

less of their mode of origin, *P. semimarmorata* now behaves as a semi-species toward both *P. bibroni* and *P. dendyi*. It is accordingly ranked as a species despite the fact that it hybridizes freely with these other taxa wherever their ranges contact. Species concepts need revision to allow for the fact that genetic isolation cannot invariably be directly equated with reproductive isolation. As others (16) have shown, strongly integrated and coadapted gene complexes may be effectively protected against introgression even in the apparent absence of premating reproductive isolation.

DAVID S. WOODRUFF

Department of Biological Sciences,
Purdue University,
West Lafayette, Indiana 47907

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8. D. S. Woodruff, *Syst. Zool.* 22, 213 (1973).
9. Typical *P. semimarmorata* have a hybrid index of 0, typical *P. bibroni* and typical *P. dendyi* both scored 10 (on different scales); specimens of intermediate coloration received intermediate scores. Independent reclassification of the specimens showed the system to be highly replicable (10).
10. L. J. McDonnell, D. F. Gartside, M. J. Littlejohn, *Evolution* 32, 602 (1978).
11. Observation of saproovoric fungal infestations in 50 batches of eggs collected away from the hybrid zones permitted the determination of natural embryonic mortality patterns. From 98 to 100 percent of the ova were fertilized and commenced development. Embryonic mortality was generally < 5 percent (range 0 to 11 percent) and most failures occurred during gastrulation.
12. M. J. Littlejohn, in *Australian Inland Waters and their Faunas: Eleven Studies*, A. H. Weatherley, Ed. (Australian National University, Canberra, 1967), pp. 163-171.
13. Studies of anurans (*Limnodynastes*, *Geocrinia*, *Litoria*) and birds (*Gymnorhina*) are cited by M. J. Littlejohn and J. D. Roberts [*Aust. J. Zool.* 23, 113 (1975)] and T. C. Burton and A. A. Martin [*Emu* 76, 30 (1976)].
14. J. A. Endler, *Geographic Variation, Speciation, and Clines* (Princeton Univ. Press, Princeton, N.J., 1977).
15. Endler argues that the few differences between the three *Pseudophryne* result from the operation of a few color-controlling genes and that the hybrid zone effect results from a few coadapted modifiers together with a clinal selection pattern between the different habitats of each species. According to this view the hybrid zones represent complex step clines, separating areas characterized by different coadapted modifiers which interact so as to reduce the fitness of some major gene heterozygotes. Assortative mating (involving premating isolating mechanisms) may be selected for under these circumstances, but the spread of assortative mating genes will be severely limited by the slope of the selection gradient around the null point, by low levels of gene flow from allopatric homospecific populations, and by the fact that the hybrids are subject to developmental problems. The latter tends to further steepen the cline as selection operates before migration.
16. W. G. Hunt and R. K. Selander, *Heredity* 31, 11 (1973); J. M. Szymura, *Bull. Acad. Pol. Sci. Cl.* 24, 355 (1976).
17. Supported by a Commonwealth of Australia postgraduate award. I thank M. J. Littlejohn (who discovered these zones); D. F. Gartside, and M. J. D. White for help in Australia; J. A. Endler, E. O. Wilson, and E. E. Williams for subsequent useful discussions; and A. J. Cain and M. Levy for critically reading the manuscript.

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The *Salmonella* Mutagenicity Assay: Recommendations

A number of recommendations have recently been made (1) concerning the use of the *Salmonella* plate assay for determining chemical mutagenicity. This assay was described in detail by Ames and his co-workers in 1975 (2), and since that time, many variations of the procedure have been incorporated into the test as it is used in individual laboratories. In ongoing efforts to identify mutagenic chemicals that may pose a hazard to human health, the diversity of existing pro-

ocols presents several questions. Among them are: (i) What are the most likely sources of variation in results among laboratories? (ii) What protocol is currently best suited for routine testing? (iii) What constitutes an adequate test? The recommendations made therefore provide basic guidelines for conducting the assay and constitute minimum criteria by which reports of mutagenicity testing in the *Salmonella* plate assay can be evaluated.

Bacterial strains. For routine testing, strains TA1535, TA1537, TA1538, TA98, and TA100 should be used. The recommendation that TA1538 might be deleted (2) was not considered advisable since this strain may show greater sensitivity than TA98 with some chemicals. The use of other strains is considered optional.

Preparation of suspensions of tester strains. The growth of bacterial strains in overnight nutrient broth cultures has been found to vary considerably depending on the nutritional quality of the medium. There seem to be marked differences not only between media from different sources but also among batches from a single source. Overnight cultures which have just reached a density of 1 to 2×10^9 viable cells per milliliter are considered most desirable for mutagen testing. Instead of using washed cells, investigators should take inocula directly from nutrient broth cultures. A fresh cell suspension should be used for each day's experiments; use of cultures kept overnight in a refrigerator should be avoided. Cell suspensions should be maintained through the day at ice-bath temperature since storage at room temperature may result in loss of viability or mutagen sensitivity.

Checking tester strain genotypes. The procedures outlined by Ames *et al.* (2) are satisfactory with regard to confirming histidine requirement, deep-rough character, and ultraviolet sensitivity of tester strains. However, tests for the presence of the R factor conferring ampicillin resistance in strains TA100 and TA98 can be conducted more conveniently by using commercially available filter-paper disks containing 10 μ g of ampicillin. Ampicillin-containing disks are placed in the center of petri dishes overlaid with each of the tester strains. Zones of inhibition should be observed with strains TA1535, TA1538, and TA1537, but not with TA98 or TA100. For the three sensitive strains, the diameter of the zone of inhibition has been found to be reproducible and characteristic within a given laboratory, although some differences have been found between laboratories.

Because this procedure will not determine what fraction of the culture has lost the R factor, it is important to check stock cultures periodically to ensure that close to 100 percent of the bacteria contain the R factor. This can be done by replica plating (preferably with 100 or more colonies) onto ampicillin-free plates and plates containing 25 μ g of ampicillin per milliliter of medium.

Preparation of S9. For routine screen-

Table 1. Recommended strain-specific positive control compounds. In the presence of S9, 2-aminoanthracene can serve as the positive control for all strains.

Strain	Compound
TA1535	Methyl methanesulfonate or sodium azide
TA1537	9-Aminoacridine
TA1538 and TA98	4-Nitro- <i>o</i> -phenylenediamine, 2-nitrofluorene, or hycanthone methane-sulfonate
TA100	Sodium azide, methyl methanesulfonate, or nitrofurantoin

ing of chemicals, there is little evidence to justify the use of activation preparations other than the Aroclor 1254-induced rat liver S9 described in (2). It is important that the concentration of S9 used be within the range originally recommended and that the cofactors and their ratios also be those recommended. However, numerous variations of the activation mix can be used for intensive studies and in specific cases to alter mutagenic responses both quantitatively and qualitatively.

It is recommended that the mutagenicity of 7,12-dimethylbenz[*a*]anthracene (DMBA) be tested with each batch of S9 preparation to ensure that the S9 mix exhibits optimal activity. Although no evidence has been published, it is claimed that DMBA gives only minimal response with uninduced or suboptimal S9 preparation and mixes. Additional compounds such as 2-acetylaminofluorene can be used for routine monitoring of the activity of the S9 preparation.

The S9 preparations should be stored at or below -70°C . The period for which S9 preparations can be stored is an unresolved issue. Storage periods used have ranged from 1 week to 2 years. Although some evidence is available on the stability of a few specific enzymes during storage, there are no data concerning the effect of storage on the general ability of the S9 to activate promutagens.

Spontaneous mutability of tester strains. The number of spontaneous revertants per plate for each strain is a factor that must be considered when the acceptability of an experiment is being evaluated. Strain TA100 is particularly prone to problems in this regard because of its high background of spontaneous revertants. Any given laboratory should establish an acceptable range of spontaneous revertants per plate within which it is confident that the strains are responding properly.

Plate number and incubation time. A minimum of two plates per dose level is recommended. When positive results are obtained, the test should be repeated using the strains and dose levels in which positive results were initially observed. If all strains give negative responses, re-

tests are recommended in all strains. Any conclusions should be based on consistent results in at least two independent tests.

Although results with most compounds can be obtained with 48 hours of incubation at 37°C , the toxicity of some chemicals may delay the appearance of revertants. Investigators should be aware of this possibility and when such a phenomenon is suspected, plates should be incubated for 72 hours. It has been observed that the number of revertant colonies will increase during the 48- to 72-hour incubation period.

Background "lawn." It is important to examine, with a dissecting microscope, the general appearance of the growth. A confluent growth of nonrevertant bacteria should be present. At dose levels that are highly toxic to the cells, such a lawn will not be present and surviving cells can give rise to small colonies which might be misinterpreted as revertants. When there is any question about the nature of colonies scored as revertants the genotypes should be checked by transferring colonies onto medium containing no histidine. When positive mutagenic results are obtained, the genotype of revertant colonies should also be spot-checked by picking and streaking or by replica plating on histidine-free plates.

Plate counters. Automatic plate counters are now used routinely in many laboratories. The accuracy of these counters tends to vary with time and with the density and size of colonies. Such counters should be calibrated against hand counts on a routine basis, and calibrations should include plates of each strain used over the range of colony densities normally encountered.

Dose range. It is generally agreed that dose levels up to at least 5 mg per plate should be tested unless limitations are imposed by toxicity or solubility. A reasonable lower limit in routine testing is $0.2\text{ }\mu\text{g}$ per plate. Initial experiments to determine toxicity and solubility are necessary to select a testable dose range. When initial experiments reveal that a chemical is toxic, then the dose range chosen for subsequent experiments should include at least one observably

toxic dose. Similarly, if preliminary experiments reveal that a compound precipitates at dose levels below 5 mg per plate, subsequent experiments should include a dose at which the precipitate occurs.

Preincubation. Perhaps the most widely used and successful modification of the plate assay is the preincubation method described by Yahagi *et al.* (3). This method is now used routinely in some laboratories and is recommended for use in cases where results from the standard plate assay are inconclusive.

Controls. Negative controls with the appropriate solvent, controls for S9 sterility, and positive controls should be run with each experiment. The compounds listed in Table 1 are recommended positive controls for each strain. Other compounds that may be used satisfactorily as positive controls in the presence of S9 include *N*-nitrosomorpholine, 2-acetylaminofluorene, and benzo[*a*]pyrene, but these may be less satisfactory than 2-aminoanthracene because of their potent carcinogenicity or failure to revert all strains.

For the positive control chemicals it is good practice to use freshly prepared solutions. Exception can be made in the case of sodium azide, which is highly stable in aqueous solution.

Data presentation. Any report of the testing of a chemical for mutagenicity in the *Salmonella*/microsome plate assay should include the means and indications of variability (for example, standard deviation) of the plate counts for the negative control, the positive controls, and each dose of the test compound. The number of replicate plates included in the mean must be indicated. When the volume of data is not prohibitive, it is also desirable to report individual plate counts. When this is not feasible, the complete data should be available from the investigator. Data that have been transformed by subtracting spontaneous counts, or that are expressed as revertants per nanogram or nanomole or as a ratio of colonies on treated and control plates make independent evaluation impossible.

Definition of positive and negative results. In most tests the results are either clearly positive or clearly negative. A positive result is usually defined as a reproducible, dose-related increase in the number of histidine-independent colonies. A negative result is defined as the absence of a reproducible increase in the number of histidine-independent colonies. However, results are sometimes obtained which cannot be satisfactorily interpreted as positive or negative. In

such cases, further tests should be conducted in which modifications of the standard plate assay are used, such as preincubation or modification of the S9 preparation with regard to source, inducer, and cofactors.

FREDERICK J. DE SERRES

MICHAEL D. SHELBY

*Office of the Associate Director
for Genetics, National Institute of
Environmental Health Sciences,
Research Triangle Park, North Carolina*

References and Notes

1. The meeting at which these recommendations were made was held in Arlington, Va., 28 to 30 August 1978. The meeting was organized by F. J. de Serres and M. D. Shelby under the auspices of the National Institute of Environmental Health Sciences and was sponsored in part by Imperial Chemical Industries, Ltd. The recommendations herein complement and extend those of I. E. Mattern and H. Greim [see *Mutat. Res.* **53**, 369 (1978)].
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Manganese Nodules on the Sea Floor: Are Economic Mining Operations Feasible?

Menard and Frazer deserve congratulations for making the first statement in what will undoubtedly be a long controversy over the magnitude of copper and nickel resource estimates in deep-ocean manganese nodules (1). By finally analyzing statistically all of the available data in the public domain, their statement neatly squashes the optimistic resource estimates of Mero (2), McKelvey and Wang (3), and others who first raised interest in the economic potential of the nodules, or does it? Menard and Frazer's report also helps to dispose of the myth that vast wealth is available from the nodules for the common heritage of mankind.

Menard and Frazer do not provide sufficient information or evidence to dispel the expectation that nodules may be mined economically for their nickel, copper, and manganese content. However, their conclusions may indeed add to the discouragement of investors and policymakers, who are already frustrated by the Law of the Sea Conference.

The negative correlation between nodule abundance and copper-nickel concentrations, the central conclusion of the report, supports the expectation that high concentrations of copper are rare in the oceans as on land. This fact has been pointed out by Skinner (4), among others. This makes economic concentrations of minerals (mineral deposits) on land distinct from ordinary rock. If replotted on a log-log scale, the data in figure 2 of (1) would be similar to a very noisy version of the plot of copper grade versus concentration in a region such as

the copper province of the southwestern United States (the vertical component in the three-dimensional character of land deposits becomes negligible in an area the size of this province) (5).

The vast scale of the sample population to which Menard and Frazer's conclusions apply also deserves more attention. Perhaps it should be pointed out that the Scripps/International Decade of Oceanographic Exploration data bank contains only a few thousand samples to represent an ocean floor area of several tens of millions of square nautical miles, that this area can hardly be expected to be geologically homogeneous, and that most of the data were collected for other purposes than manganese nodule sampling. The data are thus not standardized.

According to the latest estimates, the area of potential mining sites will be of the order of 12,000 square nautical miles (1 square nautical mile = 3.4×10^6 m²) (6). The 74 samples (apparently without concentration data in kilograms per square meter) referred to in (1) represent the most economically interesting part of the northwest equatorial Pacific Ocean, which covers approximately 6 million square nautical miles. This is roughly one uncontrolled sample for each of 74 areas the size of Nevada (85,600 nautical square miles). Are there no concentration data for this most important area? The negative correlation observed by Menard and Frazer [figure 2 in (1)] is either not present or is not statistically significant for the two smaller localities mentioned by Schatz (7) and Piper *et al.*

(8) where potential economic deposits occur. As on land, economic concentrations of nodules on the ocean floor are expected to be anomalies. In figure 2 of (1), approximately 15 percent of the points are above a minable nickel and copper abundance of 0.25 kg/m² (9). Are we to conclude from this figure that 10 to 20 percent (95 percent confidence interval) of the area sampled consists of mineral deposits to be economically mined?

Although Menard and Frazer's report should sober some of the optimistic views of representatives of the lesser developed countries present at the March 1978 session of the Law of the Sea Conference, it should not deter ocean miners who are awaiting a U.S. go-ahead or a regime under which to proceed. A more thorough analysis based on geostatistical techniques (10) should be applied to the data to discover possible geographic trends or clusters. The unwarranted conclusion of Menard and Frazer serves only to point out the paucity of good data in the public domain and the need for more information so that matters of national and international interest can be debated more realistically.

FRANCOIS J. LAMPIETTI

*Post Office Box 151,
Purcellville, Virginia 22122*

LESLIE F. MARCUS

*Department of Biology, Queens College,
Flushing, New York 11367,
and American Museum of Natural
History, New York 10024*

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