purged of that element fails to grow normally and to complete its life cycle or (ii) if the element is a constituent of a molecule that is known to be an essential metabolite. The soybean plants in the $Ni_0$ treatments completed their life cycle and produced an undiminished yield of viable seed, but they accumulated toxic concentrations of urea, which is not normal. Nickel is a necessary constituent of the enzyme urease (*5*), but thus far the essentiality of this enzyme has not been demonstrated. Welch (*4*) has summarized several known biochemical pathways that could result in urea production; urea, however, is difficult to detect in plant tissue (*16*). Our results show that urea is produced during normal nitrogen metabolism in higher plants, and that nickel as a component of urease, is required to prevent the accumulation of toxic concentrations of urea.

DAVID L. ESKEW*
*Department of Agronomy,*
*Cornell University,*
*Ithaca, New York 14853*
ROSS M. WELCH
EARLE E. CARY
*U.S. Department of Agriculture,*
*Plant, Soil, and Nutrition Laboratory,*
*Agricultural Research Service,*
*Ithaca, New York 14853*

### References and Notes

1. W. Mertz, *Science* **213**, 1332 (1981).
2. T. C. Broyer, A. B. Carlton, C. M. Johnson, P. R. Stout, *Plant Physiol.* **29**, 526 (1954).
3. D. Mishra and M. Kar, *Bot. Rev.* **40**, 395 (1974).
4. R. M. Welch, *J. Plant Nutr.* **3**, 345 (1981).
5. N. E. Dixon, C. Gazzola, R. L. Blakeley, B. Zerner, *J. Am. Chem. Soc.* **97**, 4131 (1975); W. N. Fishbein, M. J. Smith, K. Nagarajan, W. Scurzi, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25**, 1680 (1976).
6. J. C. Polacco, *Plant Physiol.* **59**, 827 (1977); *Plant Sci. Lett.* **10**, 249 (1977); W. R. Gordon, S. S. Schwemmer, W. S. Hillman, *Planta* **140**, 265 (1978); N. Shimada, T. Ando, M. Tomiyama, H. Kaku, *Nippon Dojo Hiryogaku Zasshi* **51**, 487 (1980).
7. N. Shimada and T. Ando, *Nippon Dojo Hiryogaku Zasshi* **51**, 493 (1980).
8. The full-strength nitrogen-containing nutrient solution had the following composition: 3.8 m*M* $Ca(NO_3)_2$, 3.8 m*M* $KNO_3$, 1.5 m*M* $NH_4NO_3$, 2 m*M* $MgSO_4$, 1 m*M* $NH_4H_2PO_4$, 50 $\mu$*M* $CaCl_2$, 50 $\mu$*M* FeEDTA, 6.25 $\mu$*M* $H_3BO_3$, 1 $\mu$*M* $MnSO_4$, 2 $\mu$*M* $ZnSO_4$, 0.5 $\mu$*M* $CuSO_4$, 0.5 $\mu$*M* $(NH_4)_2MoO_4$, and 0.01 $\mu$*M* $CoSO_4$. The nitrogen-free solution had the following composition: 2.0 m*M* $CaCl_2$, 2.0 m*M* $K_2SO_4$, 3.0 m*M* $MgSO_4$, 0.2 m*M* $KH_2PO_4$, and iron and micronutrients as in the nitrogen-containing solution. The nitrogen-free solution was buffered with 5 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) purified as described below, and the solution *p*H was maintained between 5.5 and 6.0 by additions of *p*H 7.6 purified sodium MES [the MES technique was adapted from J. Imsande and E. J. Ralston, *Plant Physiol.* **68**, 1380 (1981)]. Deionized water with a resistance of greater than 10 megohms was used throughout. Concentrated solutions of the macronutrient salts were adjusted to between *p*H 4 and 6 with NaOH; then 25 $\mu$Ci of $^{63}Ni$ per liter (as $NiCl_2$) was added and the solution was passed through a 0.22-$\mu$m filter onto a column (0.6 by 13 cm) containing 8HQ-CPG. Boric acid was Baker Ultrex grade, $(NH_4)_2MoO_4$ was from Hedrich Chemical, and iron and micronutrients were Johnson-Mathey Specpure grade. Seeds were germinated in deionized water on filter paper. After 5 days, seedlings were transferred, two plants per 3.4-liter polyethylene pot, to quarter-strength nutrient solution. The solution strength was subsequently increased to half and then to full strength at 1-week intervals. Plants were grown in a growth chamber with a 14-hour light period (770 $\mu E\ m^{-2}\ sec^{-1}$), with a 25° to 16°C day-night temperature cycle. Makeup air was filtered through a 99 percent efficiency high-efficiency particulate air filter.
9. J. M. Hill, *J. Chromatogr.* **76**, 455 (1973).
10. Nickel was supplied by adding 0.34 ml of either a 10- or 100-mg $liter^{-1}$ solution, prepared by diluting an atomic absorption standard solution (Fisher Scientific) with 1.2*M* HCl for the first-generation experiment. In the second-generation experiment, 0.34 ml of a 10 mg $liter^{-1}$ solution of Johnson-Mathey Specpure $NiSO_4$ in 0.12*M* HCl was used.
11. Dried leaflet tips were extracted with ten volumes of 0.1*M* 2-(*N*-morpholino)propanesulfonic acid (MOPS), 1 m*M* EDTA at *p*H 7.0. Urea was hydrolyzed with Sigma type VII jack bean urease, and $NH_4^+$ was determined by Nesslerization.
12. H. G. Smith and A. J. Ohlrogge, in *Soil Testing and Plant Analysis*, L. M. Walsh and J. D. Beaton, Eds. (Soil Science Society of America, Madison, Wisc., 1973), p. 315.
13. N. E. Dixon *et al.*, *Can. J. Biochem.* **58**, 474 (1980).
14. R. J. Thomas and L. E. Schrader, *Phytochemistry* **20**, 361 (1981).
15. E. Epstein, *Mineral Nutrition of Plants: Principles and Perspectives* (Wiley, New York, 1972), p. 55.
16. E. G. Bollard, *Symp. Soc. Exp. Biol.* **13**, 304 (1959).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
18. J. C. Polacco, A. L. Thomas, P. J. Bledsoe, *Plant Physiol.* **69**, 1233 (1982).
19. We thank N. Washer for technical assistance, J. F. Thompson and J. T. Madison for advice, and R. D. Graham for suggestions on the manuscript. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.
* Present address: International Atomic Energy Agency, Wagramerstrasse 5, Post Office Box 200, A1400 Vienna, Austria.

4 February 1983; accepted 24 June 1983

## Human Endothelial Cells: Use of Heparin in Cloning and Long-Term Serial Cultivation

Abstract. *Endothelial cells from human blood vessels were cultured in vitro, with doubling times of 17 to 21 hours for 42 to 79 population doublings. Cloned human endothelial cell strains were established for the first time and had similar proliferative capacities. This vigorous cell growth was achieved by addition of heparin to culture medium containing reduced concentrations of endothelial cell growth factor. The routine cloning and long-term culture of human endothelial cells will facilitate studying the human endothelium in vitro.*

The endothelium forms the luminal surface of the vascular system and is an integral component in such physiologic functions as wound healing, hemostasis, selective transfer of substances to and from the circulation, and synthesis of numerous metabolically active compounds. Correspondingly, endothelial involvement is prominent in pathologic conditions including atherosclerosis, dia-

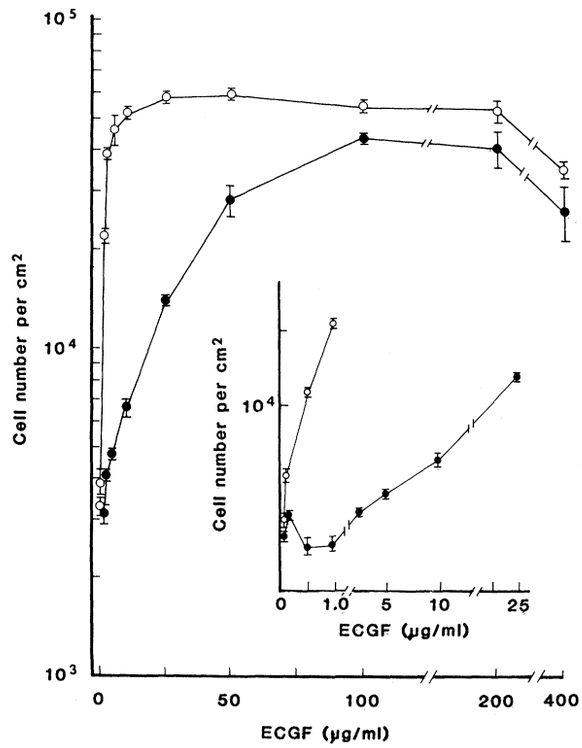


Fig. 1. Effect of heparin on human endothelial cell growth. HUVE cells (cumulative PD level, 23) were seeded in Medium 199 with ECGF (20 $\mu$g/ml) and heparin (90 $\mu$g/ml) at $5 \times 10^3$ cells per square centimeter in 25-$cm^2$ flasks coated with gelatin. Cells were allowed to attach for 1 hour, washed three times, and again fed with Medium 199 supplemented with various concentrations of ECGF in the presence (○) and absence (●) of heparin (90 $\mu$g/ml). After 4 days, cells were trypsinized and counted. Data are reported as mean cell density ± standard deviation (S.D.) for three replicate cultures. Inset: Data replotted on an expanded scale for ECGF concentrations between 0 and 25 $\mu$g/ml.

betes, thrombosis, hemorrhagic disorders, tumor metastasis, hypersensitivity, and inflammation (*1*). The need for a greater understanding of endothelial function has prompted methodological improvements for culturing this cell in vitro. Bovine endothelial cells have been studied widely because of the ease with which they can be serially subcultivated (*1, 2*). Human endothelial cells, however, have more fastidious growth requirements and, despite a suggestion (*3*) that fibroblast growth factor and thrombin stimulate their growth, little progress has been made in the long-term serial subcultivation of these cells (*4–6*).

A significant advance in this field was the use of an endothelial cell growth factor (ECGF) and fibronectin to improve the replicative capacity of human umbilical vein endothelial (HUVE) cells (*4*). These factors allowed HUVE cells to be subcultivated; however, multiplication was slow (doubling time, 2 to 3 days) and life-span short [27 to 34 population doublings (PD's)]. Human endothelial cells from adult iliac artery grown under the same conditions had an even shorter life-span (15 to 18 PD's) (*5*). In no case have cloned strains of human endothelial cells been reported. We now report that heparin greatly potentiates the stimulatory effect of ECGF on the proliferation of HUVE cells and of endothelial cells from adult human blood vessels. By growing cells on a gelatin matrix in medium supplemented with both ECGF and heparin, we have cloned and serially subcultivated human endothelial cells from various blood vessels for 42 to 79 PD's, with doubling times of 17 to 21 hours.

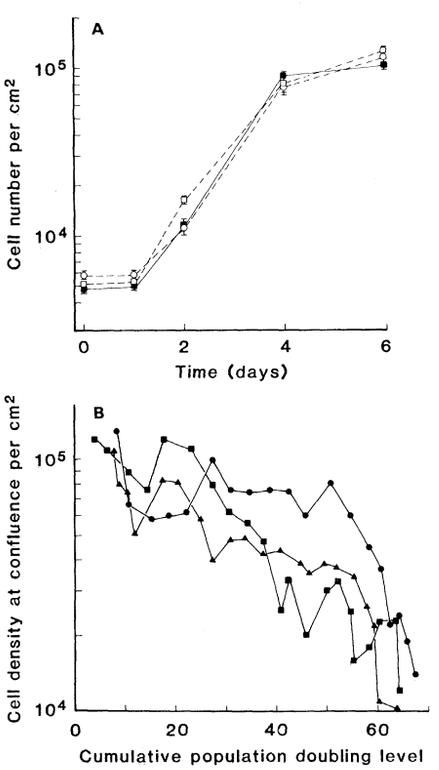


Fig. 2. Proliferative characteristics of cultured human endothelial cells. (A) Growth curves for three HUVE cell lines (cumulative PD level, 10). Cultures were seeded at $5 \times 10^3$ cells per square centimeter in 25-cm$^2$ flasks and incubated under standard conditions. At various intervals, cells were trypsinized and counted. Data are reported as mean cell densities ± S.D. for three replicate cultures. (B) Cell density at subculture versus cumulative PD level for three HUVE cell lines seeded as in (A) and subcultured weekly. The cumulative PD level was calculated as described in the text.

Endothelial cells were isolated from blood vessels by use of collagenase (*2*) and seeded into gelatin (1 percent)-coated flasks (*7*) containing Medium 199, 20 percent fetal bovine serum, ECGF (20 μg/ml), and heparin (90 μg/ml). At confluence, cultures were trypsinized (0.25 percent trypsin and 0.09 percent EDTA) and reseeded at desired cell densities. In addition to the umbilical vein, other donor vessels included the superior mesenteric, iliac, carotid, pulmonary, femoral, and splenic arteries, the thoracic and abdominal aortas, and the iliac and portal veins (*8*). Clones were derived from secondary cultures by seeding at ten cells per square centimeter and isolating single cells with cloning rings; approximately half of the isolated cells grew and were serially propagated. Eleven cloned HUVE cell strains and four abdominal aorta endothelial cell strains were established. All cultures were characterized as endothelial according to morphological and functional [expression of factor VIII–related antigen (*2*) and production of angiotensin-I-converting enzyme (*1, 9*)] criteria.

Enhanced proliferation and increased life-span of human endothelial cell cultures resulted from addition of heparin to the culture medium (Fig. 1). In the presence of heparin, significant growth was observed with as little ECGF as 1 μg/ml, with maximal growth at 25 μg/ml; in the absence of heparin, 100 to 200 μg of ECGF per milliliter was required for significant growth. Cultures supplemented with heparin grew to consistently higher densities than those without heparin. Heparin at concentrations as high as 900 μg/ml did not support replication in the absence of ECGF (*8*), showing that both factors were required for optimum growth.

Three lines of HUVE cells cultured with heparin (90 μg/ml) and ECGF (20 μg/ml) achieved cell densities at confluence of $10^5$ cells per square centimeter with doubling times of 17 to 21 hours (Fig. 2a). In contrast, cells cultured with ECGF (100 μg/ml) without heparin were reported (*4*) to grow to a maximum of $4 \times 10^4$ cells per square centimeter, with a doubling time of 64 hours. Proliferative life-span was determined by measuring harvest cell density at each subculture until proliferation ceased (Fig. 2B). The PD's undergone at each subculture were calculated with the formula PD = $\log_2$[(number of cells harvested)/(number of cells seeded × attachment efficiency)], and summed to give the cumulative PD level (*2*). In all cases the harvest cell densities decreased with increasing cumulative PD level as greater numbers of large nondividing cells appeared. Cultures were considered senescent when less than one PD occurred within 3 weeks after subculture. By this definition, the life-span of 12 uncloned lines and 11 cloned strains of HUVE cells ranged from 42 to 79, with a median cumulative PD level of 58. Endothelial cells from adult human vascular tissue had doubling times similar to those of HUVE cells and maintained higher harvest cell densities for a greater percentage of their life-span than the HUVE cells (*8*).

To confirm that heparin is responsible for promoting proliferation, we examined the effect of the heparin antagonist protamine. Cell growth in the presence of ECGF was enhanced markedly by heparin (Table 1). This effect of heparin was completely blocked by a neutralizing dose of protamine. Growth-promoting activity was reestablished by addition of a tenfold excess of heparin. At high ECGF concentrations (200 μg/ml) significant growth was observed in the

Table 1. Effect of protamine on human endothelial cell growth. Additions to the culture medium included ECGF prepared as described previously (*4*), heparin (sodium salt, grade 1, from porcine intestinal mucosa; 168 U/mg; Sigma), and protamine sulfate (sodium salt, grade X, from salmon sperm; Sigma); 150 μg of protamine neutralizes 100 μg of heparin. Cell counts [± S.D. ($N = 3$)] were made 7 days after seeding at $5 \times 10^3$ cells per square centimeter. Bovine lung heparin (sodium salt, U.S.P.; Upjohn) was as stimulatory as porcine intestinal mucosal heparin.

| Additions to culture medium (μg/ml) | | | Harvest cell density ($10^4$ cells per square centimeter) |
|---|---|---|---|
| ECGF | Heparin | Protamine | |
| 20 | | | 1.4 ± 0.1 |
| 20 | 90 | | 5.8 ± 0.3 |
| 20 | 90 | 230 | 1.1 ± 0.1 |
| 20 | 900 | 230 | 6.2 ± 0.2 |
| 200 | | | 5.4 ± 0.4 |
| 200 | | 1200 | 0.7 ± 0.01 |
| 200 | 990 | 1200 | 9.7 ± 0.01 |

absence of added heparin. One explanation for this observation might be that crude ECGF preparations contain a heparin-like substance present in sufficient quantity to promote growth when high ECGF concentrations are used. Consistent with this possibility, the stimulatory activity of ECGF at 200 μg/ml was completely blocked by addition of a high dose of protamine (1200 μg/ml); cell proliferation was restored by addition of excess heparin (990 μg/ml). In addition to heparin, the glycosaminoglycans chondroitin sulfate, hyaluronic acid, and keratin sulfate were tested for growth-promoting capabilities and had no effect on endothelial cell growth (*8*). The sulfated polysaccharide, dextran sulfate, significantly enhanced proliferation, although dextran itself was inactive (*8*).

The mechanism by which heparin promotes human endothelial cell proliferation is unknown. Azizkhan *et al.* (*10*) showed that heparin (and dextran sulfate) increased bovine capillary endothelial cell migration but had no effect on proliferation, a finding that is consistent with reported differences between large vessel and capillary endothelium (*11*). Other reports on the effects of heparin on various cell types have yielded conflicting results (*12–14*). Several investigators have shown that heparin binds to the cell surface (*14, 15*) and thus may influence intercellular communication (*16*) and membrane receptor accessibility (*17*). Morphologic changes (*18*) and modifications of cellular behavior (*10, 19*) consistent with cell membrane–heparin interactions have been reported. In vivo, the extracellular matrix of vascular tissue contains high concentrations of glycosaminoglycans (*20*). In vitro, heparin-like molecules are secreted by endothelial cells (*21*) and inhibit smooth muscle cell growth (*13*). Our demonstration that heparin enhances endothelial cell proliferation suggests that heparin-like substances may play an important role in cell growth regulation in normal and injured vessels.

In the past, existing culture techniques permitted only restricted proliferation of human endothelial cells, and therefore many basic and applied studies had to be performed on endothelial cells from other animal species. Theoretically, the procedures described above for serial subcultivation can increase the yield of HUVE cells by $10^8$-fold and of adult vessel endothelial cells by $10^{12}$-fold over previously published methods (*4, 5*). This will permit minimal amounts of human vascular tissue to be used for the generation of large numbers of cultured endothelial cells, and thus problems of human pathology involving the endothelium now can be approached directly by means of a human endothelial cell model. In addition, this cell system should prove valuable for various clinical applications, such as in vitro testing of vasoactive agents and the coating of artificial graft materials.

SUSAN C. THORNTON
STEPHEN N. MUELLER
ELLIOT M. LEVINE*

*Wistar Institute,*
*Philadelphia, Pennsylvania 19104*

### References and Notes

1. For review, see E. M. Levine, S. N. Mueller, J. B. Grinspan, J. P. Noveral, E. M. Rosen, in *Biochemical Interactions at the Endothelium*, A. Cryer, Ed. (Elsevier, Amsterdam, 1983), pp. 313–342.
2. S. N. Mueller, E. M. Rosen, E. M. Levine, *Science* **207**, 889 (1980);E. M. Rosen, S. N. Mueller, J. P. Noveral, E. M. Levine, *J. Cell. Physiol.* **107**, 123 (1981).
3. D. Gospodarowicz, K. D. Brown, C. R. Birdwell, B. R. Zetter, *J. Cell Biol.* **77**, 774 (1978).
4. T. Maciag, G. A. Hoover, M. B. Stemerman, R. Weinstein, *ibid.* **91**, 420 (1981).
5. M. K. Glassberg *et al.*, *In Vitro* **18**, 859 (1982).
6. A. R. Johnson, *J. Clin. Invest.* **65**, 841 (1980); M. A. Gimbrone, Jr., R. S. Cotran, J. Folkman, *J. Cell Biol.* **60**, 673 (1974); E. A. Jaffe, R. L. Nachman, C. G. Becker, C. R. Minick, *J. Clin. Invest.* **52**, 2745 (1973); C. C. Haudenschild, R. S. Cotran, M. A. Gimbrone, Jr., J. Folkman, *J. Ultrastruct. Res.* **50**, 22 (1975); P. B. Gordon and V. B. Hatcher, *J. Cell Biol.* **91**, 205A (1981); G. L. Fry *et al.*, *Circulation* **86**, 177 (1982).
7. Gelatin supports attachment and proliferation as well as fibronectin and is used for routine subculture.
8. S. C. Thornton, B. Jarrell, S. N. Mueller, S. Shapiro, P. Thiagarajan, E. M. Levine, in preparation.
9. J. W. Ryan, A. Chung, F. Ammons, M. L. Carlton, *Biochem. J.* **167**, 501 (1977).
10. R. G. Azizkhan, J. C. Azizkhan, B. R. Zetter, J. Folkman, *J. Exp. Med.* **152**, 931 (1980).
11. B. R. Zetter, *Diabetes* **30** (Suppl.), 24 (1981).
12. O. Costachel, L. Fadei, M. Nachtigal, *Exp. Cell Res.* **34**, 542 (1964); T. K. Yang and H. M. Jenkin, *Proc. Soc. Exp. Biol. Med.* **159**, 88 (1978).
13. J. J. Castellot, Jr., M. L. Addonizio, R. Rosenberg, M. J. Karnovsky, *J. Cell Biol.* **90**, 372 (1981).
14. M. Lippman, in *Epithelial-Mesenchymal Interactions: 18th Hahnemann Symposium*, R. Fleischmayer and R. E. Billingham, Eds. (Williams & Wilkins, Baltimore, 1968), pp. 208–229.
15. L. Kjellen, A. Oldberg, K. Rubin, M. Hook, *Biochem. Biophys. Res. Commun.* **74**, 126 (1977); B. Glimelius, C. Busch, M. Hook, *Thromb. Res.* **12**, 773 (1978).
16. R. Roblin, S. O. Albert, N. A. Gelb, P. H. Black, *Biochemistry* **14**, 347 (1975); T. Ohnishi, E. Ohshima, M. Ohtsuka, *Exp. Cell Res.* **93**, 136 (1975).
17. P. M. Kraemer and D. A. Smith, *Biochem. Biophys. Res. Commun.* **56**, 423 (1974).
18. A. Abro and K. A. Abraham, *Experientia* **31**, 1453 (1975).
19. W. Regelson, in *Advances in Chemotherapy*, A. Goldin *et al.*, Eds. (Academic Press, New York, 1968), vol. 3, pp. 303–370.
20. A. Gardais, J. Picard, B. Hermelin, *Comp. Biochem. Physiol. B* **44**, 507 (1973).
21. V. Buonassisi, *Exp. Cell Res.* **76**, 363 (1973); C. Busch, C. Ljungman, C. M. Heldin, E. Wasksson, B. Obrink, *Haemostasis* **8**, 142 (1979); G. Gamse, H. G. Fromme, H. Kresse, *Biochim. Biophys. Acta* **544**, 514 (1978).
22. Supported by NIH grant AG-00839 (E.M.L.). S.C.T. was an NIH predoctoral trainee (T32-CA-09171) at the Wistar Institute. We gratefully acknowledge the help and encouragement of B. Jarrell, S. Shapiro, and P. Thiagarajan.
* To whom all correspondence should be addressed.

25 July 1983; accepted 8 September 1983

## Long-Lived Oxidants Generated by Human Neutrophils: Characterization and Bioactivity

Abstract. *Human neutrophils were found to generate an unusual class of oxidants with a half-life of approximately 18 hours and with characteristics similar to, if not identical with, those of* N*-chloroamines. These neutrophil-derived* N*-chloroamines have sufficient oxidizing potential to attack sulfhydryl- or thioether-containing compounds and can react with both a methionine-containing chemotactic peptide and a plasma protease inhibitor. As judged by their stability and selective reactivity, the* N*-chloroamines generated by stimulated neutrophils may play an important role in the local and systemic regulation of inflammatory events in vivo.*

Human neutrophils can generate reactive oxygen metabolites and use them to destroy microorganisms and normal or neoplastic mammalian cells and to modulate the inflammatory response (*1*). After specific membrane perturbation, the neutrophil produces superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), hypohalous acids (HOX), and possibly the hydroxyl radical ($OH^{\cdot}$). In vitro, the generation of these oxygen metabolites by neutrophils is self-limited and is nearly complete within 60 minutes after the addition of a stimulus (*1*). Under physiological conditions, $O_2^{\cdot -}$ rapidly dismutates to $H_2O_2$ spontaneously or enzymically via superoxide dismutase, and $H_2O_2$ is reduced by endogenous catalase, glutathione peroxidase, or myeloperoxidase (*1*). In addition, highly reactive oxidants like HOX or $OH^{\cdot}$ disappear rapidly as they react with multiple oxidizable substrates in the complex cell systems (*1*). Thus, the concentration of oxygen metabolites is expected to fall to undetectable levels shortly after the neutrophil terminates its production of these species. We have now shown (i) that human neutrophils can generate large quantities of a long-lived oxidant, with characteristics similar to, if not identical with, those of *N*-chloroamines and (ii) that this oxidant is powerful enough to attack thioether-containing peptides and proteins thought to be important in the inflammatory response.

Human neutrophils were isolated from venous blood by a Ficoll-Hypaque sepa-