Expression of hydrogenase activity in free-living *Rhizobium japonicum*

(nitrogen fixation/legumes/soybeans/mutant)

ROBERT J. MAIER*, NORMAN E. R. CAMPBELL†, FRANK J. HANUS*, FRANK B. SIMPSON*,
STERLING A. RUSSELL*, AND HAROLD J. EVANS*

* Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331; and † Department of Microbiology, University of Manitoba, Winnipeg, Canada

Contributed by Harold J. Evans, April 17, 1978

ABSTRACT A medium is described on which selected *Rhizobium japonicum* strains express hydrogenase (H_2 uptake) activity under free-living conditions. Low concentrations of carbon substrates, decreased oxygen tension, and the quantity of combined nitrogen in the medium were major factors influencing hydrogenase expression. Hydrogenase activity was dependent upon a preincubation period in the presence of H_2 under conditions such that the cells did not exhibit nitrogenase activity. H_2 uptake rates were easily measured amperometrically in aerobically or anaerobically prepared suspensions from free-living cultures. Six *R. japonicum* strains that formed nodules with the ability to utilize H_2 oxidized this gas when grown in free-living cultures. In comparison six randomly chosen strains forming nodules that lost H_2 in air either showed no or low capacity to take up H_2 under free-living conditions. The reduction of triphenyltetrazolium chloride in an agar medium was used to detect strains capable of oxidizing H_2 . This method has enabled us to isolate a spontaneous *R. japonicum* mutant strain that has lost the ability to utilize H_2 . This mutant strain forms nodules that evolve H_2 but other symbiotic characteristics appear normal. This strain will be useful in evaluating the importance of the hydrogenase system in the nitrogen-fixing process of legumes.

In addition to catalysis of N_2 reduction, nitrogenases from all known sources catalyze ATP-dependent H_2 evolution. Apparently, H_2 evolution during N_2 fixation is an inherent property of the nitrogenase reaction. Energy loss through nitrogenase-dependent H_2 evolution is important because four or five ATP molecules are consumed per pair of electrons transferred (1, 2) regardless of whether N_2 or H^+ is the electron acceptor. The significance of ATP-dependent evolution of H_2 by nodules as an energy-wasteful process during N_2 fixation by legumes has been pointed out by Dixon (3), Schubert and Evans (4), and Evans *et al.* (5). Some N_2 -fixing symbionts, however, possess an uptake hydrogenase that provides a mechanism whereby H_2 evolved by the nitrogenase system is recycled and utilized. Experiments with pea nodule bacteroids by Dixon (6) indicated that the H_2 oxidation via the hydrogenase system was coupled to the synthesis of ATP. Those *Rhizobium* strains that form nodules with an active hydrogenase would be expected to have the capability of conserving energy provided by the plant. A H_2 -recycling mechanism should increase the overall efficiency of the N_2 -fixing process under conditions in which photosynthesis is limiting.

The genetic information for hydrogenase, like that of nitrogenase, appears to reside in the bacteria rather than in the legume. According to Carter *et al.* (7), the capacity to recycle H_2 produced by the nitrogenase system in the nodules of soybeans was determined by the genetic property of the *R. japo-*

nicum strain used as inoculum and was not appreciably influenced by several different soybean cultivars. Although hydrogenase activity has been identified in bacteroids from nodules of pea (6, 8) and soybean (9), hydrogenase activity in free-living cultures of *Rhizobium* has not yet been reproducibly demonstrated. An objective of our research was to define conditions that would allow hydrogenase expression in free-living cultures of rhizobia. This was needed for genetic and physiological investigations of the hydrogenase system in agriculturally important rhizobia.

MATERIALS AND METHODS

Rhizobium Strains and Media. *R. japonicum* strains were obtained from George Ham of the University of Minnesota, St. Paul, and Deane Weber of the U.S. Department of Agriculture. Strain USDA (DES) of *R. japonicum*, which is a small colony derivative of USDA strain 122 obtained by detergent treatment, was kindly supplied by David Emerich of this laboratory. *R. japonicum* 311b I-110, a derivative of 311b 110 (10) was obtained from G. Elkan, Department of Microbiology, North Carolina State University.

The H_2 -uptake medium is a further modification of the modified LNB5 medium used to detect acetylene reduction activity in free-living cultures of *R. japonicum* (11). The H_2 -uptake medium contains the following in 1 liter of distilled water: $NaH_2PO_4 \cdot H_2O$, 150 mg; $CaCl_2 \cdot 2H_2O$, 150 mg; $MgSO_4 \cdot 7H_2O$, 250 mg; iron EDTA, 28 mg; $MnSO_4 \cdot H_2O$, 10 mg; H_3BO_3 , 3 mg; $ZnSO_4 \cdot 7H_2O$, 2 mg; $NaMoO_4 \cdot 2H_2O$, 0.25 mg; $CuSO_4 \cdot 5H_2O$, 0.04 mg; $CoCl_2 \cdot 6H_2O$, 0.025 mg; KI, 0.78 mg; inositol, 100 mg; thiamine hydrochloride, 10 mg; nicotinic acid, 1 mg; pyridoxal HCl, 1 mg; sucrose, 0.5 g; L-arabinose, 1.0 g; sodium gluconate, 0.5 g; sodium glutamate, 0.5 g; yeast extract, 0.1 g; and Noble agar (Difco), 12 g. This was adjusted to pH 5.5 with concentrated HCl. This medium was used in all experiments unless otherwise indicated.

Hydrogenase Assays. *R. japonicum* cells were cultured in 16 × 150 mm test tubes each containing 7 ml of the H_2 -uptake medium as a 1.2% agar slant. Approximately 1×10^7 cells of an exponential liquid culture of USDA 122 (DES) (used in all experiments unless otherwise indicated) was distributed over the surface of the slant. The inoculated slant was incubated in air, unless otherwise indicated. After 5 days of growth at 27°, the tube cap was replaced with a sterile serum stopper and 1 ml of 10% H_2 (in air) was injected into each tube. After equilibration, a 0.5-ml gas sample was removed from each tube and analyzed by H_2 gas chromatography to determine the initial H_2 concentration. After 18 hr of incubation at 27°, unless otherwise indicated, a second 0.5-ml sample of gas was removed and assayed for H_2 by using a 5836 Hewlett-Packard gas chromatograph equipped with a 6.4 mm × 1.8 m column of

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

molecular sieve 5A (40–60 mesh). Other conditions were: column temperature, 120°; carrier gas, N₂ at a flow rate of 24 ml/min. After H₂ was determined, stoppers were removed and replaced by caps to permit the escape of the remaining H₂ prior to the nitrogenase assays.

Nitrogenase Assays. Caps were replaced again with serum stoppers and each tube then received 1.0 ml of C₂H₂. After incubation at 27° for 2–3 hr, a 0.5-ml gas sample was removed for C₂H₄ determination. Other conditions for measurement of C₂H₄ have been described (12).

Protein Determination. Cells were removed from agar slants with two 2.5-ml washes of 0.85% NaCl. The two suspensions of cells in saline were pooled, and the cells were removed by centrifugation at 8000 × *g* for 20 min. Each cell pellet was resuspended in 1 ml of 0.05 M potassium phosphate, pH 7.0/2.5 mM MgCl₂, and a 0.5-ml sample was digested as described by Stickland (13). Protein was determined by the biuret method of Goa (14). The specific activity of hydrogenase (nmol of H₂ taken up per hr per mg of cell protein) was calculated for each individual slant culture.

Amperometric Determinations. H₂ and O₂ uptake rates by cell suspensions were measured as described (9). Cells were removed from slants in 0.05 M potassium phosphate, pH 7.0/2.5 mM MgCl₂, dispersed by agitation, and then placed in the electrode chamber. Rates of H₂ uptake were measured under conditions in which saturation concentrations of both H₂ and O₂ were provided.

RESULTS

Conditions for Hydrogenase Expression. Initial attempts to detect H₂ uptake in free-living *R. japonicum* USDA 122 (DES) were conducted with cells cultured on a medium developed for expression of nitrogenase activity in free-living *R. japonicum* (11). Very low rates of H₂ uptake were detected on this medium; however, C₂H₂ reduction activity was observed. Concentrations of yeast extract and carbon and nitrogen substrates were varied in an attempt to optimize conditions for hydrogenase expression. A decrease in the concentration of yeast extract from 1.0 to 0.1 g/liter resulted in a doubling of hydrogenase activity (data not shown). The most dramatic increase in H₂-uptake rates resulted from decreasing the carbon sources (sucrose, gluconate, arabinose) to 10% of their original concentrations (Table 1). Although the amount of cell growth was not significantly affected by the high amounts of carbon substrates in the medium, these concentrations did not promote the expression of hydrogenase activity. Increases in H₂-uptake rates also were obtained by decreasing the glutamate from 1.0 to 0.5 g/liter. The optimal H₂-uptake activity was obtained on the H₂-uptake medium described in *Materials and Methods*.

Rates of C₂H₂ reduction <5 nmol of C₂H₄ produced per hr per mg of protein were observed in the cells grown on either the medium containing 50% or on that containing the standard amount of carbon substrates. No nitrogenase activity was detected in cells cultured on the medium containing 10% of the standard carbon substrate concentration. Attempts to obtain an optimal pH for expression of H₂-uptake activity were made in tests in which 25 mM potassium phosphate, 2-(*N*-morpholino)ethanesulfonic acid, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffers (15) were used at pH values ranging from 5.0 to 7.5. The greatest H₂-uptake rates were observed by use of the H₂-uptake medium without additional buffer. After a growth period of 6 days on this medium, the pH of the cell suspension washed from the slants with distilled water varied between 7.3 and 7.5.

Table 1. Effect of concentrations of carbon substrates and glutamate on the rate of hydrogen uptake by free-living *R. japonicum*

Carbon substrate,* % of standard medium	Specific activity at various glutamate levels†		
	1.0 g/liter	0.5 g/liter	0.1 g/liter
100	<1.0	4 ± 2	26 ± 11
50	51 ± 9	22 ± 2	16 ± 1
10	110 ± 10	195 ± 23	37 ± 6
5	87 ± 17	80 ± 11	57 ± 4

* The standard medium was a modification of the LNB5 medium of Maier and Brill (11). In addition to the components described for the H₂-uptake medium (see *Materials and Methods*) it contained, in g/liter, the following: sucrose, 5; arabinose, 10; and gluconate, 5. Each of the carbon substrates was added at the percentages indicated. Results are means ± SEM for five replicate cultures.

† Specific activity is defined as nmol of H₂ taken up per hr per mg of cell protein. The total cell protein in cultures grown on medium containing 5 or 10% carbon substrates was approximately the same as that produced on a medium containing the standard amount of carbon substrates. Cells were grown in agar slant tubes for 5 days, and H₂-uptake rates were determined by gas chromatography.

Because high hydrogenase activity of *Rhizobium* bacteroids isolated from the low-O₂-tension environment of nodules has been shown (8, 9), *R. japonicum* was cultured at a series of different O₂ concentrations for the 5-day growth period. H₂-uptake activity increased dramatically as the O₂ concentration was decreased in the gas phase above the agar slants (Table 2).

To quantitate H₂-uptake rates of *R. japonicum* grown under free-living conditions, cell suspensions of USDA 122 (DES) cultured on the H₂-uptake medium were prepared and assayed for rates of O₂ and H₂ uptake by use of the amperometric method. No H₂ was evolved during the 6-min assay period, but O₂ was taken up at rapid rates (Fig. 1A). Free-living *R. japonicum* cells exhibited O₂-dependent H₂ uptake (Fig. 1B). The rates of H₂ uptake were linear with time and were not increased by increasing the concentration of either O₂ or H₂. Hydrogenase activity was readily demonstrated in aerobically or anaerobically prepared suspensions from free-living cultures of *R. japonicum*.

In order to demonstrate hydrogenase in *R. japonicum* cells grown on the H₂-uptake medium, a preincubation period in the presence of H₂ was required (Table 3). In contrast, when *R. japonicum* cells were cultured in a medium in which nitrogenase activity was detected and undoubtedly some H₂ was produced, no preincubation period with H₂ was required. From these results, H₂ appears to be required for hydrogenase expression.

Variation of Hydrogenase Activity among Strains. Nodules formed by *Rhizobium* strains containing hydrogenase show greater relative efficiencies of energy utilization than those formed by strains without hydrogenase (9). Six *R. japonicum* strains that previously were shown to form nodules with high relative efficiencies and capacities to take up exogenous H₂ were tested for H₂-uptake capability when grown on the H₂-uptake medium under free-living conditions (Table 4). All six of the relatively efficient strains utilized H₂, but six randomly chosen *R. japonicum* strains that formed nodules showing relatively low efficiencies oxidized little or no H₂. Strain USDA 16, which is classified as relatively inefficient, took up H₂ at the low rate of 2.6 nmol/hr per mg of protein. Cell growth (as indicated by total protein content) was not affected by the magnitude of the relative efficiencies of the two groups of strains. The capability for symbiotic H₂ uptake therefore is

Table 2. Effect of O₂ concentration on hydrogenase activity of *R. japonicum* cultured on agar slants

O ₂ concentration, % of gas phase	Hydrogenase specific activity*
20	91 ± 17
10	126 ± 10
4	214 ± 36
2	806 ± 88
1	1319 ± 219

* Shown as nmol of H₂ taken up per hr per mg of cell protein (mean ± SEM, five replicate cultures). The differences in activities were not correlated with amount of cell growth because the tubes incubated under 1% and 20% O₂ levels contained approximately the same amounts of cell protein. Specific activities for cells grown on cultures provided with 20, 10, and 5% O₂ concentrations were computed on the basis of an 8-hr assay period. To obtain rates at lower O₂ concentrations, a 2-hr assay period was necessary. Cells were grown in agar slant tubes of the H₂-uptake medium for 5 days under the O₂ concentration indicated, and H₂-uptake rate was determined by gas chromatography.

positively related to the capacity of *R. japonicum* strains in bacteroids to utilize internally produced H₂ within the nodule.

Because a screening method for the detection of hydrogenase activity in free-living cultures of *R. japonicum* strains is needed, a group of relatively efficient and a group of relatively inefficient *R. japonicum* strains (Table 4) were tested on agar that contained triphenyltetrazolium chloride. This dye was reduced readily by the six strains containing hydrogenase but poorly or not at all by the six strains with low or no hydrogenase activities (Fig. 2). We have used the reduction of triphenyltetrazolium chloride as a screening procedure, along with a method that selectively kills those strains containing hydrogenase, to isolate a spontaneous mutant strain of *R. japonicum* lacking hydrogenase activity. This mutant strain forms nodules that evolve H₂ and exhibits no H₂-uptake activity as a cell suspension from a free-living culture (Fig. 3). Details of the procedure for isolation of the mutant will be presented elsewhere.

FIG. 1. H₂ and O₂ uptakes by suspensions of strain USDA (DES) 122. Cells from slants were suspended in 0.05 M potassium phosphate, pH 7.0/2.5 mM MgCl₂ and O₂ and H₂ concentrations were measured amperometrically. Each reaction contained 0.90 mg of cell protein per ml. (A) O₂ was added at the start of the assay. (B) Both O₂ and H₂ were added to initiate the assay.

Table 3. Effect of medium and addition of H₂ on nitrogenase and hydrogenase activities in free-living *R. japonicum*

Conditions	Rate of C ₂ H ₂ reduction, nmol/hr/mg protein*	Hydrogen- ase specific activity*
1) H ₂ -uptake medium alone	0	0
2) H ₂ -uptake medium + H ₂ [†]	0	109
3) Nitrogenase expression medium [‡]	10	36

* Rates of C₂H₂ reduction were determined by using a 2-hr incubation period; rates of H₂ uptake are during a 5-min amperometric measurement of H₂ uptake by suspension of cells removed from agar cultures. The minimum detectable rates (reported as zero) for C₂H₂ reduction and H₂ uptake were judged to be 0.1 and 5.0 nmol/hr, respectively. Replicates of this experiment showed similar results.

[†] To each culture slant (12 ml gas volume) was added 0.1 ml of H₂ 4 hr prior to the initiation of the electrode assay.

[‡] The high-carbon LNB5 medium as described by Maier and Brill (11) was used with the exception that it contained 5 g of sucrose and 0.5 g of yeast extract per liter.

DISCUSSION

The results presented show conclusively that those *R. japonicum* strains with the capability of forming nodules that recycle H₂ produced by the N₂-fixing reaction have the capacity to synthesize hydrogenase when cultured under free-living conditions. The genetic information for hydrogenase synthesis therefore is located within the *R. japonicum* cells. Sufficient information is now available to provide a logical basis for use of free-living *R. japonicum* cultures for genetic experiments designed to incorporate the uptake-hydrogenase into those rhizobial strains that possess other desirable characteristics. To consistently demonstrate high hydrogenase activity in free-

Table 4. Relationships of hydrogenase activities in free-living *R. japonicum* strains to efficiency of energy utilization for N₂ fixation by soybean nodules

Strain of <i>R. japonicum</i>	Hydrogenase in free-living cultures, specific activity*	Relative efficiencies of energy utilization by nodules [†]
3I1b I-110	46 ± 5	0.99
3I1b 6	12 ± 3	0.99
3I1b 142	18 ± 3	0.99
3I1b 143	45 ± 8	1.00
USDA (DES) 122	39 ± 6	1.00
USDA 136	38 ± 4	1.00
3I1b 144	0 [‡]	0.60
USDA 117	0	0.71
USDA 120	0	0.67
USDA 38	0	0.50
USDA 62	0	0.61
USDA 16	2.6 ± 0.9	0.66

Cells were grown and assayed as described for Table 1.

* Shown as nmol of H₂ taken up per hr per mg of cell protein (mean ± SEM, five replicate cultures).

[†] Relative efficiency has been defined as the decimal fraction of the total electron flux through the nitrogenase system that is utilized for N₂ reduction. All relative efficiency values except those for 3I1b I-110 and USDA (DES) 122 were taken from Carter *et al.* (7).

[‡] Rates of H₂ uptake are reported as zero if they differ from uninoculated control culture slants by less than the minimum detectable rate, 0.8 nmol/hr.

FIG. 2. Triphenyltetrazolium chloride reduction by *R. japonicum* strains. Cells were grown for 5 days on the H_2 -uptake medium containing sucrose (5 g/liter), arabinose (10 g/liter), gluconate (5 g/liter), and triphenyltetrazolium chloride (ICN Pharmaceuticals, Cleveland, OH) at 0.1 g/liter. (Left) These six strains have the ability to take up H_2 in both free-living cultures (Table 4) and as bacteroids isolated from soybean nodules. (Right) H_2 -uptake activity could not be detected in five of these six strains (Table 4). Strain USDA 16, however, utilized some H_2 .

living cultures of *R. japonicum*, it was necessary to limit the supply of both carbon substrates and O_2 to the culture. These conditions are similar to those that undoubtedly occur inside legume nodules (16, 17) where bacteroids actively synthesize hydrogenase if they possess the genetic capability (8, 9). The optimal conditions necessary for expression of hydrogenase activity in *R. japonicum* resemble those required for expression of activity of this enzyme system in the chemolithotrophic H_2 -oxidizing bacteria (18). In some of these organisms, ample supply of organic carbon substrates and high O_2 concentration

in the medium also were detrimental to hydrogenase expression (18).

Our experiments show that *R. japonicum* cells removed from slants under aerobic conditions contained hydrogenase activity that was approximately equal to that of comparable cells removed from slants under anaerobic conditions. The inhibitory effect of O_2 on hydrogenase expression therefore probably was due to repression of hydrogenase synthesis rather than inactivation of preformed hydrogenase. The complexity of the environmental and nutritional requirements for hydrogenase expression by free-living rhizobia provides an explanation for the difficulties in demonstrating activity of this enzyme in laboratory media by previous workers (19, 6).

We have demonstrated H_2 uptake by *R. japonicum* grown asymbiotically under conditions in which the carbon substrates were limited and nitrogenase activity was not detected. Under these conditions, however, it was necessary to pretreat the cultures with H_2 before the expression of hydrogenase activity was detected. These results indicate that H_2 is required for induction of the synthesis of hydrogenase. As an alternate interpretation, however, H_2 may be needed as a substrate for the generation of the energy required for the synthesis of the hydrogenase system (18). When nitrogenase is active, H_2 is produced as a by-product and presumably this plays a role in hydrogenase expression.

Those strains of *R. japonicum* that possess the capacity to synthesize hydrogenase in nodules reduce triphenyltetrazolium chloride (in an agar medium) more rapidly than do those strains that lack the capability to form hydrogenase (Fig. 2). Whether the reduction of this dye is directly or indirectly related to hydrogenase activity has not been established. Use of triphenyltetrazolium chloride as a method for detecting colonies with hydrogenase activity is advantageous because this dye may be used in presence of O_2 and it is relatively nontoxic to rhizobia.

A mutant strain lacking H_2 -uptake ability was isolated by using the reduction of triphenyltetrazolium chloride as a screening procedure. This mutant strain forms nodules that evolve H_2 but other symbiotic characteristics appear normal. This strain does not exhibit H_2 -uptake capability when grown under free-living conditions (Fig. 3). Mutant strains lacking hydrogenase will be helpful in the biochemical characterization of the hydrogenase complex.

FIG. 3. Hydrogenase activities in *R. japonicum* USDA (DES) 122 and Hup^- (hydrogenase uptake negative phenotype) mutant of USDA (DES) 122. Both parent and mutant strains were induced for hydrogenase by preincubation under 0.7% H_2 for 16 hr. Protein concentrations were: 0.29 mg/ml for DES 122, and 0.59 mg/ml for the Hup^- mutant.

The advice and criticism of Professor John Postgate is greatly appreciated. We acknowledge Drs. David Emerich and Tomás Ruiz-Argüeso for providing strain USDA (DES) 122 and relative efficiency values for this strain. We thank Mrs. Flora Ivers for typing the manuscript. This research was supported by National Science Foundation Grant PCM 77-08784. One of us (R.J.M.) thanks the Rockefeller Foundation for a postdoctoral fellowship. The research also was supported by the Oregon Agricultural Experiment Station, from which this is paper 4820.

- Hadfield, L. K. & Bulen, W. A. (1969) *Biochemistry* 8, 5103–5108.
- Burris, R. H. (1977) in *Genetic Engineering for Nitrogen Fixation*, ed. Hollaender, A. (Plenum, New York), pp. 9–18.
- Dixon, R. O. D. (1975) in *Nitrogen Fixation by Free-Living Microorganisms*, ed. Stewart, W. D. P., pp. 421–435.
- Schubert, K. R. & Evans, H. J. (1977) in *Recent Developments in N₂ Fixation*, eds. Newton, W. E., Postgate, J. R., Rodriguez-Barrueco, C. (Academic, New York), pp. 469–487.
- Evans, H. J., Ruiz-Argüeso, T., Jennings, N. T., & Hanus, J. (1977) in *Genetic Engineering for Nitrogen Fixation*, ed. Hollaender, A. (Plenum, New York), pp. 333–335.
- Dixon, R. O. D. (1972) *Arch. Mikrobiol.* 85, 193–201.
- Carter, K. R., Jennings, N. T., Hanus, J. & Evans, H. J. (1978) *Can. J. Microbiol.* 24, 307–311.
- Ruiz-Argüeso, T., Hanus, J. & Evans, H. J. (1978) *Arch. Microbiol.* 116, 113–118.
- McCrae, R. E., Hanus, J. & Evans, H. J. (1978) *Biochem. Biophys. Res. Commun.* 80, 384–390.
- Kuykendall, L. D. & Elkan, G. H. (1976) *Appl. Environ. Microbiol.* 32, 511–519.
- Maier, R. J. & Brill, W. J. (1976) *J. Bacteriol.* 127, 763–769.
- Schwinghamer, E. A., Evans, H. J. & Dawson, M. D. (1970) *Plant and Soil* 33, 192–212.
- Stickland, L. H. (1951) *J. Gen. Microbiol.* 5, 698–703.
- Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* 5, 218.
- Cole, M. A. & Elkan, G. H. (1973) *Antimicrob. Agents Chemother.* 4, 248–253.
- Streeter, J. G. (1974) *J. Exp. Bot.* 25, 189–198.
- Appleby, C. A., Turner, G. L. & MacNicol, P. (1975) *Biochim. Biophys. Acta* 387, 461–474.
- Schlegel, H. G. & Eberhardt, U. (1972) *Adv. Microbiol. Physiol.* 7, 205–243.
- Shug, A. L., Hamilton, P. B. & Wilson, P. W. (1956) in *Inorganic Nitrogen Metabolism*, eds. McElroy, W. D. & Glass, B. (Johns Hopkins Press, Baltimore, MD), pp. 344–360.