Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6369–6373, September 1987
Biochemistry

Macrophage synthesis of nitrite, nitrate, and *N*-nitrosamines: Precursors and role of the respiratory burst

(L-arginine/^{15}N enrichment/*N*-nitrosomorpholine)

RADHA IYENGAR, DENNIS J. STUEHR*, AND MICHAEL A. MARLETTA†

Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

Communicated by Robert A. Alberty, May 29, 1987

ABSTRACT **The macrophage cell line RAW 264.7 when activated with *Escherichia coli* lipopolysaccharide and interferon-γ synthesized nitrite (NO_2^-) and nitrate (NO_3^-). Medium change after the activation showed that L-arginine was the only amino acid essential for this synthesis. D-Arginine would not substitute for L-arginine. Other analogues that could replace L-arginine were L-homoarginine, L-arginine methyl ester, L-arginamide, and the peptide L-arginyl-L-aspartate. L-Argininic acid, L-agmatine, L-ornithine, urea, L-citrulline, and ammonia were among the nonprecursors, while L-canavanine inhibited this L-arginine-derived NO_2^-/NO_3^- synthesis. When morpholine was added to the culture medium of the activated RAW 264.7 macrophages, N-nitrosation took place, generating *N*-nitrosomorpholine. GC/MS experiments using L-[*guanido*-$^{15}N_2$]arginine established that the NO_2^-/NO_3^- and the nitrosyl group of *N*-nitrosomorpholine were derived exclusively from one or both of the terminal guanido nitrogens of arginine. Chromatographic analysis showed that the other product of the L-arginine synthesis of NO_2^-/NO_3^- was L-citrulline. The role of the respiratory burst in NO_2^-/NO_3^- synthesis was examined using the macrophage cell lines J774.16 and J774 C3C. Both cell lines synthesized similar amounts of NO_2^-/NO_3^-. However, J774 C3C cells do not produce superoxide and hence do not exhibit the respiratory burst. Additional experiments also ruled out the involvement of the respiratory burst in NO_2^-/NO_3^- synthesis.**

We have previously reported that primary cultures of murine macrophages, when treated with *Escherichia coli* lipopolysaccharide (LPS), synthesize NO_2^- and NO_3^- (1). The addition of T lymphocytes to macrophage cultures enhanced this synthesis (1) and was due primarily to the T-cell-derived lymphokine interferon-γ (IFN-γ) (2). Regardless of the stimulant used, a time lag of 6–12 hr was observed for NO_2^-/NO_3^- synthesis (2, 3), during which protein synthesis required for the response occurred (D.J.S. and M.A.M., unpublished observations). NO_2^- and NO_3^- are stable in the presence of macrophages in culture and do not inhibit additional synthesis (2). When NO_2^- was added to macrophage cultures, it was not oxidized to NO_3^-, suggesting that the NO_3^- synthesized is not derived from NO_2^- (2). When secondary amines, such as morpholine, were added to cell culture medium of activated macrophages synthesizing NO_2^-/NO_3^-, it was found that the macrophages also carry out N-nitrosations, generating carcinogenic *N*-nitrosamines (4). Results from the nitrosamine synthesis studies showed that NO_2^- was not the nitrosating agent but suggested that the amines were reacting with an intermediate in the pathway from the precursor to NO_2^- (4).

Although the toxicity of NO_2^- to some bacteria is well established (5), until very recently the biological role for NO_2^-/NO_3^- synthesis was unknown. Previous studies suggested a link between NO_2^-/NO_3^- synthesis and the acquisition of increased nonspecific bacterial resistance (1). In addition, Hibbs *et al.* have reported that L-arginine is required for the selective metabolic inhibition of tumor target cells by macrophages (6). They have also reported findings similar to ours that show L-arginine is the precursor for NO_2^-/NO_3^- in macrophages (7–9). The studies reported here show that the NO_2^-/NO_3^- synthesized by activated macrophages is derived specifically from the two equivalent guanido nitrogens of L-arginine. In addition, the nitrosation of morpholine requires L-arginine, and the *N*-nitrosyl group is also derived exclusively from these same guanido nitrogens. A number of arginine analogues were examined as either precursors or inhibitors. The role of the respiratory burst—namely, the production of superoxide, hydrogen peroxide, and other cytocidal products—was investigated.

MATERIALS AND METHODS

L-Arginine, D-arginine, L-canavanine, L-arginine hydroxamate hydrochloride, L-agmatine, L-arginine methyl ester, L-homoarginine, L-argininic acid, L-arginamide, and the peptide L-arginyl-L-asparate were purchased from Sigma. L-[*guanido*-$^{15}N_2$]Arginine ([*amidino*-$^{15}N_2$]N^5-ornithine) (95% ^{15}N) was obtained from Cambridge Isotope Laboratory (Woburn, MA). L-[U-^{14}C]Arginine (specific activity, 323.5 mCi/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Dowex AG 50W-X8 (200–400 mesh) was obtained from Bio-Rad. It was converted to the Na^+ form by washing with 1 M NaOH. Low endotoxin defined calf serum was obtained from HyClone (Logan, UT) and it was heat-inactivated at 60°C for 30 min prior to use. Murine recombinant IFN-γ (specific activity, 1.9×10^7 units/ml, 1.5 mg/ml) was a gift of Genentech (South San Francisco, CA). Stock solutions of *E. coli* LPS (serotype 0127:B; Sigma) and IFN-γ (1×10^4 units/ml in culture medium) were stored at 4°C and diluted as needed (2). All other chemicals are commercially available and when necessary were of tissue culture grade.

Two different culture media were used for experiments reported here: (*i*) SMEM was powdered Eagle's minimal essential medium without phenol red (Flow Laboratories) supplemented with sodium bicarbonate (2.0 g/liter), sodium pyruvate (110 mg/liter), glucose (3.5 g/liter), L-glutamine (584 mg/liter), penicillin (50 units/ml), streptomycin (50 μg/ml), Hepes (15 mM), and 10% calf serum (final pH 7.3–7.4); and (*ii*) MEM was free of amino acids and contained the same concentration of salts and supplements as SMEM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: LPS, *Escherichia coli* lipopolysaccharide; IFN-γ, interferon-γ.

*Present address: Department of Medicine, Cornell University Medical Center, New York, NY 10021.

†To whom reprint requests should be addressed at: Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065.

but was vitamin and L-glutamine free. The macrophage cell line RAW 264.7 (certified mycoplasm free at the time of purchase) was obtained from American Type Culture Collection. The cell lines J774.16 and J774.C3C were a gift of Barry Bloom (Albert Einstein Medical College, New York). The cells were grown as described (10).

To obtain activated macrophages, RAW 264.7 cells were plated at 1.5×10^6 cells per ml in 24-well tissue culture plates (Costar, Cambridge, MA). After a 2-hr incubation, nonadherent cells were removed by aspiration and the adherent cells were incubated with SMEM containing LPS (1 μg/ml) and IFN-γ (500 units/ml) for 18 hr. The supernatant was removed from the macrophage monolayers (at this stage, the cells typically produced 125 nmol of NO_2^-/NO_3^-), and the adherent cells were washed with Hank's balanced salt solution containing calcium, magnesium, and bicarbonate but without phenol red. The activated macrophages were incubated with fresh SMEM or MEM or with MEM supplemented with L-arginine (2 mM) or other substrates as described with each specific experiment in *Results*. The culture supernatants were collected after 48 hr and stored at −10°C until analysis. In all the experiments reported, control cultures were macrophages that had not been activated with LPS and IFN-γ for 18 hr. Unless specifically stated, all the values reported have been corrected for the background synthesis (typically 18 nmol of NO_2^-/NO_3^- per 10^6 cells) that occurred in these controls and are the mean ± SD for at least two experiments. Macrophage viability after the second incubation was determined by trypan blue exclusion. Macrophage cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Nitrite and Nitrate Analysis. Nitrite and nitrate concentrations in the supernatant samples were determined using an automated procedure based on the Greiss reaction (11).

***N*-Nitrosomorpholine Analysis.** MEM was supplemented with morpholine (5 mM). After 48 hr an equal volume of 5 M NaOH was added to cell culture supernatants to stop the nitrosation reaction. *N*-Nitrosomorpholine was analyzed by gas chromatography and detected by a thermal energy analyzer using identical conditions reported by Miwa *et al.* (4).

^{15}N Enrichment of Nitrite and Nitrate. ^{15}N enrichment of NO_2^-/NO_3^- in cell culture supernatants was measured by GC/MS after conversion to nitrobenzene by the procedure of Tesch *et al.* (12). To the cell culture supernatant (1 ml) was added 1 M H_2O_2 (0.3 ml) and benzene (5 ml) followed by the slow addition of concentrated H_2SO_4 (5 ml). The solution was shaken at room temperature for 10 min. The benzene layer was passed through silica Sep-Pak (Waters Associates) and concentrated under a stream of N_2 to ≈100 μl. Samples (2 μl) were injected into a Hewlett–Packard 5992 GC/MS system. The gas chromatography separation was on a 3-m DB 5 column using a carrier flow of helium at 25 ml/min and an initial oven temperature of 60°C for 2 min followed by a temperature increase to 110°C at 10°C/min. The nitrobenzene emerged from the column at 6.2 min. The ratio of $^{15}N/^{14}N$ nitrobenzene was determined by selective ion monitoring at $m/z = 123$ and $m/z = 124$ as reported by Green *et al.* (11).

^{15}N Enrichment of *N*-Nitrosomorpholine. An aliquot of the dichloromethane extract of the culture supernatants was injected into the Hewlett–Packard 5992 GC/MS system. The gas chromatograph separation was on a 3-m DB 5 column using a carrier flow of helium at 25 ml/min and an initial oven temperature of 50°C for 2 min followed by a temperature increase to 150°C at 20°C/min. The *N*-nitrosomorpholine emerged from the column at 6.2 min. The $^{15}N/^{14}N$ ratio was determined by selective ion monitoring at $m/z = 116$, $m/z = 117$, and $m/z = 118$. [^{14}N]-*N*-Nitrosomorpholine gave a M + 1 peak (4.8%). Therefore, for the calculations of the ^{15}N enrichment, the $m/z = 117$ has been corrected by this value.

HPLC Analysis of Amino Acids. Prior to analysis, the cell supernatants were centrifuged through a Centricon 10 microconcentrator (Amicon Division, W. R. Grace, Danvers, MA) to remove the calf serum proteins. To quantitate the amino acids, α-amino butyrate (10 nmol) was added as the internal standard to an aliquot (0.1 ml) of the serum-free culture supernatant. The solution was derivatized with *o*-phthalaldehyde and an aliquot (25 μl) was analyzed by HPLC using the identical conditions reported by Fernstrom and Fernstrom (13). Under those conditions, citrulline had a retention time of 25 min; arginine, 32 min; α-amino butyrate, 33 min; ornithine, 60 min.

TLC Analysis. Aliquots (10 μl) of the cell culture supernatants were applied to silica gel 60 plates (20 × 20, aluminum; Alltech Associates, Los Altos, CA). Two solvent systems were used.

(*i*) $CHCl_3/MeOH/NH_4OH/H_2O$, 1:4:2:1 (vol/vol) R_f Arg = 0.38, R_f Cit = 0.83.

(*ii*) $CHCl_3/MeOH/NH_4OH$ 2:2:1 (vol/vol) R_f Arg = 0.22, R_f Cit = 0.77.

The location of arginine and citrulline was determined by staining with ninhydrin spray. When L-[U-^{14}C]arginine was used, each lane was cut into 10-mm sections and radioactivity was measured by scintillation counting.

Column Chromatography for Citrulline. Calf serum proteins were removed from the cell culture supernatants as described above. Serum-free supernatant (0.5 ml) was diluted to 1.0 ml and the pH was adjusted to 6.0. The sample was applied to a 3-ml plastic syringe containing Dowex AG 50W (Na^+ form, 1.5-ml bed volume) kept on top of a column of Dowex AG 50W (H^+ form, 1.5-ml bed volume) so that the sample would pass through both columns in that order (14). The columns were washed with 5 ml of H_2O and then the Dowex AG 50W (H^+ form) column alone was washed with 2 M HCl (8 ml). Appropriate fractions were combined and lyophilized to dryness.

RESULTS

Stuehr and Marletta (2) have reported that activated mouse peritoneal macrophage cultures receiving daily medium renewal (without LPS and IFN-γ) synthesized greater amounts of NO_2^-/NO_3^- over a 96-hr period than control cultures (no medium change). These results were confirmed using the macrophage cell line RAW 264.7. Experiments were then carried out to determine which components in the culture renewal medium were essential for the NO_2^-/NO_3^- synthesis. Results showed that the macrophages remained viable in a culture medium without amino acids and vitamins but containing salts, antibiotics, glucose, pyruvate, and 10% calf serum (MEM). These cultures continued to produce a small amount of NO_2^-/NO_3^- (typically ≈31% based on the SMEM). Each of the 13 amino acids present in the SMEM was added individually to the MEM. Only L-arginine reproduced the effect of the complete amino acid mixture. In fact at 2 mM, L-arginine gave 116% NO_2^-/NO_3^- based on the SMEM (see Table 1). The NO_2^-/NO_3^- ratio was 3:2, consistent with results when SMEM was used (2, 3). The concentration dependence for L-arginine was examined by varying the concentration of L-arginine added to the MEM. Macrophage NO_2^-/NO_3^- synthesis had an apparent K_m of 0.15 mM for L-arginine.

The specificity of this L-arginine synthesis was studied by replacing L-arginine in the MEM with arginine analogues and measuring the NO_2^-/NO_3^- that had been synthesized. D-Arginine did not substitute as a precursor and was not an inhibitor when added along with L-arginine, each at 2 mM. The following compounds were also found to be precursors with the results expressed as a percentage with L-arginine as 100%: L-homoarginine (80%), L-arginine methyl ester (82%),

Table 1. L-Arginine requirement for NO_2^-/NO_3^- and *N*-nitrosomorpholine by RAW 264.7 macrophages

Additions to activated RAW 264.7 macrophages	$NO_2^- + NO_3^-$, nmol per 10^6 cells	Nitrosomorpholine, nmol per 10^6 cells
SMEM	98.5 ± 1.9	
SMEM + morpholine	76.8 ± 1.4	1.4
MEM	34.3 ± 1.5	
Morpholine	23.9 ± 1.2	0.62
Arginine	114.5 ± 3.0	
Arginine + morpholine	90.6 ± 2.2	2.3
Arginine + morpholine (nonactivated macrophages)	12.3 ± 0.4	0.08

Activated RAW 264.7 macrophages (1.5×10^6 cells per ml) were incubated with L-arginine (2 mM) and morpholine (5 mM) in MEM for 48 hr. Cell culture supernatants were analyzed for NO_2^-/NO_3^- and *N*-nitrosomorpholine as described in *Materials and Methods*.

L-arginamide (72%), and the peptide L-arginyl-L-aspartate (84%). L-Argininic acid, L-agmatine, L-ornithine, urea, L-citrulline, ammonia, and L-canavinine were among the nonprecursors. When L-canavanine was added in the presence of L-arginine, NO_2^-/NO_3^- synthesis was inhibited with an apparent K_i of 0.22 mM.

Miwa *et al.* (4) have shown that activated macrophages cultured in the presence of secondary amines were capable of forming *N*-nitrosamines as well as NO_2^-/NO_3^-. To establish that L-arginine was the only amino acid necessary for the synthesis of *N*-nitrosamines, activated RAW 264.7 macrophages were incubated with both L-arginine (2 mM) and morpholine (5 mM) present in the renewal MEM. Table 1 shows *N*-nitrosomorpholine (2.3 nmol) was formed in the presence of arginine, reproducing the effect of the SMEM containing all the amino acids. MEM without any amino acids but containing morpholine (5 mM) formed 0.62 nmol of *N*-nitrosomorpholine, consistent with the small amount of NO_2^-/NO_3^- synthesis that occurred in MEM.

To determine which nitrogen atom present in L-arginine was being converted to NO_2^-/NO_3^- and nitrosating morpholine, the activated macrophages were incubated in the presence of various concentrations of L-[*guanido*-$^{15}N_2$]arginine ([*amidino*-$^{15}N_2$]N^5-ornithine) (95% ^{15}N). The ^{15}N enrichment in the products was determined by GC/MS with selective ion monitoring (Table 2). The NO_2^-/NO_3^- in the culture supernatants was converted to nitrobenzene as described in *Materials and Methods*. The measured ^{15}N enrichment in the nitrobenzene was corrected for the presence of endogenous $^{14}NO_2^-/^{14}NO_3^-$ (9.9 μM) in the MEM (no precautions had been taken to remove NO_2^-/NO_3^- present in the reagents used). The ^{15}N enrichment in the *N*-nitrosomorpholine was measured directly. The observed high ^{15}N enrichment in the products shows that one or both of the two terminal guanido nitrogens of arginine are being converted to NO_2^-/NO_3^- and the nitrosating agent. Also, the ^{15}N enrichment in nitrobenzene and *N*-nitrosomorpholine at the same arginine concentration was identical and always slightly lower than the initial ^{15}N enrichment (95% ^{15}N). This result confirmed that the small amount of NO_2^-/NO_3^- synthesis occurring in MEM without any amino acids was from endogenous L-arginine and not from another source.

Cell culture supernatants were analyzed by HPLC to determine the other products of the reaction. Only one major product accumulated in the medium over 48 hr and it had the same retention time as L-citrulline. No synthesis of L-citrulline occurred in the absence of L-arginine. To confirm the formation of L-citrulline the experiment was repeated using L-[U-^{14}C]arginine plus unlabeled L-arginine (2 mM). The cell culture supernatant was purified by column chromatography on Dowex AG 50W. The ^{14}C product was shown by TLC in two different solvent systems to have the same R_f value as L-citrulline. The experiments showed that in macrophages L-arginine is being converted to L-citrulline and NO_2^-/NO_3^-.

Quantitation of the L-citrulline produced using L-[U-^{14}C]arginine showed that the amount of L-citrulline formed was always greater than the NO_2^-/NO_3^- synthesized (Fig. 1). Another possible source of L-citrulline could be via L-ornithine. Activated macrophages are known to produce arginase (15), which converts L-arginine to L-ornithine and urea. Incubation of activated macrophage cell cultures in the presence of L-ornithine followed by HPLC analysis of the culture supernatants resulted in the formation of L-citrulline but not NO_2^-/NO_3^- (data not shown).

To investigate the role of reactive oxygen species in macrophage NO_2^-/NO_3^- synthesis, cell culture experiments comparing NO_2^-/NO_3^- synthesis by the superoxide-proficient J774.16 cell line with that of the superoxide-deficient J774 C3C cell line in response to LPS, IFN-γ, and IFN-γ plus LPS were carried out (Table 3). Phorbol 12-myristate 13-acetate-stimulated superoxide formation by both cell lines was also determined (data not shown). J774.16 cells synthesized superoxide when stimulated with phorbol 12-myristate 13-acetate, whereas J774 C3C cells did not. Superoxide was measured either as an initial rate in cell suspensions or in monolayers over 60 min, and the amount formed by this cell line agrees with published data (16, 17). J774.16 and J774 C3C cells synthesized similar amounts of NO_2^-/NO_3^- when treated with IFN-γ plus LPS. NO_2^- represented ≈60% of the total NO_2^- plus NO_3^- in either case. Treatment with either LPS or IFN-γ alone resulted in the formation of only a small amount of NO_2^-/NO_3^- by either cell line.

Table 2. GC/MS analysis for ^{15}N enrichment of nitrobenzene and *N*-nitrosomorpholine

Additions to activated RAW 264.7 macrophages	$NO_2^- + NO_3^-$, nmol per 10^6 cells	Nitrosomorpholine, nmol per 10^6 cells	^{15}N enrichment	
			Nitrobenzene	Nitrosomorpholine
SMEM	124.3 ± 1.2			
MEM	36.3 ± 1.0			
Arginine (0.4 mM)	76.3 ± 1.0		81.6 ± 0.1	
Arginine (0.4 mM) + morpholine	64.6 ± 1.3	1.53		82.0 ± 0.3
Arginine (0.6 mM)	82.2 ± 3.0		83.6 ± 0.4	
Arginine (0.6 mM) + morpholine	80.2 ± 1.6	1.66		84.1 ± 0.4
Arginine (1 mM)	88.3 ± 2.6		86.7 ± 0.1	
Arginine (1 mM) + morpholine	82.1 ± 1.3	1.96		86.4 ± 0.8
Arginine (2 mM)	99.2 ± 2.3		88.5 ± 0.4	
Arginine (2 mM) + morpholine	92.9 ± 3.0	2.03		89.8 ± 0.2

Activated RAW 264.7 macrophages (1.2×10^6 cells per ml) were incubated with various concentrations of L-[*guanido*-$^{15}N_2$]arginine and morpholine (5 mM) for 48 hr. Cell culture supernatants were analyzed as described in *Materials and Methods*.

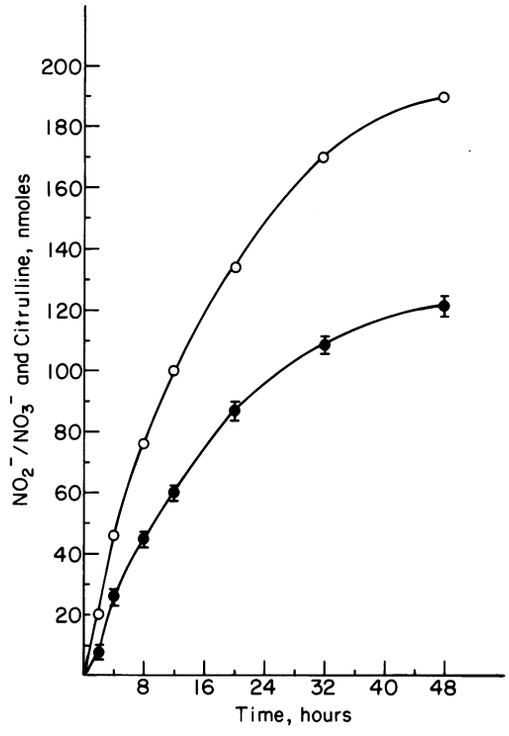


FIG. 1. Time course for the formation of L-citrulline (○) and NO_2^-/NO_3^- (●) from L-arginine. Activated RAW 264.7 macrophages (1×10^6 cells per ml) were incubated with L-[U-^{14}C]arginine (2.2×10^6 cpm, 3.1 nmol) plus unlabeled L-arginine (2 mM) for various times. [^{14}C]Citrulline formed was quantitated by TLC analysis (solvent system ii) followed by scintillation counting. NO_2^-/NO_3^- was analyzed by an automated procedure. See *Materials and Methods* for details.

A series of additional experiments also indicated no involvement of reactive oxygen species. With the same two cell lines and primary cultures of C3H/He macrophages, superoxide dismutase (300 units/ml), catalase (2000 units/ml), or mannitol (100 mM) when added to the cell culture medium had no effect on the total NO_2^-/NO_3^- synthesized or on the NO_2^-/NO_3^- ratio. Phorbol 12-myristate 13-acetate treatment of the J774.16 and J774 C3C lines or C3H/He macrophages either alone or in cells actively synthesizing NO_2^-/NO_3^- via IFN-γ/LPS treatment did not produce or enhance NO_2^-/NO_3^- synthesis. Also without effect was the generation of O_2^-/HOOH in the culture medium via xanthine (0.1 mM) and xanthine oxidase (0.2 unit/ml) or the addition of allopurinol (0.1 mM) to the culture medium.

Table 3. Comparison of NO_2^-/NO_3^- synthesis by J774.16 and J774 C3C macrophages

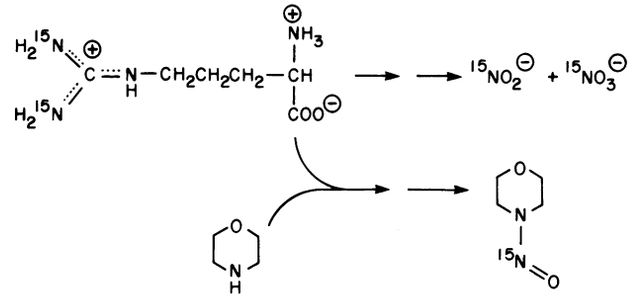
Additions to SMEM	$NO_2^- + NO_3^-$, nmol per 10^6 cells	
	J774.16	J774 C3C
IFN-γ + LPS	127 ± 14	118 ± 8
	(82 ± 1)	(73 ± 8)
IFN-γ	9 ± 4	12 ± 4
LPS	9 ± 4	23 ± 3
Control	0 ± 3	0 ± 1

Macrophages (1×10^6 cells per ml) were treated with IFN-γ (500 units/ml), LPS (5 μg/ml), or IFN-γ (500 units/ml) + LPS (1 μg/ml) for 72 hr. Values are the means ± SD for four cultures. Values given in parentheses are the NO_2^- levels (nmol per 10^6 cells) in the samples given above.

DISCUSSION

The studies reported here clearly establish the amino acid L-arginine as the precursor to NO_2^-/NO_3^- in immunostimulated macrophages. Replacement of the SMEM with medium containing L-arginine continually gave slightly more NO_2^-/NO_3^- (110–120%) when compared to the SMEM. L-Arginine was the only amino acid to yield NO_2^-/NO_3^-. Individual addition of each of the other 13 amino acids present in the SMEM yielded negative results. Furthermore, the synthesis was specific for the L configuration at the α-carbon as D-arginine was not a precursor and when present at equimolar (2 mM) concentrations with L-arginine did not inhibit NO_2^-/NO_3^- synthesis. Except for L-homoarginine, all of the other precursors could be metabolically converted to L-arginine. L-Canavanine was an effective inhibitor of NO_2^-/NO_3^- synthesis. In many arginine-requiring reactions, L-homoarginine can substitute as a substrate and L-canavanine acts as an inhibitor (18). Our results are consistent with L-arginine as a specific precursor for NO_2^-/NO_3^- synthesis.

The cell culture experiments carried out with L-[*guanido*-$^{15}N_2$]arginine confirmed L-arginine was the only precursor but more importantly showed that the NO_2^- and NO_3^- synthesized comes exclusively from the two guanido nitrogens (see Scheme I). As shown in Table 2, the ^{15}N enrichment



I

in NO_2^- and NO_3^- is high and dependent on the L-arginine concentration. At the highest L-arginine concentration tested (2 mM), the NO_2^-/NO_3^- ^{15}N enrichment of 88.5% ± 0.4% approached that of the [^{15}N]arginine (95%). In total, these results showed that the source of NO_2^-/NO_3^- was one or both of the guanido nitrogens and that there was a small amount of endogenous [^{14}N]arginine diluting the added [^{15}N]arginine. Since both *guanido* nitrogens of arginine were labeled, it was impossible to determine whether one or both nitrogens lead to the formation of NO_2^-/NO_3^-. The NO_2^-/NO_3^- ratio was typically 3:2 (2, 3). At present, we cannot explain this ratio. However, we know that NO_2^- and NO_3^- are stable under the cell culture conditions and that the NO_3^- synthesized is not derived from NO_2^- (2). Activated macrophages also synthesized *N*-nitrosamines when secondary amines were added to the cell culture medium (4). Table 2 also shows that the *N*-nitrosyl group of *N*-nitrosomorpholine is exclusively derived from the same guanido nitrogens of L-arginine (Scheme I). The concentration dependence of the enrichment observed here suggests the same dilution by endogenously synthesized L-arginine.

The actual chemistry of the synthetic pathway is not yet clear. However, our precursor/product studies are illuminating. In particular, with L-[U-^{14}C]arginine, the only product observed was L-citrulline. Figure 1 shows the L-citrulline yield relative to the NO_2^-/NO_3^- synthesized. This suggests that only one of the two terminal guanido nitrogens is oxidized to both NO_2^- and NO_3^-. At all time points, there was more citrulline produced than NO_2^- and NO_3^-. Therefore, while the other product from NO_2^-/NO_3^- synthesis appears to be citrulline, it is clear that another source of citrulline exists.

Activated macrophages are known to produce arginase (15), which converts arginine to ornithine and urea. Subsequent acetylation of ornithine would yield citrulline. As reported above, we did find evidence for the formation of citrulline from ornithine. Hence, the excess citrulline, at least in part, may be accounted for by this pathway. Neither L-ornithine or urea served as precursors for NO_2^-/NO_3^-. It has recently been suggested that NO_2^- derived from L-arginine occurs by the action of an arginine deimidase producing L-citrulline and ammonia followed by oxidation of the ammonia to NO_2^- (7). Arginine deimidase has not been found in mammals and the authors were unable to correlate ammonia formation in the cell culture medium with the L-arginine-dependent cytotoxic action of macrophages (7). Furthermore, as reported here ammonia is not a precursor for NO_2^-/NO_3^-, suggesting that another pathway is responsible for arginine oxidation to NO_2^- and NO_3^-.

One pathway that has to be considered is the respiratory burst evidenced by macrophages and other phagocytic cells when they encounter an IgG-coated microorganism or certain soluble agents (19). This respiratory burst first produces superoxide, which then leads to the formation of hydrogen peroxide and potentially other oxidants, all of which are involved in the microbicidal activity of these cells. The chemical nature of these species suggests that this pathway may have a role in NO_2^-/NO_3^- synthesis. When comparing two macrophage cell lines, the J774.16 that is competent for superoxide production and the J774 C3C line that has a mutation and cannot synthesize superoxide, we find both cell lines form similar amounts of NO_2^-/NO_3^- in response to IFN-γ and LPS (Table 3). Furthermore, addition of the soluble agent phorbol 12-myristate 13-acetate to cells already synthesizing NO_2^-/NO_3^- was without effect as well as scavenging experiments with superoxide dismutase, catalase, and mannitol. Therefore, all of our results show that the respiratory burst is not involved in NO_2^-/NO_3^- synthesis.

A possible intermediate in the L-arginine to NO_2^-/NO_3^- pathway could be an *N*-guanido-hydroxylated arginine. Our results are consistent with such an intermediate. *N*-Hydroxylated guanidines have been reported to have a wide range of biological activity, including anti-tumor and anti-viral activity (20). For example, *N*-hydroxyguanidine is as potent an inhibitor of DNA synthesis as *N*-hydroxurea (21). The L-arginine-dependent cytotoxic action of macrophages on tumor cells reported by Hibbs *et al.* (6) involves DNA synthesis inhibition. It seems reasonable at this point to conclude that this arginine metabolism represents a pathway of cytocidal activity, perhaps effective against a wide range of targets.

We are very grateful to Dr. John S. Wishnok for his help with the GC/MS instrumentation and to Joseph Glogowski for his expert maintenance of the NO_2^-/NO_3^- analyzer and GC/TEA. This work was supported by Public Health Service Grant CA26731 awarded by the National Cancer Institute. The mass spectrometer was purchased with funds from the Department of Health and Human Services-shared instrument Grant IS10 RR0190.

1. Stuehr, D. J. & Marletta, M. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7738–7742.
2. Stuehr, D. J. & Marletta, M. A. (1987) *J. Immunol.* **139,** 518–525.
3. Stuehr, D. J. & Marletta, M. A. (1987) in *Ninth International Symposium on N-Nitroso Compounds: Relevance to Human Cancer*, eds. Bartsch, H. & O'Neill, I. K. (Int. Agency for Res. on Cancer, Lyon, France), in press.
4. Miwa, M., Stuehr, D. J., Marletta, M. A., Wishnok, J. S. & Tannenbaum, S. R. (1987) *Carcinogenesis* **8,** 955–958.
5. Castellani, A. G. & Niven, C. F., Jr. (1955) *Appl. Microbiol.* **3,** 154–159.
6. Hibbs, J. B., Jr., Vavrin, Z. & Taintor, R. R. (1987) *J. Immunol.* **138,** 550–565.
7. Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) *Science* **235,** 473–476.
8. Iyengar, R. & Marletta, M. A. (1987) *Proc. Am. Assoc. Cancer Res.* **28,** 134.
9. Iyengar, R. & Marletta, M. A. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46,** 1973 (abstr.).
10. Stuehr, D. J. & Marletta, M. A. (1987) *Cancer Res.*, in press.
11. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. & Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138.
12. Tesch, J. W., Rehg, W. R. & Sievers, R. E. (1976) *J. Chromatogr.* **126,** 743–755.
13. Fernstrom, M. H. & Fernstrom, J. D. (1981) *Life Sci.* **29,** 2119–2130.
14. Gopalkrishna, R. & Nagaranjan, B. (1980) *Anal. Biochem.* **107,** 318–323.
15. Currie, G. A. (1978) *Nature (London)* **273,** 758–759.
16. Damiani, G., Kiyotaki, C., Soeller, W., Sasada, M., Peisach, J. & Bloom, B. R. (1980) *J. Exp. Med.* **152,** 808–822.
17. Kiyotaki, C., Peisach, J. & Bloom, B. R. (1984) *J. Immunol.* **132,** 857–866.
18. Fowden, L., Lewis, D. & Tristram, H. (1967) in *Advances in Enzymology and Related Areas of Molecular Biology*, ed. Nord, F. F. (Wiley, New York), Vol. 29, pp. 89–163.
19. Babior, B. M. (1984) *J. Clin. Invest.* **73,** 599–601.
20. Tai, A. W., Lien, E. J., Lai, M. M. C. & Khwaja, T. A. (1984) *J. Med. Chem.* **27,** 236–238.
21. Young, C. W., Schochetman, G., Hodas, S. & Balis, M. E. (1967) *Cancer Res.* **27,** 535–540.