are means ± S.E.M. from six independent experiments; *$P < 0.05$ versus Tris control; ns, not significant versus Tris control.

(reversible by SOD) such that, at 30 μM DETA/NO, the NO plateau was reduced by about 75 % (Figure 2B). The difference in absolute NO concentration at 30 μM DETA/NO, however, was less (0.13 μM) than at 100 or 300 μM (both 0.2 μM). With a fixed DETA/NO concentration (300 μM) the consumption of NO increased with increasing Hepes concentrations over the range 12.5–200 mM (Figure 2C).

The influence of Hepes at DETA/NO concentrations lower than 30 μM could not be measured easily because the resulting NO concentration approached the detection limit of the electrochemical probe (10 nM). To determine whether Hepes-dependent NO consumption occurred at NO concentrations relevant to physiological signalling, the activity of the NO receptor enzyme, sGC, was measured. Activation of sGC by NO catalyses the production of cGMP from GTP, and occurs at low nanomolar NO concentrations [18,19]. In Tris-buffered reaction mix, DETA/NO (3 μM) stimulated cGMP accumulation from sGC at a rate of 10.1 ± 1.6 μmol/min per mg of protein and there was no significant change in the rate in the presence of SOD (Figure 2D). In contrast, in Hepes-buffered reaction mix, sGC activity was reduced by approx. 65 % compared with that seen in Tris, and the control activity could be restored by SOD.

The inhibition of NO consumption in Hepes buffer by SOD suggests an involvement of $O_2^{\bullet-}$, which reacts extremely rapidly with NO to form $ONOO^-$ [4]. A possible source of $O_2^{\bullet-}$ would be the autoxidation of contaminating metal ions, particularly iron and copper ions [4]. To examine this possibility, DETA/NO (100 μM) was added to Tris or Hepes buffer (25 mM) containing the Cu^+ chelator neocuprione (100 μM), the Cu^{2+} chelator cuprizone (100 μM), or the iron chelators DTPA (100 μM) or deferoxamine (300 μM). Addition of neocuproine, cuprizone or DTPA had no effect on NO levels in Hepes (all remained significantly lower than in Tris controls), and subsequent additions of SOD restored NO to the concentrations found in Tris (Figure 3). In the presence of deferoxamine, however, NO levels attained in Hepes buffer were the same as those formed in Tris buffer, and subsequent additions of SOD had no further effect. A similar result was obtained in the presence of the $ONOO^-$ scavenger uric acid (300 μM; Figure 3).
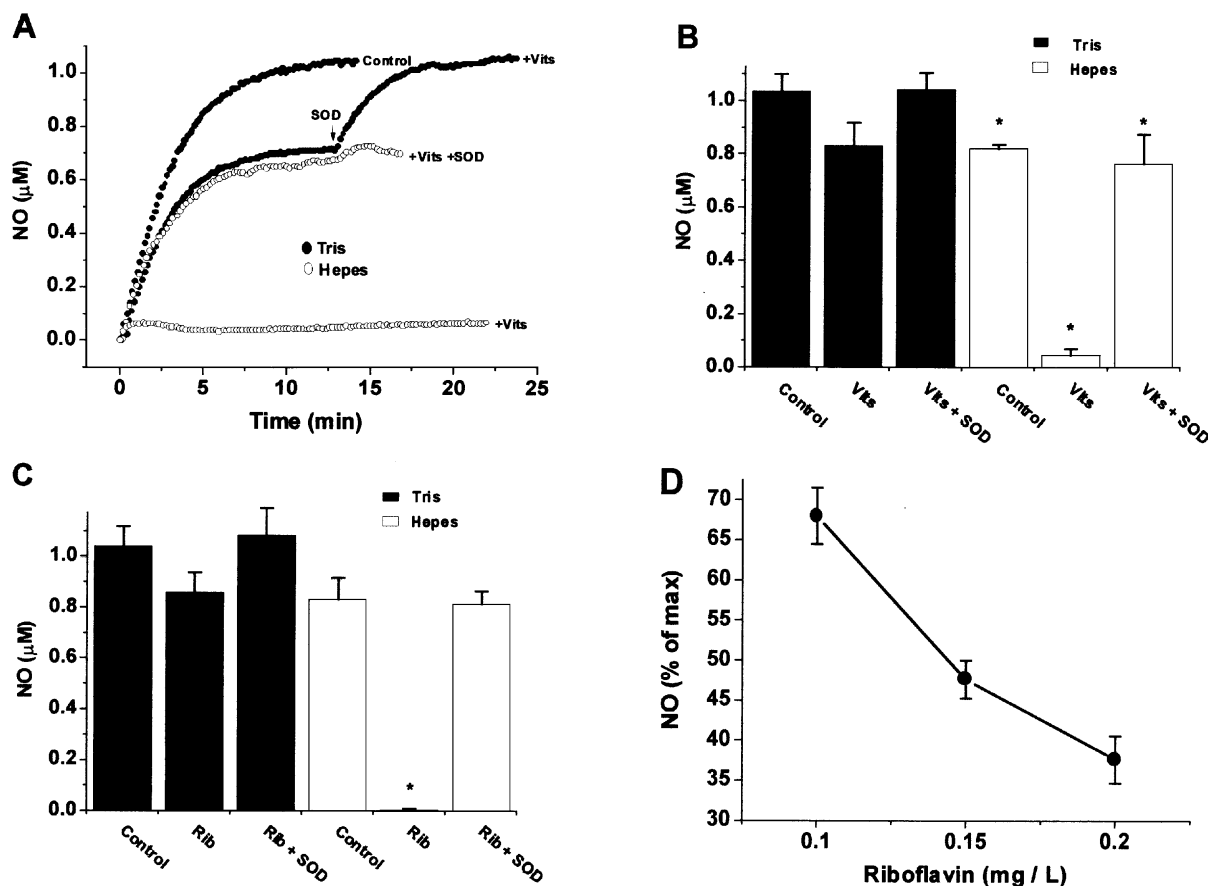
Effect of vitamins

The consumption of NO in Hepes buffer alone cannot explain the extent of NO inactivation observed when 300 μM DETA/NO
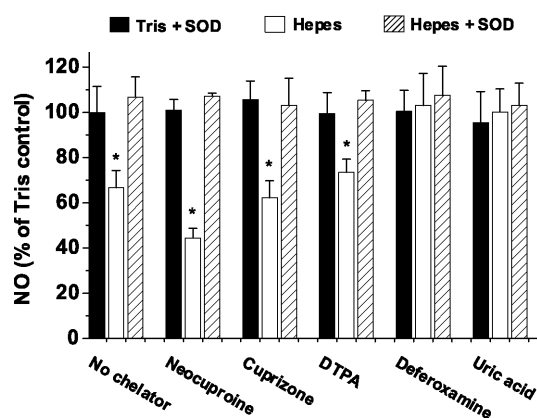
Figure 3   Effect of metal chelators and uric acid on NO concentrations

DETA/NO (100 μM) was added to Tris or Hepes (25 mM) in the presence of DTPA, neocuproine, cuprizone (all at 100 μM), deferoxamine (300 μM) or uric acid (100 μM). Once a steady-state NO concentration was achieved, SOD (1000 units/ml) was added and the recording continued until the NO concentration was again steady. Data are from 3–6 independent experiments and show the test NO concentrations expressed as a percentage of the control NO concentration found in Tris buffer (approx. 300 nM). *$P < 0.05$ versus control.
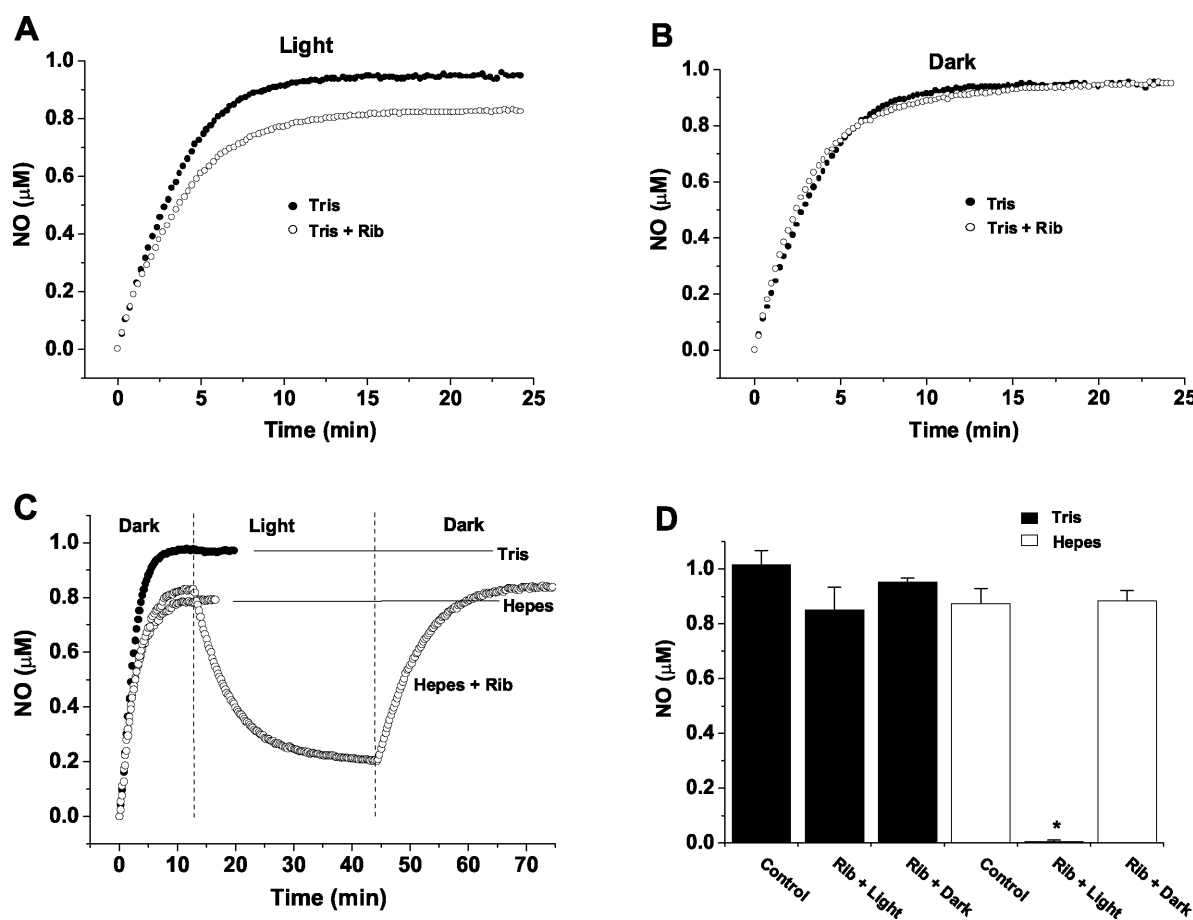
was added to MEM. In pursuit of the other ingredients responsible, the mix of vitamins present in MEM was examined (Figure 4A). In Tris buffer, the vitamins decreased the maximum level of NO achieved by 300 μM DETA/NO by about 0.2 μM and NO levels were restored by the addition of SOD (Figures 4A and 4B). With the vitamins in Hepes buffer, however, the NO signal was almost abolished. SOD restored the NO concentration under these conditions to about 80% of the control.

Of the components of the vitamin mix present (D-calcium pantothenate, choline chloride, folic acid, i-inositol, nicotinamide, pyroxidal HCl, riboflavin, thiamine HCl), riboflavin was considered a likely candidate because it is capable of generating $O_2^{\cdot-}$ [20]. When DETA/NO (300 μM) was added to Tris in the presence of riboflavin at the concentration found in MEM (0.1 mg/l; Figure 4C) the maximum concentration of NO was reduced by 0.18 ± 0.03 μM ($n = 3$). SOD restored NO to the control level (1.1 ± 0.1 μM). Increasing the riboflavin concentration led to an approximately proportional increase in NO consumption that was, in all cases, inhibited by SOD (Figure 4D). When examined in Hepes buffer, the NO concentration reached in the presence of riboflavin was below detection levels, consistent with the very low concentration found in the vitamin

Figure 4   Inactivation of NO by vitamins

(**A**) Representative traces of NO accumulation upon addition of DETA/NO (300 μM) to Tris ($\bullet$) or Hepes ($\bigcirc$) in the presence of vitamins ($+$Vits). SOD (1000 units/ml) was added as indicated by the arrow or, with Hepes, was present throughout. The control trace (labelled Control) contained no additions. (**B**) Summary data from three independent experiments; *$P < 0.05$ versus Tris control. (**C**) Summary data from similar experiments ($n = 3$–4) using riboflavin (Rib, 0.1 mg/l) instead of the mixture of vitamins; *$P < 0.05$ versus Tris control. (**D**) Steady-state NO concentrations generated from DETA/NO (300 μM) in different riboflavin concentrations (0.1–0.2 mg/l) in Tris buffer. Data ($n = 3$–4) are expressed as a percentage of the maximum NO signal (approx. 1 μM in all cases) generated following a further addition of SOD (1000 units/ml).

Figure 5   Effects of light on the consumption of NO in Tris or Hepes in the presence or absence of riboflavin

(**A**) and (**B**) show representative traces of NO accumulation upon addition of DETA/NO (300 μM) to Tris buffer (●) or Tris + riboflavin (Rib, 0.1 mg/l; ○) in the light (**A**) or dark (**B**). (**C**) Trace showing the accumulation of NO in Tris buffer alone (●) or Hepes buffer in the absence or presence of riboflavin as indicated (○). The apparatus was initially kept in the dark but was exposed to light during the interval indicated. (**D**) Summary data from 3–6 experiments; *$P < 0.05$ versus Tris control.

Table 1   Steady-state NO concentrations in different tissue-culture media

The data ($n = 3$–10) represent the mean steady-state NO concentration formed following addition of 100 μM DETA/NO to various tissue-culture media in the light or dark.

Medium	Gibco Life Technologies catalogue number	Hepes (mM)	Riboflavin (mg/l)	NO (μM) In the dark	In the light
MEM	11012	25	0.1	0.14 ± 0.01*	< 0.01*
MEM	31095	–	0.1	0.29 ± 0.03	0.01 ± 0.005*
RPMI-1640	31870	–	0.2	0.25 ± 0.03	< 0.01*
DMEM	41965	–	0.4	0.24 ± 0.02	< 0.01*
Neurobasal	21103	10	0.4	0.24 ± 0.02	< 0.01*

* $P < 0.05$ versus Tris control (0.28 ± 0.01 μM).

mix. Again, SOD restored the NO concentration to approx. 80 % of the control.

Influence of light

Riboflavin is photosensitive and its presence in culture media has been reported to contribute to phototoxic effects [21]. To investigate whether the consumption of NO was due to photo-

sensitization of riboflavin the apparatus was enclosed in a light-proof box, the lid of which could be opened or closed as desired. As before, in the light (Figures 5A and 5D), the presence of riboflavin reduced the maximum NO concentration achieved with DETA/NO (300 μM) in Tris buffer by about 20 %. In the dark, no effect of riboflavin in Tris buffer was observed (Figures 5B and 5D). In Hepes buffer in the dark, riboflavin did not decrease the NO concentration further (Figures 5C and 5D). On

opening the box, however, the NO concentration fell by about 0.5 μM within 10 min and then continued to fall more gradually (Figures 5C and 5D). On closing the box, NO abruptly increased and, within 20 min, regained the concentration previously achieved. In Hepes buffer without riboflavin, light did not alter the NO concentration (Figure 5C).

NO consumption by different tissue-culture media

Several commonly used tissue-culture media containing various concentrations of Hepes and riboflavin (MEM 11012 and 31095, Neurobasal medium, DMEM and RPMI-1640) were tested for their ability to consume NO released by DETA/NO (100 μM) in the light or dark. In the dark, only MEM 11012 (which contains 25 mM Hepes) was effective, whereas in the light NO concentrations attained in all the media tested were below the detection limit (Table 1).

DISCUSSION

The present study shows that, over a range of concentrations covering those encountered *in vitro* following constitutive or inducible NO synthase activity [22–24], NO can be consumed as a result of the presence of two common constituents of cell-culture media: riboflavin and Hepes buffer. The combination of the two ingredients under normal laboratory lighting conditions leads to a greatly amplified quench of the NO signal. Moreover, the sensitivity of the quench to SOD in all cases implies the participation of $O_2^{\bullet-}$ and subsequent generation of $ONOO^-$, which can be a powerful oxidant and cytotoxin [4].

Hepes is a very commonly used biological buffer and the finding that NO was consumed by this buffer in a SOD-sensitive manner implies a continuous formation of $O_2^{\bullet-}$ that interacts with NO. Moreover, the effect remained marked even at the NO concentrations relevant to sGC activation, which lie in the low nanomolar range [18,19]. Consequently, Hepes buffer could introduce artifacts associated with $O_2^{\bullet-}$ formation over a wide range of biologically relevant NO concentrations.

As no such effect was observed in Tris buffer, the $O_2^{\bullet-}$ generation is not simply due to contaminants, such as metal ions. A likely mechanism comes from the results of Kirsch et al. [25] showing that $O_2^{\bullet-}$ was generated upon addition of $ONOO^-$ to Hepes. It was suggested that $ONOO^-$ (or any strong oxidant derived from it) oxidizes the piperazine ring of Hepes. Following deprotonation, reaction with O_2 leads to the formation of $O_2^{\bullet-}$ which then goes on to form H_2O_2 via dismutation. In our experiments, the inhibition of the Hepes-dependent NO consumption by SOD and by the $ONOO^-$ scavenger uric acid would be consistent with this reaction being responsible.

For such a scheme to explain the results, there would need to be a source of $O_2^{\bullet-}$ to initiate $ONOO^-$ formation. One possible source we considered was the autoxidation of trace metals, the most likely candidates being iron and copper. Neither of the copper chelators had a significant effect on the Hepes-dependent NO quench, which could indicate a lack of involvement of copper ions. However, Cu^{2+} can form an active complex with Hepes [26,27] which could render the ion unavailable for chelation. The iron chelators DTPA and deferoxamine gave differing results, with only deferoxamine inhibiting NO consumption. Again these results are difficult to interpret clearly. A lack of effect of DTPA could be explained by the iron–DTPA complex continuing to catalyse oxygen radical generation [28]. Conversely, the effect of deferoxamine may be explained by the ability of this compound to scavenge free radicals, including $O_2^{\bullet-}$ [29], rather than sequester iron. Thus, whereas metal con-
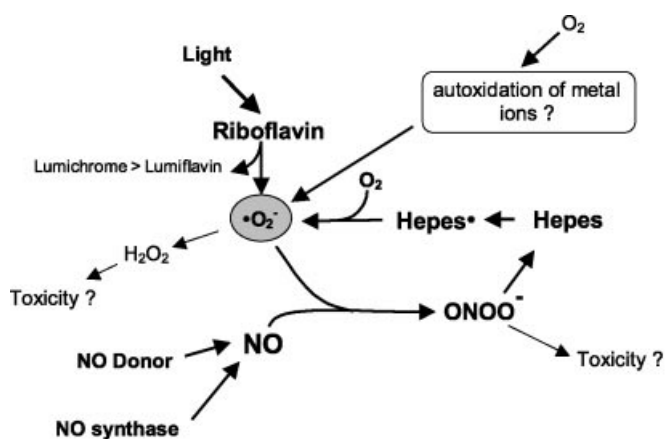
Figure 6   Possible interactions between NO, Hepes, riboflavin and light

$O_2^{\bullet-}$, initially generated by riboflavin and/or from the presence of metal contaminants, reacts with NO to form $ONOO^-$, which then oxidizes Hepes buffer forming the Hepes radical. This radical fuels further $O_2^{\bullet-}$ generation from molecular O_2. By this means Hepes sustains $O_2^{\bullet-}$ generation and the subsequent consumption of NO. The products H_2O_2 and/or $ONOO^-$ formed artifactually in this way could exert biological actions, including cytotoxicity.

taminants are still good candidates, unambiguous evidence for their involvement is difficult to obtain. Nevertheless, it is likely that there is a background formation of $O_2^{\bullet-}$ that would react with NO at a diffusion-controlled rate [30] to form $ONOO^-$ which, by oxidizing Hepes, would lead to more $O_2^{\bullet-}$, more $ONOO^-$, and so on (Figure 6). Adding further complications, the reaction of $ONOO^-$ with Hepes buffer may also result in the formation of NO donors with unknown additional reactivity [31]. Obviously, it would have been desirable to have measured the rates of formation of $O_2^{\bullet-}$ and/or $ONOO^-$ directly but the usual methods for doing so result in the consumption of these species, which would have the effect of inhibiting the reaction (compare the effects of SOD and uric acid).

The other ingredient found to be responsible for NO consumption was riboflavin, when exposed to laboratory lighting. The generation of $O_2^{\bullet-}$ by illuminated riboflavin is well documented [20,32]. Indeed, riboflavin is used to produce $O_2^{\bullet-}$ for determining the efficiency of $O_2^{\bullet-}$ scavengers [33]. However $O_2^{\bullet-}$ may not be the only species involved in riboflavin photo-sensitization. Reactions involving riboflavin are complex, and light-sensitized riboflavin may also produce singlet oxygen ($^1O_2$), which may further react with organic compounds [34]. Deleterious effects of riboflavin on mammalian cells in culture have been known for many years and have been ascribed to various mechanisms. Work by Griffin et al. [35] suggested that just 3 h exposure of riboflavin-containing medium to bench-top levels of cold white fluorescent light could produce significant toxicity in a leukaemic cell line. The principal mechanism of phototoxicity is thought to involve the generation of $^1O_2$ and probable subsequent oxidation of the amino acids tryptophan and tyrosine [35,36]. It has since been demonstrated that fluorescent light may cause a build up of toxic H_2O_2 levels in DMEM or RPMI-1640 medium, both of which contain riboflavin [21,37]. In the present experiments, the SOD-sensitive quench of the NO signal produced by riboflavin alone (in the light) in Tris buffer increased in proportion with riboflavin concentration, as expected from a simple photosensitive generation of $O_2^{\bullet-}$. At the concentrations found in MEM (11012) the NO quench exerted by riboflavin (0.1 mg/l) was similar to that produced by Hepes (25 mM),

implying similar rates of NO consumption (about 20 % of the rate of release from the donor) under these two conditions. The quench of NO down to undetectable levels observed with the combination of Hepes and riboflavin (in the light) implies a much greater than additive effect. This may be explained by there being disproportionately more $O_2^{\bullet-}$ ultimately available as a result of its additional formation following Hepes oxidation (Figure 6).

In comparison with the simple combination of Hepes and riboflavin in the light, complete MEM containing the same concentrations of these ingredients quenched NO less (80 % versus 100 % with 300 μM DETA/NO), suggesting that other components of MEM may act as radical scavengers or suppress radical generation. A likely contributor here is the pH indicator Phenol Red, which quenches photoexcited riboflavin [38]. At a lower DETA/NO concentration normally generating about 300 nM NO, however, light exposure effectively eliminated the NO signal in Hepes-containing MEM (Table 1). Under the same conditions, other common media (lacking Hepes) behaved similarly, suggesting that, in the light, the riboflavin content alone is sufficient to consume all the available NO when released at this rate (approx. 100 nM/min [19]).

Overall, the results point to potential sources of artifact that may have unwittingly complicated many *in vitro* studies. Since the inclusion of Hepes buffer is an option in most standard media formulations (MEM, RPMI-1640, DMEM) and is inherent in others (Neurobasal medium) its presence is not always published. Likewise, as the frequency and intensity of light exposure is rarely mentioned, it must be assumed that the potential problems associated with NO being present with either Hepes buffer or light-exposed riboflavin, despite being deducible from information in the literature, are not generally appreciated. The exaggerated effect of the two together multiplies the potential problems. The findings with Hepes are likely to apply to other 'Good' buffers equipped with a piperazine ring, such as Mops, Pipes and EPPS [4-(2-hydroxyethyl)-1-piperazinepropane-sulphonic acid] [25,39], and we suggest that they should be only used with caution, particularly in experiments with NO.

In the absence of the requisite information, the extent to which media factors might have generated spurious results in published studies cannot be evaluated. Blockade of cell death by NO synthase inhibitors and SOD is typically taken to indicate mediation by endogenously generated ONOO⁻. It now becomes plausible that inadvertent reactions between NO and constituents of the bathing medium are responsible. Interestingly, the cytotoxicity of dopamine has recently been attributed to artifactual oxidative reactions occurring in tissue-culture media [40], although the ingredients responsible were not identified. Alternatively, the consumption of NO by the medium may render NO non-toxic, depending on its fate therein. Whether NO appears to participate in a toxic (or other) process in cultured cells or not may depend on a simple experimental variable, such as the choice of buffer or the degree of exposure to light.

REFERENCES

1   Mayer, B. (2000) Nitric Oxide, Springer-Verlag, Berlin
2   Ignarro, L. J. (1991) Signal transduction mechanisms involving nitric oxide. Biochem. Pharmacol. **41**, 485–490
3   Waldman, S. A. and Murad, F. (1987) Cyclic GMP synthesis and function. Pharmacol. Rev. **39**, 163–196
4   Beckman, J. S. and Koppenol, W. H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol. **271**, C1424–C1437
5   Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S. and Snyder, S. H. (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc. Natl. Acad. Sci. U.S.A. **88**, 6368–6371
6   Demerle-Pallardy, C., Lonchampt, M. O., Chabrier, P. E. and Braquet, P. (1991) Absence of implication of L-arginine/nitric oxide pathway on neuronal cell injury induced by L-glutamate or hypoxia. Biochem. Biophys. Res. Commun. **181**, 456–464
7   Hewett, S. J., Corbett, J. A., McDaniel, M. L. and Choi, D. W. (1993) Inhibition of nitric oxide formation does not protect murine cortical cell cultures from N-methyl-D-aspartate neurotoxicity. Brain Res. **625**, 337–341
8   Pauwels, P. J. and Leysen, J. E. (1992) Blockade of nitric oxide formation does not prevent glutamate-induced neurotoxicity in neuronal cultures from rat hippocampus. Neurosci. Lett. **143**, 27–30
9   Aizenman, E., Brimecombe, J. C., Potthoff, W. K. and Rosenberg, P. A. (1998) Why is the role of nitric oxide in NMDA receptor function and dysfunction so controversial? Progr. Brain Res. **118**, 53–71
10   Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. and Lipton, S. A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. U.S.A. **92**, 7162–7166
11   Brorson, J. R., Schumacker, P. T. and Zhang, H. (1999) Nitric oxide acutely inhibits neuronal energy production. J. Neurosci. **19**, 147–158
12   Dawson, V. L., Kizushi, V. M., Huang, P. L., Snyder, S. H. and Dawson, T. M. (1996) Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J. Neurosci. **16**, 2479–2487
13   Feelisch, M., Ostrowski, J. and Noack, E. (1989) On the mechanism of NO release from sydnonimines. J. Cardiovasc. Pharmacol. **14** (suppl. 11), S13–S22
14   Butler, A. R., Flitney, F. W. and Williams, D. L. (1995) NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. Trends Pharmacol. Sci. **16**, 18–22
15   Lipton, S. A. and Stamler, J. S. (1994) Actions of redox-related congeners of nitric oxide at the NMDA receptor. Neuropharmacology **33**, 1229–1233
16   Ford, P. C., Wink, D. A. and Stanbury, D. M. (1993) Autoxidation kinetics of aqueous nitric oxide. FEBS Lett. **326**, 1–3
17   Schmidt, K., Desch, W., Klatt, P., Kukovetz, W. R. and Mayer, B. (1997) Release of nitric oxide from donors with known half-life: a mathematical model for calculating nitric oxide concentrations in aerobic solutions. Naunyn Schmiedebergs Arch. Pharmacol. **355**, 457–462
18   Bellamy, T. C., Wood, J., Goodwin, D. A. and Garthwaite, J. (2000) Rapid desensitization of the nitric oxide receptor, soluble guanylyl cyclase, underlies diversity of cellular cGMP responses. Proc. Natl. Acad. Sci. U.S.A. **97**, 2928–2933
19   Griffiths, C. and Garthwaite, J. (2001) The shaping of nitric oxide signals by a cellular sink. J. Physiol. (Cambridge) **536**, 855–862
20   Joshi, P. C. (1985) Comparison of the DNA-damaging property of photosensitised riboflavin via singlet oxygen ($^1O_2$) and superoxide radical $O_2^{\bullet-}$ mechanisms. Toxicol. Lett. **26**, 211–217
21   Zigler, Jr, J. S., Lepe-Zuniga, J. L., Vistica, B. and Gery, I. (1985) Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. In Vitro Cell Dev. Biol. **21**, 282–287
22   Bal-Price, A. and Brown, G. C. (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J. Neurosci. **21**, 6480–6491
23   Clementi, E., Brown, G. C., Foxwell, N. and Moncada, S. (1999) On the mechanism by which vascular endothelial cells regulate their oxygen consumption. Proc. Natl. Acad. Sci. U.S.A. **96**, 1559–1562
24   Lewis, R. S., Tamir, S., Tannenbaum, S. R. and Deen, W. M. (1995) Kinetic analysis of the fate of nitric oxide synthesized by macrophages in vitro. J. Biol. Chem. **270**, 29350–29355
25   Kirsch, M., Lomonosova, E. E., Korth, H. G., Sustmann, R. and de Groot, H. (1998) Hydrogen peroxide formation by reaction of peroxynitrite with HEPES and related tertiary amines. Implications for a general mechanism. J. Biol. Chem. **273**, 12716–12724
26   Hegetschweiler, K. and Saltman, P. (1986) Interaction of Copper(II) with N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES). Inorg. Chem. **25**, 107–109
27   Simpson, J. A., Cheeseman, K. H., Smith, S. E. and Dean, R. T. (1988) Free-radical generation by copper ions and hydrogen peroxide. Stimulation by Hepes buffer. Biochem. J. **254**, 519–523
28   Egan, T. J., Barthakur, S. R. and Aisen, P. (1992) Catalysis of the Haber–Weiss reaction by iron-diethylenetriaminepentaacetate. J. Inorg. Biochem. **48**, 241–249
29   Davies, M. J., Donkor, R., Dunster, C. A., Gee, C. A., Jonas, S. and Willson, R. L. (1987) Desferrioxamine (Desferal) and superoxide free radicals. Formation of an enzyme-damaging nitroxide. Biochem. J. **246**, 725–729
30   Koppenol, W. H. (1998) The basic chemistry of nitrogen monoxide and peroxynitrite. Free Radicals Biol. Med. **25**, 385–391

31   Schmidt, K., Pfeiffer, S. and Mayer, B. (1998) Reaction of peroxynitrite with HEPES or MOPS results in the formation of nitric oxide donors. Free Radicals Biol. Med. **24**, 859–862

32   Naseem, I., Ahmad, M. and Hadi, S. M. (1988) Effect of alkylated and intercalated DNA on the generation of superoxide anion by riboflavin. Biosci. Rep. **8**, 485–492

33   Roubaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P. and Zweier, J. L. (1997) Quantitative measurement of superoxide generation using the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide. Anal. Biochem. **247**, 404–411

34   Spikes, J. D. (1989) Photosensitization. In The Science Of Photobiology (Smith, K. C., ed.), pp. 79–110, Plenum Press, New York

35   Griffin, F. M., Ashland, G. and Capizzi, R. L. (1981) Kinetics of phototoxicity of Fischer's medium for L5178Y leukemic cells. Cancer Res. **41**, 2241–2248

36   Stoien, J. D. and Wang, R. J. (1974) Effect of near-ultraviolet and visible light on mammalian cells in culture II. Formation of toxic photoproducts in tissue culture medium by blacklight. Proc. Natl. Acad. Sci. U.S.A. **71**, 3961–3965

37   Wang, R. J. and Nixon, B. R. (1978) Identification of hydrogen peroxide as a photoproduct toxic to human cells in tissue-culture medium irradiated with 'daylight' fluorescent light. In Vitro **14**, 715–722

38   Grzelak, A., Rychlik, B. and Bartosz, G. (2001) Light-dependent generation of reactive oxygen species in cell culture media. Free Radicals Biol. Med. **30**, 1418–1425

39   Grady, J. K., Chasteen, N. D. and Harris, D. C. (1988) Radicals from 'Good's' buffers. Anal. Biochem. **173**, 111–115

40   Clement, M. V., Long, L. H., Ramalingam, J. and Halliwell, B. (2002) The cytotoxicity of dopamine may be an artefact of cell culture. J. Neurochem. **81**, 414–421