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SHORT COMMUNICATIONS

Direct quenching of adriamycin radicals by coenzyme Q_{10} and tetrazolium salts

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Adriamycin radicals (ADM·)* are hypothesized to play a central role in both the tumor and side toxicities induced by the clinically useful antineoplastic agent, adriamycin. These radicals generate spontaneously from adriamycin (ADM) [1], and their formation is catalyzed at many subcellular sites including nuclei [2], mitochondria [3, 4] and endoplasmic reticulum [5–7]. Once formed, they may rearrange and be further metabolized to aglycones [6, 8] and possibly aglycone radicals [9]. Alternatively, they may react with oxygen or attack cellular targets directly. It is via these latter pathways that ADM· are suggested to mediate ADM toxicities [7, 10–12]. Their interaction with O_2 forms "active oxygen species", i.e. O_2^-, ·OH and H_2O_2, which damage membranes [13] and DNA [14, 15]. Evidence supporting the involvement of ADM· mediated active O_2 formation in the cardiotoxicity induced by ADM has been reviewed [16]. Based on these findings, clinical trials of ADM in combination with vitamin E and *n*-acetylcysteine have been initiated.

It has also been suggested that direct ADM· attack on nucleic acids and membranes mediates ADM tumor cell toxicity [7, 12, 17, 18]. Although ADM· formation is stimulated by intact Ehrlich ascites cells [5] and tumor subcellular fractions [7], the only documented direct reactivity of ADM· is with O_2 [1, 6, 7, 13, 14].

We now demonstrate that ADM· can react rapidly with species other than O_2.

Materials and methods

α-Tocopherol free base, *n*-acetylcysteine, ascorbic acid, superoxide dismutase (SOD) Type 1, catalase and nitroblue tetrazolium (NBT) were obtained from the Sigma Chemical Co., St. Louis, MO. Iodonitrotetrazolium chloride (INT), thiazolyl blue tetrazolium bromide (MTT) and 2-(2′-benzothiazolyl)-5-styryl-3-(4′-phthalhydrazidyl) tetrazolium chloride (BSPT) were obtained from the Polysciences Co., Warrington, PA. Promethazine·HCl was obtained from the Knoll Pharmaceutical Co., Whippany, NJ, and ICRF-159 from NCI. Fenamole, coenzyme Q_{10} (IBR20089) and TEMPONE (2,2,6,6-tetramethyl-4-piperidinone-1-oxyl) were gifts from Dr. Charles Bossinger, Dr. Karl Folkers and Dr. Ira Pullman respectively. Both the pharmaceutical and pure doxorubicin forms of adriamycin were supplied by Adria Laboratories, Dublin, OH. All other chemicals were reagent grade.

Adriamycin radicals were generated spontaneously at 25° and measured directly using a Varian X-band E-3 electron paramagnetic spectrometer with a TE_{102} cavity and supercil quartz flat cell for aqueous solutions, as previously described [1]. ADM (0.5 mg in 0.425 or 0.465 ml H_2O) was bubbled with nitrogen for 10 min in a septum-fitted screw cap vial (Pierce Chemical Co., Rockford, IL), at which time radical generation was initiated by anoxic injection of 25 μl of 1 M Tris·HCl, pH 7.9, at 25° (t = 0). After an additional 3 min of N_2 bubbling, quenching activity was tested by anoxic injection of suitable vehicle: H_2O (50 μl), ethanol (ETOH) (10 μl), or dimethyl sulfoxide (DMSO) (50 μl), carrying 50 nmoles or 250 nmoles of each substance tested. The combination was bubbled with N_2 for 2 min, positioned into the quartz cell, and examined for the presence of an ADM· signal for 30 min when H_2O or ETOH was the vehicle and for 40 min when DMSO was the vehicle. The signal heights used for calculating percent quenching are the maximums obtained in the presence and absence of each agent:

$$\% \text{ Quenching} = \left(1 - \frac{\text{ADM· signal height in presence of compound}}{\text{ADM· signal height in presence of vehicle}}\right) \times 100$$

Machine parameters were: field set, 3390 G; scan range, 50 G; microwave power, 5 mW; time constant, 3.0 sec; modulation amplitude, 1.6 G; scan time, 8 min; and receiver gain, 10×10^5. The pH of each sample was determined immediately after each experiment using a model 801A Orion Research digital pH meter.

The approximate ADM radical concentration (assuming 1 spin/molecule) was calculated from TEMPONE standard curves in which spin concentration was plotted against peak-to-peak width^2 × height for each signal.

The anaerobic reduction of nitroblue tetrazolium was studied by monitoring the increase in absorbance at 560 nm [19, 20]. ADM (0.8 mg/0.06 ml H_2O) and NBT (30 nmoles/0.045 ml H_2O) were added to a Type 71Q bubble-forming anaerobic cuvette (Precision Cells, Inc.) and the volume was adjusted to 0.76 ml with H_2O. Anoxic Tris·HCl (40 μmoles/0.04 ml), pH 7.2, 7.8 or 8.0, at 33°, was added to the hollow stopper. The reference cuvette was prepared in exactly the same manner except for the exclusion of NBT. The contents of both cuvettes were bubbled with N_2 for 15 min. The cuvettes were then placed in a DB spectrophotometer and allowed to temperature equilibrate to 33°. The reaction was initiated by tipping both cuvettes simultaneously, thus rinsing the Tris·HCl from the hollow stopper into the body of the cuvette. The pH of each sample was determined immediately after each experiment. An LKB recorder plotted percent trasmission with respect to time. These tracings were replotted as absorbance versus time using a linear absorbance scale. Initial rates were determined as the initial slopes of these tracings.

Results

Adriamycin radicals are generated spontaneously in adriamycin solutions adjusted to physiologic pH [1]. We have used Tris·HCl to initiate ADM· formation. Tris·HCl also maintains pH in this system, which is important since the rate of ADM· formation dramatically increases with rising pH over the range 7.0 to 8.8 [1].

When Tris·HCl was added to ADM solution under

* Abbreviations: ADM·, adriamycin radicals; ADM, adriamycin; SOD, superoxide dismutase; NBT, nitroblue tetrazolium; INT, iodonitrotetrazolium chloride; MTT, thiazolyl blue tetrazolium bromide; BSPT, 2′-benzothiazolyl)-5-styryl-3-(4′-phthalhydrazidyl) tetrazolium chloride; DMSO, dimethyl sulfoxide; TEMPONE, 2,2,6,6-tetramethyl-4-piperidinone-1-oxyl; NBT·, NBT radical; and NBT_{red}, reduced form of NBT.

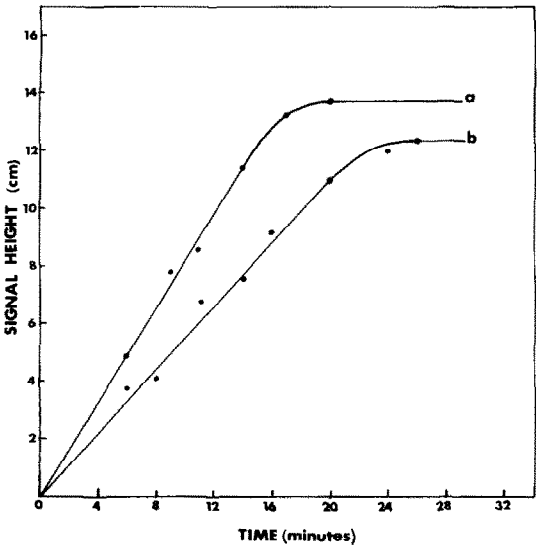

Fig. 1. Kinetics of adriamycin radical formation. Tris·HCl, 1 M, pH 7.9 (25 μl), was added to adriamycin (0.5 mg in 0.425 ml or 0.465 ml H_2O) to initiate radical formation (t = 0). Three minutes later, the following additions were made: (a) none, or 50 μl H_2O, or 10 μl ETOH; (b) 50 μl DMSO. Two minutes later, the samples were positioned and examined for the presence of radicals. Machine settings are described in Materials and Methods.

Table 1. Adriamycin radical quenching activity*

	% Quenching†
Antioxidants	
α-Tocopherol, 0.1 mM, 0.5 mM	0
N-Acetylcysteine, 0.1 mM, 0.5 mM	0
Ascorbic acid, 0.1 mM, 0.5 mM	0
SOD, 1 μg/ml	0
SOD + catalase, 1 μg/ml each	0
Coenzyme Q_{10}, 0.1 mM	100
Tetrazolium salts	
NBT, 0.1 mM	100
MTT, 0.1 mM	100
INT, 0.1 mM	100
BSPT, 0.04 mM	27.0 ± 4.2‡
Miscellaneous	
Fenamole, 0.1 mM, 0.5 mM	0
Promethazine·HCl, 0.1 mM	0
ICRF-159, 0.07 mM	0

* Quenching activity was assayed by the methodology and timing described in Fig. 1 and in Materials and Methods. Coenzyme Q_{10} and α-tocopherol were tested in both ETOH and DMSO vehicle; all other compounds used H_2O vehicle. Each compound was tested at least three times.

$$\dagger\ \%\ \text{Quenching} = \left(1 - \frac{\text{ADM}\cdot\ \text{signal height in presence of compound}}{\text{ADM}\cdot\ \text{signal height in presence of vehicle}}\right) \times 100$$

‡ Standard deviation, N = 3.

anoxic conditions, ADM· concentration increased linearly for approximately 14 min and reached a maximum at 20 min (Fig. 1a). ADM· concentration was maintained at this level for at least 1 hr. Addition of 50 μl H_2O or 10 μl ETOH had no effect on radical formation. DMSO (50 μl) prolonged the time required to reach maximal ADM· concentration to 26 min and decreased radical yield by 10% (Fig. 1b). The small inhibition at high DMSO concentration (~1.4 M) was probably due to an alteration in the formation and/or disappearance rate of ADM· rather than a direct weak quenching effect. The stable free radical TEMPONE was used as a standard to determine ADM· concentration. Using the machine settings described, a signal height of 13.5 cm corresponds to approximately 1.4 μM.

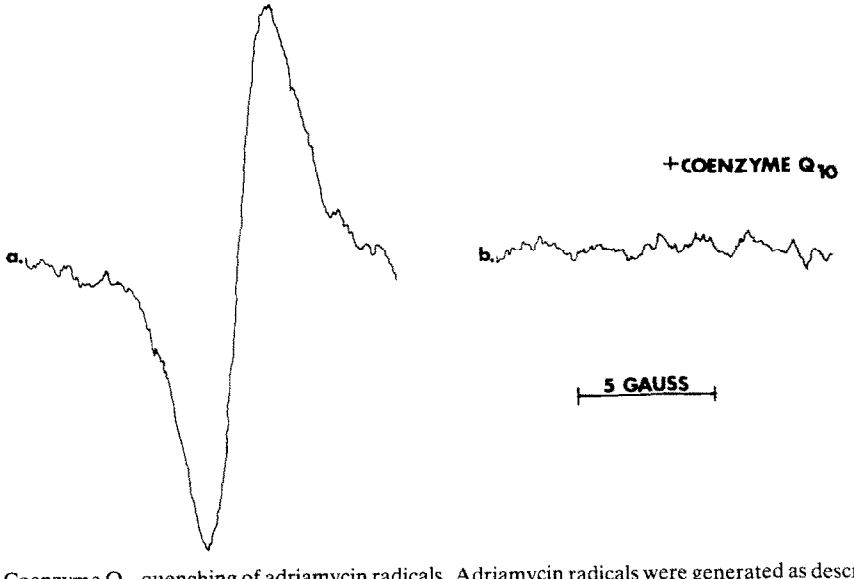

Fig. 2. Coenzyme Q_{10} quenching of adriamycin radicals. Adriamycin radicals were generated as described in Fig. 1. Additions subsequent to Tris·HCl were: (a) 10 μl ETOH; (b) 10 μl ETOH containing 50 nmoles coenzyme Q_{10}. Electron paramagnetic spectra were recorded every 2 min starting at t = 6. The signals shown represent the maximum signals obtained. Machine settings are described in Materials and Methods.

A number of antioxidants and other compounds were assayed for direct ADM· quenching activity. Coenzyme Q_{10} is a direct ADM· quencher which totally eliminated all radicals at 0.1 mM (Fig. 2 and Table 1). Coenzyme Q_{10} quenching was independent of the vehicle employed (ETOH or DMSO). The tetrazolium salts NBT, MTT, INT and BSPT are also direct ADM radical quenchers. At 0.1 mM NBT, MTT and INT quenched 100%. Due to its low H_2O solubility, BSPT was tested at 0.04 mM. Under these conditions the ADM· signal was not totally eliminated. However, the signal did not appear until 24 min and did not reach its final maximum height of 9.8 cm until 42 min. This corresponds to 27% quenching. The antioxidants α-tocopherol, *n*-acetylcysteine and ascorbic acid were inactive even at 0.5 mM. Since a tetrazole ring is the site of electron transfer in the tetrazolium salts, we assayed fenamole, another tetrazole-containing compound. Fenamole, however, was inactive, as were promethazine·HCl and ICRF-159. SOD was inactive both alone and in combination with catalase.

Concomitant with the quenching of ADM· by NBT and MTT, there was a dramatic darkening and color change (towards dark blue) in the red ADM solutions. The reduced counterparts of NBT and MTT, i.e. formazans, are dark blue [20].

We therefore decided to investigate the possibility that the reduction of tetrazolium salts was linked to their ADM· quenching activity. Results are shown in Table 2. NBT was reduced by adriamycin solutions above neutral pH. The increase in NBT reduction rate with increasing pH correlates closely to the increased rate of ADM· production in these samples [1]. Furthermore, at pH 5.8 (before the additions of Tris·HCl), there was no increase in A_{560} and, in the absence of ADM, NBT exhibited no increase in A_{560}.

Table 2. Anaerobic reduction of nitroblue tetrazolium by adriamycin*

	Δ_{560}/min
pH 7.2	0.007 ± 0.003 (4)
pH 7.8	0.046 ± 0.008 (7)
pH 8.0	0.062 ± 0.007 (5)

* Shown is the rate of increase in A_{560} in a sample cuvette containing ADM (1 mg/ml), NBT (37.5 μM) and Tris·HCl (50 mM), versus a reference cuvette prepared identically except for the absence of NBT. The reaction was initiated with Tris·HCl which was added simultaneously to both cuvettes. Each run was in an anaerobic atmosphere at 33°. Each value is the mean ± S.D. (N).

Discussion

Adriamycin radicals are generated spontaneously at physiologic pH. Coenzyme Q_{10} and tetrazolium salts directly interact with these species. In each case, the interaction results in the elimination of ADM·.

In exerting quenching activity, NBT is reduced to formazans (NBT_{red}). That the reduction of NBT is the result of its direct interaction with ADM· is supported by the finding that the rate of NBT reduction increases in proportion to the rate of ADM· production [1]. Thus, NBT quenches ADM· by a direct electron scavenging mechanism, i.e.

$$NBT + ADM\cdot \longrightarrow ADM + NBT\cdot,$$

The NBT radical (NBT·) rapidly disproportionates ($K \sim 3.0 \times 10^9 M^{-1} sec^{-1}$),

$$NBT\cdot + NBT\cdot \longrightarrow NBT + NBT_{red}$$

eliminating all radicals from the system [21].

Since MTT, INT and BSPT are even more facile electron acceptors than NBT [20], it seems likely that these tetrazolium salts also quench ADM· by an electron scavenging mechanism. In this regard, the color change found in association with MTT quenching is consistent with formazan formation. Unlike NBT and MTT, reduction of INT and BSPT would not be expected to produce a dramatic color change in ADM solution since their formazans are orange-red. Furthermore, the delayed but eventual appearance of ADM· subsequent to BSPT quenching is also consistent with an electron scavenger mechanism. In this regard, due to its low concentration, BSPT would be converted to inactive formazan in a relatively short time, after which ADM· would exhibit their usual lifetime.

The mechanism by which coenzyme Q_{10} exerts ADM· quenching activity is at present unknown. It is also unknown whether direct interaction between coenzyme Q_{10} and ADM· takes place in physiologic conditions, i.e. when coenzyme Q_{10} is bound to biological membranes. Nevertheless, coenzyme Q_{10} has been suggested as a possible site of ADM-induced cell damage [22, 23]. Mitochondrial perturbations involving coenzyme Q-dependent enzymes have been reported [23, 24], and coenzyme Q_{10} administration inhibits ADM-induced cardiotoxicity [22, 25, 26]. The direct interaction between ADM· and coenzyme Q_{10} may, in part, be responsible for these effects.

In summary, coenzyme Q_{10} and tetrazolium salts directly quench adriamycin radicals. Unlike "non-quenching" antioxidants, their ability to eliminate ADM· makes them capable of interfering with events mediated by ADM· directly, in addition to those dependent upon O_2. For instance, it has been suggested that covalent binding of ADM to DNA and protein *in vitro* is mediated by ADM· [27]. If this is true, then direct ADM· quenchers should inhibit this binding. Direct adriamycin radical quenchers should be useful tools in experimental situations where ADM· can be monitored [1, 5–7, 14, 17, 18, 27].

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Departments of Neurosurgery Environmental Medicine and Pathology
New York University Medical Center
New York, NY 10016, U.S.A.

DENNIS D. PIETRONIGRO*
JOHN E. MIGNANO
HARRY B. DEMOPOULOS

* Send correspondence to: Dr. Dennis D. Pietronigro, Neurosurgical Research Laboratories, New York University Medical Center, 550 First Ave., New York, NY 10016, U.S.A.

REFERENCES

1. D. D. Pietronigro, J. E. McGinness, M. J. Koren, R. Crippa, M. L. Seligman and H. B. Demopoulos, *Physiol. Chem. Physics* **11**, 405 (1979).
2. N. R. Bachur and M. V. Gee, *J. Pharmac. exp. Ther.* **197**, 681 (1976).
3. J. H. Doroshow, *J. Pharmac. exp. Ther.* **218**, 206 (1981).
4. W. S. Thayer, *Chem. Biol. Interact.* **19**, 265 (1977).
5. S. Sato, M. Iwaizumi, K. Handa and Y. Tamura, *Gann* **68**, 603 (1977).
6. N. R. Bachur, S. L. Gordon and M. V. Gee, *Molec. Pharmac.* **13**, 901 (1977).
7. N. R. Bachur, S. L. Gordon and M. V. Gee, *Cancer Res.* **38**, 1745 (1978).
8. T. Oki, T. Komiyama, H. Tone, T. Inui, T. Takeuchi and H. Umezawa, *J. Antibiot., Tokyo* **30**, 613 (1977).

9. R. P. Mason, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), p. 151, Elsevier/North-Holland Biomedical Press, Amsterdam (1979).
10. C. E. Myers, W. P. McGuire, R. H. Liss, I. Ifrim, K. Grotzinger and R. C. Young, *Science* **197**, 165 (1977).
11. J. H. Doroshow, G. Y. Locker and C. E. Myers, *J. clin. Invest.* **65**, 128 (1980).
12. N. Bachur, *Cancer Treat. Rep.* **63**, 817 (1979).
13. J. Goodman and P. Hochstein, *Biochem. biophys. Res. Commun.* **77**, 797 (1977).
14. V. Berlin and W. A. Haseltine, *J. biol. Chem.* **256**, 4747 (1981).
15. J. W. Lown, S. K. Sim, K. C. Majumdar and R. Y. Chang, *Biochem. biophys. Res. Commun.* **79**, 705 (1977).
16. R. C. Donehower, C. E. Myers and B. A. Chabner, *Life Sci.* **25**, 1 (1979).
17. B. K. Sinha, *Chem. Biol. Interact.* **30**, 66 (1980).
18. B. K. Sinha and C. F. Chigell, *Chem. Biol. Interact.* **28**, 301 (1979).
19. M. Nikishimi, N. A. Rao and K. Yagi, *Biochem. biophys. Res. Commun.* **46**, 849 (1972).
20. F. P. Altman, *Prog. Histochem. Cytochem.* **9**, 1 (1976).
21. B. H. J. Bielski, G. G. Shiue and S. Bajuk, *J. phys. Chem.* **84**, 830 (1980).
22. C. Bertazoli and M. Ghione, *Pharmac. Res. Commun.* **9**, 235 (1977).
23. Y. Iwamoto, I. L. Hansen, T. H. Porter and K. Folkers, *Biochem. biophys. Res. Commun.* **58**, 633 (1974).
24. T. Kishi, T. Watanabe and K. Folkers, *Proc. natn. Acad. Sci. U.S.A.* **73**, 4653 (1976).
25. N. Domae, H. Sawada, E. Matsuyama, T. Konishi and H. Uchino, *Cancer Treat. Rep.* **65**, 79 (1981).
26. E. P. Cortes, M. Gupta, C. Chou, V. C. Amin and K. Folkers, *Cancer Treat. Rep.* **62**, 887 (1978).
27. B. K. Sinha and J. L. Gregory, *Biochem. Pharmac.* **30**, 2626 (1981).

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Reductive fragmentation of 2-nitroimidazoles in the presence of nitroreductases—glyoxal formation from misonidazole

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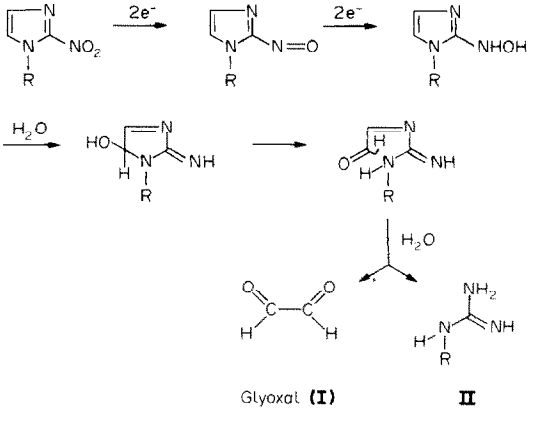
Nitroimidazoles are effective radiosensitizers in experimental systems but their clinical effectiveness is compromised by their neurotoxicity [1]. The origin of this toxicity is not known but may arise from reductive metabolism of the drug. Nitroheterocycles are known to fragment upon reduction [2–7], and it is possible that the molecular fragments are the toxic entities. They could account for both the enzyme inhibition we have observed in the presence of reduced 2-nitroimidazoles [8] and the production of the guanosine adduct which is formed in the presence of reduced misonidazole [9].

A molecular fragment (**II**, Scheme 1) has been identified from the biological reduction of misonidazole [10]. This product leaves two of the three ring carbons unaccounted for. On the basis of Scheme 1, we deduced that these ring carbons could appear as aldehydes and have now found that one of these aldehydes, glyoxal (**I**), is formed when misonidazole is reduced by xanthine oxidase and xanthine in the absence of air. Glyoxal reactivity with proteins and nucleic acids is well-known [11, 12] and is probably related to its mutagenic activity [13]. Glyoxal is shown in this report to inhibit a selection of enzymes and to be toxic to cultured mammalian cells.

Xanthine oxidase (Grade III, chromatographically purified), acetyl cholinesterase and lactic dehydrogenase, along with analytical kits for these enzymes, were obtained from the Sigma Chemical Co. and used as received. Xanthine and uric acid were also obtained from the Sigma Chemical Co. 1-(2-Hydroxy-3-methoxypropyl)-2-nitroimidazole (misonidazole) and 1-(2,3-dihydroxypropyl)-2-nitroimidazole (desmethylmisonidazole) were donated by Hoffmann-La Roche Ltd. Glyoxal was obtained from the Fisher Chemical Co. as a 40% solution in water. A 0.145 g aliquot of the solution was mixed with 10 ml of distilled water and added to a solution of 0.75 g of 2,4-dinitrophenylhydrazine in 300 ml of 6 N HCl. After standing for 2 hr at room temperature, the solution was filtered, and the orange precipitate that was collected was washed with water and a small quantity of 95% ethanol. After drying overnight in a vacuum desiccator over P_2O_5, the chromatographically pure glyoxal bishydrazone weighed 0.40 g (theoretical for a 40% solution, 0.39 g).

In a typical enzymic reduction of the 2-nitroimidazoles by xanthine oxidase, 204 mg of misonidazole was added to 760 mg of xanthine in 80 ml of 0.1 N NaOH, and the mixture was diluted with 920 ml of 0.01 M phosphate buffer, pH 7.8. The pH was adjusted to 8.0 with 70% phosphoric acid, and the solution was bubbled with nitrogen gas for 30 min.



Scheme 1.