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Autoradiographic Study of the Distribution and Cellular Uptake of (^{14}C)-Streptozotocin in the Rat

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Summary. The distribution and cellular accumulation, in the rat, of three specifically ^{14}C-labelled forms of streptozotocin were investigated. A significant pancreatic accumulation of radioactivity was observed with (3′-methyl-^{14}C)-streptozotocin only. Autoradiographic studies revealed high levels of bound radioactivity in the islet tissue following the administration of (3′-methyl-^{14}C)-streptozotocin whereas much lower levels of radioactivity were detected in the pancreatic tissue following the administration of either (1-^{14}C)-streptozotocin or (2′-^{14}C)-streptozotocin. In the pancreas, the amount of radioactivity bound to islet tissue was always significantly higher than that bound to acinar tissue. In addition to the islet tissue, the kidney cortex showed a very high level of bound radioactivity after the administration of (3′-methyl-^{14}C)-streptozotocin. The results suggest that streptozotocin is rapidly metabolised by the rat. The apparent specificity for the accumulation of radiolabel from (3′-methyl-^{14}C)-streptozotocin suggests that a metabolite derived from the methyl bearing ureido side chain of the drug may be specifically involved in the induction of tissue damage and the consequent development of diabetes.

Key Words: Streptozotocin-diabetes, autoradiography, radioactive, pancreas, liver, kidney, blood, metabolism, distribution.

Streptozotocin is a broad spectrum antibiotic produced by *Streptomyces achromogenes var* 128 [1]. In 1963 Rakienten et al., [2] reported that streptozotocin induces diabetes in rats and dogs and exhibits a specific toxicity for the B-cells of the pancreatic islets of Langerhans. The diabetogenic action of the drug in a number of laboratory animals has been confirmed by several authors [3–6] and streptozotocin has now replaced alloxan as the drug of choice for the induction of experimental diabetes [7–9]. However, despite its extensive usage, the metabolic fate and the mode of action of streptozotocin have not been satisfactorily explained.

In an attempt to elucidate the distribution, metabolism, excretion and mode of action of streptozotocin we have recently devised and reported [10] the chemical synthesis of streptozotocin specifically and independently radiolabelled with ^{14}C at three positions in the molecule (Fig. 1). In these studies we also reported that whole-body autoradiographic experiments revealed that following the administration of radiolabelled streptozotocin there was marked but transient hepatic and renal uptake of injected radioactivity, followed by a rapid renal excretion. Other than a greater residual bound radioactivity after 24 h, for (3′-methyl-^{14}C)-streptozotocin, no major differences

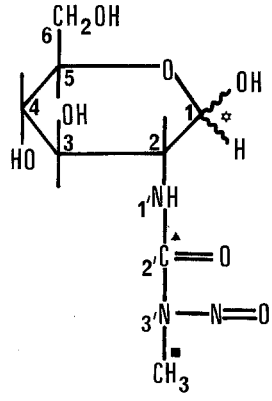

Fig. 1. 2-Deoxy-2(3′-methyl-3′-nitrosoureido)-D-glucopyranose (Streptozotocin). Streptozotocin was synthesized specifically labelled with ^{14}C. (✡), (1-^{14}C)-streptozotocin; (▲), (2′-^{14}C)-streptozotocin; (■), (3′-methyl-^{14}C)-streptozotocin

in the distribution patterns were evident for the three labelled forms of streptozotocin. No pancreatic accumulation of the radioactivity was apparent. In view of the reported [2] marked specificity of streptozotocin for pancreatic islets, this latter observation was somewhat surprising. However, our inability to observe radioactivity in the pancreas using whole-body autoradiography did not preclude the possibility of the accumulation of small, yet significant, quantities of the drug or its metabolites [11] and it could well be that accumulation did occur, but that its detection was masked by the relatively high levels of extra-pancreatic radioactivity.

The possibility of a small accumulation of radioactivity in the pancreas following the administration of (^{14}C)-streptozotocin was initially evaluated in a tissue distribution study in which the level of radioactivity in the pancreas and blood was measured for 6 h after the administration of the drug. Then, in order to characterise further the pancreatic, hepatic and renal involvements in the metabolism and mode of action of streptozotocin, and also to distinguish between any accumulation of radioactivity in the acinar and islet tissue of the pancreas, an autoradiographic study was undertaken.

Materials and Methods

Experimental Animals

Male Sprague-Dawley rats (200 ± 10 g body weight) maintained on a standard laboratory diet were used in all studies.

Radiolabelled Streptozotocin

(1-^{14}C)-streptozotocin, (2′-^{14}C)-streptozotocin and (3′-methyl-^{14}C)-streptozotocin (specific activity 2–3 μCi/mg) were synthesised as reported by Karunanayake et al. [10]. The radiolabelled drug was administered (70 mg/kg body weight, 30–40 μCi/animal) via a lateral tail vein while the animal was under light diethyl ether anaesthesia. Due to the instability of streptozotocin in aqueous media, the drug was dissolved (70 mg/ml) in citrate buffer (0.01 M, pH 4.5) immediately before administration.

Distribution of Radioactivity in Blood and Pancreas

Rats (n = 12) were separately injected with each of the labelled forms of streptozotocin. At the end of the experimental period (ranging from 1 min to 6 h) the animals were lightly anaesthetized with diethyl ether and the abdominal cavity was opened. The abdominal aorta was cannulated and the blood collected into heparinised tubes. The pancreas was then rapidly removed, frozen in liquid nitrogen and powdered in a percussion mortar. The finely powdered pancreas (0.5–1.0 g) and blood (1 ml) were separately homogenized with 25 ml of citrate buffer (0.01 M, pH 4.5). Samples (3 × 1 ml) were assayed for radioactivity in a Beckman LS-200B liquid scintillation counter, using a Cab-O-Sil/dioxan scintillation system [10].

Autoradiography

Two techniques of tissue preparation were used. The first was a conventional fixation method in which only tissue bound radioactivity was retained and detected. The second was a freezing method which retains both soluble and the non-diffusible, tissue bound radioactivity.

a. Fixation Method [12]. In this method, at the end of the experimental period and prior to collection, the tissues (kidney, liver and pancreas) were perfused in a retrograde direction, via the aorta, with Locke's solution (37° C, 100 mmHg, 5 min) followed by fixative (3% glutaraldehyde in cacodylate buffer, pH 7.0, 0.1M, 10 min). The tissues were collected and placed in buffered formalin (10% w/v, pH 7.0) for 24 h. The specimens were then washed in Sorensen's phosphate buffer (pH 7.0), dehydrated in an ascending series of alcohols, cleared in xylene and embedded in Fibrowax (melting point 56° C). Aliquots (1 μl) of all processing solutions were retained for scintillation counting. Sections (4 μ) were cut and were then coated with Kodak AR 10 stripping film and exposed in a silica gel dry atmosphere at 4° C for 12 weeks. The autoradiographs were developed in Kodak D 19 developer and stained with haemotoxylin and eosin.

b. Freezing Method [12]. In this method Arcton 12 (Imperial Chemical Industries, London, U. K.), cooled with liquid nitrogen, was used to freeze the tissues. In order to retain the nephron patency, the kidneys were frozen *in situ*. Other tissues (liver and pancreas) were rapidly excised before freezing. Cryostat sections (5 μ) were cut and autoradiographs prepared by the method of Appleton [12], with the modification that sections were mounted on to pre-emulsioned slides (rather than cover slips, as described in the original method). The preparations were exposed at –30° C for 6 weeks. The autoradiographs were developed in Kodak D 19 developer and were stained as described above.

Quantitation of Autoradiographs

For quantitation, sections were examined and silver grains counted using a Leitz Ortholux microscope with a X10 eye piece containing 0.37 mm^2 graticule

and X100 oil immersion objective. Six fields, covering two or more sections, were chosen randomly for liver, kidney and pancreas. The results are given as mean ± SEM.

Results

Distribution of Radioactivity in Blood and Pancreas

The level of radioactivity in blood and pancreas following the administration of each of the three labelled forms of streptozotocin is illustrated in Figure 2. With all three radiolabelled forms of the drug only a very small percentage (0.15–1.5%) of the injected dose of radioactivity remained in the blood 10 min after injection. This observation emphasises the very rapid rate of clearance of the drug from the blood stream. Small amounts of radioactivity were detected in all pancreatic preparations from rats receiving each of the three radiolabelled forms of the drug. Due to the vascular volume of the pancreas a pre-requisite for evidence of tissue accumulation of radioactivity at any time must therefore be that the level of radioactivity in the pancreas is higher than that in the blood at that time. With (1-^{14}C)-streptozotocin, the level of radioactivity in the pancreas was very low and did not exceed the blood levels during the entire experimental period. With (2′-^{14}C)-streptozotocin the level of radioactivity in the pancreas was slightly greater than that in the blood after 120 min. However, with (3′-methyl-^{14}C)-streptozotocin, in marked contrast to the other two labelled forms, there was a clear cross-over after 90 min, with a significantly higher specific radioactivity in the pancreas than in the blood for the remainder of the experimental period.

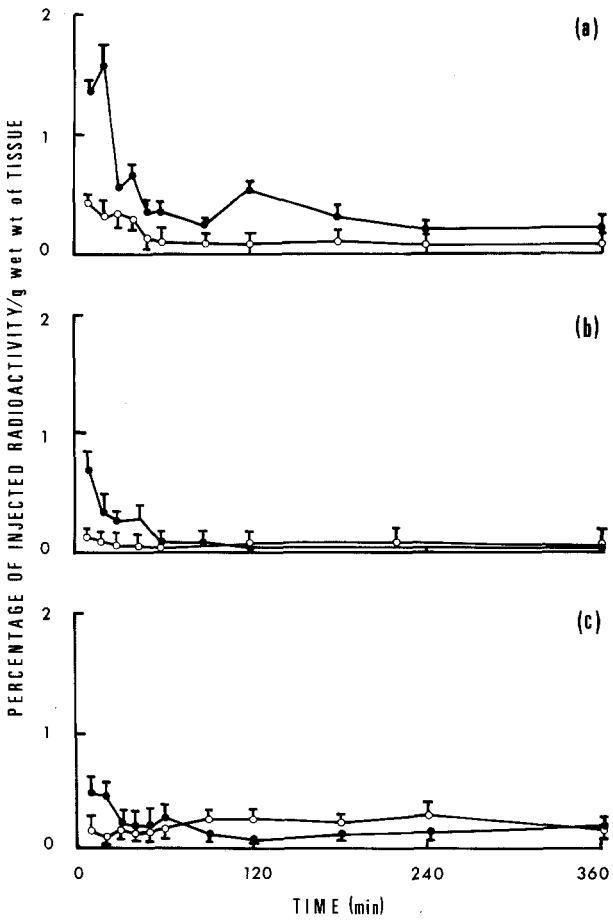

Fig. 2. Radioactivity in blood and pancreas after the administration of ^{14}C streptozotocin. Rats (= 12) were given ^{14}C-streptozotocin (70 mg/kg intravenously). (a) (1-^{14}C)-streptozotocin; (b) (2′-^{14}C)-streptozotocin; (c) (3′-methyl-^{14}C)-streptozotocin. Tissues were removed after various time intervals and assayed for radioactivity (% of injected radioactivity/g wet weight of tissue). The SEM values are indicated by bars. (•), blood; (○), pancreas

Autoradiography

The results have been assessed on the basis that the autoradiographs prepared from frozen sections contain both freely diffusible and tissue-bound radioactivity, whereas in autoradiographs prepared from fixed sections, the processing procedure washes out all freely diffusible isotope and only tissue-bound radioactivity remains. The complete elution of soluble isotope from the fixed sections was confirmed by scintillation counting of sequential washing solutions used in the fixation process. While the frozen section method offers the advantage of revealing total tissue radioactivity, this procedure does have several limitations. It is probable that the quantitation of the autoradiographs from frozen tissue is somewhat less reliable than that from fixed material, since the freeze-drying of the sections creates a variable topography (D. T. Clarkson-personal communication). Under these conditions, non-cellular regions (e. g. nephron lumina) dry down to an extremely thin layer while the cells present a thicker section on the slide.

Examination of frozen sections (free plus bound radioactivity) revealed a marked concentration of radioactivity for all tissues after 1 h (see renal cortex, Fig. 3). After 6 h this radioactivity declined greatly. Grain counting revealed that the extent of this loss was compatible with the results obtained from whole-body autoradiography and tissue distribution studies [10]. The amounts of radioactivity detected in frozen tissues after 6 h were very similar to those detected in the fixed sections, and suggests that after 6 h only tissue-bound radioactivity remains.

The grain-counting results for fixed sections of each tissue with each labelled form are shown in Figure 4a for 1 h and Figure 4b for 6 h. With (1-^{14}C)-

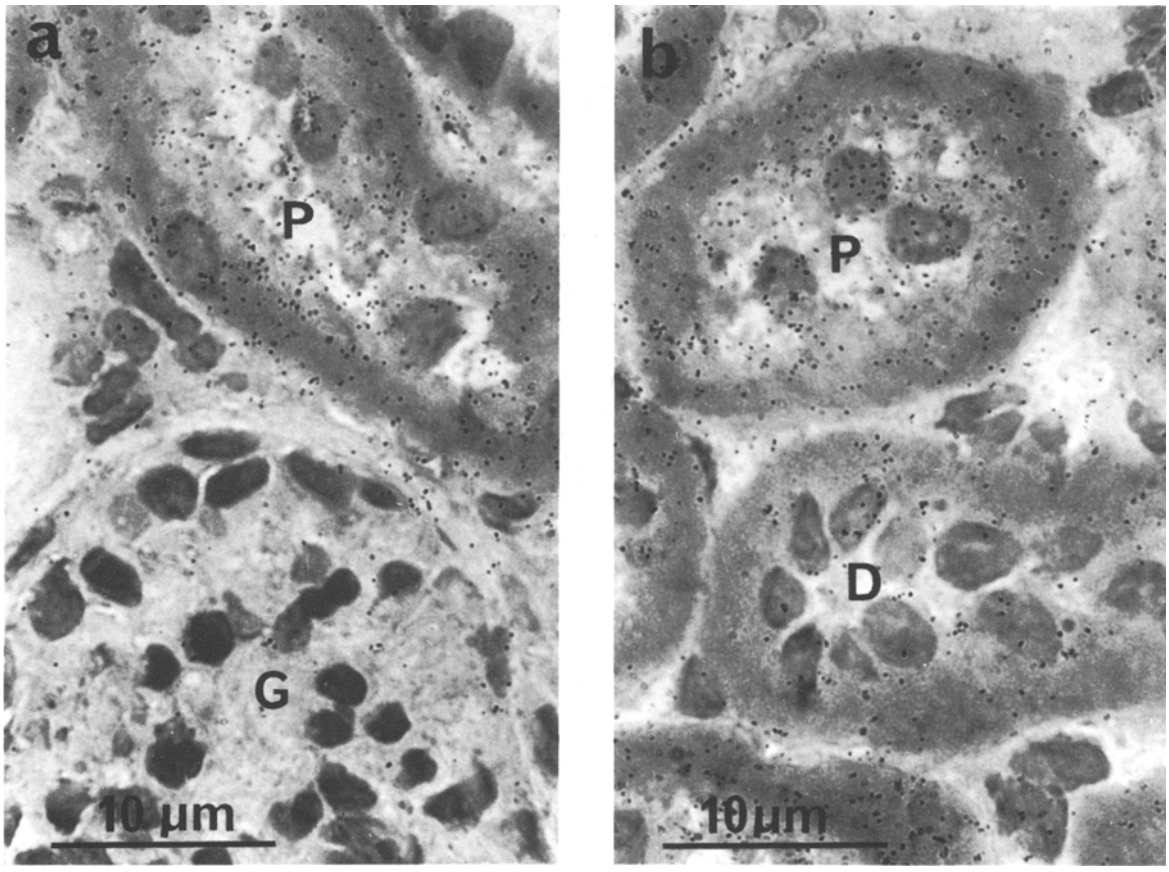

Fig. 3. (a) Autoradiograph of renal cortical tissue (frozen section) collected 1 hour after the administration of (3′-methyl-^{14}C)-streptozotocin. The label in the proximal tubule (P) appears more dense than in the glomerulus (G). Magnification × 3000. (b) Autoradiograph of the same tissue but showing the relative high density of label in the proximal tubule (P) as compared to the distal tubule (D). Magnification × 3000

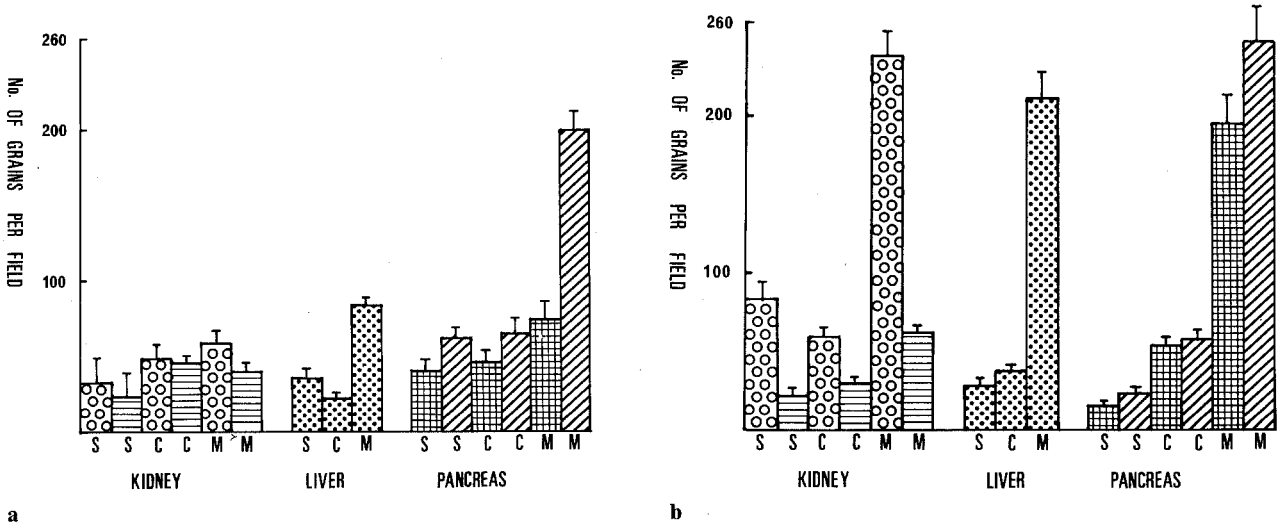

Fig. 4. ^{14}C radioactivity (as determined by grain-counting) in various rat tissues (fixed sections) (a) 1 h (b) 6 hours after the administration of each of the three radio labelled forms of streptozotocin. S = (1-^{14}C)-streptozotocin; C = (2′-^{14}C)-streptozotocin; M = (3′-methyl-^{14}C)-streptozotocin; ( ) = kidney cortex; ( ) = kidney medulla; ( ) = liver; ( ) = pancreatic acinar tissue; ( ) = pancreatic islet tissue. Multiple samples were analysed for each tissue type (see the text) and the bars represent the standard errors of the mean

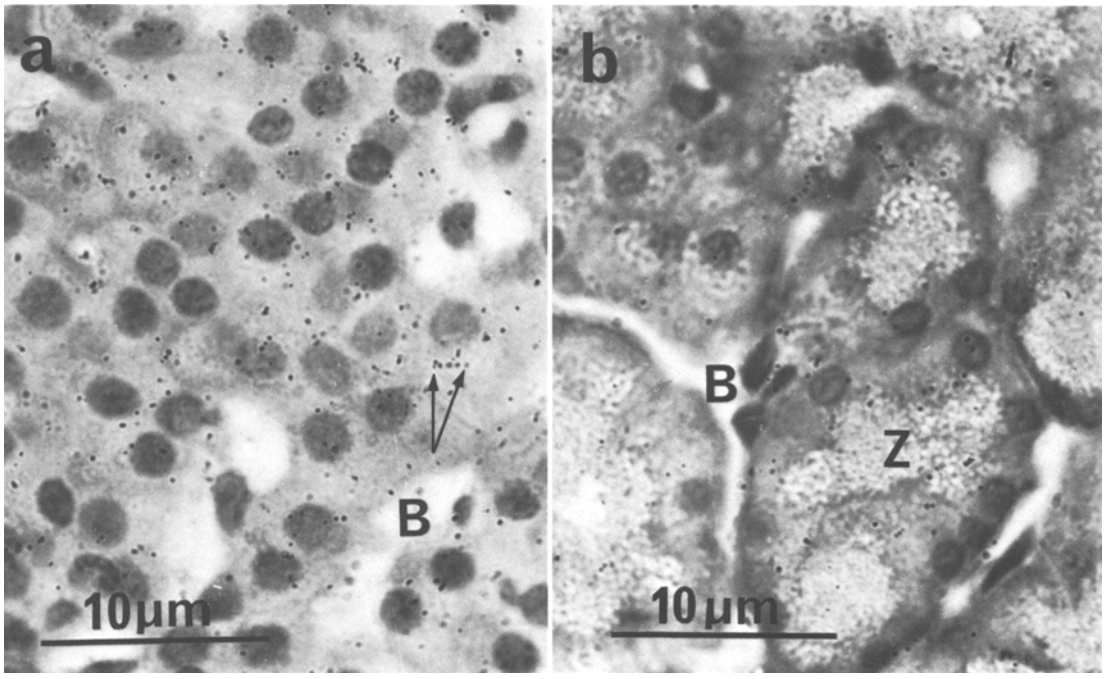

Fig. 5. Autoradiographs of pancreas (fixed section) collected 1 hour after the administration of (3′-methyl-^{14}C)-streptozotocin. Islet tissue (a) is more intensely labelled than the adjacent acinar region (b). B, blood space; Z, zymogen; arrows, one of many beta-tracks which were counted as one disintegration only for quantitation of the autoradiographs. Magnification × 3000

streptozotocin and (2′-^{14}C)-streptozotocin after 1 h only a small amount of radioactivity was bound to hepatic, renal and pancreatic tissue. In contrast, with (3′-methyl-^{14}C)-streptozotocin significantly more binding was observed in most tissues, particularly pancreas. Further, in the pancreatic preparations the amount of radioactivity bound to islet tissue was always significantly higher than that bound to acinar tissue. After 6 h only small amounts of (1-^{14}C)-streptozotocin and (2′-^{14}C)-streptozotocin remained bound to hepatic, renal and pancreatic tissue. In marked contrast, with (3′-methyl-^{14}C)-streptozotocin all three tissues showed large amounts of bound radioactivity (see pancreas in Fig. 5) and in addition, these amounts were greater than that observed after 1 h. This high residual radioactivity is in accordance with our previous observations [10]. It is also noteworthy that in the kidney the cortical tissue showed a very high level of bound radioactivity 6 h after the administration of (3′-methyl-^{14}C)-streptozotocin.

Discussion

The tissue distribution studies reported in this paper have revealed that, following the administration of each of the three radiolabelled forms of streptozotocin, only (3′-methyl-^{14}C)-streptozotocin leads to a pancreatic accumulation of radioactivity which is significantly in excess of that found in the blood. These results were confirmed and extended in the autoradiographic studies where high levels of bound radioactivity were detected in the islet tissue following the administration of (3′-methyl-^{14}C)-streptozotocin, whereas much lower levels of radioactivity were detected in the pancreas following the administration of either (1-^{14}C)-streptozotocin or (2′-^{14}C)-streptozotocin. The time course that we have observed for the accumulation of radioactivity is very similar to the time course of the pathogenesis of streptozotocin-induced B-cell necrosis that has been reported by Howell and Whitfield [13]. Furthermore, the apparent specificity for the accumulation of the (3′-methyl-^{14}C)-streptozotocin may suggest that a metabolite derived from the methyl-bearing ureido side chain of the drug may be specifically involved in the induction of tissue damage and the consequent development of diabetes.

In the light of the proposed [14] metabolic cleavage of the terminal methyl group, particularly in the pancreas, it may be possible to make some deductions about the mode of action of the drug. In view of the known [15] biological interactions of N-alkyl-N-nitroso compounds with cellular components and the ability of such compounds to alkylate DNA, RNA and

proteins [16], it is possible that streptozotocin or one of its metabolites may be responsible for tissue damage through a process of methylation. However, such a general methylation of renal, hepatic and pancreatic tissue would not explain the reported specificity of streptozotocin for the pancreatic B-cells. It may well be that the sugar moiety acts as a carrier, thereby facilitating the transport of the drug to some binding site on the B-cell membrane. Metabolic degradation may then result in the release of the N-methyl-N-nitroso group, possibly as diazomethane, which may then penetrate the cell membrane and exert its cytotoxic effect. After the cleavage of this group, the rest of the molecule may then be released from the B-cell membrane and this may account for the lack of any significant pancreatic uptake of radioactivity from either (1-^{14}C)-streptozotocin or (2′-^{14}C)-streptozotocin. This interpretation of our data would be consistent with, and support, the findings of Gunnarsson et al. [17] in which the B-cell cytotoxicity of streptozotocin and its aglycone, N-nitroso-N-methyl urea, was compared in mice. From these studies Gunnarsson concluded that the glucose residue in streptozotocin potentiates the B-cytotoxic properties of the molecule.

The present studies also indicated high levels of bound radioactivity from (3′-methyl-^{14}C)-streptozotocin in the liver and kidney, particularly in the cortical tissue. As discussed earlier, these high levels may represent the methylated forms of DNA, RNA and proteins. Since the alkylation of cellular DNA and RNA is believed [15] to be the initial biochemical lesion induced by N-alkyl-N-nitroso carcinogenic compounds, the observed high levels of bound radioactivity for (3′-methyl-^{14}C)-streptozotocin in the liver may possibly explain the occurrence of streptozotocin-induced hepatic tumours reported by Sibay & Hayes [18]. Similarly, high levels of bound radioactivity in the cortical tissue may explain acute renal toxicity of streptozotocin [19].

In conclusion, the findings described in this paper provide further evidence in support of the specificity of streptozotocin for the B-cells of the Islets of Langerhans. The present work also suggests that the diabetogenic activity of streptozotocin may result from the action of a metabolite derived from the ureido side chain. The presence of the sugar moiety in streptozotocin may facilitate the transport and metabolic cleavage of the ureido side chain, resulting in the formation of this metabolite in close proximity to the B-cells of the islets of Langerhans.

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