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A HISTORY OF ATTEMPTS TO QUANTIFY ENVIRONMENTAL MUTAGENESIS

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ABSTRACT It became obvious in the early 1960's that the ready recognition of mutations produced by chemicals could have a profound influence on the refinement of methods to detect environmental mutagens. The experience derived over the previous 30 years in characterizing the effects of ionizing and ultraviolet radiation on the genetic mechanism came to serve us in good stead. Although the effects of chemicals are considerably more complicated and often require the analysis of individual substances, nonetheless, the area has developed rapidly in recent decades. The establishment and historical background of the International Association of Environmental Mutagen Societies (IAEMS) will be discussed. An attempt at the quantitation of chemical effects has been developed in comparison with radiation mutagenesis. As a first step, a definition of the "Mutagen Burden" or unavoidable exposure to chemicals will be discussed. A mathematical approach (Haynes/Eckhardt) will be considered and finally an outline for the comprehensive investigation of detailed interscience study will be made of less than six chemicals.

INTRODUCTION

The study of induced mutagenesis had its beginning more than 54 years ago, as all of us are well aware, with the work of Herman Muller (1) who developed a quantitative method for the recognition of mutations in Drosophila and

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who was able to establish the mutagenic effect of ionizing radiation. During the 1930's, the field of ultraviolet induction of mutagenesis developed very well, especially toward the end of the 1930's with the recognition that the most effective wavelength is the one absorbed by the nucleic acids. I had many interesting discussions with Dr. Muller in regard to the importance of these areas and it became obvious to us that the next field to be studied would be the effects of chemicals that result in mutation in exposed tissue. The recognition of chemical mutagenesis should be credited to Auerbach and Robson (2), to Rapoport (3), and to Oehlkers (4) whose work during the second World war was published in 1946.

The detection and evaluation of environmental mutagens was a very logical development but it took almost 20 years before practical methods became available for their routine measurement. When Milislav Demerec retired from the Directorship of the Cold Spring Harbor Laboratory he concentrated all of his research on developing Salmonella as organisms for the detection of chemical mutagens (5). Later, his elegant mutation techniques were further developed by Philip Hartman (6) of Johns Hopkins University. Many chemicals were missed at that time which later turned out to be mutagens since it was found that liver extract is needed for the activation of numerous mutagens. This requirement was first identified by Heinrich Malling (7). Liver microsomes are now used routinely in chemical mutation work.

The most significant development came when Bruce Ames of Berkeley developed Salmonella as a most ready tool for the detection of many mutagens (8). Ames developed many new strains of Salmonella which have specific ability to detect certain types of mutagens. The extensive work coming from Ames' laboratory has resulted in many important findings including the recognition of the broad overlap between mutagenesis and carcinogenesis. This is not to say that Salmonella hasn't any limitations, despite its use in very many laboratories all over the world. Salmonella is most useful for very preliminary screening and as a part of a well chosen battery of tests.

In the early 1960's it became obvious to some of us that the time had come to do something systematic so as to apply the knowledge derived through improving methods

of mutagen detection to the problems of environmental pollutants. The first question was, where could the work be done most effectively? The Ford Foundation gave me a small travel grant in the mid-sixties to explore the possibilities at several universities where research centers could be developed for chemical mutagen testing. I was helped very much in this exploratory period by Marvin Legator (then at the Food and Drug Administration) and Samuel Epstein (then at the Children's Cancer Hospital of the Harvard University Medical School). Workshops were initiated at Brown University[a] and at the University of Zurich[b]. Thereafter, our appeal became a little easier and others became more and more convinced that something should be done in an organized way.

Through my friend, Joe Slater, I applied to the newly established Anderson Foundation for support of my ideas and the urgent need for giving this field an immediate boost. I received a grant of $25,000 with no real strings attached to use as I saw fit to promote the field of environmental mutagenesis. In 1969, I turned over these funds to the newly organized Environmental Mutagen Society[c] (EMS). While it gave the necessary boost to get the EMS underway, the Society itself developed rather slowly, hitting its stride in the last several years.

After 1969, as all of you know, societies were organized in Europe, Japan, and India. By 1973 we had four major societies for environmental mutagenesis. At the EMS meeting at Asilomar which followed the International Congress of Genetics, the International Association of Environmental Mutagen Societies (IAEMS)[d] was, logically, instituted. This was followed by the international conference in Edinburgh in 1977, and now our third conference here in Japan. The Australian-New Zealand EMS has just joined the other four societies of the IAEMS. A number of additional societies have been organized and are in the process of applying to join the IAEMS.

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[a]Providence, Rhode Island (1971) "Workshop on Mutagenicity"
[b]University of Zurich, Switzerland (1973) "Workshop on Mutagenicity"
[c]Environmental Mutagen Society, 4720 Montgomery Lane, 506, Bethesda, Maryland 20014 (Verne Ray, President)
[d]International Association of Environmental Mutagen Societies, c/o Institute of General Genetics, Box 1031, Blindern, Oslo, Norway (Per Oftedal, President)

A number of very important things had to be done in connection with the development of the field of environmental mutagenesis. Many members of the Society were very much interested to promote the field as an essential part of the study of the environment. We then established an information collective, the Environmental Mutagen Information Center (EMIC)[e], which is now one of the most successful information sources in the area of environmental studies. EMIC was organized in 1969 under the direction of John Wassom with the support of Heinrich Malling.

In the meantime, many new methods for the detection and characterization of environmental mutagens were developed. As a matter of fact, the number increased so that we have today a great abundance of methods including some very excellent ones. The training of scientists so that they can use these methods for the intelligent detection of mutations has become increasingly important to this area. In earlier days, not many researchers saw the importance of this field. However, Galveston[f] instituted such a curriculum and has since become an important center for training in this field. Today we have many good methods for the detection of mutagens developed in many laboratories. However, in the long run, it is up to industry to use these methods. They have been most cooperative in this area, becoming an essential cog in the development of environmental mutagenesis research.

A number of workshops and training courses were organized to help individuals in developing countries by demonstrating techniques for the recognition of potentially dangerous chemicals used extensively in the environment (i.e., pesticides). We now have three important centers in the developing countries of Mexico, the Philippines, and Egypt.

The field of Environmental Mutagenesis had gone through the usual chain of development of any relatively new field, first, with the recognition of the phenomenon, then the

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[e]Environmental Mutagen Information Center (EMIC) Oak Ridge National Laboratory, P. O. Box Y, Oak Ridge, Tennessee 37830

[f]University of Texas Medical Branch, Galveston, Texas, Annual Genetic Toxicology Workshops

development of methods for its detection, followed by the qualitative observations, and finally a quantitative evaluation which contributes to an understanding of the underlying basic mechanisms. We had gone through these same steps to understanding radiation mutagenesis with many interesting results. Even after more than 50 years of intensive study, there are still quite a number of unsolved problems related to radiation mutagenesis, just as there are very many problems facing us with environmental mutagenesis.

There is really a great difference between radiation and chemical mutagenesis. One has to consider that most of the background radiation is attributed to sources outside of the human organism, whereas chemical exposures can occur from both outside influences or, since chemicals represent the building blocks of which we are made, the different internal pathways by which these compounds are absorbed and become an integral part of the body. By comparison, radiation is relatively simple with only a few types and chemicals are very numerous, with many specific metabolic pathways. Some chemicals are readily eliminated, others go through a number of stages of metabolism before they break down and then combine with other compounds or tissues. Unfortunately, these complications are not always realized as one becomes involved in working with chemicals. Occasionally, the effect of some compounds may be transitory and it has been found that some are even found in the gonads, despite their identity as somatic mutagens without germinal effect.

When it became necessary to define the basis for radiation mutagenesis some 30 years ago, it was thought essential to determine the unavoidable radiation to which man is exposed (i.e., cosmic radiation, radiation from potassium in the body, and essential medical X-rays). "Permissible limits" were based on multiples of unavoidable background radiation exposure. As a matter of fact, this served as an elegant way to proceed in determining the idea of a "permissible dose", or multiples of unavoidable background radiation.

Some of us thought that a similar approach could be applied to defining chemical exposure and effects. We know that there are certain chemicals in our bodies and many others which might be regularly ingested, inhaled, or

absorbed through the skin. A few are well established mutagens. Task Force 4 of the International Commission for Protection against Environmental Mutagens (ICPEMC)[g], consisting of Takashi Sugimura, Verne Ray, Lorenzo Tomatis, and myself (as Chairman), has been working to define a few of the basic chemical mutagens which all of us carry within us and also those which we cannot avoid from external sources or which are produced internally. Of the multitude of chemicals with which man is in daily contact, we decided to begin with three common compounds (which are also established mutagens), Aflatoxin, Benzo(a)pyrene, and Nitrosamine.

AFLATOXIN is a fungus which grows on agricultural products and, therefore, is easily ingested in foods. This mutagen is of particular importance to developing countries where fungal infection can spread through crops that are stored, often without refrigeration. Aflatoxin is a very serious mutagen and also a very strong carcinogen for both somatic and germinal cells.

BENZO(A)PYRENE is a by-product of combustion and is present in smoke and in automobile exhaust and is therefore most prevelant. This compound has been detected in body tissues and is a well-established somatic mutocarcinogen, although it has not been established as being mutagenic to germinal tissue where it is found in small quantities. It probably doesn't reach the chromosomes in the form which is detected by chemical analysis in the gonads. It is easily detectable and data are available from a variety of sources, especially from laboratories in Germany (9). Some figures are given in Table I.

NITROSAMINES are ingested and are possibly also produced by the nitrites in saliva or by intestinal bacteria. However, nitrosamines are also found in germ-free animals. There must, then, be some enzyme system which is responsible for internally producing nitrosamine. The enzymes responsible for this are not yet clear. There is considerable literature in this area and much controversy. One problem, of course, is that nitrosamines pass through the body very

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[g]ICPEMC Task Force 4, studying the "Mutagen Burden". Three meetings held in 1979-80 to establish preliminary measurements of chemical intake and effect.

Table I
An Estimate of Man's Exposure to Mutagenic Chemicals Carried Within the Human System and Unavoidably Ingested, Absorbed, or Inhaled from the Environment

Chemical		Measurement
AFLATOXIN[a] (B1 = 312)		250 ng/day/person - 3000 ng/day/person (= 8×10^{-7} mmoles)
		10 ng/hr/person - 120 ng/hr/person (= 3×10^{-8} mmoles)
	Intake:	
		C_1 (plasma) 10 ng/kg - 120 ng/kg (= 3×10^{-8} mmoles)
		C_2 (tissue) 29 ng/kg - 480 ng/kg (= 9×10^{-8} mmoles)
BENZO(A)PYRENE[b]		0.2 g - 15.6 ng/100 g dry tissue (= 8×10^{-7} mmoles - 6×10^{-5} mmoles)
NITRITE (NO_2)[c]		0.012 - 0.88 mmoles/person/day
NITRATE (NO_3)[c]		0.3 - 9.3 mmoles/person/day
NITROSAMINE[d]		0.001 - 0.05 mmoles/person/day

The exposure data supplied above were compiled by: [a]Dennis Hsieh, Univ. California, Davis; [b]James Selkirk, Oak Ridge National Laboratory, Tennessee; [c]Philip Hartman, Johns Hopkins University, Baltimore; [d]Anthony Pegg, Pennsylvania State University.

readily and are probably present not only in the intestine but also in the fecal matter. As a matter of fact, a special symposium following this conference[h] will consider the different problems of these compounds.

If we consider the three obvious mutagens as a whole, it is remarkable how few detrimental effects have been recognized with the relatively significant quantities of

[h]Seventh International Meeting on Analysis and Formation of N-Nitroso Compounds, Tokyo, September 28 - October 1, Helmut Bartsch, IARC, Lyon, France (Chairman)

mutagens or carcinogens we carry within the body while still maintaining our present integrity of genetic mechanism.

This is an interesting problem which requires further analysis. There is also the possibility that we carry sufficient "anti-mutagens" to stop the activity of the mutagens within. There is good indication that such compounds exist, but whether these are adequate to take care of the stability of our genetic make-up is not certain (i.e., ascorbic acid, uric acid).

As a basis for consideration, these three chemical compounds represent common quantities and exposures. Those found in smaller quantities ought not to be overlooked as potentially effective mutagens as, for example, William L. Russell found of Ethylnitrosourea (ENU) (10), the strongest mutagen ever found in the mouse yet formerly considered rather harmless. There may be other substances that are just as effective but which exist in lesser quantities and have not yet been recognized to contribute to the Mutagen Burden. This is another important aspect which deserves further investigation.

I would like now to discuss some of the quantitative aspects of Environmental Mutagenesis as I see it from my narrow point of view. An interesting approach has been used by Committee 17 of the Environmental Mutagen Society ("Environmental Mutagenic Hazard" In Sciences (1975) 187: 503-514) in developing the "REC" or rem-equivalent-chemical on the basis of the very extensive data which are available in radiation mutagenesis. Although this attempt is a very promising one, it is not adequate. A cautionary note is attached stating that the calculation implicitly assumes the response of the organism, while modulated by numerous pharmocological factors, remains constant in time. This, of course, is not entirely correct because the physiology of the human is a very dynamic system.

Therefore, reliance solely upon data derived from means determined for radiation mutagenesis are not adequate for an accurate evaluation of the more complex steps in chemical mutagenesis. At least this 1975 study was a good attempt and should be encouraged with some modification. The systems we are discussing are based primarily on animal work. Extrapolating from the mouse to man is a

somewhat dangerous transition. However, at the present time, this is the best that we can do if no other data are available on which to proceed. This is the reason I thought that the "Mutagen Burden" concept would offer some kind of base for human evaluation and I will come back to this point later.

The highly significant mutation data obtained on the specific locus method by Russell and Lyon and Morris on somewhat different loci in the mouse gave, until lately, the best data on the quantitative genetic effects of ionizing radiation on certain hybrid strains of mice. The genetic effects of chemicals on mammals in vivo is considerably more difficult for the reasons which we have already discussed. There is a shortage of quantitative data on the in vivo effects of chemicals in mammals. Studies show that the chemical effects on the mouse produce a very low number of gene mutations measured by the specific locus method of Russell. This was changed somewhat with Russell's finding that ENU is the most effective mutagen ever found.

When Ehling reported the effect of ionizing radiation on the skeleton as a ready way to study dominant mutations in the mouse, the picture changed. These studies have been considerably extended by Selby at Oak Ridge and some of the results are shown on Table II.

Table II
Frequency of Presumed Dominant Mutations
Affecting the Skeleton of the Mouse

Dose (R)	Interval between dose fractions	Stage	No. of F_1 skeletons	Presumed mutations	
				(No.)	(%)
0			1739	1	0.06
600	0	Post-spermatogonial	569	10	1.8
600	0	Spermatogonial	754	5	0.7
100 + 500	24 hours	Spermatogonial	277	5	1.8
500 + 500	10 weeks	Spermatogonial	131	2	1.5

Reproduced from "Radiobiological Equivalents of Chemical Pollutants", Int. Atomic Energy Agency, Vienna, p 78 (1980).

Table III
Specific Enzyme Activities (Expressed in % Activity of Normal Lenses) in the Lenses of Mice with Nzc-Cataract

Enzyme	Cataract Mutants	
	Heterozygote	Homozygote
Glucose-6-phosphatdehydrogenase	96	102
Malatdehydrogenase	111	132
Isocitratdehydrogenase	97	119
Hexokinase	161	243
Lactatdehydrogenase	130	159
Glyceraldehydphosphatdehydrogenase	116	121
Pyruvatkinase	89	111
Enolase	126	143
Phosphoglyceratkinase	102	124

Reproduced from "Elektrophorese Forum '80", 2. Diskussionstagung Technische Universitat, Munchen, Munchen (1980).

Still newer developments include the observation of the inherited cataract method by Ehling's group who recognized that these can be observed easily with a slit lamp on a large number of animals especially since these again are dominant effects. It was first developed with the evaluation of ionizing radiation but has proven most promising with chemicals. I believe that we can look forward to many interesting data available with in vivo genetic effects of chemicals in the mouse.

One of the most interesting discussions in regard to quantitative effects has been given by Haynes and Eckhardt who developed mathematical equations which take into account not only the mutation rate, but the survival rate and repair mechanisms as well. The set of curves thus developed show that even with the present rather primitive data, it is possible to get significant results. Some of the first statistics obtained are given in Figure 1. This approach would, of course, be more successful if more publications will include, besides mutation data, also exact survival curves. Joyce McCann, in a Chapel Hill discussion, recently pointed out very well the difficulties in obtaining such data by the plate method.

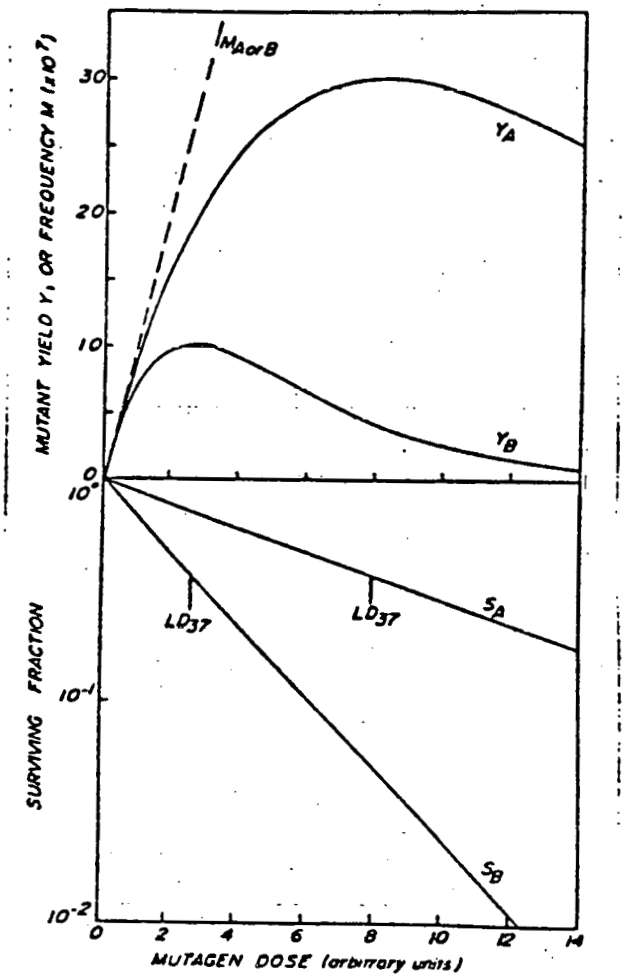

Fig. 1. Mutant yields Y(x), frequencies M(x) and survivals S(x) plotted over dose in arbitrary units for the purely linear kinetic response pattern. For both case A and B, $m_1 = 10^{-6}$ (units)$^{-1}$. In A, $k_1 = 1.23 \times 10^{-1}$ units)$^{-1}$; in B, $k_1 = 3.7 \times 10^{-1}$ (units)$^{-1}$; that is, the cross-section for killing in B is 3-fold greater than in A. Note that the frequency curve (dashed line) is the same for both A and B whilst the areas under the yield curves are very different. In each case Y_{max} is at the dose for 1 lethal hit (=LD37). Reproduced from "Quantitative Measures of Mutagenicity and Mutability based on Mutant Yield Data" Friederike Eckhardt and Robert H. Haynes. Mut. Res. 74: 439-458 (1980).

At a workshop on Statistical Analysis of In Vitro Tests for Mutagenicity held this Spring at Chapel Hill, chaired by David Hoel and managed by Michael Shelby with Fred de Serres, a careful analysis was made of some of the best data available especially on Salmonella but also of a few tissue culture tests. (Proceedings sponsored by NIEHS & NTP, in press).

This workshop illustrated again the difficulty of interpretation of mutation data as they are now appearing in the literature, especially since survival data are usually not given. However, it should be pointed out that more and more investigators are becoming conscious of the complications of the plate test for Salmonella. It is especially difficult with this test to control and determine the survival ratio, the condition that individual organisms experience during the test and many other points discussed by McCann during this workshop. We will be looking forward to the publication of this very interesting workshop.

Ehrenberg's group in Stockholm, in a very interesting paper, recommended the use of the appearances of alkylating amino acids in hemoglobin to be used as a measure of exposure to genotoxic agents, alkylating agents per se, and compounds that are metabolized to alkaylating agents. They illustrated the methods on ethylene oxide and dimethyl nitrosoamine. The latter compound respires metabolic oxygenation which gives rise to alkylating compounds which are responsible for the genotoxic effects. This method is of considerable promise for practical application in mice and could be very useful for the detection of exposure of people inadvertantly exposed to massive doses of these compounds (11).

Per Oftedal, in a review of Problems in the Reevaluation of Genetic Risk from Radiation and Other Environmental Hazards, gave a very important discussion of the shape of the mutation curve produced by radiation in Drosophila but also discussed the effects of chemicals and their relation to radiation effects. He also compared the risk estimated by the UNSCEAR and the BIER committees. Even after the many investigations to date, it was not possible for these two very important committees to agree on the doubling dose at low level exposure. So it is not surprising that we have little agreement in regard to chemical exposure.

In all of these discussions, I have tried to avoid the

problems of the recovery phenomena. Of course, the problem of repair is one of the most fundamental ones first observed after exposure of bacteria and yeast to ultraviolet radiation. It was first recognized by carefully controlling conditions of exposure to radiation at temperatures where practically no metabolism takes place and exposing the culture after to conditions of a broad variety which makes possible the recognition of the recovery phenomenon. All such techniques which make quantitative evaluation possible are somewhat difficult to control with chemicals.

Another area which I have not touched on is the work in cytogenetics - not that I think that it is not important, rather, it has been discussed so very well in symposia and special papers at this conference. The cytogenetic approach lends itself readily to quantitative work as it was first developed by Carl Sax almost 50 years ago and carried on so well by his co-workers. The leadership of H.E. Evans and his group, the new developments initiated by J.H. Taylor and later by S. Latt and then further developed and extended into new lines by S. Wolff are most impressive.

Most of the attempts in regard to evaluation of the quantitative aspects of the effects of chemical mutagens are based on experience on radiation effects. There the success for quantitative estimation was developed by a systematic planning approach that tackled the problem in a carefully planned way which took advantage of all of the different methods that radiation biology offered. This approach to radiation effects utilized all possible angles - working with viruses, microorganisms, plants, Drosophila, and mouse - from nucleic acid to proteins, using all possible tools available 35 years ago, taking advantage of the developments which were new at that time, the use of tracers included. Thus, it was possible to develop a fairly comprehensive picture of the effects of ionizing and ultraviolet radiation. Since this approach was done from a very fundamental point of view, it had also considerable impact on other biological sciences. Something of this type will have to be done with chemical mutagenesis.

Where do we go from here? I would like to see an agreement, possibly sponsored by the IAEMS, on the following points:

1. Let a group of us, who are interested in the basic aspects of chemical mutagenesis, agree on a

very short list of compounds to be investigated as thoroughly as possible, including all traditional as well as modern biological methods. If we concentrate on a few compounds, we will make more progress than if we spread our efforts on the wide variety of compounds we are presently studying.

2. What are the compounds on which one should concentrate? A committee of the IAEMS should prepare the recommendation. I would like to see us start with the three compounds which form the basis for the Mutagen Burden, i.e., Aflatoxin, Benzo(a)pyrene, and Nitrosamine. One or two additional compounds should be recommended.

3. The IAEMS should take the leadership and coordinate the investigators, collect all the data, see if they are adequate for risk assessment possibly for use in setting tolerance limits. H. Bartsch will have a workshop on Nitrosamines following this conference in Tokyo. However, this type of workshop is very useful, but if, too scattered, may fail to emphasize the basic genetic approaches. The approach by the Lyon group is extremely useful.

4. If the investigation of these few compounds is done well enough, I visualize that it could serve as a model to other studies. I believe it is time to get away from reports on one or two Salmonella strains as a means of evaluation of such a wide variety of compounds. Each of these types of reports should make at least an attempt to study the underlying mechanisms.

This should not distract us from all of the important genetic and toxicological work going on. I feel the completion of the Gene-Tox work of the U.S. Environmental Protection Agency (EPA), the different approaches of the ICPEMC , the efforts of the European Economic Community, and the U.S. National Academy of Sciences Committee (NAS) should be encouraged in every way possible. All of these efforts, I believe, will contribute important data to the larger picture which is forming on the complex problems of environmental mutagenesis. I should also compliment

the many individual investigators who have done so much to promote this field and who are helping to bring order into this complex field. Indeed, we have in front of us some very promising developments in this relatively new field which hardly existed twenty years ago. I want to pay my special compliments to our Japanese colleagues who have done such a magnificent job in making the study of environmental mutagenesis a very prominent field.

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