

- says were performed as described [J. E. Kuster, J. I. Stevenson, S. J. Ward, T. E. D'Ambra, D. A. Haycock, *J. Pharmacol. Exp. Ther.* **264**, 1352 (1993)]. Using forebrain membranes from $CB_1^{+/+}$ mice, we found a dissociation constant (K_d) of 0.73 ± 0.12 nM and a maximum binding capacity (B_{max}) of 1.17 ± 0.16 pmol of [3H]WIN55,212-2 per milligram of protein for [3H]WIN55,212-2 and a K_d of 0.74 ± 0.11 nM and a B_{max} of 1.07 ± 0.04 pmol of [3H]SR141,716A per milligram of protein for [3H]SR141,716A. No binding was detected on forebrain or cerebellar membranes from $CB_1^{-/-}$ mice.
5. Animals were housed at $21^\circ \pm 1^\circ\text{C}$ with free access to food and water. Experiments were conducted in accordance with local ethical guidelines. The measurement of locomotor activity, and the open-field and elevated plus maze tests were performed as described (24). Mice were exposed to the open field for three consecutive days. The number of squares crossed was as follows: wild type: 170 ± 12 , 127 ± 10 , and 91 ± 11 for first, second, and third days, respectively; knockout: 256 ± 20 ($P < 0.01$), 158 ± 15 (NS), and 120 ± 16 (NS) for first, second, and third days, respectively (t test, $n = 15$ per group).
 6. The spontaneous alternation test was conducted as described (24). The percentage of alternation was measured as the number of times the animal visited consecutively all three arms, divided by the total number of visits during a 10-min period.
 7. Nociceptive thresholds were monitored by applying thermal (tail-immersion and hot-plate tests), mechanical (tail-pressure test), or chemical stimuli (writhing test) (24). For the tail-immersion test, mice were maintained in a cylinder, and their tail was immersed in water at 50°C ; latency to tail withdrawal was recorded. In the hot-plate test, mice were placed on a surface heated to 50°C , and the latencies for licking their paws and jumping were recorded. For the tail-pressure test, increasing pressure (tip diameter: 1 mm) was applied to the tails of the mice until a withdrawal response was elicited. In the writhing test, mice received 0.1 ml per 10 g of body weight of a 0.6% acetic acid solution by the intraperitoneal route, and contractions of abdominal musculature (writhes) were counted between 5 and 15 min after the injection.
 8. A. Calignano, G. La Rana, A. Giuffrida, D. Piomelli, *Nature* **394**, 277 (1998).
 9. Self-administration of WIN55,212-2 and morphine was performed as described [M. C. Martellotta, G. Cossu, L. Fattore, G. L. Gessa, W. Fratta, *Neuroscience* **85**, 327 (1998)].
 10. For Δ^9 -THC dependence and withdrawal, mice were injected twice daily for 5 days with Δ^9 -THC (20 mg/kg, intraperitoneally) or vehicle (ethanol/chromophor EL/distilled water, 1:1:18). On day 6, they received the morning injection and 4 hours later the cannabinoid antagonist SR141,716A [10 mg/kg, subcutaneously (sc)]. Δ^9 -THC withdrawal was evaluated for 35 min as reported [D. M. Hutcheson et al., *Br. J. Pharmacol.* **125**, 1567 (1998)].
 11. K. D. Lake, D. R. Compton, K. Varga, B. R. Martin, G. Kunos, *J. Pharmacol. Exp. Ther.* **281**, 1030 (1997).
 12. Blood pressure and heart rate were recorded in conscious animals 4 hours after surgical implantation of catheters [T. Pedrazzini et al., *Nature Med.* **4**, 722 (1998)]. Agonists (100 μl of a 30% ethanol solution) were injected through a catheter inserted into the jugular vein. Basal values were as follows: blood pressure, 113 ± 2.4 ($CB_1^{+/+}$) and 123 ± 4.1 ($CB_1^{-/-}$) mmHg; $P > 0.05$; heart rate, 522 ± 43 ($CB_1^{+/+}$) and 494 ± 38 ($CB_1^{-/-}$) beats per minute; $P > 0.05$, t test, $n = 9$.
 13. S. P. Welch and D. L. Stevens, *J. Pharmacol. Exp. Ther.* **262**, 10 (1992).
 14. G. Tanda, F. E. Pontieri, G. Di Chiara, *Science* **276**, 2048 (1997).
 15. C. Ledent et al., data not shown.
 16. Opiate tolerance was induced by daily sc injection of morphine (20 mg/kg) for 5 days followed by a sc 6 mg/kg injection of morphine.
 17. A. Kuzmin, E. Zvartau, G. L. Gessa, M. C. Martellotta, W. Fratta, *Pharmacol. Biochem. Behav.* **413**, 497 (1992).
 18. Opiate dependence was induced by repeated intraperitoneal injection of morphine (or saline in controls) at an interval of 12 hours, over 6 days (20 mg/kg on day 1 to 100 mg/kg on days 5 and 6). Withdrawal was precipitated by injecting naloxone (1 mg/kg, sc) 2 hours after the last morphine administration and was evaluated as reported [R. Maldonado et al., *Science* **273**, 657 (1996)]. A global score was calculated for each animal [R. Maldonado, S. Negus, G. F. Koob, *Neuropharmacology* **21**, 1231 (1992)].
 19. H. W. D. Matthes et al., *Nature* **383**, 819 (1996).
 20. F. Rodríguez de Fonseca, M. R. A. Carrera, M. Navarro, G. F. Koob, F. Weiss, *Science* **276**, 2050 (1997).
 21. M. J. Christie, J. T. Williams, R. A. North, *Mol. Pharmacol.* **32**, 633 (1987); E. L. Way, in *Opioids II*, A. Herz, Ed. (Springer-Verlag, Berlin, 1993), pp. 573–592.
 22. P. B. Smith, S. P. Welch, B. R. Martin, *J. Pharmacol. Exp. Ther.* **268**, 1381 (1994).
 23. R. A. Lahti, P. F. Von Voigtlander, C. Barsuhn, *Life Sci.* **31**, 2257 (1982).
 24. F. Simonin et al., *EMBO J.* **17**, 886 (1998).
 25. We thank A. Nagy for R1 embryonic stem cells, C. Dewolf and M. J. Simons for technical assistance, and D. Penninck for discussions. Supported by the Inter-university Poles of Attraction (Belgian State, Prime Minister's Office, Federal Service for Science, Technology and Culture), the Fonds de la Recherche Scientifique Médicale, the EU BIOMED II programme, and the Fondation Médicale Reine Elisabeth. The scientific responsibility is assumed by the authors. C.L. is Chercheur Qualifié of the Fonds National de la Recherche Scientifique.

1 September 1998; accepted 7 December 1998

Diminishing Returns from Mutation Supply Rate in Asexual Populations

J. Arjan G. M. de Visser,*† Clifford W. Zeyl, Philip J. Gerrish, Jeffrey L. Blanchard, Richard E. Lenski

Mutator genotypes with increased mutation rates may be especially important in microbial evolution if genetic adaptation is generally limited by the supply of mutations. In experimental populations of the bacterium *Escherichia coli*, the rate of evolutionary adaptation was proportional to the mutation supply rate only in particular circumstances of small or initially well-adapted populations. These experiments also demonstrate a "speed limit" on adaptive evolution in asexual populations, one that is independent of the mutation supply rate.

Surveys of natural populations of pathogenic (1) and commensal (2) bacteria indicate that more than 1% are dominated by mutator genotypes with increased mutation rates. Such genotypes are even more prevalent among populations of *E. coli* evolving in the laboratory (3) and in certain tumors (4). Mutators may be favored because they produce rare beneficial mutations more often than do normal genotypes and thereby allow a faster response to selection (5). But the actual relation between mutation rates and adaptive evolution may be more complicated, especially in asexual populations that are subject to strong effects of genetic linkage. Indeed, the logic that drives any empirical association between mutators and rapid adaptive evolution can be reversed: Rapid adaptation to a novel or changing environment provides

more frequent opportunities for mutators to "hitchhike" to high frequency along with beneficial mutations to which they are genetically linked, even when mutators themselves have little effect on the rate of adaptation (3).

Moreover, population genetic models predict that the rate of adaptive evolution in asexual populations will increase proportionately with mutation rate only if populations spend most of their time waiting for beneficial mutations (6). Otherwise, two or more beneficial mutations may be simultaneously present in different lineages within a population; they will interfere with one another's spread, and ultimately only the superior mutation prevails while all others are driven extinct (6, 7). Therefore, an increase in the supply rate of beneficial mutations might of-

Table 1. Estimates of relative mutation rates of the six strains used in the evolution experiment, on the basis of eight separate fluctuation tests for each strain (14).

Mutator allele	Relative mutation rate	
	Nonadapted background	Adapted background
Wild type	1	1
<i>mutY</i>	3.3	3.3
<i>mutS</i>	34.9	32.4

*Present address: Laboratory of Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands.

†To whom correspondence should be addressed. E-mail: arjan.devissier@algemeen.micr.wau.nl

ten be subject to diminishing returns, as the extent of clonal interference increases with the number of beneficial mutations produced in an interval. This effect can be so pronounced that the rate of adaptation reaches an effective "speed limit" (6).

Consider an asexual population that is small and spends most of its time waiting for the next beneficial mutation. An increment in either mutation rate or population size should shorten the waiting time and thereby accelerate adaptive evolution. However, a further increase in the supply of beneficial mutations (because of changes in either parameter) should yield less acceleration as a consequence of more clonal interference (8). Now consider the effect of starting out with a different genotype, one better adapted to the selective environment. This population should spend more time waiting for beneficial mutations than one that is poorly adapted, all else equal, and thus clonal interference should be less important. Well-adapted populations may therefore experience a proportional acceleration of their adaptive evolution over the same range of mutation rates and population sizes that reveal a speed limit in populations founded by an inferior genotype.

We tested these hypotheses by examining how mutation rate, population size, and the level of adaptedness affect the rate of adaptive evolution in asexual populations of the bacterium *E. coli*. Forty-eight populations evolved for 1000 generations in a simple laboratory environment (9). We manipulated initial adaptedness by using two founding strains: One had not previously experienced

the selective environment, whereas the other had already adapted to that environment for 10,000 generations (10). We manipulated mutation rates by moving repair-deficient *mutY* and *mutS* alleles into repair-proficient genetic backgrounds (11). We manipulated effective population size by varying the bottleneck during propagation of the evolving populations (12). The rate of adaptive evolution was determined by measuring changes in competitive fitness relative to the corresponding ancestral strain and in the same simple environment (13). Fluctuation tests (14) indicate a moderate increase in mutation rate for the *mutY* strains and a greater increase for the *mutS* strains (Table 1). Each combination of mutation rate (wild-type, *mutY*, or *mutS*), effective population size (6.6×10^5 or 3.3×10^7), and evolutionary history (nonadapted or adapted) was replicated fourfold.

Fitness increased under each combination of mutation rate and population size in the nonadapted strains (Fig. 1). For each population, we calculated its time-averaged rate of adaptation from the regression of fitness against time. We then regressed these rates against the product of relative mutation rate and effective population size, which equals the mutation supply rate, using two different models (Fig. 2A). The first is a linear model, in which the rate of adaptation is directly proportional to the mutation supply rate. The

second is a rectangular hyperbola, such that the rate of adaptation is subject to an upper limit because of clonal interference. The *y* intercept is fixed at 0 in both models, because an asexual population with no mutations cannot adapt genetically. The linear model indicates that the rate of adaptation increases with the mutation supply rate ($F_{1,23} = 7.47$, $P = 0.0118$). But the additional degree of freedom required for the hyperbolic model provides a substantial improvement over the linear model ($F_{1,22} = 397.53$, $P < 0.0001$) (15). Our experiment thus demonstrates a speed limit on adaptive evolution in asexual populations, which was predicted by a population genetic model with clonal interference (6).

This model also predicts that the speed limit should be shifted to much higher values of the mutation supply rate for a founding strain that is so well-adapted that it spends most of its time waiting for further beneficial mutations. The rate of adaptation was much slower for the 24 populations founded by the adapted strain (Fig. 2B) than for those founded by the nonadapted strain (Fig. 2A). Even so, the rate of adaptation in these well-adapted populations increased significantly with mutation supply rate, on the basis of linear regression with the *y* intercept held at 0 ($F_{1,23} = 5.90$, $P = 0.0234$). But the hyperbolic model provides no statistical improvement for the well-adapted populations ($F_{1,22} = 0.57$, $P = 0.4565$), in contrast to the strong improvement for the nonadapted populations. Thus, the contrasting predictions for the effects of the mutation supply rate on the rate of adaptation in well-adapted versus poorly adapted populations are supported.

Our findings have three evolutionary implications. First, higher mutation rates need not accelerate the pace of evolutionary adaptation in asexual populations. Acceleration proportional to the mutation rate occurs only in limited circumstances, such as small effective population size, where an evolving population spends most of the time waiting for beneficial mutations (16). This is particularly relevant to bacterial pathogens that may experience severe population bottlenecks during colonization of the host (17). Second, mutators are common in asexual populations under conditions of rapid adaptive evolution because such conditions provide numerous opportunities for mutators to hitchhike to high frequency with beneficial mutations to which they are linked (3, 5). Mutators need not—and often will not—substantially accelerate adaptive evolution. Third, clonal interference imposes a speed limit on adaptive evolution in asexual populations, because two or more beneficial mutations that arise in different lineages cannot be combined into the same lineage. An advantage of sex is that it allows beneficial mutations to be combined into the same lineage, minimizing clonal in-

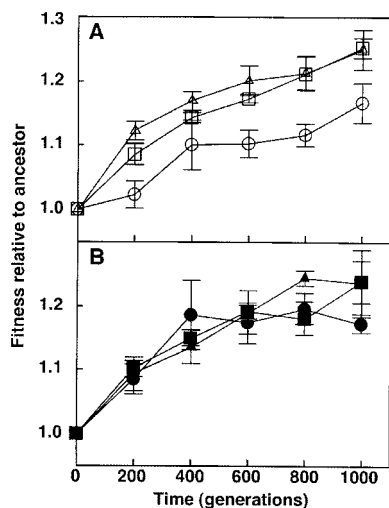


Fig. 1. Fitness trajectories of wild-type and mutator populations, founded by the nonadapted strain, during experimental evolution. Points indicate the average fitness of four populations in each treatment relative to the corresponding ancestor; error bars show standard errors. Circles are wild type, squares *mutY*, triangles *mutS*. (A) Small effective population size ($= 6.6 \times 10^5$). (B) Large effective population size ($= 3.3 \times 10^7$).

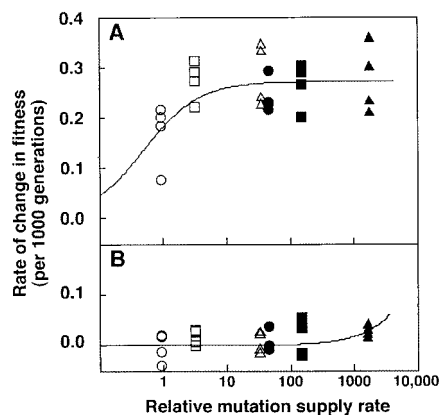


Fig. 2. Effect of mutation supply rate on rate of adaptive evolution. Mutation supply rate is the product of mutation rate and effective population size; values are expressed relative to the treatment with the lowest rate and are shown on a log-transformed scale to spread the treatments along the *x* axis (statistical regressions use untransformed values). Open symbols are small populations, filled symbols large populations; circles are wild type, squares *mutY*, triangles *mutS*. (A) Populations founded by nonadapted strains. The curve is a hyperbolic regression, which fits the data better than a linear regression ($P < 0.0001$). (B) Populations founded by previously adapted strains. The curve is a linear regression ($P = 0.0234$), which appears exponential because of the logarithmic scale. A hyperbolic regression provides no significant improvement ($P = 0.4565$).

interference and removing the speed limit on adaptive evolution that constrains asexual populations (7, 18, 19).

References and Notes

- J. E. LeClerc, B. Li, W. L. Payne, T. A. Cebula, *Science* **274**, 1208 (1996).
- I. Matic et al., *ibid.* **277**, 1833 (1997).
- P. D. Sniegowski, P. J. Gerrish, R. E. Lenski, *Nature* **387**, 703 (1997).
- L. A. Loeb, *Cancer Res.* **51**, 3075 (1991); P. Modrich, *Philos. Trans. R. Soc. London Ser. B* **347**, 89 (1995).
- F. Taddei et al., *Nature* **387**, 700 (1994).
- P. J. Gerrish and R. E. Lenski, *Genetica* **102/103**, 127 (1998).
- H. J. Muller, *Am. Nat.* **66**, 118 (1932); R. A. Fisher, *The Genetical Theory of Natural Selection* (Clarendon, Oxford, 1930); S. P. Otto and N. H. Barton, *Genetics* **147**, 879 (1997).
- This model (6) makes certain simplifying assumptions. In particular, it ignores deleterious mutations and multiple beneficial mutations. The rate of adaptation may decelerate and even become negative at very high mutation rates, especially in asexual populations [A. S. Kondrashov, *Genet. Res.* **66**, 53 (1995)]. Very high mutation rates might also produce genotypes with two or more beneficial mutations that could overcome clonal interference (although we might then expect interference to arise between genotypes that each carried multiple beneficial mutations). The former assumption depends on per capita mutation rate but not population size, whereas the latter assumption rests on both mutation rate and population size. By manipulating these two factors independently, we should be able to discern if violations of the assumptions are important over the wide range of values explored, which does not appear to be the case.
- The populations were serially propagated at 37°C in test-tubes containing 10 ml of Davis minimal medium (DM) supplemented with glucose (25 µg/ml). Every 200 generations, samples were taken and stored at -80°C for later study.
- Escherichia coli* B strain REL606 (Ara⁻ MutS⁺ MutY⁺) is the progenitor of all strains used in this study. REL4548 was derived from REL606 after 10,000 generations in the glucose-limited environment, during which time its fitness increased by ~50% relative to REL606 because of the substitution of several beneficial mutations [R. E. Lenski and M. Travisano, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6808 (1994)]. REL4548 retained functional DNA repair, unlike some other lines in an earlier experiment (3). These strains had no plasmids or functional viruses, so populations were strictly asexual.
- Repair-deficient alleles *mutY::mini-Tn10* (strain NR9373 [J. P. Radicella, E. A. Clark, M. S. Fox, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9674 (1988)]) and *mutS::Tn5* (strain ES1301) [E. C. Siegel, S. L. Wain, S. F. Meltzer, M. L. Binion, J. L. Steinberg, *Mut. Res.* **93**, 25 (1982)] were moved into each background by P1 transduction [J. W. Zyskind and S. I. Bernstein, *Recombinant DNA Laboratory Manual* (Academic Press, San Diego, CA, 1989)]. The *mutY* allele increases G:C to T:A transversions, whereas *mutS* increases G:C to A:T and A:T to G:C transitions as well as frame shifts [J. H. Miller, *Annu. Rev. Microbiol.* **50**, 625 (1996)]. The mini-Tn10 insertion was inactive, but Tn5 (or its IS element, IS50) was active in some evolving *mutS* populations. However, there was no association between number of changes detected by Southern (DNA) blots, with IS50 as a probe, and final extent of fitness gains, on the basis of analysis of covariance ($P = 0.333$, data not shown).
- Twenty-four "Large" populations were propagated for 150 days by daily 100-fold dilution into fresh medium, and 24 "Small" populations for 75 days by 10,000-fold dilution. These dilution factors allow 6.6 and 13.3 cell generations per day, respectively. Effective population sizes for the substitution rate of beneficial mutations (20) are 3.3×10^7 and 6.6×10^5 , respectively. The different dilution factors might influence the selective environment, as well as effective population size, because the treatments spend different periods in lag, growth, and stationary phases. This difference is unimportant if faster exponential growth is primarily responsible for fitness gains, as reported previously for this system [F. Vasi, M. Travisano, R. E. Lenski, *Am. Nat.* **144**, 432 (1994)]. We tested the similarity of the selective environments by measuring the final fitness of all populations under both demographic regimes. There were significant positive correlations in fitness between the two regimes for population derived from nonadapted ($r = 0.696$, $n = 24$, one-tailed $P < 0.0001$) and adapted ($r = 0.369$, $n = 24$, one-tailed $P = 0.0379$) founders, confirming that the two regimes present similar selective environments.
- Fitness is defined as the ratio of the number of doublings of a derived population relative to its ancestor (carrying the alternative Ara marker; see below), on the basis of direct competition. The protocol is described elsewhere (20); we modified this protocol by using a 10,000-fold dilution for the small-population treatment and by performing all assays in test-tubes instead of flasks. To distinguish derived from ancestral strains in the competitions, we isolated spontaneous Ara⁺ revertants of the six ancestral strains (two backgrounds $\times$ three mutator alleles). Ara⁻ and Ara⁺ strains form red and white colonies, respectively, on tetrazolium arabinose plates. The evolution experiment was balanced with respect to the Ara marker (two of the four replicate populations in each treatment were Ara⁺ and two were Ara⁻). Samples from the 24 populations founded by the nonadapted strain were assayed at 200, 400, 600, 800, and 1000 generations, with fourfold replication. Assays were performed for the 24 populations founded by the well-adapted strain only after 1000 generations, but with 10-fold replication. All fitness values were adjusted for the effect, if any, of the Ara marker on the basis of control experiments with isogenic strains differing only in their marker state.
- Fluctuation tests [S. E. Luria and M. Delbrück, *Genetics* **28**, 491 (1943)] were performed to estimate the mutation rate of founding strains, following protocols described elsewhere (3). Tests were replicated 24-fold for resistance to nalidixic-acid and 12-fold for resistances to T4, T5, and rifampicin. Maximum-likelihood estimates were calculated by a local program (3). As an overall measure of mutation rate, we used the geometric mean of eight (four loci tested $\times$ two Ara marker strains) estimates for each combination of mutator allele (wild type, *mutY*, or *mutS*) and initial fitness (nonadapted or adapted). Rates are expressed relative to wild type in Table 1.
- We screened populations after 1000 generations to see if any mutator populations had reverted to wild-type mutation rate. Two populations (both in the nonadapted, *mutY*, and small-population treatment) were indistinguishable from the wild type. When these two populations are excluded, the hyperbolic model still provides a much better fit to the data than does the linear model ($F_{1,20} = 350.61$, $P < 0.0001$).
- Small populations should also fix mutations with smaller beneficial effects, on average, than those substituted in large populations (6, 21).
- E. R. Moxon and P. A. Murphy, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1534 (1978); E. R. Moxon, P. B. Rainey, M. A. Nowak, R. E. Lenski, *Curr. Biol.* **4**, 24 (1994).
- R. L. Malmberg, *Genetics* **86**, 607 (1977).
- In idealized sexual populations that lack any genetic linkage, the rate of adaptive evolution should be proportional to mutation supply rate over its entire range. In reality, sexual organisms have linkage that prevents completely free recombination and may produce some weaker form of a speed limit on adaptive evolution.
- R. E. Lenski, M. R. Rose, S. C. Simpson, S. C. Tadler, *Am. Nat.* **138**, 1315 (1991).
- C. Burch and L. Chao, *Genetics*, in press.
- We thank L. Ekinwe, N. Hajela, J. Mongold, and M. Stanek for technical assistance, and B. Bohannon, V. Cooper, A. Kondrashov, P. Moore, D. Rozen, P. Sniegowski, M. Stanek, and an anonymous referee for suggestions and discussion. This work was supported by a fellowship from the Netherlands Organization for Scientific Research to J.A.G.M.d.v. and a grant from the NSF to R.E.L.

18 August 1998; accepted 8 December 1998

Enhance your AAAS membership with the Science Online advantage.

- Full text Science**—research papers and news articles with hyperlinks from citations to related abstracts in other journals before you receive *Science* in the mail.
- ScienceNOW**—succinct, daily briefings, of the hottest scientific, medical, and technological news.
- Science's Next Wave**—career advice, topical forums, discussion groups, and expanded news written by today's brightest young scientists across the world.

- Research Alerts**—sends you an e-mail alert every time a *Science* research report comes out in the discipline, or by a specific author, citation, or keyword of your choice.
- Science's Professional Network**—lists hundreds of job openings and funding sources worldwide that are quickly and easily searchable by discipline, position, organization, and region.
- Electronic Marketplace**—provides new product information from the world's leading science manufacturers and suppliers, all at a click of your mouse.

All the information you need.....in one convenient location.

Visit Science Online at <http://www.scienceonline.org>, call 202-326-6417, or e-mail membership2@aaas.org for more information.

AAAS is also proud to announce site-wide institutional subscriptions to Science Online. Contact your subscription agent or AAAS for details.



AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE