MUTAGENICITY STUDIES WITH IRRADIATED MEATS

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INTRODUCTION

WITHIN the last few years, the use of microbial systems to screen substances for mutagenic potential has received considerable attention. Test procedures using the yeast, Saccharomyces cerevisiae, the fungus Neurospora, and the bacteria Salmonella typhimurium and Escherichia coli have been developed. Of these procedures, the Salmonella/mammalian microsome mutagenicity assay, developed by Dr. Bruce Ames and co-workers, has been used most extensively. This assay has demonstrated an approximate 90% accuracy in the prediction of a variety of carcinogenic chemicals as mutagens. It is equally accurate in predicting that a non-mutagenic compound is non-carcinogenic. Mutagens identified by this assay range from flame retardants used in fabrics to cigarette smoke condensate.

The short-term mutagenesis assay developed by Ames employs mutant strains of Salmonella typhimurium designated TA98, TA100, TA1535, TA1537, and TA1538. Due to a specific mutation in the histidine operon, these strains are unable to grow in the absence of histidine. When grown on media containing a level of histidine sufficient for a few cell divisions, only cells able to revert to histidine independence are able to form colonies easily visible against the background lawn. Each of these mutant strains has a fairly constant rate of spontaneous reversion, however, the mutation frequency is significantly increased when a chemical mutagen is added to the system.

In addition to the previously described mutation within the histidine operon, the five tester strains contain additional mutations to increase their usefulness in the assay. All strains contain the rfa mutation which results in increased permeability of the cell wall allowing the entrance of more substances. All of the tester strains contain the uvrB mutation which results in defective excision repair of damaged DNA molecules. This mutation enhances the sensitivity of the strains to some mutagens. By incorporating all five tester strains into each assay, the type of mutation can be distinguished. Strain TA1535 is useful in the detection of mutagens which cause base-pair substitutions. Strains TA1537 and TA1538 are for detection of frameshift mutagens. Strains TA98 and TA100 were derived by the addition of a plasmid to strains TA1538 and TA1535, respectively. Carriage of this plasmid makes these two strains more sensitive to certain mutagens in addition to providing resistance to ampicillin. Some mutagens are inactive unless they are metabolized to active forms. Liver enzymes possess the capability of metabolizing many of these compounds to their mutagenic form. For this reason, a mammalian microsomal activation (S-9 activation) system prepared from rat liver is included in the test.

Of the three formats for performance of the assay, the one most frequently used is the plate incorporation procedure. This is a quantitative test run by combining the test substance with about 10^8 bacteria and rat liver homogenate, when S-9 activation is desired, in 2 ml of molten 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin. The mixture is poured onto the surface of a minimal glucose agar plate and hardens as a thin overlay. Plates are subsequently incubated at 37 C for 48 hours. Since the medium contains a limited supply of histidine and biotin, the entire population undergoes only a few divisions before the supply is exhausted and cell division ceases. From this point, only cells capable of reverting to histidine independence will grow and produce macroscopic colonies on the plate. Following the incubation period, these revertant colonies are scored and their number is compared with the number of spontaneous revertants for the strain being used. If the number of revertants produced by the test substance is twice that of the spontaneous reversion rate, the test is considered positive.

The spot test procedure is a qualitative test. In this modification, the test substance is placed on the surface of the minimal glucose agar plate already containing the agar overlay with the test organism and microsomal activation system when desired. The test substance diffuses into the agar, setting up a concentration gradient. Revertant colonies surrounding the spotted chemical constitute a positive test. Use of this procedure is limited to compounds which diffuse readily.

The liquid preincubation procedure was developed for use with compounds which do not readily diffuse. In this case, the test solution, microsomal activation system, when desired, and bacterial culture are combined in sterile tubes and incubated with shaking at either 37 C for 20 minutes or 30 C for 30 minutes. Following this preincubation, top agar is added and the tube contents are poured onto the surface of a minimal glucose agar plate, allowed to harden and incubated 48 hours at 37 C before revertants are scored.

Included with the performance of each assay are several controls. First, quality control tests are performed to assure that each assay strain has retained all of its genetic markers. Second, a short assay is performed with a known mutagen, appropriate for each assay strain, to demonstrate the strain's ability to perform properly in

the presence of a chemical mutagen. Third, negative controls are included with the test run and are simply a measure of the spontaneous reversion rate for each tester strain. Fourth, during the scoring of revertant colonies, lawns on plates are examined microscopically (low power 100X). Absence of bacterial lawn from a test plate is indicative of a toxic test compound. This suggests that a retesting of the compound at a lower concentration should be conducted. A lawn which is more dense than the negative control is suggestive of excess histidine present in the system. Finally, colonies scored as revertants are verified as true revertants by transferring them to a minimal glucose agar plate containing no histidine (or biotin). Growth on plates following 24-hour incubation at 37 C is indicative of true reversion to histidine independence.

The assay is considered to be operating properly if the strains have their markers, exhibit the appropriate level of mutagenesis when treated with a known mutagen, and show a spontaneous reversion rate within the range suggested by Dr. Ames. If these conditions have been met, a positive test is indicated when the average of revertants on the test plates exceeds the average of revertants on the negative control plates by at least a factor of two. Test substances demonstrated to be positive in the Ames assay are usually retested at varying concentrations to establish a dose response curve.

Foods can and do contain chemical mutagens. Aflatoxin B_1 may be found in contaminated corn and peanuts. With the Ames assay procedure, 100 ng of this toxin generates 1000 histidine revertants in strain TA100. This is nearly seven times the spontaneous reversion rate for this strain. Other mycotoxins have been identified as mutagens with the use of the Ames assay.

Following negative tests for carcinogenicity in rats, furylfuramide (AF2) was used extensively by the Japanese from 1965-1973 as an antibacterial food additive. In 1973, AF2 was found to be highly mutagenic in strains of E. coli, S. typhimurium, yeast, and Neurospora. These findings initiated new animal carcinogenicity tests which revealed that this compound was a carcinogen. Based on these data, Japanese officials banned the use of AF2 in foods. It is, however, still too soon to tell if the widespread usage of AF2 will increase the cancer rate in Japan.

Nitroso compounds are potent carcinogens as demonstrated in animal studies. Many of these compounds are detected as mutagens by the Ames test. The most commonly found preformed nitrosamine in foods is dimethylnitrosamine which has been identified as a potent carcinogen and can be isolated from hot dogs, ham, and luncheon meats.

The use of irradiation as a food sterilization process has been defined by the U. S. Food Additive Amendment of 1958 to the Federal Food, Drug, and Cosmetic Act as adulteration unless the use has been cleared for safety. Thus, foods processed by irradiation must undergo the same rigorous testing that food additives must undergo. The Ames assay was chosen as one of the tests to be performed on irradiated meats. Experiments were undertaken to assess the mutagenic potential of thermally processed, frozen, electron-irradiated, and gamma-irradiated beef and chicken. The meat products used in these studies were supplied by the U.S. Army Natick Research and Development Command, Natick, MA 01760.

Initially, experiments using meat samples diluted and blended with water were conducted. This slurry-like material was then used both in the standard Ames plate incorporation procedure and in the spot test.

PRELIMINARY FINDING

THE SPOT TEST is applicable only to water-soluble compounds. When the spot test was applied to the meat items, there was no indication of mutagenic effect on any of the five tester strains, but the lawn and revertant pattern were significantly changed. Normally, revertant colonies are dispersed uniformly over the plate, but when meats were spot-tested there were no revertant colonies within 2-3 cm of the meat. The lawn near the meat spot was quite dense when viewed with a low-power microscope. The lawn density decreased as the distance from the meat increased; the lawn density eventually appeared normal. In the area just distal from the meat spot and where the plate appeared to have a normal lawn, small revertant colonies appeared. As the distance from the meat spot increased, the size of the revertant colonies increased to what was normally expected. In the outer areas of the plate the revertant colonies were normal.

The interpretation of the abnormal lawn and revertant colony pattern around the spot is, that near the spot, complex substances have diffused from the meat and were supplying nutrients. Thus, the non-revertant bacterial cells compete with revertant cells for the nutrients contained in the minimal glucose agar and the revertant cells are unable to overgrow the lawn and form macrocolonies. Revertant colonies appearing at the border of the zone of dense lawn were quite small because they were competing with the non-revertant lawn for nutrients. The revertant colonies increased in size near the periphery of the plate because there was no competition from the non-revertants for nutrients. The zone beyond where revertant colonies were normal was indicative of the end of diffusion of meat substances and the start of proper performance by the test strain. Since the area distal to the zone associated with the spot was smaller than the equivalent area on a pour plate, revertant counts with the spotting procedure were lower than those counts obtained with the plate incorporation technique.

When positive control mutagens were added to the top agar with tester strain, the same abnormal lawn and revertant colony pattern appeared, except that on the outer portion of the plate the number of revertants was greatly increased. There were no revertant colonies in the zone near the meat spot. This helps to verify the theory that non-revertants are successfully competing with revertants for nutrients and that the revertants are unable to form macrocolonies because of this competition. In the light of the nutrient effect of the meats on the growth of tester strains which were all histidine-dependent for growth, it was theorized that the free histidine in the meat would permit an increased number of cell generations. Thus, the chances for cells to revert and form non-histidine-dependent macrocolonies would be significantly increased.

Difficulties were also encountered when using the plate incorporation procedure. There were occasions when revertant counts exceeded twice the spontaneous revertant level. In every case, there was a plausible explanation

and with further investigation, the cause was removed and the test proceeded to show non-mutagenicity. There was no sustainable indication of mutagenic effect by any of the meat items or any of the five tester strains. Particulate matter and fat globules from the meats made the plates difficult to count. The particulate matter from the whole meats made the use of automated colony counting machines impossible. The chemical 2,3,5-triphenyl tetrazolium chloride, when incorporated into the media, causes the colonies to be red and easily differentiated from the debris.

EXPERIMENTAL

EXPERIMENTS were designed and conducted with modification of the standard Ames assay procedures to eliminate potential sources of false positive results. Those experiments included: (a) eliminating of histidine and/or biotin from top agar, (b) determining the amount of free histidine in the meat items and reducing the amount added to the top agar, (c) testing and evaluating the chemical 2,3,5-triphenyl tetrazolium chloride to determine its mutagenic potential and potential as a means of differentiating colonies from debris, (d) removing of meat debris by centrifugation and filtration and testing the filtrate with the standard plate incorporation procedure with normal and reduced levels of histidine in the top agar, (e) verifying "revertant" colonies by streaking on histidine free media, (f) pre-incubating of tester strain with meats and meat filtrates, and (g) adding known mutagen to meats and meat filtrates with varying amounts of histidine in the top agar.

RESULTS AND DISCUSSION

WHEN no histidine was added to the top agar, the lawns appeared normal and revertant levels approximated those of the negative controls for both meat and meat filtrates. When no biotin was incorporated in the top agar, the number of revertant was reduced and the lawns were reduced. When both biotin and histidine were omitted from the top agar the revertant count was quite low and the lawns were quite sparse. These results appear to indicate the meats and meat filtrates may supply sufficient histidine for the assay, but that they do not contain sufficient biotin.

The histidine level of the meats was determined by the use of an amino acid analyzer and by microbiological assay. Then the level of histidine in the top agar was reduced by 15%, approximately the same level that was contributed by the meats. The lawn was still more dense when meat and reduced histidine were added to the top agar than when the normal amount of histidine was added without meat (as on the negative control plates). Revertant counts on plates with reduced histidine and test meats were within the normal range for all strains. Meat filtrates were tested using top agar that had only 50% of the normal amount of histidine and the lawn was still slightly more dense than the lawn on negative control plates. Revertant counts were again in the normal range.

Revertant colonies were tested and verified as true revertants by their subsequent inoculation onto minimal glucose agar plates without histidine or biotin. Growth on such plates is indicative of the return of organism to prototrophy and can be used as a quality control measure. Due to the difficulty in counting plates, this type of quality assurance procedure should be used when foods are assayed. True revertant rates ranged from 20 to 95%, with the lowest levels of true revertants coming from meat or meat filtrate plates with revertant colony counts higher than the negative control. Negative control true revertant rate was about 95%. In some cases, the revertant counts were high enough to incriminate the test compound as a mutagen, but when the extremely high proportion of false revertant was taken into consideration, the meats could not be considered mutagens. Also on occasions when few cells are present, there is more histidine available for each cell; hence, the background lawn undergoes more generations and may produce non-revertant microcolonies. When testing food for mutagenicity with the Ames assay, it is imperative to verify revertant colonies and make suitable allowance for false positives.

Meat filtrates were exposed to tester strains with S-9 before top agar was added. After exposure, the filtrates and cells were centrifuged. The liquid was decanted and the cells were reconstituted and used in the standard plate incorporation procedures. Revertant counts were lower than the spontaneous revertant counts in all cases. The explanation was theorized that cells were lost or destroyed during centrifugation and washing so that fewer than the number required for optimum sensitivity survived. The addition of known mutagens to the filtrates produced mixed results, ranging from toxicity, mutagenicity, to no effect. The toxicity was probably related to concentration. The reason for no effect was probably insufficient or unavailable mutagen because of insolubility in water, insufficient amount of mutagen added or it was chemically bound and not active. The chemical 2,3,5-triphenyl tetrazolium chloride (TTC) was an effective means of dyeing revertant colonies red so that they could be easily differentiated from the meat debris. Thus, the mutagenic potential of TTC was assayed by using the standard plate incorporation procedure. The compound showed no mutagenicity but revertant counts were lower than controls, which is indicative of toxicity.

SUMMARY

STUDIES were undertaken to determine the mutagenic activity of beef and chicken that had been frozen, thermally processed, electron irradiated, and gamma irradiated. The Salmonella/mammmalian microsome mutagenicity assay, as described by Ames and with several modifications, was performed on beef and chicken that had undergone the four different processing procedures. Considerable difficulty in performing the test and interpreting the results were encountered. After experiencing high counts of revertants from the homogenized meats and meat extracts, experiments were conducted that showed on some occasions up to 80% of the apparent revertants were not true revertants. It was presumed that the meats contained water-soluble growth factors, specifically identified was histidine which apparently supported greater than normal growth and macrocolony formation. Subsequently, the level of histidine in the media was reduced by an amount equal to that contributed by the meats. Also, extracts of the meat were substituted for whole meats as test material for evaluation. Particulate matter from the whole meats made automated colony counting impossible and complicated manual counting. Data collected failed to demonstrate that any of the meats or processing techniques as producing mutagens. We concluded that the test had limited applicability to whole food items and that the use of thermal, frozen, electron-irradiated, and gamma-irradiated processing do not induce mutagenic potential in chicken or beef.

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