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The Organ-Specific Mammalian Host-Mediated Microbial Mutagen Assay (OSHMA)

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THE ORGAN-SPECIFIC MAMMALIAN HOST-MEDIATED MICROBIAL MUTAGEN ASSAY (OSHMA)

by

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Roger D. Beyer

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INTRODUCTION

An organism is an inseparable part of and is at the mercy of its environment.[17] A living organism gradually consumes the beneficial parts of its surroundings and, thereby, slowly changes its surroundings to a more hostile environment. Since this process normally occurs at a slow rate, the species, because of its genetic endowment, is able to compensate for the environmental change. A steady state is maintained by the process of natural selection. However, the genetic changes of a species are finite, while the possible environmental changes are infinite. If the changes are too rapid or extensive, the species either evolves into a new species or becomes extinct.[17,31]

Man follows this same pattern. However, since the beginning of the Industrial Revolution, man has been injecting a greater amount of foreign materials at a higher rate into his environment than ever previously.[26] Some of these materials, whether they are chemical or high energy, increase the frequency of the deleterious mutations in the human gene pool.[4] At the same time, man has been decreasing selection from his environment by such means as improved medical care and sanitation. As man decreases selection, he also, though unintentionally, maintains and accumulates deleterious mutations.[13,26] This is because the frequency of mutant genes in a population is primarily determined by: a) mutation rate and b)

degree of selection exerted on new mutations.[61,63]

Since most societies, including ours, are committed to the maintenance of a high standard of living, the trend to use materials in large quantities in the absence of selection will continue. To help preserve the human species as we know it, we must identify those chemicals that cause mutations before they become widespread. The detection of chemical mutagens among many non-mutagens is a difficult task since the effect of a chemical may be very slight quantitatively when observed for a generation and in an experimental sample of limited size. Nevertheless, even a slight increase in mutation rate can change a species irreversibly over the generations. This thesis deals with the mutagenic aspects of one of these chemical foreign materials.

Two categories of test systems to detect chemical mutagens have been devised: a) those that employ mammals and, b) those that employ non-mammalians. Inasmuch as our concern is primarily with the effects of mutagens on humans, a test system utilizing mammals is the most desirable.

The large population, the inherent variability from individual to individual, and the facts that mutations are rare events and detection depends on a large number of organisms (10,000 and up), make it desirable to use large numbers of organisms in a test system. Mammals are expensive and difficult to raise and test on such a large scale. The non-mammalian (especially microorganism) system

offers an advantage because of the ease of raising and handling large numbers of organisms. However, the results obtained by the non-mammalian system may not be relevant to man.[4,16]

Recently, a test system was developed that employed the two systems together - the host-mediated assay[21] - so that the advantages of the two systems could be combined. This method employs the use of several million bacteria in which the mutation frequencies could be measured. These are introduced into the peritoneal cavity of mammals, which in turn are injected or fed with a test compound. With this method, the ability of the mammal's system to potentiate, detoxify, excrete or otherwise neutralize a compound is taken into account. A small number of animals is needed for this test. The limitation of the host-mediated assay is that it is capable of measuring genetic response in only one area of the animal's body, the peritoneal cavity, an area that is not directly involved with heredity. Thus, a chemical that is mutagenic in the peritoneal cavity may or may not be mutagenic in the gonads.

In this thesis a successful modification of the host-mediated assay is presented in which the bacteria, placed in diffusion bags, were implanted in the testes of mammals. These mammals were than injected with a known mutagen,[21,24,34] streptozotocin. Streptozotocin was found to increase the frequency of mutations in bacteria located in the testes.

REVIEW OF LITERATURE

Streptozotocin

History of streptozotocin

The antibiotic streptozotocin[25,28,37,60,64] (Figure 1) was first isolated and purified from a Streptomyces achromogenes fermentation broth by Herr et al[28] in 1959, and its antibiotic properties were described by Vavra et al.[64] Streptozotocin is produced by Streptomyces achromogenes variant 128, which was isolated from a soil sample taken at Blue Rapids, Kansas. The organism was deposited with the Northern Utilization Research Branch of the United States Department of Agriculture as culture NRRL 2697.[64]

Physical and chemical properties

In 1967, Jahnke and Argondelis[29] were the first to synthesize streptozotocin. This process was later modified by Hessler and Jahnke[30] into a two-step synthesis. First, N-methyl isocyanate is reacted with an aqueous solution of D-glucosamine at -2°C to yield D-glucosamine N-methylurea which is in turn reacted with liquid nitrogen trioxide to afford streptozotocin (Figure 2). This reaction produces an 80 percent yield of streptozotocin in yellow crystalline form which has a melting point of 115-115.5°C with decomposition and evolution of gas. Streptozotocin is freely soluble in water and

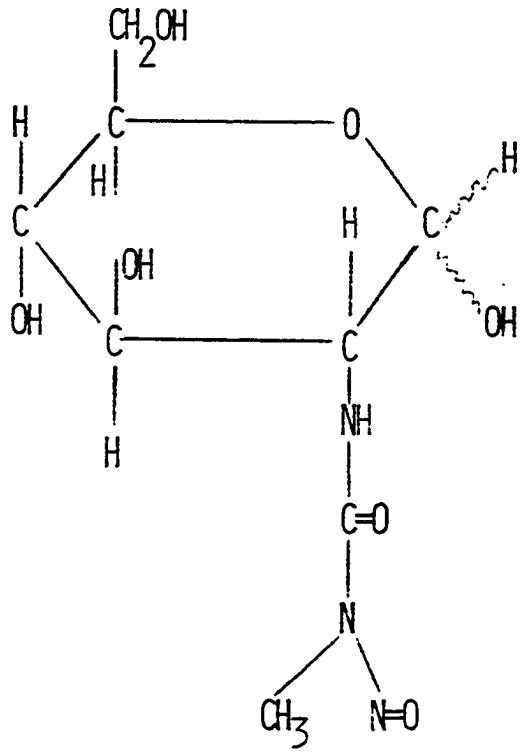


Figure 1. Streptozotocin ($C_8H_{15}N_3O_7$), N-carbamyl-N-methyl-N-nitroso-D-glucosamine, has a molecular weight of 265 with 115-115.5°C mp associated with decomposition and evolution of gas.

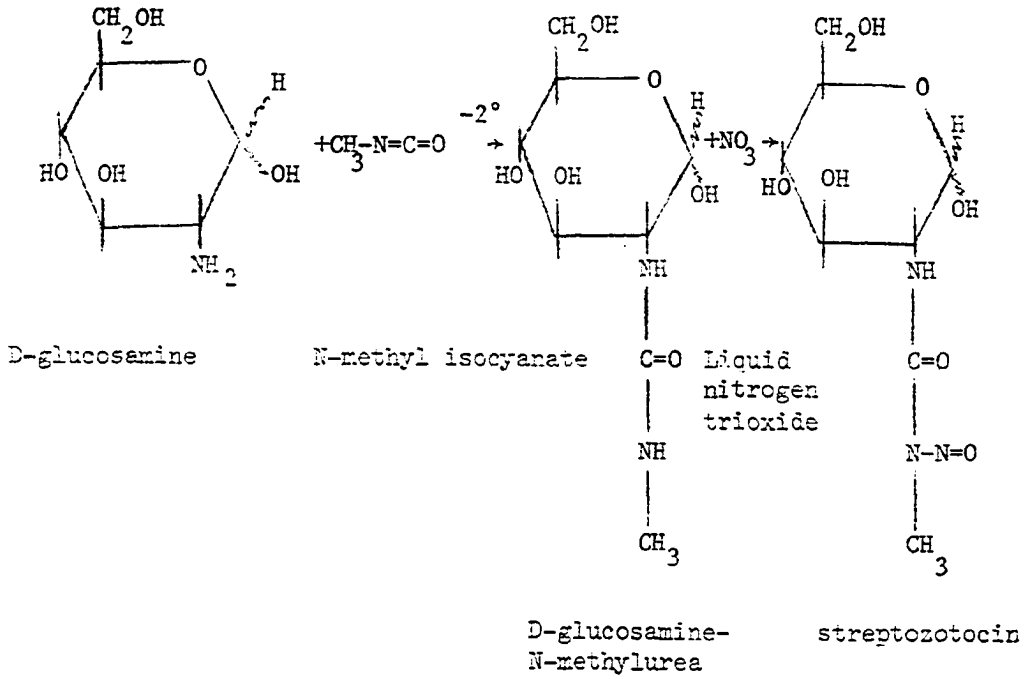


Figure 2. Synthesis of streptozotocin.

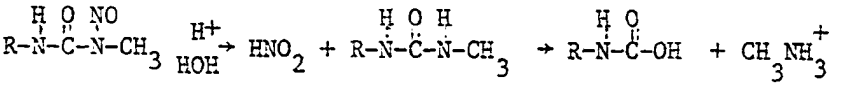
shows mutarotation to an equilibrium value of $[\alpha]_D^{25}$ 39 degrees. The solid is usually a mixture of α- and β- isomers with regard to C_1 in the glucose moiety of the molecule.[46]

The stability of streptozotocin in solution was studied by Garrett in 1960.[23] He found that streptozotocin readily degrades in an aqueous solution as a function of hydrogen and hydroxyl concentrations (Figure 3) with a maximum stability at pH 4.0, where the antibiotic has a half life of 90 hours at 30°C. He further found that at pH 1.0 the half life is 14 hours and at pH 7.0, 3.84 hours at 30°C. Garrett's prediction of the in vivo stability of streptozotocin indicates that although gastric degradation would be of little importance on oral administration, degradation in the intestines and blood would be important because the half life of the antibiotic at pH 7.0 at 37°C is calculated to be one hour. Thus, the half life of streptozotocin in the blood would be one hour or less regardless of the metabolic or excretory rates of the organism given the drug. The solid state of streptozotocin is also unstable at room temperature and must be stored below 20°C.[46]

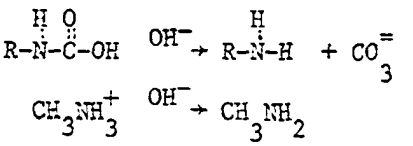
Spectrophotometric determination

A spectrophotometric determination of streptozotocin was developed by Forist in 1964.[18] The method involves acid cleavage of the N-nitrosomethylamide to yield nitrous acid, diazotization of sulfanilic acid with the resulting nitrous acid, and coupling of the diazonium salt with N(1-naphthyl)ethylenediamine dihydrochloride to produce an azo compound with maximum absorption at 550 mμ, which is

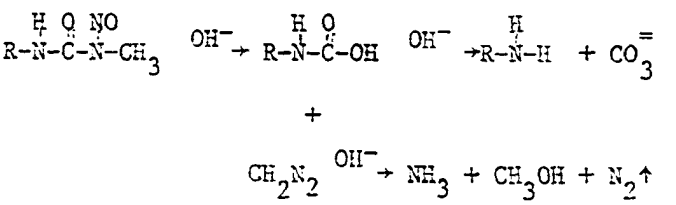
(a) Acid degradation of streptozotocin



(b) Alkaline digestion of acid degraded streptozotocin



(c) Alkaline degradation of streptozotocin



(d) Acid digestion of alkaline degraded streptozotocin

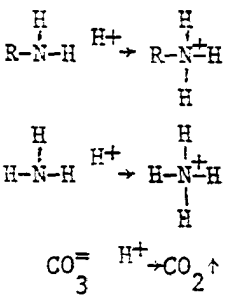
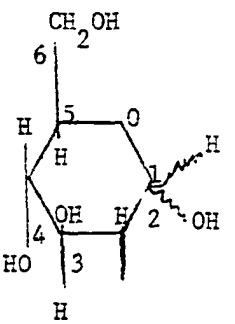


Figure 3. Mechanism for the degradation of streptozotocin.

R is



The N-nitrosomethylurea group is attached to the second position carbon on the 2-deoxy-D-glucose molecule.

read on a spectrophotometer. This method was found to be able to recover 97.8 ± 3.0 percent of streptozotocin in a solution (30 to 120 µg/ml).

Antibacterial activity

Streptozotocin is active in vitro against both gram-positive and gram-negative bacteria and demonstrates in vivo activity against most of these organisms in mice.[25,28,37,60,64] This activity is thought to be due to streptozotocin's ability to inhibit deoxyribonucleic acid (DNA) synthesis.[48,49] Streptozotocin also inhibits this synthesis in mammalian cells.[6]

Diabetogenic activity

Of the vast amount of literature pertaining to streptozotocin, the majority concerns its diabetogenesis. In 1963, Rakieten, Rakieten and Nadkarni[45] reported that the antibiotic streptozotocin was diabetogenic because intravenous administration produced frank diabetes in dogs and rats. In addition to the rat and the dog, streptozotocin has been found to induce diabetes in a number of species including the hamster, monkey, mouse and guinea pig.[7,38,45,54] The rabbit and man have been found to be resistant to streptozotocin's diabetogenesis.[35,51] The toxicity and diabetogenic doses are species dependent. A single intravenous dose of 50 mg per kg is needed to induce diabetes in the dog, but this dosage causes about 50 percent of the animals to die. Diabetes can be conveniently produced in the dog with no deaths by repeated daily injections of

lower doses; the minimum necessary was reported to be 15 mg per kg per day for three days.[45] In the rat, diabetes is produced 100 percent of the time with a single injection of 50 mg per kg. The LD_{50} in the rat to streptozotocin was found to be about 140 mg per kg.[46] The mouse, however, requires a dose of 175 to 200 mg per kg for induction of diabetes.[47,53,54] The LD_{50} in the mouse is estimated to be about 275-400 mg per kg.[46] The overdoses of the drug appear to affect, apart from the islet β-cells, the kidney tubules, the liver and the exocrine portion of the pancreas.[2,32] Cataracts and eye lesions were also found in rats rendered diabetic by streptozotocin.[2,52]

Streptozotocin causes degranulation and frank necrosis of the β-cells and disruption of the islets of Langerhans. The α-cells appear normal during the course of the diabetes induction.[46] A triphasic pattern of the blood glucose, depletion of liver glycogen at about the time of the peak of the initial hyperglycemia, and the elevated plasma insulin levels during the hypoglycemia are also caused by streptozotocin.[46]

The glucose moiety in the streptozotocin molecule is necessary for streptozotocin's diabetogenic activity, but not its antitumor activity.[46] The glucose moiety of streptozotocin is thought to have a potential "carrier function" to bring about contact with or the transport across the β-cell membrane.[55]

Several general conclusions can be made about the poorly understood mechanism of the diabetogenic action of streptozotocin: the site of diabetogenic action is the islet-cell, the binding of the

drug to its site of action is completed within a very short time, and the histological and biochemical changes observed later than about 15 minutes after intravenous injection are secondary changes and are not due to a direct streptozotocin effect.[26]

Nicotinamide has been found, when given intraperitoneally 10 minutes before streptozotocin in a 500 mg per kg dose, to completely protect against diabetogenesis in mice and rats.[46,54,55] Pyrazinamide and 2-deoxyglucose were also found to be protective against streptozotocin diabetes.[10] In mice, a single diabetogenic dose of streptozotocin depletes NAD and NADH for about 24 hours. Initially NAD is removed from the liver followed by a decrease in NAD formation probably resulting from a reduction in tissue uptake of the precursors.[9] Nicotinamide's diabetic protection is due to its stimulation of liver NAD and NADH synthesis de novo.[9] This pretreatment, which protects completely against streptozotocin diabetes, does not abolish streptozotocin's antitumor activity.[46]

Carcinogenic activity

In 1967, Arison and Feudale[3] demonstrated that streptozotocin could induce renal tumors in rats and was thus carcinogenic. The male rats were found to be more susceptible to tumorigenic action than female rats, and Rakietan et al[44] suggested that this difference may be due to sex difference in drug metabolism and excretion or to some hormonal factors. Sibay and Hayes[58] were also able to demonstrate that streptozotocin can induce tumors and other lesions in the liver of the Chinese hamster. Pancreatic islet-cell tumors

were also found to be induced in the rat, according to Rakietan et al.[43]

Carcinostatic activity

As predicted from its phage induction ability.[27,41] streptozotocin has been found to have carcinostatic activity in a variety of animal systems. Phage induction has been correlated with antitumor activity.[27,41] Streptozotocin is effective in inhibiting growth of: Walker 256 carcinosarcoma, sarcoma 180, Ehrlich carcinoma, and murine leukemias L1210, P388 and L5178Y. These results were also confirmed by the Cancer Chemotherapy National Service Center screening tests (NSC-37917).[11,54] It was because of streptozotocin's activity against mouse leukemia L1210 that the drug was selected for clinical trial.[8] Murray-Lyon et al[40] was the first to use streptozotocin to control intractable hypoglycemia in a human with a metastatic carcinoma of the pancreatic islets. This treatment was found to prevent hypoglycemia and to reduce the tumor size. Since then, a number of patients have been treated for the islet-cell carcinoma with varying degrees of success.[50,56,59,62] In addition, the streptozotocin treatment of advanced gastro-intestinal cancer was studied and the cancer was found to respond only slightly to the treatment.[39] Streptozotocin causes varying side effects, including nausea, vomiting, and transient and reversible renal, hepatic and pancreatic β-cell toxicity.[8] Several deaths due to renal failure have been attributed to streptozotocin treatment.[51,56] Since these deaths, the dosage of streptozotocin has been recommended as an

intravenous dose of 1 to 2 grams of streptozotocin per square meter of body surface area per week for a period of five to six weeks.[56] In none of the reported cases did any patient develop insulin-dependent diabetes nor was there any marrow damage observed.

Mutagenic activity

In 1968, Gichner, Veleminsky and Krepinsky[24] and Kolbye and Legator[34] reported that streptozotocin was mutagenic. Gichner's group detected streptozotocin's mutagenic activity in plants, while Kolbye's group detected its mutagenic activity in bacteria. Kolbye and Legator found that streptozotocin and N-nitrosoguanidine had comparable activity *in vitro*. Gabridge and Legator[21] and Gabridge, Denunzio and Legator[20] in 1969 were able to demonstrate streptozotocin's mutagenic activity in an *in vivo* system (the host-mediated microbial assay). Since then streptozotocin has been found to be mutagenic in other systems, including mammalian cell cultures and tissue homogenates.[14,15,33]

Host-mediated Microbial Assay

The host-mediated microbial assay was developed by Gabridge and Legator, in 1969,[21] to determine the ability of laboratory animals either to potentiate or to detoxify compounds in regard to their mutagenic activity.[22,36] This assay incorporates the advantages of bacterial test systems (observation of large numbers and several generations over a short period of time) with those of a mammalian system, which is more representative of man.[21] In the host-mediated

assay, as originally developed, the indicator microorganism is injected into the peritoneal cavity, while the compound to be tested for mutagenicity is introduced into the mammal by a different route (e.g. orally, intravenously or intramuscularly). Subsequently, the genetic indicator organism is recovered and plated on appropriate selective media for the determination of mutation frequencies. A significant increase in mutation frequency over control levels following treatment with a test compound indicates that the compound was present in the mammal in a mutagenically active form.

The histidine auxotrophs of Salmonella typhimurium, the forward mutation system in Neurospora crassa and auxotrophs of the yeast Saccaharomyces cerevisiae have been the major indicator organisms utilized in this technique. The histidine operon of Salmonella is one of the best characterized operons, and over a thousand histidine-requiring mutants have been located in a fine structure map of this operon. The structural genes for the enzymes of the histidine operon (10 enzymes that convert the 5-carbon chain of phosphoribosyl pyrophosphate to histidine) are located in a cluster on the Salmonella chromosome.[65]

A detailed protocol for the host-mediated assay was recently published by Zeiger and Brusick.[66]

Since its development, the host-mediated assay has been used by a number of investigators,[12,19,42,67] and their results have supported the original finding that the assay would detect whether the host organism potentiated or detoxified the test compound.

Among the chemicals that have been tested in the assay using Salmonella his G-46 are: streptozotocin, which was found to be positive in both the *in vitro* and the *in vivo* tests; 2-amino purine nitrate, which was found to be positive only in the *in vitro* test; dimethylnitrosamine, which was found to be positive only in the *in vivo* assay; and caffeine and LSD, both found to be negative in the *in vivo* and *in vitro* assays.[20,21]

METHODS AND MATERIALS

Bacterial strain

Salmonella typhimurium tester strain G46, which is a spontaneous histidine requiring missense mutation, was used throughout these experiments. The his G46 strain has a base substitution which alters one codon in the mRNA from the gene coding for the first enzyme of histidine biosynthesis making it ineffective in the conversion of 5-phosphoribosyl-1-pyrophosphate to N-(5'-phosphoribosyl)-ATP in the histidine biosynthetic pathway[1] (also see REVIEW OF LITERATURE). Bacteria carrying the his G46 mutation are unable to grow on minimal media (see Media) while its spontaneous or induced revertants to histidine independence can. The strain was obtained from Dr. Bruce Ames, Department of Biochemistry, University of California at Berkeley.

Media

Overnight cultures of Salmonella typhimurium his G46 were grown in nutrient broth (8 grams Difco Nutrient Broth per liter of distilled water). The minimal media (MM) used contained 15 g Bacto Agar (Difco), 20 g glucose, 9.85 g K_2HPO_4, 4.22 g KH_2PO_4, 0.94 g $(NH_4)_2SO_4$, 0.44 g $Na_3C_6H_5O_7$ and 0.05 g $MgSO_4$ per liter of distilled water.

To determine the mutagenicity of a compound, bacteria were spread on MM plates enriched with a trace (0.1 μ mole per plate) of histidine. The purpose of the small amount of histidine in the MM was to assure a few cell divisions on the MM plates following mutagenic treatment so that mutations present in a premutational form (such as an alkylation) or in only one strand of DNA could be expressed. After a few rounds of cell replication, the histidine enrichment is exhausted and only the histidine-independent revertants could form colonies which then were counted to determine the mutagenicity of the compound (for computation of mutation frequency see Surgical technique and bacterial plate assay). Nutrient agar (Difco) plates and appropriate dilutions of bacteria in glucoseless MM were used for viable counts.

Animals

The animals used were Upjohn strain male albino rats, weighing between 300 and 350 grams. They were obtained from the Upjohn Company, Kalamazoo, Michigan.

Diffusion bag construction and inoculation

The diffusion bags were constructed from Millipore filters purchased from the Millipore Company, Bedford, Massachusetts. Although several different size bags were experimented with, the bags constructed from the 9 X 14 mm rectangles that were cut from sheets of 25-μ thick filters (0.45-μ pore size) were found to be the best

compromise between the amount of bacteria that they could hold and practical size for implantation.

In order to prevent damage during subsequent handling, the rectangles were wetted with water and handled with blunt-nosed forceps. A wetted filter was wrapped around the plunger of a 0.25 ml glass syringe which was mounted on a stand about 40 mm above the table surface. The overlapping edges were sealed with Millipore MF Cement No. 2, applied with a syringe fitted with a 27G needle.

The resulting filter tube was pulled halfway off the plunger and sealed by applying MF Cement to the inner surface of the tube and by compressing it between two Teflon blocks. The partly sealed tube was removed from the plunger. Cement was then applied to the remaining open end, and the end was sealed on a plane perpendicular to the first seal. This resulted in a closed bag (about 7 mm long, 3.5 mm in diameter) having a triangular shape on all sides and a volume of about 30 µl.

The diffusion bags, placed individually in small vial caps, were arranged in petri dishes in groups of five and were sterilized with ethylene oxide (steam sterilization distorts them). The sterilized diffusion bags were stored in the refrigerator until needed.

On the day of the experiment, a culture of Salmonella typhimurium his G46 grown overnight in nutrient broth (Difco) was centrifuged and resuspended in one-fifth of its original volume in nutrient broth. 20 µl of the resulting 5-fold concentrated culture were placed in each sterile diffusion bag by puncturing the filter bags with a 27G needle fitted on a 100 µl syringe. Following the inocul-

ation, the puncture holes were sealed with MF Cement No. 2. The bags were examined for proper sealing by means of a stereoscopic microscope. They were then flooded with 0.2 ml MM to prevent drying out and were stored at 4°C for one to three hours until implantation.

Surgical technique and bacterial plate assay

Just prior to surgery, all the animals were shaved of their hair from the areas in which incisions would be made. These areas were scrubbed with pHisohex. The diffusion bags containing the tester bacteria were implanted under aseptic condition into the testes and peritoneal cavities of the rats, which had been anesthetized with ether. The testicular implantation was always done first because of the greater time needed.

An incision large enough to allow the testis to pass through was made in the scrotum just above the left testis with a scalpel. A similar incision was made in the tunica vaginalis using scissors. The testis was exposed through the incisions by means of a crile retractor. Throughout the procedure, the testis was kept moist with normal saline. A puncture was then made in the exposed testicular capsule, and extra fine point dissecting microscissors were used to make an incision from the puncture wound approximately 4 mm in length. Care was taken to avoid damage to the vasculature and seminiferous tubules. The diffusion bag was eased through the incision and placed among the seminiferous tubules with a slight rocking motion. Subsequently, the incision was tightly sutured with Cuticulas 668 G 5-0 Ethilon nylon. The testis was returned to the scrotum, and the

scrotal incision was closed with wound clips.

The peritoneal implantation was accomplished by making an incision in the lower right section of the abdominal wall with a scalpel. A diffusion bag inserted through the incision into the peritoneal cavity was placed near the spleen. This incision was closed with wound clips.

The animals were allowed to come out of the ether; and one hour following the placement of the diffusion bags, the animals were injected intramuscularly in the right thigh with one of three solutions: saline, or 1, or 10 mg/kg body weight streptozotocin suspended in saline. Three hours later, the animals were killed by cervical dislocation, and the diffusion bags were recovered aseptically. First the wound clips in the scrotal incision were removed and the testis was re-exposed. Scissors were used to expand the testicular capsule incision until the diffusion bag was exposed. The bag was then removed with blunt forceps and was placed in 5 ml MM to remove adhering fluids and tissues. The peritoneal diffusion bags were removed in a similar manner and were also placed in separate vials containing 5 ml MM.

Each bag was transferred to another vial containing 5 ml MM and was cut into very small pieces with two sharpened spatula before being shaken vigorously for 2 minutes with a Vortex mixer to release the bacteria into the media. After the filter fragments settled to the bottom of the tube, the bacterial suspension was decanted and was washed twice in 5 ml portions of MM by means of centrifugation at

6000 rpm for 15 minutes. At the end of the second washing, the cells were resuspended in 2 ml MM. The resuspended bacteria were then plated on both histidine-enriched MM and nutrient agar to determine mutation frequency and survival.[14,66] The plates were incubated at 37°C; the nutrient agar plates were incubated for about 24 hours and scored, and the histidine-enriched minimal agar plates were scored up to 72 hours at 24 hour intervals. The bacteria was scored and mutation frequency to histidine independence was determined by dividing the number of histidine-enriched colonies (appearing on histidine-enriched minimal media plates during 48 hours of incubation) by the number of survivors plated. Mutation frequency to small or medium colony types was determined by dividing the number of small or medium colonies (appearing on nutrient agar plates during 48 hours of incubation) by the number of colonies per plate.

RESULTS

Mice were originally used as the test animals, but their testes were too small to implant diffusion bags of a size to hold sufficient numbers of bacteria. Rats were then used, and their testes were found to be of adequate size. The testes were easily lifted from and returned to the scrotum. The arrangement and consistency of the seminiferous tubules allowed the insertion of the diffusion bag with a slight rocking motion. This quality is species dependent, for in the dog the testis could not be returned to the scrotum, once removed from it, without great difficulty. The seminiferous tubules of the dog are so densely packed that the tubules had to be cut before the bag could be eased into the testis.[5] With experience, the testis implantation of the diffusion bag in the rat could be done in 10 minutes with one assistant.

Control experiments (Table 1) showed that 86% of the cells could be recovered from the diffusion bags. Incubation of equivalent-size inoculum in nutrient broth in or out of diffusion bags resulted in about a tripling or quadrupling of the cell count during four hours of incubation at 37°C. When diffusion bags containing bacteria were implanted in either the peritoneal cavity or testis for four hours, the number of cells recovered remained about the same as at the beginning of implantation. The frequency of mutation to histidine independence was similar under the various control conditions. The

TABLE 1

The effect of diffusion bag on S. typhimurium in vitro and in vivo.

Handling of inoculum[a]	Number of samples tested[b]	Survival[c] (%)	Histidine-independent revertants per 10^6 cells plated
Plated immediately	5	100	0.53
Placed in diffusion bag, immediately recovered and plated	7	86	0.73
Suspended directly in 5 ml nutrient broth, incubated at 37° for 4 h, recovered and plated	2	280	0.13
Placed in diffusion bag, suspended in 5 ml nutrient broth, incubated at 37° for 4 h, recovered and plated	2	440	0.10
Placed in diffusion bag, implanted in the peritoneum of rats for 4 h, recovered and plated	2	129	0.47
Placed in diffusion bag, implanted in the testes of rats for 4 h, recovered and plated	2	88	0.14

[a]Inoculum is prepared by resuspending an overnight culture in one-fifth its original volume of fresh nutrient broth (5 times concentrated culture) of which 20 µl are used either for immediate plating or to fill diffusion bags.

[b]Each sample was plated in triplicate.

[c]100% survival represents approximately 6.6×10^6 cells per plate.

survival of the bacteria showed large sample-to-sample variation under comparable control or treatment conditions (Table 2). Several of the bags in the peritoneal cavity were observed to have been surrounded with fatty tissue.

Bacteria samples incubated in the peritoneal cavity and in the testis of the same animal had similar frequencies of mutation to histidine independence at both concentrations of streptozotocin in each of the two trials (Table 2). At 10 µg streptozotocin/kg body weight, the mutation frequency to histidine independence increased about 10-fold as compared to a treatment of 1 µg streptozotocin/kg body weight. When streptozotocin-treated cells were plated for viable count on nutrient agar plates and incubated for 48 hours at 37°C, some small (barely visible with the naked eye) and medium-sized (about a third to a half of the normal size) colonies were observed in addition to the expected normal-sized colonies. Small and medium colonies, like histidine-independent revertants, were induced in approximately the same frequency in both the peritoneal cavities and the testes. However, they were induced from 100 to 1000 times more frequently than histidine-independent revertants. The induction of small and medium colony types, like the induction of the histidine-independent revertants, was dose-dependent (Table 2). Small and medium-sized colonies were induced in approximately equal frequencies.

TABLE 2

The frequency of mutations induced in S. typhimurium during 3 h exposure to streptozotocin in vivo per 10^6 survivors.

Treatment of inoculum in diffusion bag[a]	Streptozotocin (mg/kg body weight)	Survival (%)	Histidine-independent revertants	Small- and medium-size colonies
Experiment 1				
Unincubated control[b]		100[c]	0.0	0
Implanted in peritoneum	0	29	1.3	0
Implanted in testis	0	88	0.2	0
Implanted in peritoneum	1	40	10.5	3,000
Implanted in testis	1	16	10.9	9,000
Implanted in peritoneum	10	22	111.0	128,000
Implanted in testis	10	45	93.3	271,000
Experiment 2				
Unincubated control[b]		100[d]	0.5	0
Implanted in peritoneum	0	134	0.5	0
Implanted in testis	0	11	4.8[e]	0
Implanted in peritoneum[f]	1	119	45.9	13,000
Implanted in testis[f]	1	64	52.4	10,000

[a]Inoculum prepared the same way as in control experiments (Table 1).

[b]Inoculum recovered from diffusion bag immediately after inoculation and plated.

[c,d]100% survival represents approximately 7.6 and 4.7 x 10^6 cells per plate, respectively.

[e]The relatively high spontaneous frequency noted here is probably due to the low number of cells plated.

[f]Average of two samples.

DISCUSSION

This thesis presents evidence that the OSHMA not only possesses the advantages of the host-mediated assay, but also improves upon this system. Both the host-mediated assay and the OSHMA are advantageous in that they incorporate the advantages of bacterial test systems (observation of large numbers and several generations over a short period of time) with those of a mammalian system, which is more representative of man.[21] Both assays are used to determine the ability of laboratory animals either to potentiate or to detoxify compounds in regard to mutagenic activity.[22,36]

The OSHMA has at least three advantages over the host-mediated assay.[21,66] The first is that the host-mediated assay monitors mutagenicity in an area of the test animal, the peritoneal cavity, which is not involved in the transmission of the hereditary material. In contrast, the OSHMA as applied in the experiments reported here is able to detect mutagenic activity in the area of gonads and in the close vicinity of the seminiferous tubules within which meiosis and spermatogenesis take place. While mutations can take place in both somatic (e.g. peritoneal cavity) and gonadal (e.g. testes) cells, in the former mutations are confined to the individual carrying them, while in the latter induced mutations stand a chance of being transmitted to future generations and may increase in number and frequency through sexual reproduction and genetic drift. Before the initiation of the present work, it has not been

clear whether or not streptozotocin can be detected in the testis through the use of implanted bacteria, although this possibility was strongly implied by the work of Ficsor and Muthiani[15] with testes homogenates from streptozotocin in treated animals. The present data (Table 2) indicates that the presence of streptozotocin in a mutagenically active form can be demonstrated in the testes with implanted indicator bacteria. Based on this observation, it may be predicted that other indicator cells, such as human leucocytes, mammalian bone marrow, blastocytes, yeast, Neurospora or other varieties of indicator cells could be implanted in the testes of the rat.

The second advantage of the OSHMA over the host-mediated assay is that with the former method various organs of the test animal's body could be surveyed for the presence of a mutagen or its mutagenically active metabolite. This type of information may be useful in locating the site of detoxification, activation, or storage of the test compound in the animal.

The third advantage of the OSHMA is that a higher percentage of the implanted bacteria can be recovered than in the host-mediated assay. The reported efficiency of recovery of bacteria with the host-mediated assay is only 10%, while the recovery of bacteria with the OSHMA was found to be 86% (Table 1). It should be noted, however, that with the host-mediated assay, a larger number of bacterial cells can be treated and recovered per animal, since many more cells can be implanted in the peritoneal cavity (ca. 10^8) than in the diffusion bag (ca. 10^6) due to the small volume of the latter

(30 µl). This volume limitation is imposed by the size of the rat testis.

Table 2 shows that the results of the OSHMA, like the host-mediated assay, are reproducible. Some sample-to-sample variation under comparable control or treatment conditions was found. This may be due to either partial loss of some samples during handling or to differences in their interaction with the in vivo environment.

A technical improvement of the OSHMA would be the use of nylon reinforced 0.45 µ pore size Millipore filter tubes of appropriate length sealed on both ends in place of Millipore filter sheets (Materials and Methods) that were used in the present experiments to construct diffusion bags. To date only one diffusion bag has been constructed from nylon reinforced Millipore filter tube. This tube was filled with bacteria and was implanted in the testis of a streptozotocin-treated dog (10 mg/kg body weight). A positive mutagenic response was obtained.[5] The diffusion bag constructed from this nylon reinforced filter tube is sturdier and easier to construct than the diffusion bag constructed from ultra-thin Millipore filter sheets. The new type of diffusion bag has not yet been tested in the rat.

In comparison to other test systems, such as the dominant lethal test, in vivo cytogenetics test and the specific locus test, both the OSHMA and the host-mediated assay offer some advantages: they are less expensive, less time-consuming, require fewer manipulations and are more sensitive in detecting point mutations.

Some disadvantages were found in the OSHMA and were not unlike those of the host-mediated assay. As with this latter method, "a negative result is most probably due to detoxification, but a non-systemic distribution or sub-optimal concentration is also possible. A negative result would also be obtained following a point mutation at a locus different used in defining the auxotroph; i.e., a negative result only indicates the absence of a mutation at the locus being examined and does not preclude other mutations. The probability of detecting mutagenic activity could thus be increased by using an organism(s) with more than one marker."[21]

Even though the presence of a chemical mutagen in the testes of rats in the close proximity of the seminiferous tubules was demonstrated, it is not known if the mutagen was also present inside the seminiferous tubules and in the germinal cells.[57] A method to detect chemical mutagens inside the seminiferous tubules is under development.[5] Most importantly, it is not known whether a mutation of bacteria implanted in the testis or the peritoneal cavity is a measure of mutagenicity in the host. Only parallel OSHMAs and animal breeding experiments could answer this question. Should there be a positive correlation between the two, the OSHMAs could be used to determine if a treated animal (to be used as a sire in breeding experiments) received the desired mutagen exposure in the testes.

The OSHMA could be used to test man-consumed drugs for mutagenicity in man in vivo. Such testing could either be done within intact human subjects or with removed organs.[15] The testing of human blood for the presence of a mutagen should raise no difficulty

since such tests have already been performed with mammalian blood.[14,15]

The small colonies mutations induced by the streptozotocin treatment in the OSHMA were subsequently investigated by David Zimmer, another student in Dr. Gyula Ficsor's laboratory. His study revealed that the small colonies remained small even after 6 days of incubation at 37°C, and they produced small-colony progeny through repeated single-colony isolations. Some of the small-colony isolates revert to normal colony size at a low frequency. Light microscopic examination showed that cells taken from the small colonies were smaller than their untreated parents. Normal colonies that have been derived from streptozotocin-treated cells contained normal-sized cells. Cells from small colonies are non-motile, as shown by the hanging-drop motility test and lack flagella, as shown by electron microscopic examination.[14] The advantage of scoring the small-colony phenotypes (Table 2) was that they increased the genetic resolution of the system since they occured in uncommonly high frequencies. In some treatments 27% of the treated cells acquired this phenotype (Table 2). Another advantage of scoring these small-colony phenotypes is that they represent genetic changes that are independent of the mutational events at the histidine G46 locus.

CONCLUSION

This thesis resulted in a number of conclusions regarding the OSHMA and its ability to detect mutagenic activity. Three advantages of the OSHMA over the host-mediated assay were found. These included:

1) the ability to monitor mutagenicity in an area of the test animal involved in the transmission of hereditary material as well as in the peritoneal cavity.

2) the ability to survey the test animal's organs for the presence of a mutagen or its mutagenically active metabolite.

3) the ability to recover a higher percentage of the implanted bacteria than in the host-mediated assay (86% as compared to 10%).

A number of additional conclusions were demonstrated by this thesis. These included:

1) diffusion bags were found to have little effect on survival and mutagenicity of the bacteria.

2) streptozotocin was found to be mutagenic in the testis as well as in the peritoneal cavity.

3) the mutation frequency induced by streptozotocin in the testis was found to be about the same as in the peritoneal cavity.

4) a 10-fold increase in the mutation frequency was found to exist when 1 µg streptozotocin/kg body weight was compared to 10 µg streptozotocin/kg body weight.

5) small- and medium-sized colonies were induced by the streptozotocin.

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