Alkylation of DNA in Rat Tissues following Administration of Streptozotocin

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ABSTRACT

Streptozotocin, an antibiotic widely used for induction of diabetes in experimental animals and for the treatment of pancreatic neoplasms, was shown to be a potent methylating agent reacting with DNA in vitro to form methylated purines. The reaction was similar in extent and relative proportions of methylation products to that produced by N-methyl-N-nitrosourea, the aglycone of streptozotocin. When streptozotocin was administered to rats by i.v. injection, DNA was methylated with the formation of 7-methylguanine, O^6-methylguanine, 3-methyladenine, and 7-methyladenine in liver, kidney, intestine, and pancreas. In contrast to N-methyl-N-nitrosourea which produced approximately equal amounts of methylation in DNA of liver, brain, and kidney, streptozotocin caused virtually no methylation in brain DNA; but, both liver and kidney DNA were alkylated to a greater extent than with N-methyl-N-nitrosourea. This methylation of renal DNA may account for the ability of streptozotocin to induce renal tumors. Streptozotocin produced significant methylation of pancreatic DNA which, if concentrated in the \( \beta \)-cells, may account for their destruction. Pretreatment with nicotinamide reduced the extent of methylation of pancreatic DNA but did not affect the methylation in the liver or kidney. Methylation of \( \beta \)-cell DNA in the pancreas may lead to the initiation of tumors if the extent of alkylation is not so great that cell death occurs.

INTRODUCTION

Streptozotocin, an antibiotic produced by a strain of \( \textit{Streptomyces achromogenes} \), is a 2-deoxy-D-glucose derivative of the carcinogen N-methyl-N-nitrosourea (9). In addition to its antibiotic properties, streptozotocin has been shown to be effective in the induction of experimental diabetes in several laboratory species by virtue of selective destruction of the pancreatic \( \beta \)-cells (31). Following the discovery of the \( \beta \)-cell cytopotoxic property of streptozotocin, islet cell tumors were obtained in rats maintained for long periods of time after the administration of a diabetogenic dose of this compound (14, 28, 38). The rat kidney is also a target for streptozotocin carcinogenesis (3, 10, 14, 23, 29, 30). Nicotinamide, when administered to rats shortly before or after a diabetogenic dose of streptozotocin, prevents the destruction of the pancreatic \( \beta \)-cells (5, 33) but potentiates streptozotocin carcinogenesis in the \( \beta \)-cells (14, 28). Neither the mechanism(s) by which streptozotocin exerts its cytopathic and carcinogenic effects nor that by which nicotinamide alters these effects is clearly understood.

It is generally accepted that the alkylation of DNA by carcinogens such as the alkylnitrosoureas is a critical event in the induction of tumors by these compounds (4, 18, 19, 21, 25, 34). Particular attention has been focused on the alkylation of the exocyclic oxygen atom of guanine in DNA since the formation and persistence of \( O^6 \)-alkylguanine residues in DNA has been shown to be generally correlated with tumor induction by alkylating carcinogens (6, 20, 24, 25, 27, 34).

In light of the observed correlation between the alkylation of DNA and tumor induction by alkylating carcinogens, we have investigated the methylation of DNA in target and non-target tissues of the rat following the administration of a carcinogenic dose of streptozotocin. The influence of prior administration of nicotinamide on streptozotocin-induced methylation of DNA in these tissues was also investigated.

MATERIALS AND METHODS

Chemicals. Streptozotocin labeled in the methyl group ([3^-methyl-^14C]streptozotocin, 14 Ci/mol) was synthesized by Amersham/Searle Corporation (Arlington Heights, Ill.) by the method of Karunanayake et al. (13) and used without further purification. Assay of this material by the manufacturer showed it to be about 55% pure, the remainder being contaminants resulting from the decomposition of the labile carcinogen. Since this study is concerned with the methylation of DNA by streptozotocin, it was important that none of the contaminants present in the material used were themselves capable of affecting such alkylation. N-Methyl-N-nitrosourea is the only alkylating species likely to be a contaminant of streptozotocin; therefore, the impure carcinogen was analyzed for the possible presence of methylaziridinourea. Analysis of the streptozotocin on silica gel thin-layer plates using a mobile phase of chloroform:dichloromethane:ethyl acetate:acetic acid, 4:1:29:29:0.6 (Rf streptozotocin = 0; Rf methylaziridinourea = 0.4), showed that less than 1% of the label present appeared as methylaziridinourea. Further purification of the streptozotocin was not attempted since the spontaneous breakdown of the carcinogen occurs very rapidly after administration.

Streptozotocin was stored at a powder at -60° until use. Immediately before use, the material was dissolved at a concentration of 1 mCi/ml in 10 mM sodium citrate, pH 4.0, made isotonic by the addition of an appropriate volume of 0.25 m NaCl.

\( N^-\text{[methyl-^3H]}^-\text{N-nitrosourea} \) (1.01 Ci/mmole) was purchased from New England Nuclear (Boston, Mass.) and used without further purification. Nicotinamide and calf thymus DNA (Sigma type I) were purchased from Sigma Chemical Company (St. Louis, Mo.). All other chemicals were of analytical reagent quality.

Animals. The animals used in this study were female Sprague-Dawley rats (Charles River Breeding Laboratories,
North Wilmington, Mass.) approximately 8 weeks old and ranging in weight from 190 to 220 g. Rats were housed in conditions of controlled lighting and temperature and were allowed free access to water and food until the evening before they were to be used in experiments. At this time, food was withdrawn from the animals; free access to water was still allowed. Rats were anesthetized with an i.p. injection of 100 µl of sodium pentobarbital (64.8 mg/ml). Ten min after injection with the pentobarbital, each animal received [14C]streptozotocin at a dose of 2 µCi/kg (21 mg/kg) administered via the tail vein. Rats were then killed by cervical dislocation either 2 hr or 24 hr after receiving the streptozotocin. The liver, kidneys, intestines, brain, and pancreas of each animal were quickly removed and frozen by immersion in liquid nitrogen. All organs were stored frozen at −20°C until used for isolation of DNA.

In some experiments, rats first received an i.p. injection of nicotinamide at a dose of 100 mg/kg. Each animal then received injections of pentobarbital and labeled streptozotocin as described above at times of 5 and 15 min, respectively, after the injection of nicotinamide. Animals were killed and organs were removed and handled as described above. N-[methyl-3H]nitrosourea was administered by i.v. injection as described above for streptozotocin. The dose was 9 mg/kg and 2.5 mCi/kg.

Isolation, Hydrolysis, and Analysis of DNA. DNA was isolated from the various organs as described previously (26). In each experiment, DNA was isolated from individual livers, but in the case of the other organs, DNA was isolated from organs pooled from pairs of rats. The DNA obtained from each sample was hydrolyzed by heating for 1 hr at 70°C in 0.1 N HCl. Hydrolyzed samples were then analyzed chromatographically by either of 2 methods. The first method consisted of chromatographic analysis on Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) as described previously (26). In the second method, samples were analyzed by high-pressure liquid chromatography on a system consisting of a Micromeritics Model 750 solvent delivery system, Model 730 universal injector, Model 731 column oven, and a Model 740 microprocessor (Micromeritics Instrument Corporation, Norcross, Ga.). Detection of major sample components and of added authentic marker compounds was accomplished at 254 nm with a Waters Model 440 absorbance monitor (Waters Associates, Milford, Mass.). Separation of the sample components was achieved using a 0.46x 25.0-cm column of Partisil 10 SCX cation exchange medium (Whatman, Inc., Clifton, N. J.) eluted at 50°C with an isocratic mobile phase of 0.020 M ammonium formate, pH 4.0, at a flow rate of 2.0 ml/min. Fractions were collected directly into scintillation vials for determination of 14C content by means of a LKB Model 2111 fraction collector (LKB Instruments, Inc., Rockville, Md.).

Those peaks corresponding to adenine and guanine in each sample were collected into preweighed vials, and the volume of each fraction was determined by weight. A portion of each of the 2 major purine fractions was then diluted to allow spectrophotometric quantitation of the corresponding major base present. Following the determination of adenine and guanine, all of the fractions in each sample were brought up to a volume of 5 ml by addition, where necessary, of mobile phase; a 10-ml volume of Formula 947 liquid scintillation cocktail (New England Nuclear) was then added to each fraction for counting. Samples were counted on either a Beckman Model LS3133T or LS8000 liquid scintillation spectrometer (Beckman Instruments, Fullerton, Calif.). Correction of cpm to dpm was accomplished by means of quench curves and the external standard channel ratio; counting efficiency was about 80% for all samples.

A comparison of the 2 chromatographic methods used in this study was performed by analyzing portions of several samples with both methods. The results of analyses done in this way failed to show any significant differences between the 2 systems. Therefore, the high-pressure liquid chromatography system was used for most of the analyses as it afforded shorter analysis times and allowed detection of smaller quantities of alkylated products in a sample.

Alkylation of DNA in vitro. Calf thymus DNA, at a concentration of 3 mg/ml in 0.05 M potassium phosphate, pH 7.5 to 7.6, was alkylated by the addition of either [3H]methylnitrosourea or [14C]streptozotocin to a concentration of 25 (N-methyl-N-nitrosourea) or 20 (streptozotocin) µCi/ml. The alkylation reactions were carried out for 1 hr at 37°C. Following the reaction, DNA was precipitated by the addition of either NaCl or sodium acetate to a concentration of 0.25 M followed immediately by 2 volumes of 95% ethanol (−20°C). Samples were held on ice for 15 min after which time the DNA was pelleted by centrifugation (10,000 x g for 10 min; 4°C). The pellets were washed 3 times, and the alkylated DNA was dried overnight in a vacuum. The samples were then hydrolyzed and analyzed as described above.

RESULTS

Labeled streptozotocin was reacted with DNA in vitro, and the amounts of methylated purines formed were compared with those produced by the reaction of DNA with N-methyl-N-nitrosourea. It was found that both in relative amounts and extent of reaction, the products were identical (Chart 1). This finding shows that, as expected from the chemical structure, strepto-
Zotocin is a potent methylating agent. Therefore, the difference between the physiological effects of streptozocin and N-methyl-N-nitrosourea must be due to factors (e.g., selective uptake by certain tissues) not related to chemical reactivity.

Table 1 shows the levels of methylated purines present in the DNA of several rat organs 2 hr after the administration of a carcinogenic dose of streptozocin with and without nicotinamide pretreatment. Results are also given for the content of methylated guanine derivatives in the DNA of some of these organs after an approximately equimolar dose of N-methyl-N-nitrosourea. N-Methyl-N-nitrosourea led to about equal extents of methylation in liver, kidney, brain, and intestine as expected from previous studies which showed that this compound penetrates equally into all tissues (15, 20, 26, 27). In contrast, streptozocin gave widely differing levels of alkylation. The liver was alkylated to the greatest extent followed by the kidney. In both of these organs, the degree of methylation (as measured by the production of 7-methylguanine which is lost sufficiently slowly from DNA that the likely decline during the 2-hr exposure period is negligible (22, 26)) was significantly greater than that observed with N-methyl-N-nitrosourea. Methylation of DNA in the intestine and pancreas was considerably less than methylation in the liver and kidney, while methylation of brain DNA by streptozocin was very low, suggesting that the compound does not penetrate well into the nervous system.

Pretreatment of the rats with nicotinamide had no significant effect on the streptozocin-induced methylation of DNA in the liver, kidney, intestine, and brain. However, there was a 40% reduction in alkylation of pancreatic DNA. The relative abundances of the methylated purines were not affected by the pretreatment with nicotinamide in any organ.

The persistence of the alkylated bases over a 24-hr period following treatment with streptozocin was studied, and it was found that, in liver, kidney, pancreas, and brain, the amount of 7-methylguanine decreased only slightly in this period in agreement with previous studies, showing that removal of this product occurs at a relatively slow rate (22, 25, 26). There was a significant decline (46%) in the content of 7-methylguanine in the intestine, but this can be explained by the rapid cell turnover in this organ. 7-Methyladenine was lost from the DNA of all tissues at the same rate, and it is known that this product is rapidly depurinated from DNA (16, 21). Table 2 shows the percentage loss of O6-methylguanine and 3-methyladenine in these organs. The fall in 3-methyladenine is consistent with its excision by both spontaneous depurination and by the action of a glycosylase (22, 25) and occurred to the same extent in all organs tested. This was not the case for O6-methylguanine, which was lost from DNA more rapidly in the liver and intestine than in the kidney and pancreas. (As mentioned above for 7-methylguanine, part of the loss in intestine may be due to cell turnover).

**DISCUSSION**

As expected from the chemical structure and its reaction with 4-(p-nitrobenzyl)pyridine (6, 37), it was found in the present work that streptozocin was a potent DNA-methylating agent. Since there is extensive evidence that methylation of DNA, particularly at the O-6 position of guanine, provides a stimulus which can initiate neoplastic growth (6, 19-21, 25-27, 34) it is likely that the pancreatic and renal tumors produced

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Methylated purines present in DNA of various organs after administration of streptozocin, streptozocin plus nicotinamide, or N-methyl-N-nitrosourea</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organ</strong></td>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>Liver</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>Liver</td>
<td>N-Methyl-N-nitrosourea</td>
</tr>
<tr>
<td>Kidney</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>Kidney</td>
<td>N-Methyl-N-nitrosourea</td>
</tr>
<tr>
<td>Intestine</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>Intestine</td>
<td>N-Methyl-N-nitrosourea</td>
</tr>
<tr>
<td>Brain</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>Brain</td>
<td>N-Methyl-N-nitrosourea</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>Pancreas</td>
<td>N-Methyl-N-nitrosourea</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Amount of O6-methylguanine and 3-methyladenine remaining in tissue DNA 24 hr after administration of streptozocin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organ</strong></td>
<td><strong>Methylated bases present in DNA (pmol/μmol guanine)</strong></td>
</tr>
<tr>
<td>Liver</td>
<td>O6-Methylguanine</td>
</tr>
<tr>
<td></td>
<td>6 (9)</td>
</tr>
<tr>
<td>Kidney</td>
<td>17 (49)</td>
</tr>
<tr>
<td>Intestine</td>
<td>3 (25)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.6 (115)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2 (39)</td>
</tr>
</tbody>
</table>

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**Table 1**

*Numbers in parentheses, percentage of base remaining. The percentage of O6-methylguanine remaining after 24 hr was calculated (26) assuming that the ratio of reaction at the O6 position was 0.12 x that at the 7-position of guanine. The percentage of 3-methyladenine was calculated assuming that the ratio of reaction at this position was 0.15 x that at the 7-position of guanine (21).*
by streptozotocin are related to this methylation. It should be noted that, on the basis of a comparison of equimolar doses, streptozotocin was even more effective than N-methyl-N-nitrosourea in the methylation of kidney DNA, and the latter is known to be a highly potent carcinogen producing a high incidence of kidney tumors in adult rats treated with the compound. Single doses of streptozotocin were found to produce a high incidence of both epithelial and mesenchymal renal tumors (3, 10, 29, 30), but the relative lack of other observations of these tumors in the many reports on the pathophysiology of streptozotocin probably relates to the short life span of severely diabetic animals. The high probability of kidney tumor initiation renders streptozotocin-treated diabetic animals a poor choice of model for studies of the long-term effects of diabetes on the kidney (e.g., in the development of basement membrane changes). Similarly, the possibility that the high extent of methylation in organs such as the liver may influence metabolism in these organs should not be overlooked. In any event, the same precautions are those taken for handling N-methyl-N-nitrosourea (35) should be applied to streptozotocin, which is very widely used as a laboratory tool for inducing diabetes.

It is tempting to assume that the rather specific destruction of the pancreatic β-cells by streptozotocin is due to their methylation, since high levels of alkylation are known to cause cell necrosis. The sugar moiety may direct the alkylation preferentially to the β-cells. Although no evidence was obtained in our experiments that the pancreas as a whole was methylated to a greater extent than other tissues, the β-cells represent only a small proportion of the total; and, if alkylation was concentrated in these cells as suggested (2), there would be a very high level of methylation. The striking differences between the alkylation of brain and liver DNA when streptozotocin and N-methyl-N-nitrosourea were compared emphasizes the effect of the sugar group on the distribution of alkylation. N-Methyl-N-nitrosourea is a very potent inducer of tumors in the brain and nervous system of young rats and is a potent methylaing agent for the brain cells (19, 20, 27, 35). The inability of streptozotocin to methylate the brain, which presumably reflects the impermeability of these cells to this drug, suggests that tumors of the nervous system (which have not been seen) would not be expected. Although streptozotocin was a very active agent in methylation of liver DNA, the level of methylation produced is similar to that given by 1- to 2-mg doses of dimethylnitrosamine per kg (26). This is not sufficient to produce liver tumors with a single dose, although such tumors are seen with prolonged exposure (4, 18, 19, 25). It has been argued that the substantial ability of hepatocytes to remove O6-methylguanine from their DNA protects against carcinogenesis (6, 20, 25–27, 34), and the present results show that, within 24 hr, 90% of the O6-methylguanine formed in the liver by streptozotocin is removed. The observations of the fate of O6-methylguanine in kidney DNA after streptozotocin treatment are also similar to that seen when comparable amounts of methylation are produced by dimethylnitrosamine (24). In both cases, after an initial fall from the expected O6-methylguanine:7-methylguanine ratio of 0.12, the loss of O6-methylguanine is much slower than that from the liver DNA, correlating with the sensitivity of the kidney to tumor formation.

Several groups have attempted to study the distribution and cellular uptake of streptozotocin by autoradiography or measurement of total radioactivity present in various tissues after administration of the compound (1, 2, 11–13, 36). The findings that high levels of radioactivity were present in the liver, kidney, and pancreatic islet tissue and that no unchanged streptozotocin could be detected in the cerebrospinal fluid (1) are in agreement with the measurement of DNA methylation in the present work. However, such techniques in which the bound radioactivity is not identified chemically are rather unsatisfactory for a compound such as streptozotocin which decomposes at neutral pH to yield a methylaing agent (8, 37), since they cannot distinguish between methylation and metabolic incorporation of radioactivity derived from the methyl group into macromolecules via the one carbon pool.

The administration of nicotinamide prior to the administration of streptozotocin had no detectable effect on the methylation of DNA in any organ except the pancreas. In this organ, pretreatment of rats with nicotinamide resulted in an approximately 40% decrease in the methylation of DNA (as determined by 7-methylguanine levels) relative to the level of methylation seen in nonpretreated rats. Although this may be regarded as a relatively modest decrease in the overall alkylation of the pancreas, it is possible that a much greater change in alkylation occurs in the pancreatic islets following nicotinamide pretreatment. The ability of nicotinamide to decrease the streptozotocin-induced methylation of pancreatic DNA may partially explain the reduced β-cell cytotoxicity produced by simultaneous exposure to nicotinamide and streptozotocin. The reduction in alkylation could then permit the survival of a greater fraction of the methylated cells which may go on to develop tumors. One possible explanation for the reduced level of pancreatic methylation in the nicotinamide-treated animals is that the nicotinamide may serve as an acceptor for the methyl group and, thus, reduce the chance of DNA or other cellular components of becoming methylated. Labeled N'-methylnicotinamide was observed in the urine of mice treated with [15N]streptozotocin (11), although this represented only a very small fraction of the injected radioactivity. However, there was no effect of nicotinamide on methylation of hepatic and renal DNA. Therefore, this explanation is unlikely to account for the marked reduction in the alkylation of pancreatic DNA in nicotinamide-pretreated rats unless the nicotinamide accumulates in the pancreas to a much greater extent than in these other tissues. The effect of nicotinamide on renal carcinogenesis by streptozotocin is unclear, since both increased and decreased tumor incidences have been reported (14, 29). Other explanations for the protective effect of nicotinamide are certainly credible. Since both N-methyl-N-nitrosourea and streptozotocin lead to a rapid fall in tissue NAD levels (7), nicotinamide may reverse this decline. Finally, some evidence has been obtained by electron microscopy suggesting that, in the mouse, streptozotocin leads to the induction of type C viruses within the β-cells (17). The histological changes seen in this study were prevented by nicotinamide (17, 32). Induction of latent viruses could also be brought about by methylation of cellular DNA, since other alkyling agents are known to have this effect (19, 21, 25).

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