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(^{14}C) STREPTOZOTOCIN : ITS DISTRIBUTION AND INTERACTION WITH NUCLEIC ACIDS AND PROTEINS

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Abstract—The tissue distribution and interaction with cellular nucleic acids and proteins of (2'-^{14}C) streptozotocin and (3'-methyl-^{14}C) streptozotocin has been investigated in the rat during the first hour following their intravenous injection. The investigations have been carried out with animals of two different age groups. With each labelled form of the drug, the injected radioactivity was cleared from the blood, most of it appearing in high activity in kidney and liver. Following the administration of (3'-methyl-^{14}C) streptozotocin, in all animals relatively large amounts of injected radioactivity were associated with the nucleic acid and protein fractions of pancreas, liver and kidney. In contrast, only very small amounts of injected radioactivity were associated with these organs following the administration of (2'-^{14}C) streptozotocin. The highest level of association of the methyl label was observed with pancreatic proteins. Between the two age groups, different radioactivity profiles for renal tissue were observed and some differences were detected in the association of the methyl label with the cellular macromolecules. These results are discussed in relation to the diabetogenic and tumourigenic properties of the drug.

INTRODUCTION

Streptozotocin (STZ), a *N*-nitrosoureido derivative of glucosamine, produced by *Streptomyces achromogenes* [1], induces diabetes in laboratory animals by selective destruction of pancreatic B-cells [2–4]. STZ is also known to induce tumours in the pancreas, kidney and liver [5–7] and to possess a strong mutagenic activity both *in vivo* [8] and *in vitro* [9]. In addition, STZ has been shown to possess marked antileukaemic [10] and antitumour activities [11, 12].

With STZ independently radiolabelled with ^{14}C-isotope at three positions of the molecule, it was shown [13, 14] that the patterns of elimination and metabolism for (1-^{14}C) STZ and (2'-^{14}C) STZ were similar but were very different from that of the (3'-methyl-^{14}C) STZ. Thus it was only with the latter form that radioactivity could be detected in the tissue 48 hours after administration. In a later study [15] using microautoradiographic techniques, a significant and specific accumulation of radioactivity in the pancreatic B-cells was observed after the administration of (3'-methyl-^{14}C) STZ. Part of this radioactivity appeared to be firmly bound to cellular components. Similar tissue-bound radioactivity was noted in kidney cortex and liver. Recent studies [16, 17] using (3'-methyl-^{14}C) STZ have confirmed the above findings.

Very little is known about the nature and extent of tissue binding of radioactivity at early stages following the administration of labelled STZ, in other words at a time when the first drug-induced morphological and functional changes can be detected.

The studies reported in this paper were therefore undertaken to explore the specific macromolecular binding of the labelled component of (^{14}C) STZ. It was hoped that this might lead to a better understanding of the fate and mechanism of action of the drug. Due to the short latency of the irreversible B-cell damage [18, 19] associated with STZ, the experiments were designed in order to characterize the distribution of the radioactivity during the first hour following drug administration. Due to the age dependency of STZ effects in the rat [18–20], this study was carried out in two different age groups.

In addition to providing more information on the diabetogenic properties of STZ it was hoped that this study would shed more light on the carcinogenic properties of the drug and the mechanism of action of *N*-nitroso-*N*-methyl-ureido compounds in general, this latter aspect of the study being important in view of the increasing use of STZ as an agent in the treatment of some forms of cancer [12, 21, 22].

MATERIALS AND METHODS

Radiolabelled streptozotocin. (2'-^{14}C)STZ and (3'-methyl-^{14}C)STZ (see Scheme 1) were synthetized as previously reported [13]. Both the labelled compounds had a specific radioactivity of 0.244 μCi/mg. For this purpose, (2'-^{14}C)STZ which had a higher specific radioactivity was diluted with an appropriate amount of cold STZ, synthesized by the same procedure.

Experimental animals. Male Wistar rats (n=80) supplied by Biogenes Pinner (Middlesex, U.K.) were

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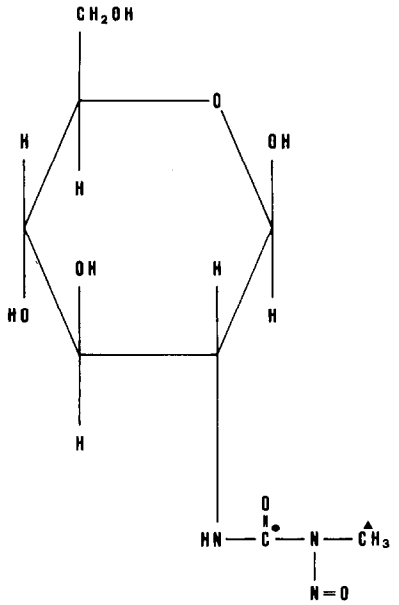

Scheme 1. The structure of streptozotocin (STZ) showing the locations of the labelled carbons (●) = 2′-^{14}C - STZ - (▲) 3′-^{14}C-methyl-STZ.

used. An experimental design of 4 randomized groups of 20 rats each, 10 rats weighing 70 g (approximate age = 30 days) and 10 rats weighing 130 g (approximate age = 45 days), was followed.

Route of administration and dose. STZ (50 mg/kg body wt) was administered intravenously via a lateral tail vein. Due to instability of STZ in aqueous media, all solutions (50 mg/ml) were prepared in 0.9 per cent saline buffered with 10 mM citrate, pH 4.5, immediately before injection.

Tissue distribution studies. Tissue samples were collected 5, 10, 20, 40 and 60 min after the administration of the labelled drug. To achieve this, animals were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (50 mg/kg body wt) 4 min before collection. Blood samples were taken by severing the femoral vessels. The exsanguinated animals were then opened by mid-abdominal incision and samples of liver, pancreas and kidney were rapidly removed and immediately frozen in liquid nitrogen and stored at −20° until the time of assay. The blood was allowed to clot, the serum was then separated by centrifugation and taken for the measurement of radioactivity. Other tissues were homogenized in 20 ml of 10 mM citrate buffer, pH 4.5, and a sample of the homogenate was taken for radioactivity determination.

Separation of nucleic acids, lipids and proteins. Aliquots of the tissue homogenate (6 ml) were mixed with 0.25 vol. of 3.5 N ice-cold perchloric acid and centrifuged in a refrigerated centrifuge. The supernatant was separated and stored as the acid-soluble fraction. The pellet was washed three times at 0° with 0.7 N perchloric acid. The nucleic acids in the sediment were then extracted by treatment with 3 ml of 0.7 N perchloric acid at 90° for 15 min [23]. The product, after cooling was centrifuged and the supernatant was collected. The nucleic acids in the supernatant were quantified by u.v. absorption spectroscopy (260 nm) and an aliquot was counted for radioactivity. From the sediment remaining after the extraction of nucleic acids, the lipids were extracted three times with 3 ml of a mixture of ethanol–diethylether (2:1, v/v) by treatment at 65° for 10 min [24]. Each time the resulting mixture was cooled and centrifuged. An aliquot of the combined supernatant was assayed for radioactivity. The final sediment, which contained the protein fraction of the tissue, was washed twice with diethylether and dried at room temperature. A sample was dissolved in 0.5 M sodium hydroxide and counted for radioactivity. The concentration of proteins was determined by the method of Lowry [25].

Radiochemical techniques. The counting of radioactivity was carried out on a Beckman LS-230 liquid scintillation counter. Both aqueous and heterogeneous samples (after neutralization) were counted using an emulsion counting system [26] which utilized toluene-TritonX-100 (2:1, v/v).

For several heterogeneous samples (i.e. tissue homogenates) a comparison was made between the toluene-Triton X-100 system and the dioxan-based scintillant with Cab-O-Sil gelling used previously [13]

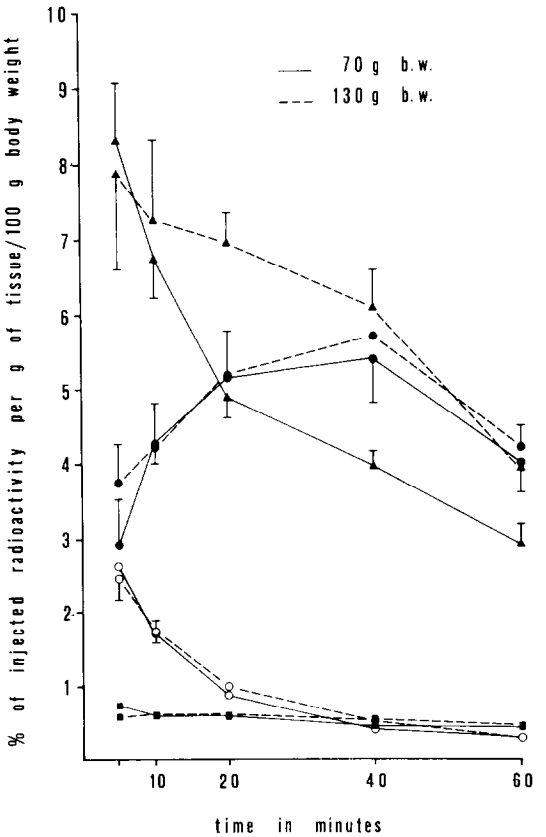

Fig. 1. Tissue radioactivity after the administration of (2′-^{14}C) streptozotocin. Twenty rats of 70 g body wt (——) and 20 rats of 130 g body wt (– –) were given (2′-^{14}C) streptozotocin (50 mg/kg) intravenously. Tissues were removed after various time intervals and assayed for radioactivity (per cent of injected radioactivity per g wet wt. of tissue/100 g body wt.). The SEM values are indicated by bars. ●, Liver; ■, pancreas; ▲, kidney; ○, blood.

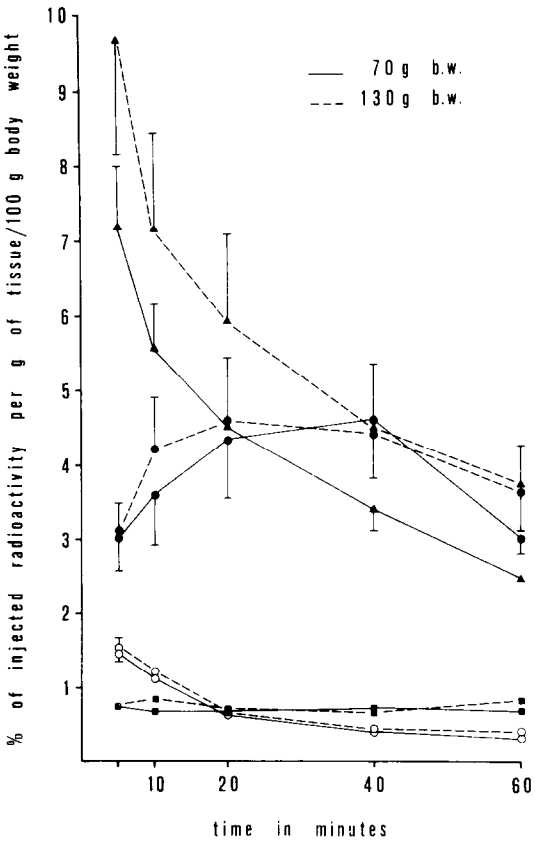

Fig. 2. Tissue radioactivity after the administration of (3′-methyl-^{14}C) streptozotocin. Twenty rats of 70 g body wt (——) and 20 rats of 130 g body wt (– –) were given (3′-methyl-^{14}C) streptozotocin (50 mg/kg) intravenously. Tissues were removed after various time intervals and assayed for radioactivity (per cent injected radioactivity per g wet wt of tissue/100 g body wt). The SEM values are indicated by bars. ●, Liver; ■, pancreas; ▲, kidney; ○, blood.

and no difference in counts per minute was found. Furthermore, the counting efficiency in the tissue homogenate samples (as measured by adding pure (^{14}C) STZ as an internal standard) was about 85 per cent, well comparable with that one achieved previously [13]. The counting efficiency for the aqueous samples was found to be 92 per cent. Toluene-soluble samples (lipid extracts) were counted in a toluene-based scintillant. The counting error of the samples was less than 3 per cent, with the only exception of lipid samples, in which the radioactivity was often not higher than the background.

Statistical analysis. Statistical significance of the results on nucleic acid and protein-associated radioactivity was evaluated by the analysis of variance (F test).

RESULTS

Tissue-distribution studies

The relative patterns of distribution of radioactivity in serum and in the whole homogenate of liver, pancreas and kidney following the intravenous administration of (2′-^{14}C) STZ and (3′-methyl-^{14}C) STZ are illustrated in Figs 1 and 2. The results obtained for each labelled form of the drug in both animal age groups are presented together in order to reveal any age-dependent difference. It is apparent that with each labelled form of STZ in both the age groups, the injected radioactivity was rapidly cleared from the bloodstream, the majority of it becoming associated with liver and kidney tissue. With each labelled form of STZ, the initial radioactivity associated with the kidney was always higher than that of the liver.

In the liver, the radioactivity increased with the time, in all cases attaining a maximum at 40 min (with the exception of 130 g rats given (3′-methyl-^{14}C) STZ in which the peak levels were reached 20 min post-administration). In the kidney, the percentage of the injected radioactivity peaked as early as 5 min after the administration of each labelled form of STZ. It was apparent that the radioactivity profile for renal tissue declined at a faster rate in young rats (70 g body wt) than in older rats (130 g body wt).

A small but appreciable accumulation of radioactivity in the pancreas was seen 20 min following

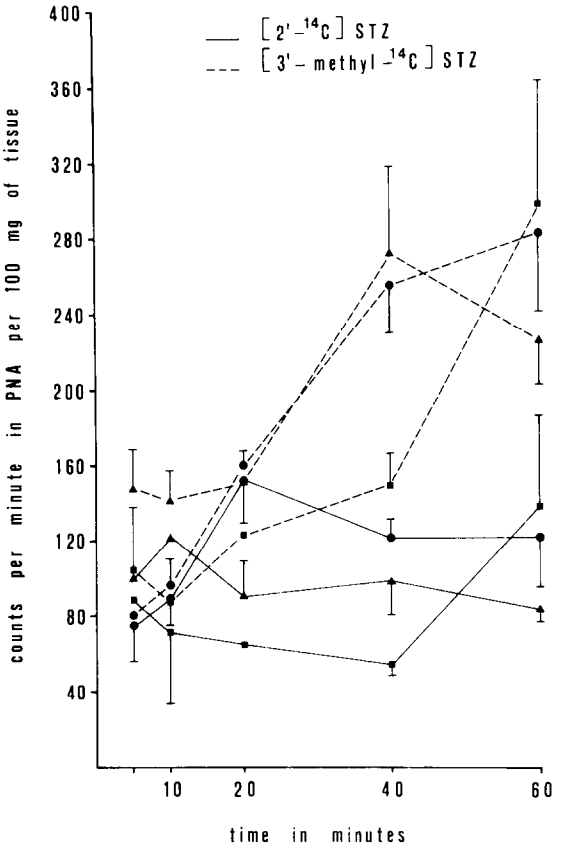

Fig. 3. Radioactivity associated with cellular nucleic acids after the administration of labelled streptozotocin to 70 g body wt rats. Rats (n = 40; 70 g body wt) were separately administered 50 mg/kg of (2′-^{14}C) streptozotocin (——) and (3′-methyl-^{14}C) streptozotocin (— —) intravenously. Tissues were removed at various time intervals, pentose nucleic acids (PNA) were separated and assayed for radioactivity (cpm/100 mg wet wt of tissue). The SEM values are indicated by bars. For statistical significance, refer to Fig. 4. ●, Liver; ■, pancreas; ▲, kidney.

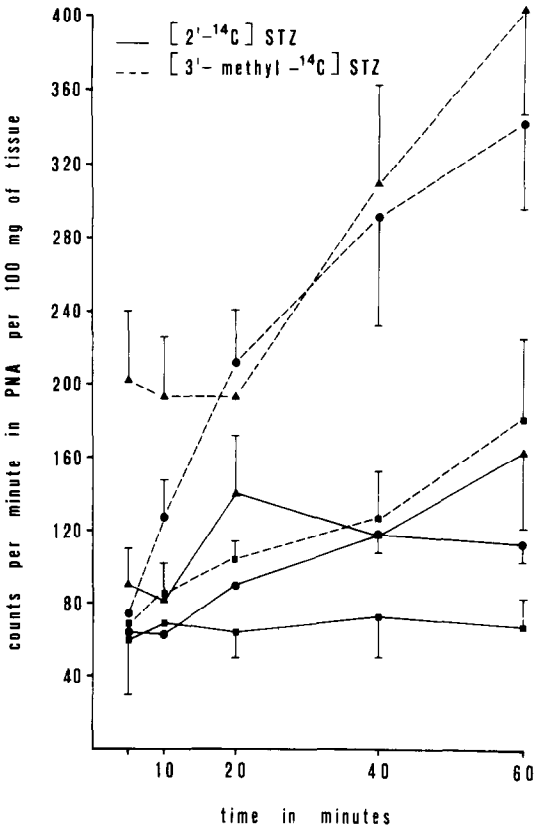

Fig. 4. Radioactivity associated with cellular nucleic acids after the administration of labelled streptozotocin to 130 g body wt rats. Rats ($n = 40$; 130 g body wt) were separately administered 50 mg/kg of (2'-^{14}C) streptozotocin (——) and (3'-methyl-^{14}C) streptozotocin (— —) intravenously. Tissues were removed at various time invervals, pentose nucleic acids (PNA) were separated and assayed for radioactivity (cpm/100 mg wet wt of tissue). The SEM values are indicated by bars. Data reported in Figs 3 and 4 were assayed together for statistical significance by the analysis of variance (F test). The effects of the following factors were significant: type of STZ ($P < 0.001$), type of tissue ($P < 0.001$), time ($P < 0.001$) and the interactions type of tissue × age ($P < 0.01$), type of tissue × type of STZ ($P < 0.05$), time × type of STZ ($P < 0.001$), time × type of tissue ($P < 0.05$), time × age × type of tissue × type of STZ ($P < 0.05$). The effect of age and all the other interactions were not significant. ●, Liver; ■, pancreas; ▲, kidney.

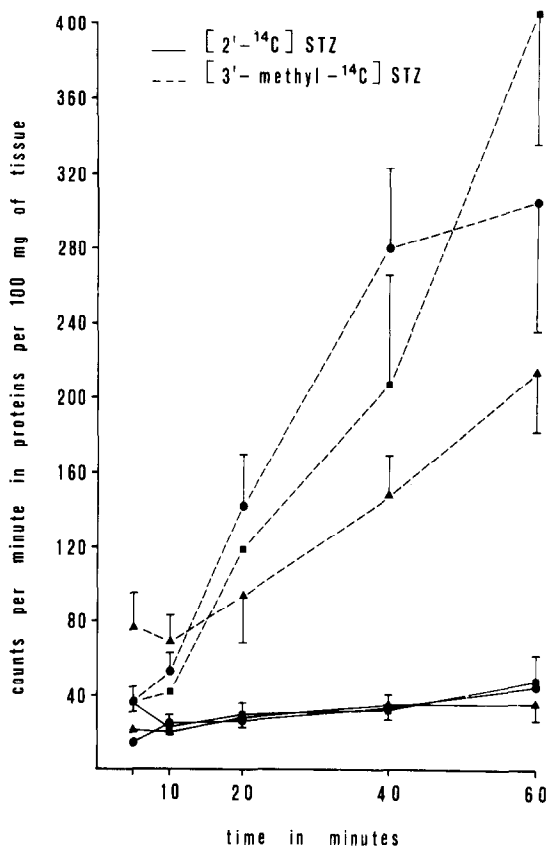

Fig. 5. Radioactivity associated with cellular proteins after the administration of labelled streptozotocin to 70 g body wt rats. Rats ($n = 40$; 70 g body wt) were separately administered 50 mg/kg of (2'-^{14}C) streptozotocin (——) and (3'-methyl-^{14}C) streptozotocin (— —) intravenously. Tissues were removed at various time intervals, proteins were separated and assayed for radioactivity (cpm/100 mg wet wt of tissue). The SEM values are indicated by bars. For statistical significance, refer to Fig. 6. ●, Liver; ■, pancreas; ▲, kidney.

the administration of (3'-methyl-^{14}C) STZ. This level then increased slowly but steadily.

Studies with proteins and nucleic acids

The relative association of injected radioactivity with cellular nucleic acid and protein of the pancreas, kidney and liver following the administration of (2'-^{14}C) STZ and (3'-methyl-^{14}C) STZ are illustrated in Figs 3–6. It should be pointed out that the absolute levels of this associated radioactivity represent only a small portion of the radioactivity of the whole homogenate (indeed, most of the radioactivity was in the acid-soluble fraction).

From an examination of Figs 3–6, it appears that only a barely detectable level of the injected radioactivity was associated with both nucleic acid and protein of the three tissues after the administration of (2'-^{14}C) STZ to either 70 g body wt or 130 g body wt rats. In contrast, with (3'-methyl-^{14}C) STZ relatively high levels of injected radioactivity were associated with the nucleic acid and protein in each of the tissue types investigated. These levels appear to be higher the longer was the exposure to the drug (but the possibility of interactions with the injected barbiturate anaesthetic in the 5 and 10 min groups should be taken into account).

In the pancreas, the radiolabel from (3'-methyl-^{14}C) STZ was incorporated to a greater extent with protein than with nucleic acid. The level of association of methyl label with pancreatic proteins was approximately linear with time and by 60 min exceeded that of liver and kidney. Since the radioactivity measured in the whole homogenate was the lowest, this indicates that the ratio between the protein-associated radioactivity and total radioactivity in the pancreas is significantly greater than in the liver and kidney. A similar, but less marked phenomenon occurs for nucleic acid.

As regards the two age groups, the amount of the

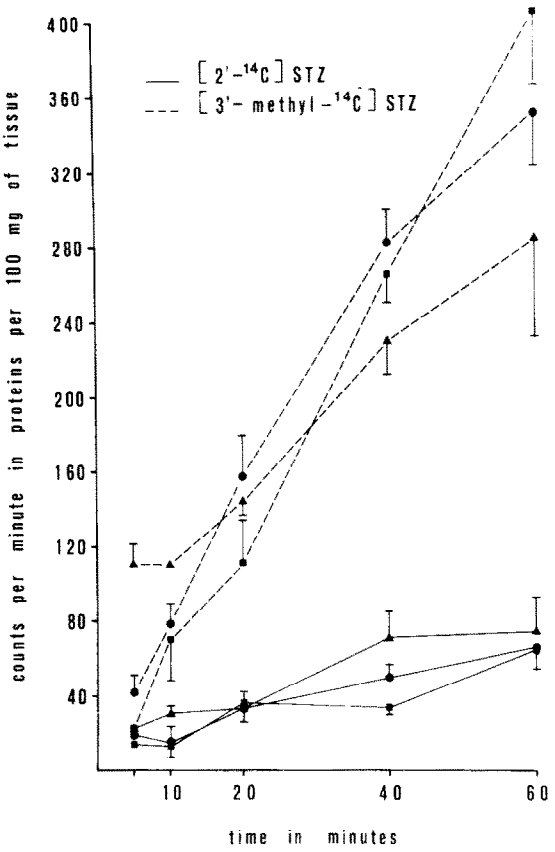

Fig. 6. Radioactivity associated with cellular proteins after the administration of labelled streptozotocin to 130 g body wt rats. Rats ($n = 40$; 130 g body wt) were separately administered 50 mg/kg of (2'-^{14}C) streptozotocin (——) and (3'-methyl-^{14}C) streptozotocin (— —) intravenously. Tissues were removed at various time intervals, proteins were separated and assayed for radioactivity (cpm/100 mg wet wt of tissue). The SEM values are indicated by bars. Data reported in Figs 5 and 6 were assayed together for statistical significance by the analysis of variance (F test). The effects of the following factors were significant: type of STZ ($P < 0.001$), time ($P < 0.001$), age ($P < 0.001$) and the interactions type of STZ × type of tissue ($P < 0.05$), time × type of STZ ($P < 0.001$) time × type of STZ × type of tissue ($P < 0.001$). The effect of the type of tissue and all the other interactions were not significant. ●, Liver; ■, pancreas; ▲, kidney.

methyl label associated with nucleic acid and protein in the kidney was higher in older animals than in younger ones. This may reflect the higher radioactivity found in the renal homogenate of 130 g body wt rats. In the pancreas, however, no age-related differences were observed in the protein-associated radioactivity, but more radioactivity was associated with nucleic acid in younger rats.

Finally, it is noteworthy that the lipid extracts of the tissue were always free of any detectable radioactivity.

DISCUSSION

The results of the tissue-distribution studies, reported in Figs 1 and 2, in accordance with previous reports [13, 14, 16, 17], are indicative of the important role played by the liver and the kidney in the metabolism and excretion of STZ. In fact, the profile for hepatic radioactivity (which is not accounted for by blood or tissue-bound radioactivity, see Figs 3–6) should reflect an intracellular accumulation of the drug and/or its metabolites, and the high initial level of renal radioactivity should account for the maximum excretion of unchanged drug during the first hour following the administration of STZ [27].

With reference to the pancreas, our results confirm the accumulation of the radioactivity in this tissue after the administration of (3'-methyl-^{14}C) STZ, but show that this accumulation can occur earlier than previously reported [14].

From the studies of the interaction of labelled STZ with the tissue macromolecules, it is apparent that in as short a time as one hour after administration of (3'-methyl-^{14}C) STZ (i.e. sufficient time for the induction of irreversible cellular damage) a very significant association of the radiolabel from the methyl group of the nitrosourea moiety of the molecule, is observed in nucleic acid and protein (but not in lipids) from pancreas, liver and kidney. This observation, together with the lack of comparable radioactive binding following the administration of (2'-^{14}C) STZ, provides further evidence for the previously proposed [28] release of the active *N*-methyl-*N*-nitroso group from STZ *in vivo*. This scission of the STZ molecule may be the result of a metabolic process, spontaneous chemical decomposition or both [3].

In order to further define the biological significance of the early retention of the methyl-^{14}C-label in tissue, we have calculated the absolute quantity of ^{14}C-atoms associated with the binding macromolecules. Thus, 60 minutes following the administration of (3'-methyl-^{14}C) STZ (specific activity 0.244 μCi/mg), the amounts of ^{14}C-atoms (n atoms/mg protein) associated with the protein from liver, pancreas and kidney were 0.16 ± 0.01, 0.27 ± 0.03, 0.14 ± 0.01 in 70 g body wt rats and 0.19 ± 0.02, 0.23 ± 0.04, 0.16 ± 0.03 in 130 g body wt rats respectively. The corresponding figures for nucleic acid were 0.025 ± 0.003, 0.010 ± 0.003, 0.023 ± 0.003 n atoms/μmol phosphate in 70 g body wt rats and 0.031 ± 0.004, 0.007 ± 0.002, 0.041 ± 0.011 n atoms/μmol phosphate in 130 g body wt rats respectively. The latter observation suggests that 1–3 extra atoms of carbon per 100,000 nucleotide residues have been bound to nucleic acid. This figure may well be an under-estimation, because some loss of labile methylated nucleic acid may have occurred during the extraction process. However, this result may indicate a significant structural change in the nucleic acid.

As regards proteins (some of which might also have been lost during the extraction), assuming for them an average molecular weight of 100,000, it can be estimated that 2 extra atoms of carbon are bound to every 100 molecules of protein in the pancreas. It could be that these bound carbon atoms induce some structural or functional change in the protein molecule which may contribute to the observed cellular damage. The calculation used was based on the assumption that the bound radioactivity is uniformly distributed throughout the pancreas. However, it is likely that the labelling of the proteins (as well as

the nucleic acids) would be greater in the endocrine than in the acinar tissue, since it has been reported that the islets of Langerhans preferentially accumulate radioactivity after the administration of (3′-methyl-^{14}C) STZ [15, 17].

Studies are in progress to investigate the chemical nature of the binding of the 3′-methyl-carbon atom of the drug to tissue DNA. Our current findings strongly suggest that alkylation reactions with the tissue macromolecules take place a few minutes after the administration of (3′-methyl-^{14}C) STZ. However, it is also possible that some of the 3′-methyl-carbon atoms may be incorporated in the tissue one-carbon pool and subsequently become bound to the macromolecules via appropriate metabolic pathways. However, this latter possibility is not thought to play a primary role in the incorporation of radioactivity during the short time periods used in this study [29].

It is likely that the mechanism of the tumourigenic action of STZ is similar to that one of *N*-methyl-*N*-nitrosourea (MNU), the aglycone of STZ. MNU, a potent carcinogen with a relative specificity for the nervous system [30, 31] has been shown to result in methylation of cellular DNA and proteins when administered to laboratory animals [31–34]. Among the methylated products of DNA resulting from MNU treatment is O^6-methylguanine, which has been suggested to be promutagenic [35, 36] and found to cause miscoding in an RNA polymerase system *in vivo* [37]. Further investigations should be performed to assess the possibility that the tumourigenic action of STZ can be also mediated via the formation of O^6-methylguanine.

The diabetogenic activity of STZ deserves additional comment. It is noteworthy that MNU is also able to induce diabetes in some species, but only if administered in high doses [38]. On the other hand, the administration of STZ alone, at low doses, can induce islet cell tumours with high frequency [7]. From these studies it has been suggested that the diabetogenic and tumourigenic properties of STZ are quite distinct. Such a hypothesis is further supported by the fact that nicotinamide can provide complete protection against the diabetogenic action without impairing the tumourigenic properties of the drug. It may be that the methylation of cellular proteins in the B-cells plays some role in the induction of diabetes. Such a possibility would be consistent with our observation of high levels of binding of methyl label to pancreatic proteins.

However, it has to be mentioned that the diabetogenic effect of STZ—which is correlated with cellular NAD depletion [39] — might be the consequence of an increased rate of poly ADP-ribosylation, which has been suggested to be strongly involved in the repair of damaged DNA [40]. In fact, an increased activity of poly (ADP-ribose) synthetase, an NAD degradating enzyme, has been shown to be induced by nitrosourea compounds including STZ, in many eukariotic cells [40–42].

It is interesting to note the lack of any labelling of lipids after the injection of (^{14}C) STZ. This might indicate that the reported early membrane alterations in the B-cells induced by STZ (either administered *in vivo* [43] or added *in vitro* to isolated islets [44], are not likely to be due to a direct interaction of STZ with the membrane phospholipids. Finally, with reference to the age-dependent differences in the tissue distribution of STZ, the radioactivity found in the kidney homogenate, following the injection of each type of (^{14}C) STZ, was higher in older rats than in younger rats (Figs 1 and 2). The radioactivity associated with renal nucleic acid and protein was also higher in older rats (Figs 3–6). However, these age-dependent differences, even if interpreted as the result of different rates of excretion of the drug and/or its metabolites, do not appear to play a role in decreasing the concentration and the biological activity of STZ in pancreas and liver, since neither the blood nor the tissue profiles of radioactivity show comparable age-related patterns. Furthermore, the incorporation of the 3′-methyl-^{14}C label in pancreatic protein appears to be quite similar between the rats of different age groups. These findings tend to rule out the possibility that age-dependent changes in the metabolism and excretion of STZ could explain the different diabetogenic effect of the drug on animals of different ages. It is therefore more likely that, as previously proposed [45], the change of sensitivity to STZ during growth reflects an age-dependent decrease of the functional reserve of the endocrine pancreas. It is also noteworthy that in younger rats a significantly larger amount of radioactivity from (3′methyl-^{14}C) STZ was associated with the pancreatic nucleic acids in contrast to the older rats. This may be related to a less well developed capacity in younger rats to remove methylated bases from pancreatic DNA, similar to that reported [46] in brain of newborn rats treated with *N*-ethyl-*N*-nitroso-urea. On the basis of this hypothesis, future investigation might show a higher incidence of pancreatic tumours in younger rats.

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