Pergamon

Biochemical Pharmacology, Vol. 50, No. 12, pp. 2015–2020, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02102-7

COMPARISON OF INHIBITION OF GLUCOSE-STIMULATED INSULIN SECRETION IN RAT ISLETS OF LANGERHANS BY STREPTOZOTOCIN AND METHYL AND ETHYL NITROSOUREAS AND METHANESULPHONATES

LACK OF CORRELATION WITH NITRIC OXIDE-RELEASING OR O^6-ALKYLATING ABILITY

CAROL A. DELANEY,*¶ ANNEMARIE DUNGER,† MARIA DI MATTEO,* JAMES M. CUNNINGHAM,* MICHAEL H. L. GREEN‡,§ and IRENE C. GREEN*

*Department of Biochemistry, School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, U.K.; †Institute of Diabetes, University of Greifswald, Karlsburg, Germany; and ‡MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton, BN1 9RR, Brighton, U.K.

(*Received* 3 *January* 1995; *accepted* 5 *September* 1995)

Abstract—We have studied inhibition of glucose-stimulated insulin secretion in islets of Langerhans isolated from adult Sprague-Dawley rats and treated with different alkylating agents. Streptozotocin (STZ), N-methyl-N-nitrosourea (MNU), and N-ethyl-N-nitrosourea (ENU) all released nitric oxide, as demonstrated by an increase in medium nitrite and cellular cyclic GMP. Methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS), which do not possess a nitroso group, did not show evidence of nitric oxide release. All five compounds, however, decreased glucose-stimulated insulin release, suggesting that nitric oxide release was not necessary for the inhibition of secretion. Lack of involvement of nitric oxide was further suggested by the failure of oxyhaemoglobin to reverse STZ and MNU inhibition of insulin secretion. Since ENU was at least as effective as MNU in inhibiting insulin secretion, it appears that alkylation of DNA at the O^6 position of guanine may not be involved in this process.

Key words: Insulin secretion; streptozotocin; N-methyl-N-nitrosourea; N-ethyl-N-nitrosourea; methyl methanesulphonate; ethyl methanesulphonate; islets of Langerhans; nitric oxide; O^6-methylguanine; O^6-ethylguanine; oxyhaemoglobin

The widely used diabetogenic agent STZ,‖ a D-glucopyranose derivative of MNU, is an antibiotic isolated from *Streptomyces achromogenes* [1]. As a methylnitrosourea derivative, STZ is a potent alkylating agent [2]. Although both STZ and MNU are highly toxic and carcinogenic, MNU is either non-diabetogenic or weakly diabetogenic (see refs. [3–5]). The specificity of STZ diabetogenicity may arise because its glucose moiety causes it to be directed more effectively to insulin-secreting cells [4], since a rat insulinoma line, lacking a glucose receptor, shows equal resistance to STZ and MNU [6]. Interestingly, STZ and MNU appear to alkylate DNA overall to an equal extent, even under conditions where STZ is substantially more cytotoxic [7]. Another possibility is that the specificity of STZ is linked in some way to its ability to induce long-lasting mitochondrial dysfunction [8].

Although the exact mechanism may be unclear, it has generally been assumed that the cytotoxicity and diabetogenicity of STZ relate to the activity of its MNU moiety as an alkylating agent. Studies using cell lines with altered DNA repair capacity provide strong evidence that the cytotoxicity of MNU relates to its capacity to alkylate DNA, especially at the O^6 position of guanine (see e.g. refs. [9–11]). An alternative hypothesis has recently been proposed that some of the diabetogenic properties of STZ may relate not to its alkylating ability, but to its potential to act as a nitric oxide donor [12]. Both STZ and MNU contain a nitroso group and may liberate nitric oxide [13, 14] in a manner similar to that of the nitric oxide donors, sodium nitroprusside, SIN-1, or S-nitrosoglutathione.

To investigate a potential role of nitric oxide in the mode of action of nitrosoureas, we have found that the alkylating agent MMS does not appear to act as a nitric oxide donor, and have confirmed its ability to inhibit insulin secretion [8]. We have also studied inhibition by the ethylating agents ENU and EMS. These differ from the corresponding methyl compounds in the proportion of different DNA alkylation products they form and in the cytotoxicity of these products. The structures of the five compounds tested are shown in Fig. 1. The proportions in which they induce four common DNA alkylation products, N^7-alkylguanine, N^3-alkyladenine, O^6-alkylguanine, and phosphotriesters are shown in Table 1 [2, 15]. It is not clear that N^7-alkylguanine and phosphotriesters [16] make any significant contribution to cytotoxicity. N^3-alkyladenine appears to be a cytotoxic lesion, but it is subject to effective repair by an N-glycosylase [17, 18], which also removes N^7-alkylguanine from DNA. O^6-methylguanine appears to be 10- to 100-fold more cytotoxic than O^6-ethylguanine [11]. Although the data in Table 1 relate to initial alkylation, and consider-

§ Corresponding author. Tel. +44 (0) 1273 678120; FAX +44 (0) 1273 678121.

‖ Abbreviations: EMS, ethyl methanesulphonate; ENU, N-ethyl-N-nitrosourea; MetHb, methaemoglobin; MMS, methyl methanesulphonate; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MNU, N-methyl-N-nitrosourea; OxyHb, oxyhaemoglobin; RIA, radioimmunoassay; SIN-1, 3-morpholinosydnonimine; STZ, streptozotocin.

¶ Current address: Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden.

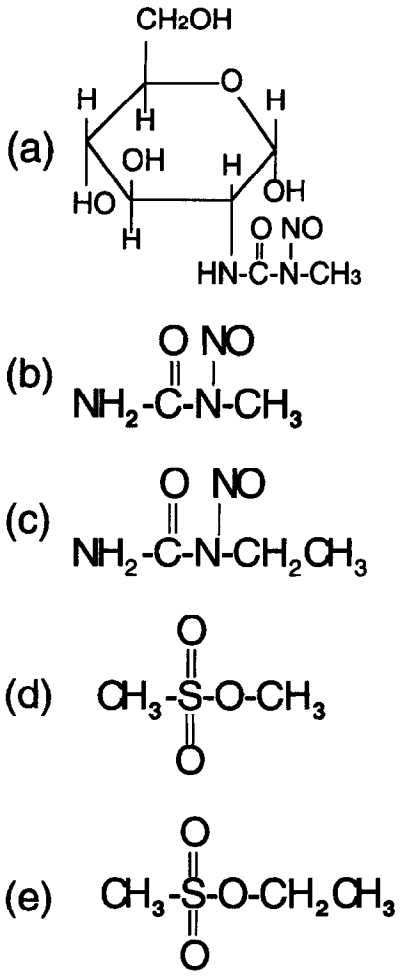

Fig. 1. Structure of the compounds used in this study: (a) STZ, (b) MNU, (c) ENU, (d) MMS, (e) EMS.

able variation in repair capacity, especially of O^6-alkylguanine, is known, repair would be unlikely to affect responses significantly over the time scale of the present experiments.

Also shown in Table 1 are Swain-Scott *s* values (obtained from ref. [19]). Agents with high *s* values attack nucleophilic sites with strong specificity, in the order S > N > O. Agents with lower *s* values show less specificity and form a higher proportion of O^6-alkylguanine in their DNA. There is a good correlation between low *s* value and carcinogenicity. It was of interest whether inhibition of insulin secretion would correlate with a low or high Swain-Scott *s* value, which could be suggestive of the nature of the nucleophilic site responsible for cytotoxicity (S vs O, for instance), or whether the ethylating agents would prove to be very much less inhibitory than their methylating counterparts, arguing for a role for O^6-alkylguanine in inhibition.

METHODS

Materials

STZ, MNU, ENU, MMS, EMS, and MetHb were all obtained from Sigma (Poole, U.K.). SIN-1 was a gift from Dr. R. Henning, Cassella AG, Frankfurt.

Islet isolation and cell preparation

Islets of Langerhans were isolated from adult Sprague-Dawley rats (200 g) by a modification of a collagenase digestion technique [20]. Islets were separated from pancreatic acinar tissue and were picked, using a finely drawn out Pasteur pipette, into a bicarbonate buffered medium [21], pH 7.4, containing 2 mM glucose.

Islet incubation for insulin secretion

Groups of freshly isolated islets were precultured for 30 min in 2 mM glucose-containing buffer. Insulin secretory responses were determined from groups of three islets challenged with buffer containing 20 mM glucose together with test compounds. At the end of a 1 hr incubation, aliquots were removed for assay of insulin. Insulin was assayed using rat insulin standards (Novo-Nordisk, Basingstoke, U.K.), guinea pig insulin antiserum, and ^{125}I-bovine insulin (both antibody and label were prepared in this laboratory [22]).

Cyclic GMP and protein determinations

Freshly isolated islets were pre-incubated for 30 min in physiological buffer containing 2 mM glucose. Groups of 30 islets were incubated in buffer containing 20 mM glucose plus test compounds for 30 min as described in a previous study [23]. The incubation medium was discarded and the islets were boiled for 3 min in sodium acetate buffer (50 mM, pH 4.75), centrifuged at 10 000 g for 3 min, following which the supernatant was removed for measurement of cyclic GMP using an 'in-house' radioimmunoassay [22]. Antibody was prepared in this laboratory in rabbit against succinyl cyclic GMP conjugated to albumin [24]. Cyclic GMP was used as standard, and succinyl cyclic GMP tyrosyl methyl ester was iodinated in our laboratory. The labelled product

Table 1. Alkylation of DNA by compounds used in this study. Data from (a) Bennett and Pegg [2] and (b) Singer and Grunberger [15]. Swain-Scott *s* values are from Vogel and Nivard [19].

Agent	Lesion (percent of total alkylation)				Swain-Scott *s* value
	N^7-alkylguanine	N^3-alkyladenine	O^6-alkylguanine	Phosphotriester	
(a) STZ	89.0	6.5	4.0	na	0.79
(b) MNU	68.0	2.4	4.9	12.5	0.42
(b) ENU	12.7	0.8	7.9	57.0	0.26
(b) MMS	84.0	6.3	0.28	0.8	0.83
(b) EMS	67.5	3.5	1.8	14.0	0.64

was purified on paper chromatography. Briefly, standards and unknowns were acetylated. The acetylation was performed with 2.5 μL of a mixture of triethylamine:acetic anhydride 2:1 in ethanol-washed soda glass tubes. An overnight incubation at 4°C of 50 μL aliquots of sample, antibody, and label was used. Bound and free label was separated using polyethylene glycol/gamma globulin mix, and the cyclic nucleotide concentrations were determined using an RIA-calc program on an LKB-Pharmacia (St. Albans, U.K.) multigamma counter [22].

The islet protein pellet from the initial centrifugation was stored frozen, then dissolved in 0.2 mL of 0.1 M NaOH, immediately prior to analysis in ELISA plates using the Bradford assay [25].

Measurement of nitrite

Incubation medium from experiments involving the treatment of cells was assayed for nitrite by the method of Green *et al.* [26]. For the nitrite assay, 75 μL samples were mixed with an equal volume of Griess reagent (0.1% naphthyl ethylenediamine and 1% sulphanilamide in orthophosphoric acid, 1:1 v/v), and absorbance read against standard sodium nitrite solutions (10–100 μM) at 546 nm.

Effect of haemoglobin on nitrosourea-induced inhibition of glucose-induced insulin secretion

The oxygenated ferrous form of haemoglobin (OxyHb) was prepared from ferric haemoglobin (MetHb) by the addition of sodium dithionite, with subsequent purification using a Sephadex G-25 desalt column as described by Murphy and Noack [27]. The OxyHb had an absorbance maximum at 413 nm, which changed to a maximum at 405 nm when saturated with nitric oxide. This change corresponds to the loss of a secondary peak at 576 nm, which can be measured to determine the extent of inactivation of OxyHb by nitric oxide. Groups of freshly isolated rat islets were preincubated in buffer containing 2 mM glucose for 30 min, followed by incubation in buffer containing 20 mM glucose plus test compounds for 60 min. The test compounds used for this experiment were STZ (1 mM), MNU (5 mM), or SIN-1 100 μM, each in the presence or absence of 10 μM OxyHb. Secreted insulin was assayed as described above. Supernatants were retained and OxyHb determined by scanning spectrophotometry, as described by Hevel and Marletta [28].

Statistical methods

Statistical differences between mean values were determined by an unpaired 2-tailed Student's *t* test, with the levels of significance established at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

RESULTS

Evidence for formation of nitric oxide

Following exposure of freshly isolated islets to the agents for 30 min, nitrite (an oxidative product of nitric oxide) was measured in the incubation medium (Fig. 2a). Using doses of agents that inhibit insulin secretion, it was found that STZ, MNU, and ENU significantly increased medium nitrite over 30 min. There was no increase in nitrite with MMS and EMS. It appears that STZ gave more nitrite than MNU and ENU. Our purpose here was to use nitrite purely as a qualitative indicator of nitric oxide formation. It would have been necessary to measure both nitrite and nitrate to obtain a quantitative indication of nitric oxide formation from the nitrosoureas.

Nitric oxide can activate the haem-containing prosthetic group of guanylate cyclase, resulting in the stimulation of guanylate cyclase and the accumulation of cyclic GMP [29]. Cyclic GMP accumulation over 30 min increased 2.5-fold with STZ, 2.5-fold with MNU, and 2-fold with ENU. Both MMS and EMS did not raise cyclic GMP (Fig. 2b).

Inhibition of glucose-stimulated insulin secretion

Groups of islets were incubated with the agents in buffer containing 20 mM glucose and insulin secreted over 1 hr was determined (Fig. 2c). There was a decrease in insulin secretion relative to control with all of the compounds tested. Thus, the capacity to release nitric oxide was not required for an alkylating agent to induce short-term inhibition of insulin secretion.

The addition of OxyHb (Fig. 3) did not reduce the inhibition of glucose-induced insulin secretion caused by STZ or MNU, whereas OxyHb significantly reversed ($P < 0.02$) the inhibition of insulin secretion caused by the nitric oxide donor SIN-1. The difference between SIN-1 + OxyHb and the OxyHb control was not significant. Interestingly, OxyHb itself significantly increased ($P < .001$) stimulation of insulin secretion by 20 mM glucose. The reason for this is unclear. Residual OxyHb was determined after each experiment. There was complete conversion of OxyHb by SIN-1, almost complete conversion by STZ, but little or no conversion by MNU. These results confirm that release of nitric oxide did occur with STZ, but it did not appear to be required for the inhibition of insulin secretion.

To compare inhibition of insulin secretion by MNU and ENU more precisely, a dose response curve was obtained. From Fig. 4 it can be seen that inhibition of glucose-stimulated insulin secretion is at least as efficient with the ethylating as with the methylating agent. To compare long-term toxicity, in four additional experiments islets were cultured for five days following treatment for 1 hr with the same doses of MNU and ENU as used in Fig. 4. They were then tested for insulin secretory responses to a 1-hr challenge with 2 mM and 20 mM glucose. No long-term inhibition of 20 mM glucose-stimulated insulin secretion was observed. With either agent there was some evidence of increased nonspecific islet damage, and basal level insulin secretion in 2 mM glucose-containing medium was increased (expressed as a % of 2 mM control, treatment with 1 mM MNU gave an increase of 47% and with 1 mM ENU gave an increase of 75% in basal insulin secretion). After 5 days' culture, islets treated with either compound at 5 mM showed considerable disaggregation; under these conditions, islet selection for secretion experiments is subject to bias.

DISCUSSION

Incubation of rat islets of Langerhans with streptozotocin, N-methyl-N-nitrosourea, and N-ethyl-N-nitro-

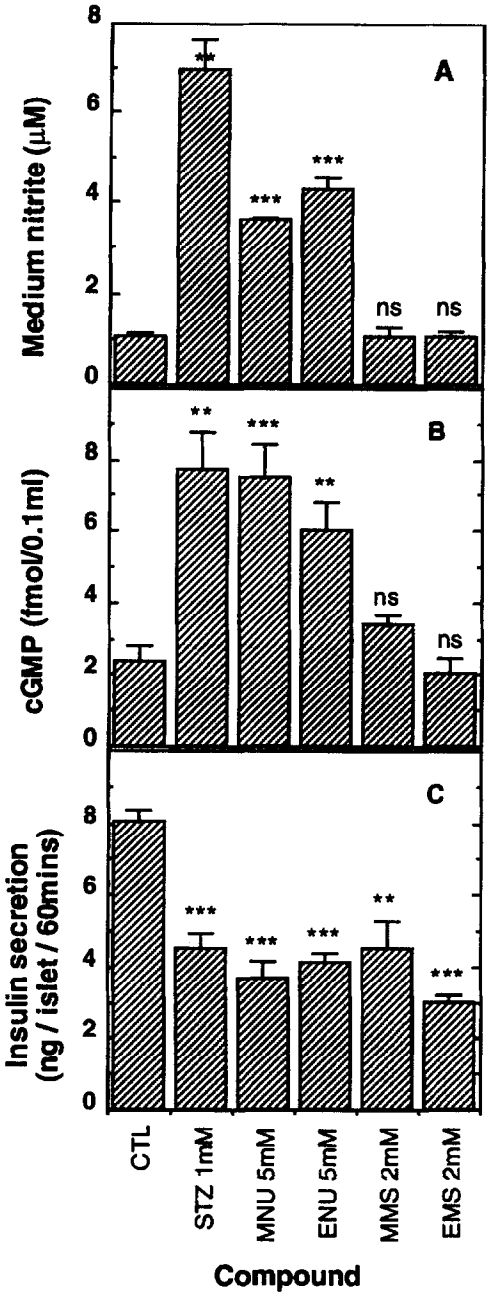

Fig. 2. Response of rat islets to alkylating agents. (A) Medium nitrite levels. (B) Islet cGMP levels. (C) Inhibition of insulin secretion. Significance levels refer to differences from control; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. Data presented from 2–3 separate experiments: $n = 8$ (A), 9 (B), and 10 (C).

sourea results in exposure of the cells to nitric oxide. This is indicated (a) by the generation of nitrite as an oxidation product of nitric oxide and (b) by a more than two-fold increase in cyclic GMP, suggesting activation of guanylate cyclase. Neither release of nitric oxide nor elevation of cyclic GMP, however, seems essential for the inhibition of glucose-stimulated insulin secretion, since EMS and MMS (which do not produce nitric oxide or raise cyclic GMP) also cause inhibition. Further evidence that nitric oxide was not necessary for nitrosourea-induced inhibition came from the experiments with

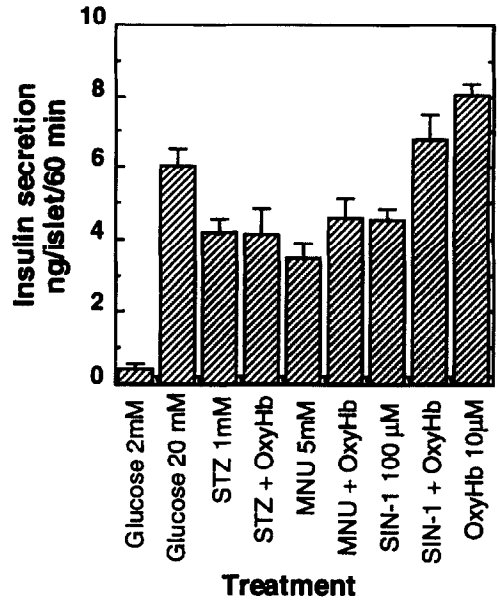

Fig. 3. Effect of Oxyhaemoglobin on nitrosourea-induced inhibition of insulin secretion. Combined results from 2 separate experiments, $n = 12$. 20 mM glucose + OxyHb gave greater insulin secretion than 20 mM glucose alone ($P < 0.001$). STZ + OxyHb gives significantly lower glucose-stimulated insulin secretion than OxyHb alone ($P < 0.001$), but not significantly higher than STZ alone. MNU + OxyHb gives significantly lower glucose-stimulated insulin secretion than OxyHb alone ($P < 0.001$), but not significantly higher than MNU alone. SIN-1 + OxyHb does not show significantly lower glucose-stimulated insulin secretion than OxyHb alone, but shows significantly higher secretion than SIN-1 alone ($P < 0.02$).

OxyHb. OxyHb is an effective protective agent against nitric oxide-induced cytotoxicity [30], and it substantially reversed the inhibition by SIN-1 of glucose-stimulated insulin secretion. It did not, however, give any significant reversal of inhibition by STZ or MNU. In the case of STZ, substantial loss of OxyHb was seen, which was further evidence for formation of nitric oxide. Our result contrasts with the observations of Kroncke *et al.*

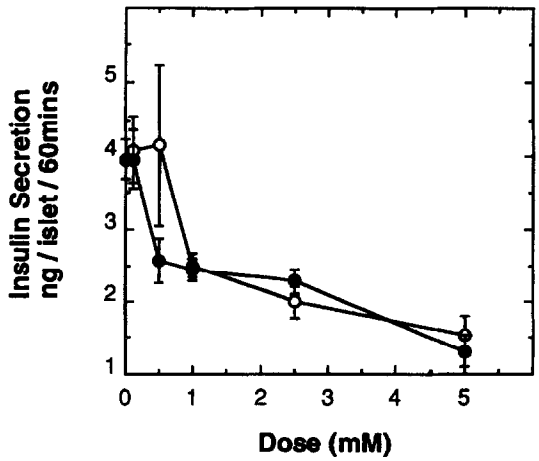

Fig. 4. Dose response for inhibition of insulin secretion by MNU and ENU. —○— MNU; —●— ENU. Data from 2 experiments, $n = 6$.

[31], who obtained protection against STZ-induced strand breakage with the nitric oxide trap Fe/DETC. Their results were obtained, however, with different cell strains, a different nitric oxide trap, and a different indicator of damage to that used in the present study. In our experiments, although nitric oxide release from nitrosoureas appears to occur, its contribution to the biological effect of these compounds is unclear.

Studies on cell lines with altered DNA repair capacities [9, 10, 32–35] suggest that O^6-alkylguanine is of major importance as a cytotoxic, as well as a mutagenic lesion. Since O^6-ethyl- is at least 10-fold less cytotoxic than O^6-methylguanine [11], if O^6-alkylation of guanine were important in inhibiting insulin secretion, MNU would be expected to be substantially more effective than ENU. In reality, ENU appears to be at least as potent as MNU. It may be that DNA is not the cellular target for this short-term effect. It has been suggested that the toxicity induced by STZ may be due to alkylation of proteins, possibly those related to the glucose-sensing mechanism of the β-cell [6] or mitochondrial proteins [8], although the actual target has not been found. If, however, alkylation of thiols were important, it would be expected that those agents with higher *s* values [19], such as MMS, would be more effective.

The results with post-treatment incubation with MNU and ENU show nonspecific toxicity, but no evidence of a persistent effect on glucose-induced insulin secretion. This is consistent with the data of Eizirik *et al.* [8], who found such an effect with STZ but not MNU. Although our results suggest that nitric oxide formation is not a necessary condition for inhibition of glucose-stimulated insulin release, it is of interest that our results, especially with OxyHb, suggest that STZ may be a more effective nitric oxide donor than MNU. Whether this is coincidence, or is related to the specificity of STZ as a diabetogenic agent, remains to be determined.

STZ and MNU have been shown to produce quite marked mitochondrial dysfunction [8], and this may be through the alkylation of mitochondrial DNA. Repair of mitochondrial DNA may be distinct from repair of overall genomic DNA [36, 37], and it is possible that O^6MeG and O^6EtG show similar toxicity in mitochondria. If so, it would remain to be determined why mitochondria should be the critical cellular target in β-cells, as opposed to alkylation of genomic DNA in other cell types. The comparison of a wider range of alkylating agents, with different spectra of damage, may be helpful in elucidating these and other questions.

Acknowledgements—We thank Dr. P. Karran for helpful advice and discussion, our colleagues Jon Mabley and Steve Thomas for their generous assistance, and one of the referees for suggesting the experiment in Fig. 3. CAD and MD were supported by BBSRC studentships. ICG was holder of the BDA-Ames Senior Research Fellowship. Work was supported in part by the British Diabetic Association, and Bundesministerium für Forschung und Technologie project 07 NBL 02-D8 (AD).

REFERENCES

1. Vavra JJ, DeBoer C, Dietz A, Hanka LJ and Sokolski WT, Streptozotocin, a new antibacterial antibiotic. In: *Antibiotics Annual, 1959–1960,* pp. 230–235. Antibiotica Inc., New York, 1960.
2. Bennett RA and Pegg AE, Alkylation of DNA in rat tissues following administration of streptozotocin. *Cancer Res* **41:** 2786–2790, 1981.
3. Voss C, Herrmann I, Hartmann K, Zuhlke H, Besch W and Keilacker H, Diabetogenic effects of N-nitrosomethylurea in rats. *Exp Clin Endocrinol* **92:** 25–31, 1988.
4. Rerup CC, Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol Rev* **22:** 485–518, 1970.
5. Gunnarsson R, Berne C and Hellerstrom C, Cytotoxic effects of streptozotocin and N-nitrosomethylurea on the pancreatic B cells with special regard to the role of nicotinamide-adenine dinucleotide. *Biochem J* **140:** 487–494, 1974.
6. Ledoux SP and Wilson GL, Effects of streptozotocin on a clonal isolate of rat insulinoma cells. *Biochim Biophys Acta* **804:** 387–392, 1984.
7. Ledoux SP, Woodley SE, Patton NJ and Wilson GL, Mechanisms of nitrosourea-induced beta-cell damage. Alterations in DNA. *Diabetes* **35:** 866–872, 1986.
8. Eizirik DL, Sandler S, Ahnstrom G and Welsh M, Exposure of pancreatic islets to different alkylating agents decreases mitochondrial DNA content but only streptozotocin induces long-lasting functional impairment of β-cells. *Biochem Pharm* **42:** 2275–2282, 1991.
9. Goldmacher VS, Cuzick RA and Thilly WG, Isolation and partial characterisation of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and N-methyl-N′-nitro-N-nitrosoguanidine. *J Biol Chem* **261:** 2462–2471, 1986.
10. Green MHL, Lowe JE, Petit-Frère C, Karran P, Hall J and Kataoka H, Properties of N-methyl-N-nitrosourea-resistant, Mex- derivatives of an SV40-immortalised human fibroblast cell line. *Carcinogenesis* **10:** 893–898, 1989.
11. Karran P and Bignami M, Self-destruction and tolerance in resistance of mammalian cells to alkylation damage. *Nucl Acids Res* **20:** 2933–2940, 1992.
12. Turk J, Corbett JA, Ramanadham S, Bohrer A and McDaniel ML, Biochemical evidence for nitric oxide formation from streptozotocin in isolated pancreatic islets. *Biochem Biophys Res Commun* **197:** 1458–1464, 1993.
13. Kwon NS, Lee SH, Choi CS, Tei K and Lee HS, Nitric oxide generation from streptozotocin. *FASEB J* **8:** 529–533, 1994.
14. Rogers NE and Ignarro LJ, Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. *Biochem Biophys Res Commun* **189:** 242–249, 1992.
15. Singer B and Grunberger D, *Molecular Biology of Mutagens and Carcinogens.* Plenum Press, New York, 1983.
16. Hall J, Kataoka H, Stephenson C and Karran P, The contribution of O^6-methylguanine and methylphosphotriesters to the cytotoxicity of alkylating agents in mammalian cells. *Carcinogenesis* **9:** 1587–1593, 1988.
17. Engelward BP, Boosalis MS, Chen BJ, Deng Z, Siciliano MJ and Samson LD, Cloning and characterization of a mouse 3-methyladenine/7-methylguanine/3-methylguanine DNA glycosylase cDNA whose gene maps to chromosome 11. *Carcinogenesis* **14:** 175–181, 1993.
18. O'Connor TR and Laval J, Human cDNA expressing a functional DNA glycosylase excising 3-methyladenine and 7-methylguanine. *Biochem Biophys Res Commun* **176:** 1170–1177, 1991.
19. Vogel EW and Nivard M, The subtlety of alkylating agents in reactions with biological macromolecules. *Mutation Res* **305:** 13–32, 1994.
20. Howell SL and Taylor KW, Potassium ions and the secretion of insulin by Islets of Langerhans incubated *in vitro*. *Biochem J* **108:** 17–24, 1968.
21. Gey GO and Gey MH, Maintenance of human normal cells and tumour cells in continuous culture. *Am J Cancer* **27:** 45–76, 1936.
22. Green IC, Delaney CA, Cunningham JM, Karmiris V and Southern C, Interleukin 1β effects on cyclic GMP and cy-

clic AMP in cultured rat islets of Langerhans—Arginine dependence and relationship to insulin secretion. *Diabetologia* **36:** 9–16, 1993.
23. Cunningham JM, Mabley JG, Delaney CA and Green IC, The effect of nitric oxide donors on insulin secretion, cyclic GMP and cyclic AMP in rat islets of Langerhans and the insulin secreting lines HIT-T15 and RINm5F. *Mol Cell Endocrinol* **102:** 23–29, 1994.
24. Green IC, Ray K and Perrin D, Opioid peptide effects on insulin release and cAMP in islets of Langerhans. *Horm Metab Res* **15:** 124–128, 1983.
25. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
26. Green L, Wagner D, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR, Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Anal Biochem* **126:** 131–138, 1982.
27. Murphy ME and Noack E, Nitric oxide assay using hemoglobin method. *Methods in Enzymology* **233:** 240–250, 1994.
28. Hevel JM and Marletta MA, Nitric-oxide synthase assays. *Methods in Enzymology* **233:** 250–258, 1994.
29. Murad F, Mittal CK, Arnold WP, Katsuki S and Kimura H, Guanylate cyclase: Activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. In: *Advances in Cyclic Nucleotide Research* (Eds. George WJ and Ignarro LJ), pp. 145–158. Raven Press, New York, 1978.
30. Walker MW, Kinter MT, Roberts RJ and Spitz DR, Nitric oxide-induced cytotoxicity: Involvement of cellular resistance to oxidative stress and the role of glutathione in protection. *Pediatric Res* **37:** 41–49, 1995.
31. Kroncke K, Fehsel K, Sommer A, Rodríguez M and Kolb-Bachofen V, Nitric oxide generation during cellular metabolization of the diabetogenic N-methyl-N-nitroso-urea Streptozotocin contributes to islet cell DNA damage. *Biol Chem Hoppe-Seyler* **376:** 179–185, 1995.
32. Samson L, Derfler B and Waldstein E, Suppression of human DNA alkylation-repair defects by *Escherichia coli* DNA repair genes. *Proc Natl Acad Sci USA* **83:** 5607–5610, 1986.
33. Ishizaki K, Tsujimura T, Yawata H, Fujio C, Nakabeppu Y, Sekiguchi M and Ikenaga M, Transfer of the Escherichia coli O^6-methylguanine methyltransferase gene into repair-deficient human cells and restoration of cellular resistance to N-methyl-N′-nitro-N-nitrosoguanidine. *Mutation Res* **166:** 135–141, 1986.
34. Brennand J and Margison GP, Reduction of the toxicity and mutagenicity of alkylating agents in mammalian cells harbouring the *Escherichia coli* alkyltransferase gene. *Proc Natl Acad Sci USA* **83:** 6292–6296, 1986.
35. Kataoka H, Hall J and Karran P, Complementation of sensitivity to alkylating agents in *Escherichia coli* and Chinese hamster ovary cells by expression of a cloned bacterial DNA repair gene. *EMBO J* **5:** 3195–3200, 1986.
36. Ledoux SP, Patton NJ, Avery LJ and Wilson GL, Repair of N-methylpurines in the mitochondrial DNA of xeroderma pigmentosum complementation group D cells. *Carcinogenesis* **14:** 913–917, 1993.
37. Driggers WJ, Ledoux SP and Wilson GL, Repair of oxidative damage within the mitochondrial DNA of RINr 38 cells. *J Biol Chem* **268:** 22042–22045, 1993.