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# Relationship of Chemical Structure and Mutagenic Activity of Streptozotocin and Its Analogs

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# RELATIONSHIP OF CHEMICAL STRUCTURE AND MUTAGENIC ACTIVITY OF STREPTOZOTOCIN AND ITS ANALOGS

by

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# INTRODUCTION

Streptozotocin is a broad-spectrum antibiotic produced by Streptomyces achromogenes var. streptozoticus.[61] In addition, it displays mutagenic activity,[20,23,27,28] a marked anti-leukemic activity[10,14] and is a diabetogenic agent being specifically toxic to the $\beta$-cells of the islets of Langerhans.[12]

This antibiotic is interesting in that it has a deoxyglucose moiety attached to N-methyl-N-nitrosourea,[57,58] a known mutagen.[16] Tetsuo Suami and Tomoya Machinami have prepared several analogs of streptozotocin with altered carbohydrate moiety. In all these analogs, the N-methyl-N-nitrosourea group, potentially responsible for mutagenic activity, remains unchanged. In the present investigation, streptozotocin and these analogs have been used to study the effect of change in the non-mutagenic part on the mutagenic activity of the molecule.

The only report available on a study of streptozotocin and its analogs concerns itself with their antibacterial, diabetogenic and cytotoxic effects;[8] it is shown that inversion of the 4-hydroxyl group of streptozotocin leads to complete loss of antibacterial activity against the test organism, Proteus vulgaris, as does methyl glycoside formation in both anomeric configurations; none of Bannister's[8] analogs of streptozotocin showed diabetogenic activity at 65mg/Kg. in the rat at which concentration streptozotocin

causes a four-fold elevation of blood sugar concentration; cytotoxic activity against L-1210 cells in culture showed that $\alpha$-methyl glycoside is twice as active as $\beta$-methyl glycoside.

Rosenkranz[51] has compared biological and metabolic effects of N-methyl-N-nitrosourea and streptozotocin in bacteria as well as in mammalian cells; he has shown that N-methyl-N-nitrosourea is more toxic than streptozotocin against Escherichia coli cells and streptozotocin affects RNA and protein synthesis much less drastically in mammalian cells than does N-methyl-N-nitrosourea.

The present study is significant in that it employs a substantial number of analogs i.e. nine, which differ from each other not only in altered carbohydrate stereochemistry but two of them carry substituent carbohydrates thus providing enough variations in the non-mutagenic carbohydrate moiety to understand its role in mutagenic action of streptozotocin.

## REVIEW OF LITERATURE

Several hundred publications dealing with different aspects of research on streptozotocin have appeared since its discovery in 1960.[61] The discovery, isolation, toxicity and chemical synthesis of streptozotocin have been recently reviewed by Beyer.[9] Information on the analogs used in the present work is supplied in Appendix I. In the present review, certain properties of streptozotocin, methods for detection of chemical mutagens and nature of permeability barriers in bacteria will be dealt with briefly. A section dealing with present concepts on relationship of mutagenesis and carcinogenesis of nitroso compounds has also been included in this review.

### Properties And Activities of Streptozotocin

Garrett[22] has reported on aqueous solution degradation of streptozotocin and has shown that streptozotocin has a half life of 90 hours at 30°C at pH 4; it has a half life of one hour at pH 7 at 37°C and the rate of degradation of streptozotocin is a function of $H^+$ and $OH^-$ ion concentrations. For a review of other properties of streptozotocin see Beyer.[9]

#### Antibacterial activity

Streptozotocin is active in vitro against both gram-positive and gram-negative bacteria[55] and this is attributed to its ability to inhibit DNA synthesis.[50,51]

Diabetogenic activity

In 1963, Rakieten et al.,[46] showed that the antibiotic streptozotocin was diabetogenic because intravenous administration produced frank diabetes in dogs and rats. Rabbit and man have been found to be resistant to streptozotocin's diabetogenesis.[32,52] Streptozotocin causes degranulation and frank necrosis of the β-cells and disruption of the islets of Langerhans in rats. The α-cells appear normal during the course of the diabetes induction.[49]

The glucose moiety in the streptozotocin molecule is necessary for streptozotocin's diabetogenic activity.[49] Nicotinamide, when given intraperitonially ten minutes before streptozotocin, has been found to completely protect against diabetogenesis in mice and rats.[53] Pyrazinamide and 2-deoxyglucose were also found to be protective against streptozotocin diabetes.[13] It is of interest to note that nicotinamide pretreatment, which protects against streptozotocin induced diabetes, does not abolish streptozotocin's antitumor activity.[49]

Carciogenic activity

In 1967, Arison and Feudale showed that streptozotocin could induce renal tumors in rats.[7] Sibay and Hayes[54] were able to demonstrate streptozotocin induced tumors and other lesions in the liver of chinese hamster. Pancreatic islet-cell tumors were also found to be induced in the rat as shown by Rakieten and co-workers.[47]

Carcinostatic activity

As predicted from its pahge induction ability,[26,45] streptozotocin has been found to have carcinostatic activity in a variety of animal systems. It is effective in inhibiting growth of : Walker 256 carcinoma, sarcoma 180, Ehrlich carcinoma and murine leukemias L1210, P388 and L5178Y. Adolphe and co-workers[1] used labelled streptozotocin in advanced cancer patients; streptozotocin was either labelled in the methyl group ($^{14}C$) of the methyl-nitrosourea moiety or it was tritium labelled on the sixth carbon of the glucose ring. They have shown that patients receiving $^{3}H$-streptozotocin and $^{14}C$-streptozotocin showed higher levels of $^{14}C$ in plasma after 48 hours than $^{3}H$ even though higher doses of $^{3}H$ were administered. $^{14}C$ and $^{3}H$-streptozotocin were detected in biopsy tissues of two patients as long as four days after administration.

## Relationship Between Mutagenicity And Carcinogenicity of Nitroso Compounds

The concept that the induction of cancer might follow mutation in a somatic cell is one of the prevalent hypotheses of cancer causation.[41] This hypothesis maintains that cancer is initiated by an unrepaired mutation of that part of the genome that codes for DNA replication-initiating polymerase. This mutation could arise by direct reaction with an ultimate carcinogen or indirectly if the ultimate carcinogen reacts first with any information handling substance e.g. DNA-dependant RNA polymerase, mRNA for information-

handling protein, tRNA codons etc.

Haddow[25] demonstrated DNA, RNA and protein alkylations in organs of animals treated with nitroso carcinogens and concluded that whatever the precise chemical mechanism of the action of carcinogens, there can be little doubt of the importance of their combination with genetic material or its precursors, if (as seems likely) this is essential to those at least which function through biological alkylation.

It has been found wherever tested, that nitrosoamides are not only strong carcinogens but fairly potent mutagens.[36] These compounds as exemplified by N-methyl-N-nitro-N'-nitrosoguanidine and N-nitroso-ethyl urethan need sulfahydryl groups for conversion to very reactive carbonium ions ($CH_5^+$) or alkyl diazonium ions ($CH_3N_2^+$).[37] On the other hand, the strongly carcinogenic nitrosamines e.g. N-methyl-N-nitrosamine, have shown only a limited range of mutagenic activity. A negative correlation between mutagenic and carcinogenic activity is exemplified by tertbutylethylnitrosamine which by virtue of a tertiary butyl group lacks an $\alpha$-carbon and cannot be oxidized to give an alkyl diazonium ion; hence this compound is not carcinogenic in rat[11] and not mutagenic in Drosophila.[43]

Nitroso compounds preferentially alkylate the N-7 and O-6 atoms of guanine residues and show less tendency to react with N-1 atom of adenine or N-3 atom of cytosine.[29] The ability of nitroso compounds to methylate the O-6 atom of guanine could account for their mutagenic potency since this could cause mispairing and hence

transition mutations. The observation that 0-6 methylated guanine is excised from Escherichia coli DNA[30] indicates that repair systems recognize the potentially miscoding group.

Positive correlations between carcinogenicity and production of DNA alkylations by ethyl- and methyl methane sulfonates have been demonstrated in the kidney of rat. A similar relationship can be hypothesized for nitroso compounds but this has been doubted as it fails to explain the strong carcinogenic and weak mutagenic action for nitrosamines.[6] Swann and Magee[59] have countered this objection by showing that similar tumors are induced in brain and kidney of the rat by both alkylating agents used in similar concentrations and administered alike. Another objection to alkylation as a basic mechanism of carcinogenicity and mutagenicity of nitroso compounds, has been the possible inability of some cyclic nitrosamines to undergo necessary metabolic activation to produce the required alkylating intermediate.[6] This has been countered by some but not all subsequent evidence. Lee and Lijinsky[33] reported alkylation of rat liver RNA by some cyclic nitrosamines in vivo which indicated that metabolic opening of heterocyclic rings must have occurred. However, subsequently, Lijinsky and Ross[34] failed to detect nucleic acid alkylation with some cyclic nitrosamines but did find it with some noncarcinogenic cyclic nitroso compounds. Stewart[56] has studied the metabolism of N-nitrosomorpholine labelled in the 2 or 3 position of the ring; he has found that, with each sample, more than 80% of radioactivity injected into rats was excreted in the urine during a

period of 30 hours following administration. In both cases, however, more than 3% of injected radioactivity appeared in the expired $CO_2$ during this period; this again indicated that metabolic opening of the heterocyclic ring must have occurred.

Since the discovery of the potent mutagenic action of N-methyl-N-nitro-N'-nitroso-guanidine (MNNG) in bacteria by Mandell and Greenberg,[39] this compound and other nitrosomides have been recognized to be among the most powerful of known mutagens.[44,63] The nitrosoamides are mutagenically active in bacteria, yeasts, Neurospora, plants and Drosophila but nitrosoamines have generally been reported to be inactive in all of the above organisms except Drosophila. This difference seems to be due to the requirement for metabolic activation of nitrosamines and lack of this requirement for nitrosamides.[45]

In order to see, if changes in pH would bring about a difference in mutagenic pattern of nitrosamines and nitrosamides, Neale[40] studied induction of revertants to tryptophan prototrophy in E. coli A58 under the action of these compounds. N-methyl- and N-ethyl-N'-nitrosourea did not show any changes in their mutagenic activity from pH 6.0 to 7.9 but at pH 5.0, the activity was drastically reduced. On the other hand, nitrosamines as exemplified by dimethyl- and diethyl nitrosamine had no detectable mutagenic effect. Similar concentrations of N-nitrosomorpholine were also nonmutagenic in this system. In another study, Zeiger and Legator[62] have shown N-nitrosomorpholine, a powerful carcinogen, to be

mutagenic in host-mediated assay but non-mutagenic for bacterium alone.

Reusser[50] has reported *in vitro* studies of streptozotocin and found it to bind preferentially with cytosine containing mononucleotides. He suggests that the interaction of streptozotocin with cytosine residues in DNA, even though taking place at a very low frequency, may constitute the primary step inducing DNA strand breakage. Kolbye and Legator[28] maintain the possibility that streptozotocin acts as an alkylating agent, most probably as a methylating agent through the formation of diazomethane. Rakieten *et. al.*[47] have reported on tumorigenic properties of streptozotocin and have hypothesized that carcinogenic action of this compound probably depends on N-nitrosoalkane end of the molecule or the *in vivo* release of this group as diazomethane. Release of diazomethane *in vitro* has already been observed by Garret.[22]

Despite conflicting evidence, it seems probable that carcinogenesis and mutagenesis by N-nitroso compounds are mainly mediated by metabolic products of these agents but that the unchanged molecules also play a part, cannot be excluded. The nature of biologically active metabolic products of the nitrosamines have not been established but much of the available evidence suggests that the formation of an alkylating agent may be involved. The use of alkylated polynucleotide templates for nucleic acid polymerases *in vitro* may be of value in evaluating the sides of nucleic acid alkylation that may be important for carcinogenesis or mutagenesis.

## Nature of Permeability Barriers in Bacteria

The cytoplasmic or plasma membranes of gram-positive and gram-negative bacteria show a triple layered appearance in cross section examined in the electron microscope. The type of proteins and lipids found in the membrane are characteristic of species and of strains of bacteria.[18]

The first indication of a differential intrinsic resistance to different drugs came primarily from studies with some antibiotics in relation to cell envelope. In 1962, Taubeneck[60] found that erythromycin and a host of other antibiotics were much more active against L-forms of Proteus mirabilis than against intact parents. The reports of Taubeneck[60] and Mach and Tatum[35] showed that some gram-negative bacteria with absent or defective cell walls had enhanced sensitivity to antibiotics. It was conceivable that the loss of or damage to the peptidoglycan layer permitted the penetration of the drugs into the cells. The use of the chelating agent, ethylenediaminetetraacetate (EDTA) in several studies, has indicated fairly conclusively that it is the lipopolysacchride (LPS) of the outer layers of these bacteria that hinders the access of drugs to the inside of the cell.

The LPS layer which is normally present on the surface of Salmonella typhimurium apparently acts as a partial barrier to the passage of mutagens through the membrane as shown by marked increase in sensitivity to mutagens of LPS defective strains.[5]

## Detection of Mutagens in Tissue Homogenates

Since 1971, several publications have appeared on detection of chemical mutagens in mammalian tissues. In general these studies have been confined to the detection of mutagenic agents in liver homogenates. The need for studying the influence of mammalian tissues on mutagenicity was demonstrated by Malling;[38] he showed that dimethyl nitrosamine when applied to bacteria directly in liquod culture is non-mutagenic but when the same compound is first exposed to liver extract, it becomes mutagenic to bacteria.

Ficsor and Muthiani[15] have investigated the presence of streptozotocin in blood, testes and liver homogenates of mice using S. typhimurium HisG46 as the tester indicator of mutagenesis. In their experiments, specially designed millipore diffusion chambers containing bacteria were immersed in tissue homogenates of treated and control animals; increased mutation frequencies over control levels were noted in bacteria exposed to blood, liver and testes of streptozotocin treated animals.

Garner et al.[21] have shown that carcinogenic compounds need metabolic activation to the ultimate reactive forms to act as carcinogens and have used for this purpose, liver microsomal pellet as the activating agent thus simulating in vivo conditions in an in vitro test. They have also shown toxicity of reactivated aflatoxin B to S. typhimurium strain 1530.

A modified version of the use of liver homogenates and

bacteria for the evaluation of carcinogens as mutagens, has recently been described by Ames and co-workers.[2] The tester strains used in these studies carry different mutations in the his region besides having deletion through uvrB region and a deep rough surface (rfa) which make these bacteria permeable to larger molecules; using this system, these authors have shown that all the carcinogens tested are mutagens.

## Different Types of Mutagenic Agents and a Method for their Detection

Different mutagenic agents can induce different types of mutations.[19] In 1971, Ames described[3] a set of three pairs of bacterial mutants of S. typhimurium that carried base substitution, base deletion or base addition mutations and could be used for detection of base substituting, base adding or base deleting agents respectively. In each pair, one strain is $uvrB^+$ and the other is $uvrB^-$ but both carry the same mutation in the his operon. Mutagenic agents produce specific types of mutations depending on their reactive groups. Therefore, by using point, frameshift or deletion mutants with and without excision repair, the nature of mutations caused by different mutagenic agents can be determined. Many alkylating agents such as ethyl methanesulfonate, ICR-191 etc. induce a higher frequency of mutations in $uvrB^-$ than in $uvrB^+$ strain carrying the same his mutation. Interestingly, N-methyl-N-nitro-N'-nitrosoguanidine, streptozotocin (Ficsor, personal communication)

and methyl methane sulfonate (Beyer, personal communication) induce somewhat fewer mutations in $uvrB^-$ than in $uvrB^+$ strain. The significance of this observation has not yet been established.

## MATERIALS AND METHODS

### Bacterial Strains

For determining the mutagenic activity of streptozotocin and its analogs, the genetic tester used was a histidine requiring missense mutant, S. typhimurium hisG46.[3,4] To determine the nature of mutations caused by streptozotocin, its analogs and N-methyl-N-nitrosourea, three pairs of S. typhimurium strains, viz. 172A, 173A; 174A, 175A; and 176A, 177A; originally described by Ames[3] were used. Each pair carries a different mutation in its his operon, and one of each pair is $uvrB^+$ and the other $uvrB^-$ (Table I). The mutagenic action of streptozotocin, its analogs and N-methyl-N-nitrosourea was also studied in S. typhimurium histidine mutant strains, 213 and 219 (Table I); of which 219 carried an rfa mutation which made its cell wall defective and hence more permeable to some chemicals.[5]

### Chemicals

The names and chemical structures of the compounds tested for their mutagenic activity are given in Fig. 1. Streptozotocin was a gift of the Upjohn Co. of Kalamazoo, Michigan. The other compounds were synthesized in the laboratory of Tetsuo Saumi of Keio University, Yokohama, Japan. A brief description of chemical synthesis of these compounds is given in Appendix I on page 38. The synthesis of compounds II and III[58] and of compounds VIII and IX[57] has already been published ; the method of synthesis of

TABLE I

Genotype, Nature of Mutation and Repair Specificity of the Tester *Salmonella typhimurium* Strains

| Strain code No. | Mutation(s) carried | Nature of Mutation | Type of mutation detected by the strain |
|---|---|---|---|
| 172A | *his*G46 | base substitution | base substitution |
| 173A | *his*G46, *gal*-*bio*-*uvr*B deletion[a] | " " " | " " " |
| 174A | *his*C207 | one or two bases deleted | addition of one or two bases |
| 175A | *his*C207, *gal*-*bio*-*uvr*B deletion[a] | " " " | " " " |
| 176A | *his*C3076 | one base added | deletion of one base |
| 177A | *his*C3076, *gal*-*bio*-*uvr*B deletion[a] | " " " | " " " |
| 213 | *his*G46, *uvr*B deletion | base substitution | base substitution |
| 219 | *his*G46, *uvr*B deletion, *rfa* mutation[b] | " " " | " " " |

[a] *uvr*B strains fail to excise T-dimers and certain types of alkylations. The *gal* and *bio* mutations have not been utilized in this study.

[b] *rfa* mutations eliminate to different extents, the polysacchride side chain of the LPS that coats the bacterial surface, making the bacteria more permeable and completely non-pathogenic.[5]

Figure 1

Identity of the compounds used:

I Streptozotocin;

II Methyl N-carbamyl-N'-methyl-N'-nitroso-β-D-glucosaminide;

III Methyl N-carbamyl-N'-methyl-N'-nitroso-α-D-glucosaminide;

IV n-Propyl N-carbamyl-N'-methyl-N'-nitroso-β-D-glucosaminide;

V n-Butyl N-carbamyl-N'-methyl-N'-nitroso-β-D-glucosaminide;

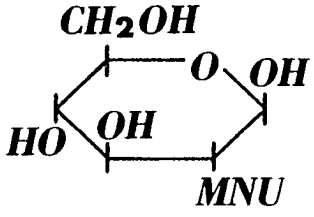
VI Ethyl N-carbamyl-N'-methyl-N'-nitroso-α-D-glucosaminide;

VII n-Butyl N-carbamyl-N'-methyl-N'-nitroso-α-D-glucosaminide;

VIII N-Carbamyl-N'-methyl-N'-nitroso-scyllo-inosamine;

IX N-Carbamyl-N'-methyl-N'-nitroso-epi-inosamine-2;

MNU N-methyl-N-nitrosourea

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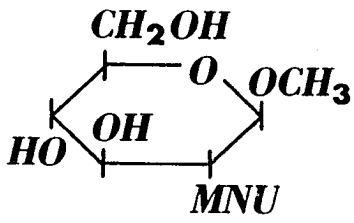


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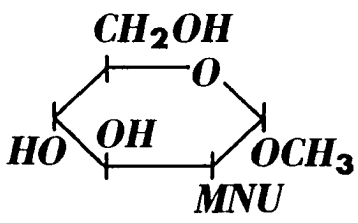


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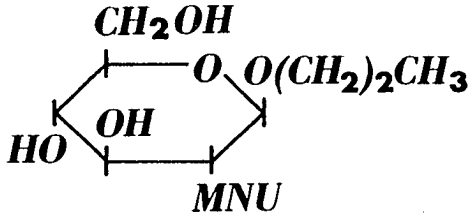


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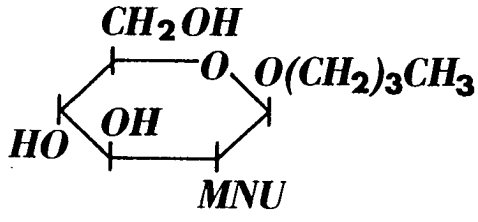


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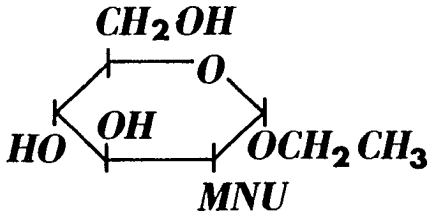


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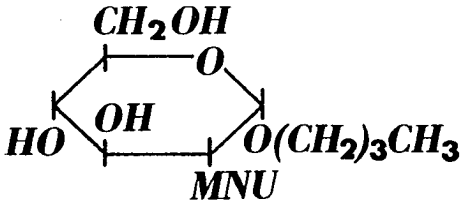
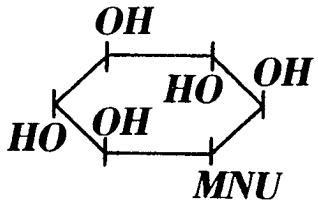
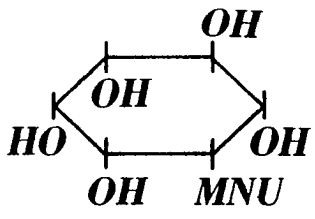



VII




VIII

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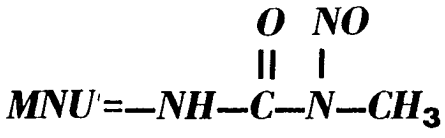



Fig. 1

compounds IV, V, VI and VII has not been reported. All of these analogs are due to anomerism or addition of alkyl groups of different sizes at C-1 of the carbohydrate moiety of streptozotocin and all contain N-methyl-N-mitrosourea attached either to glucose (I-VII) to to inositol (VIII, IX) at C-2 position.

## Media

Different types of media used in these studies are given in Appendix II on page 39.

## Comparison of Mutagenic Activity

a) Liquid culture treatments in vitro

The tester strain 172A was grown overnight at 37°C in water bath with constant shaking. The bacteria were centrifuged and resuspended in equal volume of fresh nutrient broth and divided into 4.9 ml aliquots. Each test aliquot was treated with 0.1 ml of a concentration of one of the test compounds (I - IX). To control aliquots, 0.1 ml sterile distilled water was added. All bacterial samples were incubated for three hours at 37°C with constant shaking; then washed thrice with minimal salt solution (MSS) and resuspended to original volume in the same solution. Suspensions of treated and control cells were serially diluted from $10^{-1}$ to $10^{-8}$. The first three dilutions i.e. $10^{-1}$, $10^{-2}$ and $10^{-3}$ were plated on histidine enrichment plates (HEM). The purpose of the small amount of histidine in histidine enrichment plates is to allow

a few rounds of cell replication for expression of reversion but not enough to support the growth of non-revertant auxotrophs. The last two dilutions i.e. $10^{-7}$ and $10^{-8}$ were plated on nutrient agar plates to determine viable count. In each case 0.1 ml of bacterial suspension was spread on a petri dish. All plates were incubated at 37°C. Viable counts on nutrient agar plates were made after 24 hours. Histidine independent revertants were counted on HEM plates after 48 hours. Induced mutation frequency (IMF) was calculated by the following formula:

$$IMF = \frac{\text{No. of revertants on treated plate}}{\text{Total number of viable cells plated}} - \frac{\text{No. of spontaneous revertants in corresponding control plate}}{\text{Total number of viable cells plated}}$$

b) Bacterial assay of tissue homogenates

Mutagenic activity of compounds I, II and V was examined in mouse liver and testes homogenates by the microbial mutagen assay of Ficsor and Muthiani[15] using three hour incubation period. The concentration of bacteria recovered from control chambers ranged from $2 \times 10^9$ to $3 \times 10^9$ cells per ml. Ficsor and Muthiani[15] have used bacterial diffusion chambers made with 25μ thick millipore filters. In these studies, sturdier reinforced 100μ thick filters were used. It was noted, however, that the use of thicker filters lowered the frequency of revertants compared to the previous study. Therefore, the use of 25μ non-reinforced filter is recommended for increased sensitivity of this method. After the bacteria contained

in the chambers had been exposed to liver or testes homogenates from treated or control animals, these were washed, diluted and plated as described earlier in (a). Induced mutation frequencies were calculated.

Determinations of the Nature of Mutations

Using three pairs of tester strains (127A to 177A, Table I), N-methyl-N-nitrosourea and compounds I and II (Fig. 1) were tested to determine the nature of mutations. Experiments were run using a different pair of tester strains each time. Every experiment was repeated once. For each experiment, a $uvrB^+$ and a $uvrB^-$ strain of a pair were incubated overnight separately in 20 ml nutrient broth (Difco) at 37°C on a shaker water-bath. Next morning, the cells were harvested by centrifugation and the cell pellet of each strain was resuspended in 20 ml fresh nutrient broth; this was divided into 4.9 ml aliquots in four centrifuge tubes. 0.1 ml sterile distilled water was added to Tube 1 and it was designated as control. To tubes 2, 3 and 4, 0.1 ml of solutions of streptozotocin, β-methyl-streptozotocin and N-methyl-N-nitrosourea were added respectively for a final concentration of 0.01 μmoles per ml. All tubes were incubated for one hour at 37°C in a shaker water-bath. The cells were then washed twice in cold biotin supplemented histidine enriched minimal salt solution (BSHEMSS) and finally resuspended to original volume in the same solution. Each cell suspension was serially diluted in biotin supplemented minimal salt solution (BSMSS); it was

plated and observations were recorded as described for 'Liquid culture treatments *in vitro*'. Mutation frequencies were calculated as follows:

$$MF = \frac{\text{No. of revertants in treated plate}}{\text{Total number of viable cells plated}}$$

## Susceptibility Experiments

The two tester strains used in these experiments were 213 and 219 (Table I). Both of these strains are *his*G46 mutants and carry a deletion in biotin and uvrB genes; strain 219, in addition, carries an *rfa* mutation which eliminates to a certain extent the polysacchride side chain of the lipopolysacchride that coats the bacterial surface, making the bacteria more permeable to many mutagenic compounds.[5] The two strains were grown overnight in nutrient broth and were subsequently treated in the manner described earlier in 'Liquid culture treatments *in virto*' but using a one hour incubation after addition of mutagenic compounds. In these experiments, streptozotocin was used at 0.01 µmoles per ml; N-methyl-N-nitrosourea, compounds II, V and VIII (See Fig. 1) were used at 0.1 µmoles per ml. Mutation frequencies were subsequently calculated.

## Spectrophotometric Measurements of Nitroso Group

All mutagenic compounds tested in this investigation share in common the nitroso group. Using Forist's method,[17] the disappearance of this nitroso group from solution in presence of bacteria was

measured spectrophotometrically. Compounds I, II, V, VI and VIII at a test concentration of 0.05 μmoles in 0.85% saline were incubated with $5 \times 10^9$ bacteria for 30 minutes at 37°C. Simultaneously, five "Control" concentrations of each compound vis. 0.01, 0.025, 0.05, 0.07 and 0.1 μmoles per ml were similarly incubated without bacteria. Higher and lower "Control" concentrations helped to read accurately the decrease of nitroso group in the "Test" concentration. The pH of the incubation solutions was 6.2 at the beginning and 6.3 at the end of incubation period. Following incubation, the solutions were buffered with 0.2 ml of pH 4.0 acetate buffer per ml solution. The bacteria were discarded by centrifugation and to each ml of "Test" or "Control" supernatant, 3 ml of Forist's colour reagent and one ml of 6N HCl were added. The reaction mixtures were incubated at 60°C for 45 minutes for colour development. Absorbance was read at 550 mμ on a Coleman double beam Hitachi Model 124 spectrophotometer. Concentrations were calculated from extinction coefficient.

## RESULTS

### Survival

Streptozotocin is a broad spectrum antibiotic and kills both Gram positive and Gram negative bacteria. While studying mutagenic activity of streptozotocin and its analogs in the present investigations, it was considered important to make observations on bacterial survival. Table II shows that only streptozotocin killed cells at the test concentrations. At 0.005 μmoles per ml, the survival was 46%; at 0.01 μmoles per ml, 7% and at 0.05 μmoles per ml, only 0.5%.

### Mutagenicity

a) Liquid culture treatments in vitro

An interesting relationship of structure and mutagenicity emerged during the course of this work. The presence of a β-OH (compound I) on C-1 of the glucose moiety of streptozotocin is essential for a high degree of mutagenicity; replacement of β-OH(I) by β-$OCH_3$(II) results in about 10 to 100 fold decrease in mutagenic activity over the concentrations tested. Analogs of streptozotocin containing larger side groups such as β-$O(CH_2)_2CH_3$ (IV, Fig. 2a) or β-$O(CH_2)_3CH_3$ Fig. 2b) show another 10 fold decrease in mutagenicity compared with β-$OCH_3$ analog(II). It is also apparent from Fig. 2a and 2b that the bacteria fail on the basis of mutagenicity to

TABLE II

Survival of Bacteria Following Treatment* with Streptozotocin, Streptozotocin Analogs and N-methyl-N-nitrosourea

| Compounds tested | Percent survival concentrations of compounds tested (μmoles/ml) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.005 | 0.01 | 0.05 | 0.1 |
| None | 100 | - | - | - | - |
| Streptozotocin (SZN) | - | 46 | 7 | 0.5 | - |
| β-methyl SZN | - | - | 100 | 70 | 70 |
| α-methyl SZN | - | - | - | 100 | 80 |
| α-ethyl SZN | - | - | - | 100 | 100 |
| β-propyl SZN | - | - | - | 100 | 100 |
| β-butyl SZN | - | - | - | 100 | 100 |
| N-methyl-N-nitroso-urea (MNU) | - | - | 100 | - | 100 |
| MNU-scyllionosamine | - | - | - | 100 | 100 |
| MNU-epi-inosamine-2 | - | - | - | 100 | 100 |

* Treated for three hours at 37°C as described in Materials and Methods.

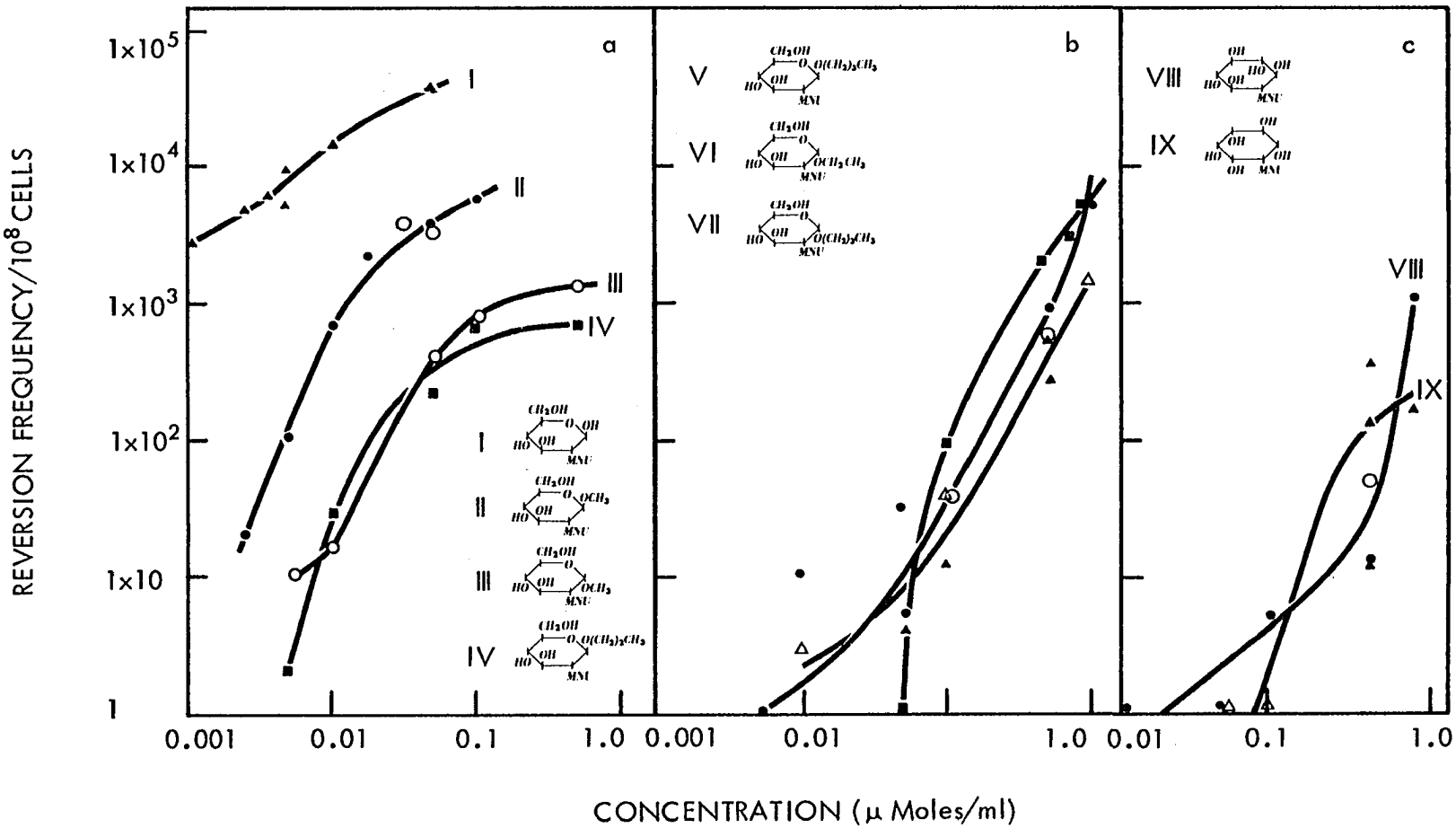



Fig. 2. Frequency of Histidine-independent Revertants Induced by Treatment with Streptozotocin (I) and Streptozotocin Analogs (II-IX). Symbols of Different Shapes and Shades Identify Different Experiments with the Same Compound.

distinguish between analogs bearing larger side groups such that compound IV has similar mutagenic activity as its heavier analog, compound V.

The $\beta$-$OCH_3$ analog(II) is about 10 fold more mutagenic than the $\alpha$-$OCH_3$ analog(III, Fig. 2a). The higher mutagenic activity of $\beta$- versus $\alpha$-isomer which is evident for the $-OCH_3$ group, does not hold for larger alkyl groups since all larger alkyl group analogs regardless of their alkyl group being in $\alpha$ or $\beta$ position, are all poorly mutagenic (IV, V, VI, VII: Fig. 2a, 2b).

Replacement of glucose with inositol in the streptozotocin molecule (VIII and IX, Fig. 1) is accompanied with several thousand fold decrease in its mutagenic activity (Fig. 2c). There is, however, no significant difference between the mutagenic activity of $\alpha$-OH and $\beta$-OH inositol analogs of streptozotocin (VIII, IX Fig. 2c).

b) Bacterial assay of tissue homogenates

Streptozotocin(I) was nearly five hundred times more mutagenic than compound II (Table III). This difference is about 5 to 50 times greater than the difference in mutagenic activity observed for compounds I and II in liquid culture treatments. Compound V has not shown mutagenicity in this assay.

## Determinations of the Nature of Mutations

Streptozotocin(I) and $\beta$-methyl streptozotocin(II) induced many mutations in the strains that revert by base substitution (Table IV);

TABLE III

Frequency of Histidine Independent Revertants Following a Three Hour Exposure of Auxotrophs to Tissue Homogenates from Treated and Control Animals

| Treatment[a] | Source of homogenate | Histidine independent revertants induced per $10^8$ survivors plated |
|---|---|---|
| Streptozotocin (I) (SZN) | Liver | 4,160 |
| | Testes | 4,730 |
| β-methyl SZN (II) | Liver | 9 |
| | Testes | 10 |
| β-butyl SZN (V) | Liver | 0 |
| | Testes | 0 |
| Control | Liver | 0 |
| | Testes | 0 |

[a] Concentration of each compound tested, 0.1 μmoles/gm. animal.

TABLE IV

Mutation Specificity of Streptozotocin (SZN), β-methyl SZN and N-methyl-N-nitrosourea[a,b]

| Strain[c] | Type of mutation reverted | Histidine independent revertants/$10^8$ cells | | | |
|---|---|---|---|---|---|
| | | Control | SZN | β-methyl SZN | MNU |
| 172A | Base substitution | 6 | 8,940 | 225 | 6 |
| 173A | Base substitution | 20 | 5,369 | 85 | 30 |
| 174A | Base deletion | 2 | 12 | 4 | 1 |
| 175A | Base deletion | 1 | 15 | 1 | 1 |
| 176A | Base addition | 1 | 13 | 4 | 2 |
| 177A | Base addition | 1 | 20 | 1 | 1 |

[a] All treatments were 0.01 μmoles/ml for one hour in nutrient broth

[b] Numbers underlined are considered to be different in mutagenicity from untreated controls.

[c] Even numbered strains are $uvrB^+$ and odd numbered strains are $uvrB^-$.

a slight decrease in the number of revertants was observed with the bacterial strain that lacks the excision repair. Streptozotocin and its analogs appear to cause mutations through base-pair substitutions since they failed to revert strains that revert by base deletion or addition. N-methyl-N-nitrosourea, a strong carcinogen, did not prove to be mutagenic at the concentration at which streptozotocin and β-methyl streptozotocin were strongly mutagenic.

## Susceptibility Experiments

The deep rough, rfa mutant strain of S. typhimurium (Strain 219, Table 1) has defective LPS; this resulted in increased mutagenic activity of streptozotocin and N-methyl-N-nitrosourea in this strain but β-methyl streptozotocin and β-butyl streptozotocin exhibited a decreased mutagenicity (Table V). In these experiments, N-methyl-N-nitrosourea was used at ten times higher concentration than in other mutagenicity experiments reported earlier (Table IV).

## Spectrophotometric Measurements of Nitroso Group

When the concentration of mitroso groups remaining in solution in presence of bacteria was measured spectrophotometrically, it was observed that nitroso group of streptozotocin was depleted two times as rapidly as that of compounds II and V and three times as rapidly as that of compounds VI and VIII (Table VI).

TABLE V

Frequency of Histidine-Independent Revertants Following Treatment with Streptozotocin, its Analogs and N-methyl-N-nitrosourea in Strains of S. typhimurium with Normal and Defective Cell Walls

| Compound | Dose µmoles/ml | Number of revertants per $10^8$ cells[a] strain 213 (normal cell wall) | strain 219 (rfa cell wall) |
|---|---|---|---|
| No compound (Control) | - | 4 | 2 |
| Streptozotocin (SZN) | 0.01 | 2,868 | 7,720* |
| β-methyl SZN | 0.1 | 1,696 | 1,422 |
| β-butyl SZN | 0.1 | 26 | 3 |
| N-methyl-N-nitrosourea (MNU) | 0.1 | 23 | 99 |
| MNU-scyllo-inosamine | 0.1 | 2 | 1 |

[a] Numbers underlined are considered to be different from control.

* Number considered to be different from normal cell wall strain.

TABLE VI

Bacterial Depletion of 0.05 μmoles/ml Streptozotocin and its Analogs from 0.85% Saline in Half Hour at 37°C

| Compound tested | Percent depletion of Compound |
|---|---|
| Streptozotocin (SZN,I) | 84 |
| β-methyl SZN (II) | 42 |
| β-butyl SZN (V) | 40 |
| MNU-Scyllo-inosamine (VIII) | 30 |
| α-ethyl SZN (VI) | 26 |
| Control (No bacteria)* | 0 |

* Each compound tested was simultaneously run as control without bacteria and its absorbance at 500 mμ was taken as 100% presence or '0' depletion.

## DISCUSSION

Streptozotocin is a 2-deoxy-D-glucose derivative of N-methyl-N-nitrosourea; the latter compound is strongly carcinogenic[24] and weakly mutagenic.[16] According to the conclusions of Kolbye and Legator[28] and those of Rakieten *et al.*,[48] the mutagenic and carcinogenic activities of streptozotocin are both associated with N-methyl-N-nitrosourea end of the molecule. Because of the observations of Garret[22] indicating *in vitro* release of diazomethane, Kolbye and Legator[28] hypothesized that both the carcinogenic and mutagenic properties of streptozotocin are expressed through methylations via diazomethane formation; they further predicted a similar mode of action for N-methyl-N-nitrosourea. Rozenkranz and Carr,[51] however, reported differences in the action of N-methyl-N-nitrosourea and streptozotocin. They showed that bacteria resistant to streptozotocin are sensitive to all of the biological effects of N-methyl-N-nitrosourea i.e. devitalization, inhibition of cellular metabolism and degradation of cellular DNA. They also showed that in bacteria as well as in mammalian cells, streptozotocin is primarily an inhibitor of DNA synthesis while N-methyl-N-nitrosourea also inhibits RNA and protein synthesis. Reusser's work,[50] previously cited, indicated cytosine binding of streptozotocin in cellular DNA and thus corraborated findings of Rozenkranz *et al.*

The apriori formation of diazomethane for alkylations by N-methyl-N-nitrosourea, however, is contradicted by recent work of

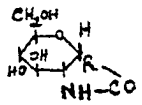
Lawley and Shah.[31] Working with two labelled N-methyl-N-nitrosourea preparations i.e. $C^{3}H_3$ $^{14}CH_3$ and $^{14}CH_3$, these authors showed that the ratio of $^{3}H/^{14}C$ in DNA products was the same and equal to that in the original reagents. This is in accord with the concept that methyl group is transferred intact and not via diazomethane ($CH_2N_2$). It is evident that if diazomethane was generated, extent of methylations measured by $^{3}H$ labelled preparation would be two-thirds of the extent measured by $^{14}C$ label as $^{3}H$ is likely to be lost as H during the reaction. It is quite likely that DNA alkylations are caused by N-methyl-N-nitrosourea (MNU) and streptozotocin in a similar manner i.e. direct methylations via the nitroso-alkane end of the molecule.

In the present study, streptozotocin and eight of its analogs have been tested for their mutagenic activity. None of these analogs carry any changes at the nitroso-alkane end of the molecule. Thus any differences in mutagenic action among these analogs cannot be attributed to the unchanged MNU part but must either be associated with the variations in the carbohydrate moiety alone or to an overall configurational change in molecular structure thus hindering biological reactivity of the nitroso-alkane end.

On the basis of their mutagenic activity these analogs fall into four groups, each being different from the other by about one order of magnitude in mutation frequency. The first or the most mutagenic group is represented by the parent compound, streptozotocin (I, Fig. 1); the next group is again represented by a single

compound, II, which differs from streptozotocin in having a β-methyl group instead of a β-hydroxyl group at C-1 of glucose moiety. The third level of mutagenicity is expressed by a number of different compounds that differ from streptozotocin by having either an α-methyl group (compound III) or heavier alkyl groups in α or β positions (compounds IV, V, VI and VII). It may be noted that with alkyl groups larger than $-OCH_3$, the effect of α or β isomerism observed for the methoxyl group cannot be differentiated on the basis of mutagenicity. In the fourth and the least mutagenic group are the two analogs (compounds VIII and IX) in which the glucose moiety of streptozotocin has been replaced by deoxyinositol. In this case, the similarity of compound VIII to streptozotocin i.e. both have a β-OH at C-1 of the carbohydrate moiety, does not make it a more effective mutagen than its α-OH isomer (compound IX). From these observations, it is concluded that structural modifications of the glucose moiety of streptozotocin or its replacement by inositol reduces the effectiveness of streptozotocin as a mutagen.

The question may then be asked whether the observed differences in mutagenic activity are due to differential uptake, to differences in reactivity or both. Spectrophotometric determinations show that the nitroso group of the more mutagenic compounds is depleted more rapidly from treatment medium in presence of bacteria. J. Wiley (The Upjohn Co., Kalamazoo, personal communication) has indicated that at pH range 6 and above, the MNU moiety of streptozotocin

may give rise to cyclic compounds of the type 

releasing highly unstable compound $CH_3.NH.NO$. This unstable compound i.e. $CH_3.NH.NO$. is converted into $CH_3.N{:}N.OH$ which in turn may form different types of ions i.e. $CH_3N_2^+$, $CH_2N_2^+$. All these unstable forms in all probability are not measured as nitroso group. Any spectrophotometric measurements of a nitroso group most likely represent the whole compound. It may also be appreciated that any of these highly reactive ions formed in solution would have reacted with other molecules outside the cell before reaching sites on DNA to cause alkylations.

Experiments on susceptibility of defective cell wall mutant (rfa) show increased mutagenicity for streptozotocin and N-methyl-N-nitrosourea(MNU), a slight decrease for β-methyl streptozotocin and β-butyl streptozotocin while MNU-scyllo-inosamine (VIII) was not mutagenic. The formation of a cyclic compound in solution as discussed above may be an important prerequisite to the breakdown of MNU side chain in compounds having a carbohydrate moiety attached to MNU. This could result in different types of behaviours in solution for streptozotocin and its analogs depending on the position and resulting reactivity of alkyl group on the anomeric carbon of the carbohydrate moiety. Also, rfa mutants lack the LPS coat partially or completely and would have less affinity for lipophilic molecules compared to a normal cell wall strain; therefore, β-methyl streptozotocin and β-butyl streptozotocin would be

expected to show less mutagenicity than streptozotocin, Table V. N-methyl-N-nitrosourea reflects a pattern of mutagenic activity similar to streptozotocin but is a poor mutagen which reflects a role of glucose moiety in enhancing the mutagenic action of streptozotocin.

One might also expect that if the barrier between mutagens and DNA is altered, the difference in mutagenicity of related compounds will change. This is indeed the case. When the mutagenicity of compounds I and II was compared by liquid culture treatment and by tissue mediated microbial assay, the difference in mutagenic activity changed from 10-100 fold to nearly 500 fold, Fig. 2a and Table III.

Differential depletion alone cannot account for the quantitative differences found in mutagenic activity among these compounds. For example, in the depletion experiments, streptozotocin is depleted only twice as fast as β-methyl streptozotocin, yet in the in vitro mutagenicity experiments, SZN is about 10-100 times more mutagenic. The lack of direct parallel between depletion and mutagenicity is even more striking if streptozotocin is compared with compounds V, VI and VIII, Fig. 2a, 2b and Table III. In this case, streptozotocin is $10^3$ times more mutagenic than any of the other compounds, yet it is depleted only about three times faster than the others. If differential depletion alone cannot account for the noted differences in mutagenicity then there must also be intracellular discrimination against some compounds and this could

be metabolic, lack of transport or poor mutagenic interaction with DNA.

## APPENDIX I

Tetsuo Suami and Tomoya Machinami have reported on preparation of streptozotocin analogs in which the pyranose ring of the glucose moiety has been replaced by a cyclohexane ring.[57] These compounds are the N-carbamyl-N'-methyl-N'-nitroso derivatives of scyllo-inosamine, ep-inosamine-2 and myo-inosamine-1.

In a subsequent paper,[58] the same authors have reported on preparation of methyl glycosides of streptozotocin by a two step reaction. Methyl β-D-glucosaminide hydrochloride was prepared by the method of Neuberger and Rivers[42] and then it was treated with methyl isocynate in the presence of silver carbonate to give methyl-N-carbamyl-N'-methyl-β-D-glucosaminide; nitrosation of this compound carried out with sodium nitrite in aqueous acetic acid gave methyl N-carbamyl-N'-methyl-N'-nitroso-β-D-glucosaminide. Similarly starting from methyl N- carbobenzoxy-α-D-glucosaminide, methyl N-carbamyl-N'-methyl-N'-nitroso-α-D-glucosaminide was obtained. Purity of these compounds was based on their melting point determined on a Mitamura micro hot stage and on NMR spectra determined with a Varian A-60D spectrometer.

## APPENDIX II

Different media used in the present work are described here:

(i) Nutrient broth and nutrient agar: Difco dehydrated media were used.

(ii) Minimal salt solution (MSS) - single strength (1-X)

| | | |
|---|---|---|
| $K_2HPO_4$ | 9.84 | Grams |
| $KH_2PO_4$ | 4.22 | " |
| $CH_3COONa)_4$ | 0.94 | " |
| $CH_3COONa$ | 0.42 | " |
| $MgSO_4$ | 0.05 | " |
| Distilled water to | 1,000 | ml. |

(iii) Histidine enrichment medium (HEM)

Double strength (2-X) minimal salt solution containing 56 µgrams of histidine and 0.4 grams of glucose per 100 ml was mixed with equal volume of double strength Difco agar (3%); this mixture was then used to prepare HEM plates.

(iv) Biotin supplemented histidine enriched minimal salt solution (BSHEMSS)

Single strength minimal salt solution (1-X) containing 28 µgrams of histidine and 3.5 milligrams of biotin in 100 ml constitutes BSHEMSS.

(v) Biotin supplemented minimal salt solution (BSMSS)

Single strength minimal salt solution (1-X) containing 3.5 milligrams of biotin constitutes BSMSS.

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