PROGRESS IN MUTATION RESEARCH

VOLUME 2

PROGRESS IN ENVIRONMENTAL MUTAGENESIS AND CARCINOGENESIS

Proceedings of the 10th Annual Meeting of the European Environmental Mutagen Society (EEMS)
Athens (Greece), 14–19 September 1980

edited by

A. Kappas

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Under the auspices of the Ministry of Culture and Science of Greece
and the Greek Atomic Energy Commission

edited by

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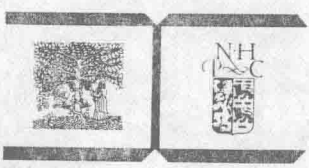

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PREFACE

The European Environmental Mutagen Society (EEMS) was founded in 1969 and its 10th annual meeting was held in Athens, Greece from 14th to 19th of September 1980.

Scientists actively involved in the field of genetic toxicology from many European countries, the United States, Japan and others were present. Their scientific contribution was shown in the form of posters, oral presentations and invited lectures. Obviously the continuous and active discussions developed during the six days of the meeting was the best result of this meeting.

The invited lectures presented during the meeting dealt mainly with the aspect of mutagenic potential of food and pesticides, the mutagenic and carcinogenic evaluation of environmental pollutants and several methodological and basic problems common to the different topics of this field of research.

The text of these lectures is given in this book and they clearly represent the present achievements in the field of environmental mutagenesis and carcinogenesis.

The abstracts of posters and oral presentations will appear in *Mutation Research* (Vol. 85, No. 4, August 1981, pp. 215—307).

The organization of the meeting was excellent and all participants could appreciate the values of the cultural tradition of Greece which has seen the birth of modern science and philosophy.

N. LOPRIENO
President of EEMS

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CONTENTS

Preface v

Scientific Programme Committee; Organizing Committee; Acknowledgement vi

Mutagens in Food and Gut Contents

Faecal mutagens: Their discovery and possible relevance to the aetiology of large-bowel cancer in man
by S. Venitt (Chalfont St. Giles) 3

Mutagenic and colicin-inducing activity of some antioxidants
by Renana Ben-Gurion (Tel-Aviv) 11

Acetaldehyde not ethanol is mutagenic
by G. Obe (Berlin) 19

Penicillium roqueforti toxin
by S. Moreau (Villeneuve d'Ascq) 25

Biological activity of the aflatoxins
by R. Colin Garner (York) 33

The role of bacterial metabolism in the gut in relation to large bowel cancer
by M.H. Thompson and M.J. Hill (London and Salisbury) 41

Mutagen—carcinogens in amino acid and protein pyrolysates and in cooked food
by T. Matsushima and T. Sugimura (Tokyo) 49

Mutagenic Pesticides

The genotoxicity of benomyl
by A. Kappas (Athens) 59

Does dichlorvos constitute a genotoxic hazard?
by Claes Ramel (Stockholm) 69

The mutagenicity of the fungicide thiram
by M. Zdzienicka, M. Zielenska, M. Hryniewicz, M. Trojanowska, M. Zalejska and T. Szymczyk (Warsaw) 79

Comparison of the mutagenic activity of pesticides in vitro in various short-term assays
by A. Carere and G. Morpurgo (Rome) 87

Case-control studies: Soft-tissue sarcomas and malignant lymphomas and exposure to phenoxy acids or chlorophenols
by L. Hardell (Umeå) 105

Mutagenic and Carcinogenic Pollutants

Cigarette smoke induced DNA damage in man
by H.J. Evans (Edinburgh) 111
How relevant are high doses in mutagenicity and carcinogenicity studies in animals?
by H. Greim, U. Andrae, W. Göggelmann, S. Hesse, L.R. Schwarz and K.H. Summer (Neuherberg) 129

Fundamental Microbiological Aspects of the Ames Test Panel Discussion

Introduction
by J.P. Seiler (Wädenswil) 151
Influence of composition and treatment of the growth media on the yield of mutant colonies
by J.P. Seiler (Wädenswil) 155
The effect of spontaneous mutation on the sensitivity of the Ames test
by M.H.L. Green (Brighton) 159
Test cell problems in the Ames test
by A. Grafe (Mannheim) 167
Culture conditions and influence of the number of bacteria on the number of revertants
by W. Göggelmann (Neuherberg) 173
Statistical problems in the Ames test
by J. Vollmar (Mannheim) 179
Basis of evaluation of an Ames test
by I.E. Mattern (Rijswijk) 187
Summary and discussion
by J.P. Seiler (Wädenswil) 191

Concluding Remarks

Concluding remarks
by Per Oftedal (Oslo) 197

Author Index 201

Subject Index 203

MUTAGENS IN FOOD AND GUT CONTENTS

Progress in Mutation Research, Vol 2
A. Kappas, editor.

FAECAL MUTAGENS: THEIR DISCOVERY AND POSSIBLE RELEVANCE TO THE AETIOLOGY OF LARGE-BOWEL CANCER IN MAN

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Introduction

The epidemiology of large-bowel cancer (colorectal cancer) has been extensively reviewed by Correa and Haenszel (1978). In summary, a high incidence of large-bowel cancer is associated with consumption of a high-fat, high-protein diet, characteristic of affluent countries in North-West Europe, U.S.A. and Australia, and with urban life, whereas large-bowel cancer is relatively rare in Africa, Asia and the Andes, where average income is very low and the diet is concomitantly low in fat and protein. Studies of migrant populations have shown that racial/genetic differences in these disparate populations make little or no contribution to the observed distribution of large-bowel cancer. It is generally accepted, therefore, that diet has a large part to play in the aetiology of colorectal cancer. The content of the diet has, of course, a marked influence on the contents of the large bowel, and the possibility arises therefore that a high incidence of colorectal cancer might be associated with the presence of chemicals carcinogenic to the epithelium lining the gut. This idea is now susceptible to direct test with the advent of reliable short-term tests for potential carcinogens, most of which are based on the now well-established qualitative relationship between carcinogenicity and mutagenicity (Venitt, 1980). Several groups of investigators are now engaged in studies of the mutagenic activity of human faeces, and the present contribution is a brief summary of published data available to the author by August 1980.

Bacterial mutation studies

W.R. Bruce and his co-workers were the first to demonstrate the presence of mutagenic activity in extracts of human faeces (Bruce et al., 1977, 1979; Varghese et al., 1978). Initial studies showed that ether-extracts of freeze-dried faeces from normal healthy male volunteers, consuming a normal "Western" diet, were mutagenic to *Salmonella typhimurium* strains TA100, TA1535, TA98 without a requirement for an external source of metabolism (i.e. in the

absence of S9). These and subsequent studies employed the classical plate-incorporation assay of Ames et al. (1975). Further studies using TA100 indicated that mutagenic activity was higher in ether extracts which had been washed in alkali. Fractionation of this material using high-pressure liquid chromatography revealed mutagenic activity associated with specific fractions. The level of mutagenic activity was found to differ markedly from sample to sample, and from individual to individual. Chemical investigations of volatile material from the freeze-drier trap and from freeze-dried stools showed the presence of both volatile and non-volatile nitroso compounds, including nitrosodimethylamine, nitrosodiethylamine, nitrosopyrrolidine and nitrosomorpholine (Wang et al., 1978). Nitrite and nitrate have also been shown to be present in human faeces (Tannenbaum et al., 1978). Bruce et al. (1979) claimed that the mutagen isolated from human faeces had the properties of a nitroso compound associated with a lipid. It is of interest to note that the nitrosamines detected in faeces all require metabolic activation (in the form of rodent S9) for detection of their mutagenic activity in *S. typhimurium*, whereas the mutagen partially characterised by Bruce and his co-workers was shown to be mutagenic in the absence of S9: if in fact this mutagen is a nitroso compound, it is more likely to be a nitrosamide, since nitrosamides are, in general, directly-acting alkylating agents.

Whatever the precise nature of the faecal mutagen(s) discovered by Bruce et al., the level of mutagenic activity appears to be changed by systematic changes in diet, and to differ in amount in faeces obtained from different populations. For example, Bruce et al., studied the level of faecal mutagen in one individual after the addition of either ascorbic acid or tocopherol to the diet: in both cases, there was a marked decrease in mutagenic activity following addition of either of these antioxidants to the diet, followed by a gradual increase to the control (no antioxidant) level over a period of about one month.

The second group (Ehrich et al., 1979) to have reported the presence of bacterial mutagens in human faeces employed the extraction methods of Bruce and his co-workers, and applied the technique to populations at different risk of colon cancer. Single faecal samples were collected from a group of urban whites and a group of urban blacks living in Johannesburg, and a group of blacks living in Ruskinburg, a rural locality. The age and sex-distribution of each group are summarized in Table 1. The urban whites represented a group at high risk of large-bowel cancer, the black groups representing two low-risk populations.

TABLE 1

AGE- AND SEX-DISTRIBUTION OF POPULATIONS DONATING FAECAL SAMPLES IN THE STUDY BY Ehrich et al. (1979)

Group	Male	Female	Total	Age (mean ±S.D.)
Urban whites	23	19	42	46 ± 8
Urban blacks	50	32	82	47 ± 9
Rural blacks	54	54	108	51 ± 15

Freeze-dried faeces were extracted with ether, which was then evaporated, the residue being dissolved in dimethyl sulphoxide, and tested for mutagenicity in *S. typhimurium* using the plate-incorporation method. Mutagenic activity was detected in both TA100 and TA98, and was not dependent on the addition of S9. 19% of urban-white samples were mutagenic in TA100, compared with 2% of urban black, and 0% of rural black samples, the difference between urban whites and blacks being significant at $p < 0.001$. This pattern was seen in TA98, with percentages of 10, 5 and 2 for whites, urban blacks and rural blacks respectively. This study suggests, therefore, that individuals within a group (urban whites) at high risk for large-bowel cancer were more likely to have faecal mutagens than individuals (urban blacks and rural blacks) well matched for age and sex, considered to be at lower risk of this disease. However, this conclusion is based on just one faecal sample per individual: bearing in mind the rather large variations in the level of faecal mutagens from day to day seen in the results of Bruce and his co-workers, it would at this stage seem prudent to await further confirmation of the work of Ehrich et al., before coming to far-reaching conclusions about the precise role of faecal mutagens in the aetiology of large-bowel cancer. Further work from Wilkins' laboratory on the characterization of faecal mutagens has recently been published (Lederman et al., 1980).

In summary, anaerobic incubation of faeces for 96 h prior to extraction substantially increased the mutagenicity of faecal extracts compared with non-anaerobically-incubated faeces. Incubation in the cold, in air, with antimicrobial agents, or sterilization of faeces with heat or γ-irradiation did not enhance mutagenicity. All this evidence suggests the involvement of the faecal flora in the formation of the mutagen. Thin-layer chromatography (TLC) of the TA100-positive material revealed bands fluorescing in long-wave UV, and with characteristic UV-absorption. Mutagen from several donors had the same UV-absorption and retention time on HPLC columns, and the amount of this material correlated with the level of mutagenicity. "Purified" mutagen had the same R_f on TLC as mutagen directly extracted from fresh faeces, and the faeces obtained from 5 donors all contained this material. No firm conclusions have been drawn as to the chemical nature of the purified mutagen.

A third group (Reddy et al., 1980) have recently published an investigation of faecal mutagens from groups representing populations at low or high risk of colon cancer, again using the extraction methods of Bruce and co-workers: ether extracts were partially purified by silica-gel chromatography and assayed by the standard procedures of Ames et al. (1975), using *S. typhimurium* TA98 and TA100, and Aroclor-induced rat-liver S9. The study-population consisted of 44 healthy men, of mean age 49 ± 5 years. 11 were Seventh-Day Adventists, resident in New York, consuming a vegetarian diet (milk and milk products, but no fish, fowl, or flesh) for more than 10 years and considered to represent a population at low risk of colon cancer. 15 were inhabitants of Kuopio, in rural Finland, an area whose male population suffers an age-adjusted incidence of colon cancer of 5.6 per 100 000, compared with 28.5 per 100 000 for the white male population of the U.S.A. The third group, representing this high-risk population, consisted of 18 New Yorkers. The Finns consumed more milk, dairy products and fibre, and less meat-fat than the New Yorkers: all 3 groups

consumed much the same amounts of total protein, and fat-intake was lowest in the Seventh-Day Adventists. Faeces were collected over a 48-h period, pooled and extracted as described above. In this study, a sample was declared mutagenic if the ratio of the number of mutants per plate on treated plates to the number on control plates was equal to or greater than 3. Ehrich et al. (1979) considered a ratio of 2 or more indicative of mutagenicity in their study.

None of the samples from Seventh-Day Adventists showed mutagenic activity by the criterion adopted by the investigators: 13% of Finnish faecal samples were mutagenic, this activity being confined to TA98 + S9. The New Yorkers had the most mutagenic faeces, 22% of samples being active in at least one test system (i.e. one strain, with or without S9): the highest mutagenic ratios were detected in TA98 without S9, followed by TA100 without S9, then TA100 with S9. All samples active in TA100 without S9 were also active in TA98 without S9. It was concluded that faecal extracts from New Yorkers (high fat, high meat, low fibre diet) were more mutagenic than samples from the Finns (high fat, high fibre diet).

This study is the second to demonstrate marked differences in the bacterial mutagenicity of faecal extracts from groups representing populations at differing risk of large-bowel cancer. The picture which emerges is complex, the differences in strain-specificity and requirement for metabolic activation between the New Yorkers and Finns suggesting the presence of at least two and probably more classes of mutagen. The percentage of positive samples for the "high-risk" group (22%) in the study of Reddy et al. agrees closely with the 19% positive found by Ehrich et al. for their "high-risk" group, but the pattern of strain-specificity and metabolic requirement is different in the two studies, again suggesting the production of several mutagenic classes.

A study of faecal mutagens in relation to large-bowel cancer in the United Kingdom was started in September 1979, by S. Venitt, S.P. Pickering, M.J. Hill and F. Fernandez. Results obtained in this study have not been published and a brief account of our preliminary results are therefore included. Our initial studies are based on the extraction procedures pioneered by Bruce et al., and we initially examined the following extracts of freeze-dried faeces for mutagenicity, using plate-incorporation tests with several strains of *S. typhimurium* and *E. coli*, and fluctuation tests using *S. typhimurium* TA100 and *E. coli* WP2uvrA(P) (Venitt and Crofton-Sleigh, 1979; Gatehouse, 1978): (a) water from the freeze-drier trap; (b) ethyl acetate extract of (a); (c) chloroform/methanol; (d) ether extract; (e) concentrated ether extract; and (f) alkali-washed ether extract. We have examined faeces from 7 healthy individuals consuming a "Western" diet, and have observed mutagenic activity in 4 of these subjects. The extracts giving the most consistent results were (e) and (f), although mutagenic activity was occasionally seen in (c) and (d). The bacteria most sensitive to faecal mutagens were *S. typhimurium* TA100, and *E. coli* WP2uvrA(P), the most sensitive method of assay being the fluctuation test. Doses of extract giving positive results were very similar to those reported by Bruce et al., by Ehrich et al., and by Reddy et al., namely within the range 100—500 mg dry-weight of faeces per plate, in plate-incorporation assays. In concordance with the results obtained by some other groups, we find that

addition of an Aroclor-induced rat-liver S9 fraction has no enhancing effect on the mutagenicity of faecal extracts. Fig. 1 shows a dose—response curve for the mutagenicity of an ether extract of freeze-dried faeces in a plate-incorporation assay using *E. coli* WP2uvrA(P) in the presence or absence of S9. Fig. 2 shows results of microtitre[R] fluctuation tests, using the same bacterial strain, and a similar faecal extract. These are pooled results from 3 separate assays of the same extract; Fig. 3 shows results where the extract was tested using 3 microtitre plates per dose in the same experiment. These results indicate the fairly reasonable consistency within and between assays of the same extract. However, we, like the other investigators, have found considerable variation in the mutagenicity of faecal samples given by the same volunteer on separate occasions. Systematic investigation of this variability is now under investigation. In order to verify that the results obtained to date reflect true mutagenic effects, rather than artefacts caused, for example, by toxicity, we have characterized the mutants obtained in fluctuation tests: samples from positive and negative wells were plated on minimal medium/agar plates supplemented with glucose, and incubated. Positive wells yielded a variety of revertant types of widely differing growth rates, which were viable upon further replating on minimal medium. The vast majority of negative wells produced to visible colonies, the occasional large colony probably representing pre-existing revertants in the original inoculum. Parallel studies using nutrient agar plates, and suitably diluted samples from negative wells allowed us to obtain information on cell survival following treatment with faecal extracts. The results so far indicate that some faecal extracts are slightly toxic at the highest doses giving mutagenic effects, and that the problem of the effect of toxicity on mutagenic yield warrants further investigation.

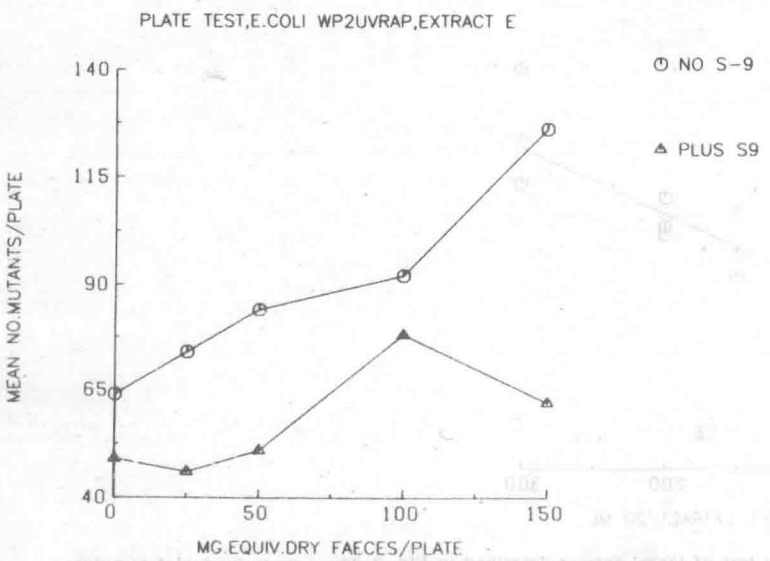

Fig. 1. Plate-incorporation assay of faecal extract (ether extract of freeze-dried stool sample), using *E. coli* WP2uvrA(P) ($trp^- \rightarrow trp^+$) in the presence or absence of S9 prepared from the livers of Aroclor-induced CB Hooded male rats. Each point represents the mean of 3 plates; ($p < 0.001$ for slope).

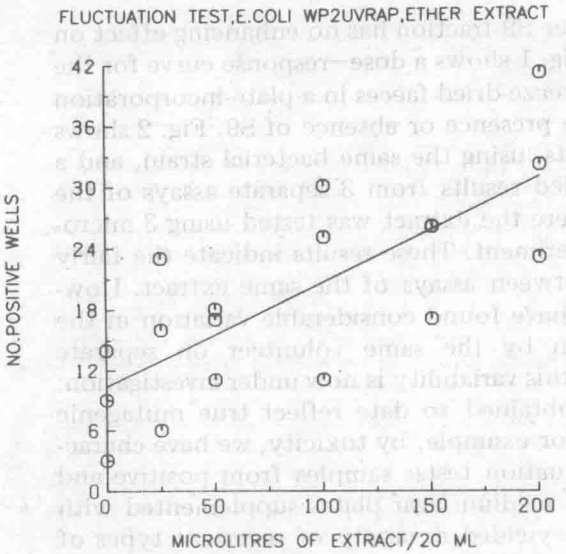

Fig. 2. Microtitre[R] fluctuation test of faecal extract made by extraction of freeze-dried stool sample with diethyl ether, and the ether extract washed in 1 N Na_2CO_3. The values on the abscissa represent the volumes of extract added to 20 ml of medium prior to dispensing 200-μl samples to each of 96 wells in the microtitre[R] plate. The extract was originally dissolved in dimethyl sulphoxide at a concentration equivalent to 1 g dry weight of stool per ml DMSO. The final concentrations in mg-dry-weight equivalent are therefore 0, 2.5, 5, 7.5 and 10 mg/ml respectively, or 0.5, 1, 1.5 and 2 mg per well. These are data pooled from 3 separate Expts. using the same extract. The line drawn through the points is the best fit by linear regression, $p < 0.01$.

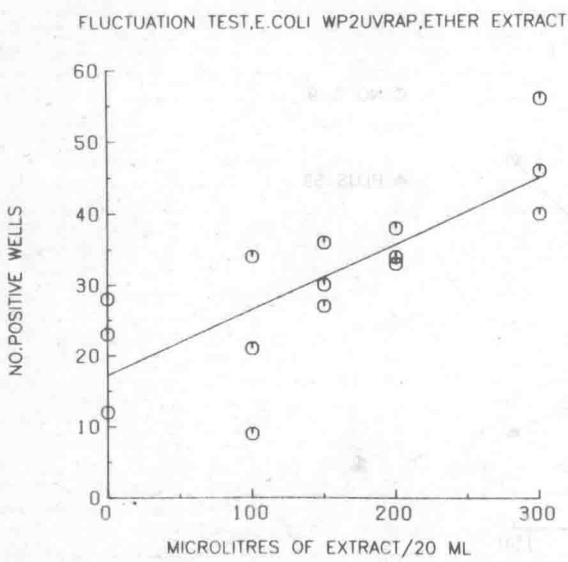

Fig. 3. Microtitre[R] fluctuation test of faecal extract described in Fig. 2. These are individual data points obtained in the same experiment, using 3 plates at each dose. Doses per well are 0, 1, 2 and 3-mg-dry-weight equivalent. The line drawn through the points is the best fit by linear regression, $p < 0.001$.