bon gases were detected, although the cores had been held for many days (Table 2). The ratio of methane to ethane plus propane ($C_1/(C_2 + C_3)$] and the $\delta^{13}C$ values of gases are used to distinguish biogenic from petrogenic hydrocarbons. A ratio greater than 1000 and $\delta^{13}C$ more negative than −60 per mil indicates bacterial methane (*2*). On the basis of these parameters, the gases in the cores are derived from deep, thermally produced sources (Table 2). The relative percentage of ethane to pentane and the percentage of propane (Table 2), as high as 75.7 and 33.5, respectively, suggest that a mechanism is available to concentrate these molecules. Anomalous seismic reflections suggest the presence of gas hydrates in western Gulf of Mexico sediments (*3*). Gas hydrate formation has been suggested as a possible mechanism for the retention of natural gas of unusual chemical composition (*13*).

R. K. ANDERSON
R. S. SCALAN
P. L. PARKER
Marine Science Institute, University of Texas, Port Aransas 78373
E. W. BEHRENS
Institute of Geophysics, University of Texas, Austin 78712

References and Notes

1. R. A. Geyer and W. M. Sweet, Jr., *Trans. Gulf Coast Assoc. Geol. Soc.* **23**, 158 (1973).
2. B. B. Bernard, J. M. Brooks, W. M. Sackett, *Earth Planet. Sci. Lett.* **31**, 48 (1976).
3. T. H. Shipley *et al.*, *Am. Assoc. Pet. Geol. Bull.* **63**, 2204 (1979); S. K. Addy and J. L. Worzel, *ibid.*, p. 668.
4. A simple long-wavelength ultraviolet source consisting of a GE-F6T5-BLB lamp in a camping lantern was used for the sample surveys.
5. High-resolution gas chromatography was carried out on the saturated and unsaturated hydrocarbon fractions with a Perkin-Elmer model 910 chromatograph with flame-ionization detector. A 25-m fused-silica, wall-coated open tubular column coated with OV-101 liquid phase was programmed from 100°C to 270°C at 7.5°C per minute. Silica-gel chromatography was done on a portion of the bitumen after the methanol-benzene solvent was removed by rotary vacuum evaporation and the material was dissolved in hexane. The hexane solution was placed on a silica-gel column (20 by 1 cm) (Activity-I, 60- to 200-mesh Woelm silica gel). The three fractions of total bitumen were successively eluted with hexane, benzene, and methanol. These fractions were then freed of solvent and used for GLC and isotope analysis.
6. Carbon isotope compositions are expressed as the difference (in parts per mil) between the standard (std) and the sample, based on the $\delta^{13}C$ notation:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{std}}{(^{13}C/^{12}C)_{std}} \times 10^3$$

When Pee Dee belemnite is used as a standard, data for organic matter will be small negative numbers. The reproducibility is ± 0.2 per mil. We converted organic carbon to CO_2 for isotope analysis by the sealed tube method, using Pyrex glass and copper oxide [S. Sofer, *Anal. Chem.* **52**, 1389 (1980)]. Isotope measurements were made on a VG-Micromass model 602E isotope-ratio mass spectrometer. Carbonate samples were converted to CO_2 with phosphoric acid according to standard procedures.
7. M. A. Northam, D. J. Curry, R. S. Scalan, P. L. Parker, *Geochim. Cosmochim. Acta* **45**, 257 (1981).
8. N. J. L. Bailey, H. H. Krouse, C. R. Evans, M. A. Rogers, *Am. Assoc. Pet. Geol. Bull.* **57**, 1276 (1973); R. Sassen, *Org. Geochem.* **2**, 153 (1980); R. Rezak *et al.*, *Trans. Gulf Coast Assoc. Geol. Soc.* **19**, 115 (1969).
9. K. A. Kvenvolden and M. E. Field, *Am. Assoc. Pet. Geol. Bull.* **65**, 1642 (1981).
10. Bitumen sample IG-46-5 (280 to 285 cm) had an organic sulfur content of 2.5 percent as compared to 3.74 percent for the Challenger Knoll oil. The API gravity of the Challenger Knoll oil was 14.8; the corresponding values for the top six samples of core IG-46-5 were 10.7, 14.5, 16.2, 14.8, 16.3, and 17.6. High sulfur content is generally associated with low API gravity values.
11. R. S. Scalan, J. K. Winters, P. L. Parker, *Energy and Environmental Chemistry*, vol. 1, *Fossil Fuels*, L. H. Keith, Ed. (Ann Arbor Science, Ann Arbor, Mich., 1982), pp. 219–231; B. Koons and P. H. Monaghan, *Initial Rep. Deep Sea Drill. Proj.* **1**, 486 (1969). The $\delta^{13}C$ values for bitumen, saturated hydrocarbons, aromatic hydrocarbons, and polar compounds were, respectively, −26.6, −26.5, −26.8, and −26.5.
12. We took gas samples by puncturing the plastic core liner with a two-way needle and allowing gas to flow into a 20-ml Vacutainer. Gas components were separated by a combination of differential adsorption on silica-gel and packed-column chromatography. The components were oxidized to CO_2 for isotopic analysis in a vacuum apparatus adapted from H. Craig [*Geochim. Cosmochim. Acta* **3**, 53 (1953)].
13. K. A. Kvenvolden, private communication. J. M. Brooks has recently reported (private communication) finding gas hydrates in sediment samples taken from this same general location in the Gulf of Mexico.
14. This research was sponsored by Getty Oil, Gulf Oil Exploration and Production, Mobil Exploration and Production, Phillips Petroleum, Shell Development, and Tenneco Oil. This report is contribution 590 of the Marine Science Institute and 561 of the Institute of Geophysics, University of Texas.

20 June 1983; accepted 22 July 1983

Nickel: An Essential Micronutrient for Legumes and Possibly All Higher Plants

Abstract. *Soybean plants deprived of nickel accumulated toxic concentrations of urea (2.5 percent) in necrotic lesions on their leaflet tips. This occurred regardless of whether the plants were supplied with inorganic nitrogen or were dependent on nitrogen fixation. Nickel deprivation resulted in delayed nodulation and in a reduction of early growth. Addition of nickel (1 microgram per liter) to the nutrient media prevented urea accumulation, necrosis, and growth reductions. This evidence suggests that nickel is essential for soybeans and possibly for higher plants in general.*

In contrast to the situation in animals, for which four new essential trace elements have been identified in the last decade (*1*), no new generally essential micronutrient for higher plants has been discovered since 1954 (*2*). Several investigators have suggested that nickel might be essential for plants, but conclusive evidence has not been reported (*3, 4*). The recent finding that urease is a nickel-metalloenzyme (*5*), however, suggested that nickel might have a specific function in higher plants. Nickel has been shown to stimulate growth when urea is the sole nitrogen source but to have slight or no effect with other nitrogen sources (*6, 7*). We report here that nickel is essential for nitrogen metabolism in soybeans [*Glycine max* (L.) Merr.], either when nitrogen is supplied as NO_3^- and NH_4^+ or when the plants are dependent on nitrogen fixation.

Soybeans (cultivar "Maple Presto") were grown in nutrient solutions that had been purified of nickel by chromatography with 8-hydroxyquinoline and controlled-pore glass beads (8HQ-CPG) (*8*). This technique, similar to that proposed by Hill (*9*), removed 99.99 percent of ^{63}Ni added. The purified nutrient solutions were estimated to have a nickel concentration of 0.06 μg liter^{-1}. Plants grown without nickel additions (Ni_0 treatment) developed necrotic lesions on 27.5 ± 3.2 percent (mean ± standard error) of their leaflet tips, and this injury was absent on plants that were supplied nickel at concentrations of either 1 (Ni_1) or 10 (Ni_{10}) μg liter^{-1} (*10*). Leaflet tip necroses were counted 56 days after imbibition, during mid-podfill (Fig. 1A). A urea concentration of 2.5 percent (weight/weight) was found in necrotic leaflet tips, whereas no urea could be detected in Ni_1 plants (*11*). Leaflet tips were also analyzed for potassium, calcium, magnesium, phosphorus, sulfur, iron, manganese, zinc, boron, and copper by inductively coupled argon plasma–optical emission spectrophotometry. All these concentrations were within the normal range for soybean leaves (*12*).

Shimada and Ando (*7*) found that nickel additions increased leaf urease activity in tomatoes and soybeans and that low-nickel plants, grown with urea as the sole nitrogen source, accumulated urea and developed necrotic leaflet tips. Thus, we conclude that the leaflet tip necrosis that we observed was caused by the accumulation of toxic concentrations of urea.

Seed yields were similar in all three treatments, and all gave 100 percent germination. Urease activity and the nickel concentration in the seed were increased when nickel was supplied (Table 1). Nickel concentrations in Ni_0 seed digests were comparable to blank values. A similar relationship in jack bean seed has been reported by Dixon *et al.* (*13*), but

Fig. 1. (A) First-generation Ni_0 soybean plant with necrotic lesions on leaflet tips due to the accumulation of 2.5 percent urea. (B) A typical trifoliate showing more severe leaflet tip necrosis due to nickel deficiency in nitrogen-fixing plants.

their low-nickel seed still had a nickel concentration of 480 ng g^{-1} and retained 6 percent of the urease activity of the parent seed.

The Ni_0 seed were used to grow a second generation. Three treatments were performed with the nitrogen-containing solution formulation (*8*): (i) no nickel added, (ii) 1 μg of nickel per liter added, or (iii) a treatment in which analytical reagent grade salts were used without further purification. Plants were also grown in purified nitrogen-free solution and inoculated with *Rhizobium japonicum* U.S. Department of Agriculture strain 110 with either no nickel added or with a nickel concentration of 1 μg $liter^{-1}$. It was suspected that nitrogen-fixing plants would have a higher requirement for nickel because soybeans transport 80 to 95 percent of the nitrogen fixed as ureides, and it has been suggested that these compounds would be broken down to urea and glyoxylate prior to further metabolism (*4, 14*).

Nodulation was delayed by 2 to 3 days without added nickel, and plant dry weight was reduced at 31 days in the nitrogen-free solution (Table 2). No growth differences were observed for plants in nitrogen-containing solutions. Moreover, no growth enhancement was observed for plants grown in unpurified nitrogen-containing solution. This indicates that the purification method did not remove any other, as yet unrecognized, essential element.

Leaf urease activity was higher in plants supplied with nickel than in those not receiving this element and was intermediate in plants grown in unpurified nutrient solutions (Table 2). These observations are in good agreement with the fact that the nickel concentration of unpurified solutions was 0.28 μg $liter^{-1}$ and suggest that nutrient solutions often used in plant nutrition studies may be only marginally adequate in nickel.

The percentage of tip necroses increased in second-generation plants, and, as predicted, the injury was more severe for plants fixing nitrogen than for those receiving inorganic nitrogen (Table 2 and Fig. 1B). No differences in final seed yield were found as a result of nickel treatments, and all seed lots gave 100 percent germination.

The evidence presented here meets, at least in part, both of the independent criteria for establishing that an element is essential for higher plants (*15*). An element may be considered essential (i) if a plant grown in a medium adequately

Table 1. Yield, nickel concentration, and urease activity of first-generation soybean seeds grown in three nutrient solutions. Urease activity [per milligram of protein (*17*)] was assayed in the Ni_1 and Ni_{10} seed as described by Polacco *et al.* [0.025 μmole mg^{-1} $hour^{-1}$, detection limit (*18*)] except that 0.1*M* MOPS buffer replaced the 0.1*M* tris-maleate used by Polacco *et al.* Assays of Ni_0 seed were performed with 10 m*M* [^{14}C]urea (0.02 μCi $\mu mole^{-1}$) instead of 900 m*M* urea. For nickel analysis, we digested 1 g of seed in polytetrafluoroethylene Teflon beakers, using 10 ml of glass-distilled HNO_3 and 2 ml of Baker Ultrex $HClO_4$. Digests were preconcentrated on 8HQ-CPG and then analyzed by heated graphite atomization–atomic absorption spectrophotometry. The values listed are the means ± the standard errors of 12 measurements.

Nickel content of nutrient solution (μg $liter^{-1}$)	Seed yield (g per plant)	Seed nickel concentration (ng g^{-1})	Urease activity (μmole mg^{-1} $hour^{-1}$)
0	27.2 ± 1.6	≤ 10	0.007 ± 0.003
1	24.8 ± 2.7	53 ± 6	2.3 ± 0.7
10	27.8 ± 1.3	637 ± 28	36.9 ± 6.3

Table 2. Effects of a nickel concentration of 1 μg $liter^{-1}$ on shoot dry weight after 31 days, leaf urease activity at 29 days, percentage of leaflet tip necrosis at 56 days, and seed yield in second-generation soybeans. To assay leaf urease activity, leaflets were extracted in three volumes of 0.1*M* MOPS (*p*H 7.0), 10 m*M* EDTA, 2 percent soluble polyvinylpyrrolidone, 10 m*M* mercaptoethanol. A 0.1-ml portion of this extract was added to 0.3 ml of 0.4*M* tris (*p*H 8.9), 1 m*M* EDTA, 7.5 m*M* mercaptoethanol. Assays were started by the addition of 0.1 ml of 50 m*M* [^{14}C]urea (0.1 μCi $\mu mole^{-1}$). After 1 hour at 30°C, 0.25 ml of 2*N* H_2SO_4 was added and $^{14}CO_2$ was determined. The values listed are the means ± the standard errors of six measurements.

Nutrient solution		Shoot dry weight (g)	Leaf urease activity (nmole mg^{-1} $hour^{-1}$)	Leaflet tip necrosis (%)	Seed yield (g per plant)
Nitrogen*	Nickel*				
−	−	1.4 ± 0.1	7.9 ± 0.7	70.7 ± 7.9	8.4 ± 0.4
−	+	2.0 ± 0.2	14.5 ± 0.3	0	8.3 ± 0.4
+	−	11.8 ± 1.8	6.2 ± 0.4	48.8 ± 5.5	23.1 ± 1.5
+	+	11.6 ± 0.2	10.8 ± 0.5	0	19.8 ± 0.8
+	U†	9.7 ± 1.4	7.5 ± 0.4	0	21.4 ± 2.2

*Plants were either grown with 13.0 m*M* NO_3^- and 2.5 m*M* NH_4^+ (+) or were dependent upon nitrogen fixation (−). †Unpurifed nutrient solution (*8*) with no additional nickel.

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 μ*M* $CaCl_2$, 50 μ*M* FeEDTA, 6.25 μ*M* H_3BO_3, 1 μ*M* $MnSO_4$, 2 μ*M* $ZnSO_4$, 0.5 μ*M* $CuSO_4$, 0.5 μ*M* $(NH_4)_2MoO_4$, and 0.01 μ*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 μCi of ^{63}Ni per liter (as $NiCl_2$) was added and the solution was passed through a 0.22-μm 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-

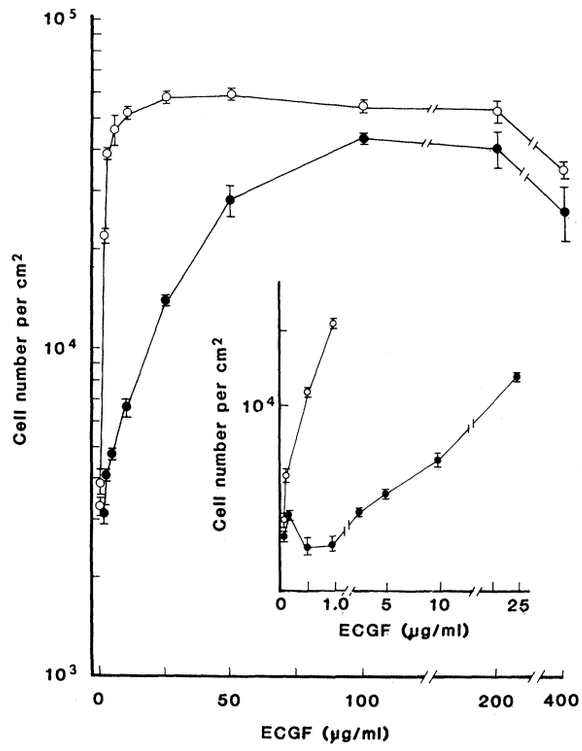

Fig. 1. Effect of heparin on human endothelial cell growth. HUVE cells (cumulative PD level, 23) were seeded in Medium 199 with ECGF (20 μg/ml) and heparin (90 μg/ml) at 5×10^3 cells per square centimeter in 25-cm^2 flasks coated with gelatin. Cells were allowed to attach for 1 hour, washed three times, and again fed with Medium 199 supplemented with various concentrations of ECGF in the presence (○) and absence (●) of heparin (90 μg/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 μg/ml.