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The Synthesis of [^{14}C]Streptozotocin and its Distribution and Excretion in the Rat

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[^{14}C]Streptozotocin was synthesized specifically labelled at three positions in the molecule. The biological activity of synthetic streptozotocin was characterised by studies *in vivo* of its diabetogenic activity and its dose–response curves. After this characterization the excretion pattern of all three labelled forms of streptozotocin was studied. With [1-^{14}C]-streptozotocin and [2′-^{14}C]streptozotocin the injected radioactivity was excreted (approx. 70% and 80% respectively) mainly in the urine, the greater part of the excretion occurring in the first 6h period; small amounts (approx. 9% and 8% respectively) were found in the faeces. In contrast, with [3′-*methyl*-^{14}C]streptozotocin a much smaller proportion (approx. 42%) of the injected radioactivity was excreted in the urine, the major proportion appearing in the first 6h, whereas approx. 53% of the injected radioactivity was retained in the carcasses. In whole-body radioautographic studies very rapid renal clearance and hepatic accumulation of the injected radioactivity was observed with all three labelled forms of the drug. There was some evidence for biliary and intestinal excretion. Major differences were apparent in the tissue-distribution studies, with each of the three labelled forms, particularly with [3′-*methyl*-^{14}C]streptozotocin. There was no accumulation of [1-^{14}C]streptozotocin in the pancreas for the 6h period after administration. However, with [3′-*methyl*-^{14}C]-streptozotocin (and also [2′-^{14}C]streptozotocin) there was evidence of some pancreatic accumulation after 2h. The results indicate that streptozotocin is subjected to considerable metabolic transformation and to rapid renal clearance. The implication of these suggestions is evaluated with particular reference to the diabetogenic action of streptozotocin.

Streptozotocin is produced by *Streptomyces achromogenes* var. 128 (Vavra *et al.*, 1959). The structure, established by degradation studies (Herr *et al.*, 1967) and synthesis (Hessler & Jahnke, 1970), has been shown to be 2-deoxy-2-(3′-methyl-3′-nitrosoureido)-D-glucopyranose. Streptozotocin exhibits a broad spectrum of antibacterial activity (Vavra *et al.*, 1959); it also displays marked antileukaemic (Evans *et al.*, 1965), and diabetogenic (Rakienten *et al.*, 1963) properties.

Rakienten *et al.* (1963) initially reported that streptozotocin induces diabetes in rats and dogs, and shows a specific toxicity for the β-cells of the islets of Langerhans. Streptozotocin has since been shown to have a similar effect in mice and guinea pigs (Petersson *et al.*, 1970), rabbits (Lazarus & Shapiro, 1972), rhesus monkeys (Pitkin & Reynolds, 1970) and Chinese hamsters (Willander & Boquist, 1972; Losert *et al.*, 1971).

Currently, streptozotocin is the drug of choice for the induction of experimental diabetes in laboratory animals (Mansford & Opie, 1968; Arison *et al.*, 1967; Rerup, 1970; Hoftiezer & Carpenter, 1973). However, despite its extensive usage, the metabolic fate and mode of action of streptozotocin has not been satisfactorily elucidated. In an attempt to explain the mechanism of action it has been suggested that diabetogenic activity of streptozotocin is mediated through its ability to lower pancreatic NAD^+ concentrations significantly (Schein *et al.*, 1967; Schein & Loftus, 1968; Schein & Bates, 1968; Junod *et al.*, 1969; Ho & Hashim, 1972; Lazarus & Shapiro, 1973). This postulate is based on the observation that nicotinamide given either 10min before or up to 2h after streptozotocin prevents the development of diabetes (Dulin & Wyse, 1969*a*,*b*; Stauffacher *et al.*, 1970; Lazarus & Shapiro, 1973).

In contrast, it has been reported (Dulin & Wyse, 1969*a*) that nicotinic acid, another biosynthetic precursor of NAD^+ (Preiss & Handler, 1958), does not prevent streptozotocin-diabetes. Dulin & Wyse (1969*a*) have interpreted this difference in terms of the existence of two pathways for the biosynthesis of NAD^+ (Ichiyama *et al.*, 1967*a*,*b*), only one of which (from nicotinamide) is thought to exist in the β-cell. Schein & Loftus (1968), however, have suggested that any inhibition of NAD^+ biosynthesis in the β-cell by streptozotocin may be similar to the azaserine type

of inhibition (Narrod *et al.*, 1961). Studies of the incorporation of [^{14}C]nicotinamide (Schein *et al.*, 1973) indicate that decreased amounts of pancreatic NAD^+ results from the combination of decreased tissue uptake of precursors and a decrease in the synthesis of NAD^+. The latter effect is thought to arise from a partial inhibition of the first step in the biosynthesis of NAD^+ from nicotinamide.

Despite the controversy over the mode of action of streptozotocin it is used extensively for the induction of experimental diabetes. Comparing streptozotocin-diabetes with alloxan-diabetes, Arison *et al.* (1967) have shown that streptozotocin produces permanent diabetes with extrapancreatic lesions that mimic the pathological status found in human diabetes. Similarly, Junod *et al.* (1969) have concluded that in contrast with alloxan-diabetes, streptozotocin-diabetes is more reproducible, more convenient and better for the induction of a diabetic state of graded severity suitable for modulated endocrine and metabolic studies.

The non-availability of specifically radioactively labelled forms of streptozotocin has doubtlessly hindered attempts to elucidate its mechanism of action. The availability of streptozotocin, specifically labelled with ^{14}C in the carbohydrate moiety and various positions in the side chain, would facilitate the elucidation of the distribution, metabolism and excretion of streptozotocin. In addition, such studies may shed some light on the biochemical basis of diabetes mellitus.

We report in this paper the synthesis of three radioactively labelled forms of streptozotocin (Fig. 1). By using these preparations in conjunction with excretion studies, whole-body radioautography and tissue-distribution studies we have investigated the distribution and excretion of streptozotocin in the rat.

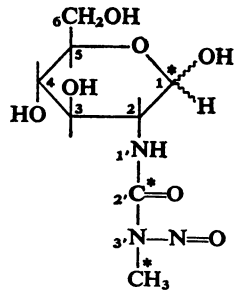


Fig. 1. 2-*Deoxy*-2-(3′-*methyl*-3′-*nitrosoureido*)-D-*glucopyranose* (*streptozotocin*)

Streptozotocin was synthesized specifically labelled with ^{14}C at the positions indicated by asterisks.

Materials and Methods

General

All chemicals used were of an analytical grade. All solvents were dried and redistilled. Sodium [1-^{14}C]-acetate, sodium [2-^{14}C]acetate and [1-^{14}C]glucosamine hydrochloride were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Melting points were determined on a Kofler block and are uncorrected. Nuclear-magnetic-resonance (n.m.r.) spectra were recorded on a Varian HA 100 spectrometer in dimethyl sulphoxide. U.v. spectra were recorded on a Unicam SP.800 spectrometer. I.r. spectra were recorded on a Unicam SP.200 spectrometer for solutions in chloroform or Nujol mulls. T.l.c. and preparative-layer chromatography (1mm thickness, loading not exceeding 100mg/20cm × 20cm plate) were performed on Kieselgel GF_{254} (E. Merck, Darmstadt, Germany) activated at 110°C for 1h. For t.l.c. development, the solvent system used was methanol–chloroform (1:3, v/v). Under these conditions glucosamine had R_F 0.1, the ureido derivative R_F 0.3 and streptozotocin had R_F 0.5. Components were detected by exposure to u.v. (254nm) and/or by spraying with the reagent described by Lemieux & Bauer (1954).

Activated sodium azide. NaN_3 was activated by the method of Nelles (1932).

Diphenylphosphoryl azide. Diphenylphosphoryl azide was synthesized from diphenylphosphoryl chloride by the procedure described by Shiori *et al.* (1972) and distilled under vacuum (22.7Pa). The fraction with a boiling point of 157°C was used in subsequent experiments.

D-*Glucosamine.* Free D-glucosamine was prepared from the hydrochloride salt by the method of Breuer (1898).

2-*Deoxy*-2-(3′-*methylureido*)-D-*glycopyranose* (*ureido derivative*). To a solution of glucosamine (8.95g) in water (40ml) at 0–2°C, redistilled methyl isocyanate (3.14g) was added dropwise during 30min. The reaction was monitored by t.l.c. After a further 30min, if t.l.c. indicated that glucosamine was still present, more methyl isocyanate (0.5g) was added and the reactants were stirred until t.l.c. showed that only the ureido derivative was present. The resultant mixture was freeze-dried to give the desired ureido compound (9.44g). The 2-*deoxy*-2-(3′-*methylureido*)-D-*glucopyranose* was crystallized from ethanol to yield white needles, m.p. 127–128°C (Found: C, 40.8; H, 6.9; N, 11.8; $C_8H_{16}N_2O_6$ requires C, 40.7; H, 6.8; N, 11.9). N.m.r.: τ 7.32 ($N-CH_3$).

2-*Deoxy*-2-(3′-*methyl*-3′-*nitrosoureido*)-D-*glucopyranose* (*streptozotocin*). An ice-cold solution of D-glucosamine (720mg) in water (4ml) was added to methyl isocyanate (230mg). The reactants were stirred for 30min at 0°C. The ureido intermediate was

converted, without isolation, into streptozotocin as described below. Nitrogen trioxide was passed through the mixture until t.l.c. indicated that all the ureido derivative had reacted. Cold butan-1-ol (20ml) was added and the mixture concentrated to approx. 10ml *in vacuo*; at this point streptozotocin began to crystallize. The flask was kept at 0–4°C for 10h. The product was collected by filtration, washed (3×10ml) with butan-1-ol–ether (1:1, v/v), followed by ether (3×10ml) and dried *in vacuo*. The crude streptozotocin (820mg) was crystallized from ethanol to give pale yellow needles, m.p. 115–116°C; $\lambda_{max.}$ (ethanol) 226nm (ε 5000), $\nu_{max.}$ 1500cm^{-1}. N.m.r.: τ 6.95, approx. 1.50*H*, s (syn CH_3); τ 6.70, approx. 1.50*H*, s (anti CH_3); τ 7.10–6.70, 1*H*, m; τ 6.64–6.30, 5*H*, m; τ 5.64, 1*H*, t (*J* 5.5Hz) (6-OH); τ 5.16, 1*H*, d (*J* 4Hz) (3-OH); τ 5.10, 1*H*, d (*J* 5Hz) (4-OH); τ 4.92, 1*H*, dd (*J* 2.4Hz) (1-H); τ 3.44, 1*H*, d (*J* 4Hz) (1-OH); τ 2.32, 1*H*, m (N–H).

Synthesis of ^{14}*C-labelled streptozotocin*

[1-^{14}*C*]*Acetic acid.* Sodium [1-^{14}C]acetate (6.0mg; 667μCi/mg) was diluted with an aqueous solution of sodium acetate (420mg), freeze-dried and dried at 100°C *in vacuo* overnight. The sodium [1-^{14}C]acetate was converted into [1-^{14}C]acetic acid by using the vacuum-manifold technique of Cox & Turner (1950).

[1-^{14}*C*]*Methyl isocyanate.* [1-^{14}C]Acetic acid as obtained above was placed in a two-neck flask (25ml) fitted with a solid CO_2–acetone condenser, a drying tube and a nitrogen inlet. After cooling to 0°C triethylamine (0.5g) was added slowly with stirring. Diphenylphosphoryl azide (1.38g) was then added and the reactants were heated under reflux at 60°C for 4.5h. The i.r. spectrum of an unlabelled preparation indicated that the reaction was completed at this stage. A steady stream of dry N_2 was then passed through the reaction mixture and the temperature of the bath gradually raised to 60°C and maintained at this temperature for 1h. [1-^{14}C]Methyl isocyanate was entrained in the N_2 gas and was collected in a trap at −78°C. The i.r. spectrum of unlabelled methyl isocyanate prepared in this way, and the m.p. (85°C) of its phenylcarbamate derivative, were identical with those of authentic specimens.

2-*Deoxy*-2-(3′-*methyl*-3′-*nitroso*[2′-^{14}*C*]*ureido*)-D-*glucopyranose* ([2′-^{14}*C*]*streptozotocin*). To the [1-^{14}C]methyl isocyanate was added an ice-cold solution (4ml) of D-glucosamine (575mg) and the whole stirred at 0°C for 30min. Nitrogen trioxide was passed through the mixture until t.l.c. indicated that the reaction was complete. After the addition of butan-1-ol (20ml) the solution was concentrated *in vacuo* until crystallization started. The concentrate was kept at 0–4°C overnight. The product was collected by filtration, washed with butan-1-ol–ether (3×5ml, 1:1, v/v) followed by ether (3×8ml) and dried *in vacuo* to give crude [2′-^{14}C]streptozotocin (575mg). The latter was crystallized from ethanol to give pale-yellow needles (400mg; yield 30% from sodium [1-^{14}C]acetate), m.p. 115–116°C, specific radioactivity 3.02μCi/mg.

2-*Deoxy*-2-(3′-[^{14}*C*]*methyl*-3′-*nitrosoureido*)-D-*glucopyranose:* ([3′-*methyl*-^{14}*C*]*streptozotocin*). This was prepared from sodium [2-^{14}C]acetate (4.12mg; 728μCi/mg) by the procedure outlined above. Crystalline [3′-*methyl*-^{14}C]streptozotocin (410mg, yield 30% from sodium [2-^{14}C]acetate) had a specific radioactivity of 2.264μCi/mg.

D-[1-^{14}*C*]*Glucosamine.* [1-^{14}C]Glucosamine hydrochloride (15.62mg; 15μCi/mg) was dissolved in water containing unlabelled glucosamine hydrochloride (864mg) and freeze-dried. The diluted [1-^{14}C]glucosamine hydrochloride was converted as described previously into [1-^{14}C]glucosamine (630mg; specific radioactivity 0.35μCi/mg).

2-*Deoxy*-2-(3′-*methyl*-3′-*nitrosoureido*)-D-[1-^{14}*C*]-*glucosamine* ([1-^{14}*C*]*streptozotocin*). [1-^{14}C]Glucosamine (630mg; 0.35μCi/mg) was converted as described above into [1-^{14}C]streptozotocin (540mg; yield 50% from [1-^{14}C]glucosamine hydrochloride) which had a specific radioactivity of 0.235μCi/mg.

Degradation of [2′-^{14}*C*]*streptozotocin*

To assess the specificity of labelling in the streptozotocin samples labelled in the side chain [2′-^{14}C]-streptozotocin was degraded as follows. [2′-^{14}C]-Streptozotocin (3.21mg; 9.694μCi) was diluted with an aqueous solution of unlabelled streptozotocin (265mg in 1ml). This solution was added dropwise to NaOH (10ml; 10%, w/v), which was covered with a layer of ether (50ml). The green–yellow ethereal phase containing the liberated diazomethane was separated (the alkaline solution was retained) and added to an ethereal solution (50ml) of β-naphthoic acid (200mg). The methyl β-naphthoate was separated from the β-naphthoic acid by preparative layer chromatography on development with benzene. The band at R_F 0.8 was eluted with benzene and the product crystallized from ethanol to give inactive methyl β-naphthoate, m.p. 77°C. The radioactivity of this ester was not significantly above that of background.

A stream of N_2 was passed through the alkaline solution after acidification with HCl (7ml; 70%, v/v). The CO_2 evolved was precipitated as $BaCO_3$ from a saturated solution of $Ba(OH)_2$. The $BaCO_3$ was collected by centrifugation, washed with water, then ethanol, and finally dried *in vacuo*. $Ba^{14}CO_3$ was counted for radioactivity by the method of Pegg & Williams-Ashman (1968); the specific radioactivity was 0.0432μCi/mg (99.9% recovery from [2′-^{14}C]streptozotocin).

Radiochemical techniques

Radioactivity counting was carried out on a Beckman LS-200B liquid-scintillation counter. For

counting radioactivity of non-aqueous samples, a toluene-based scintillant was used: 2,5-diphenyloxazole (5g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3g) in toluene (1 litre). For aqueous samples a dioxan system was used, comprising naphthalene (60g), 2,5-diphenyloxazole (4g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.2g), methanol (100ml) and ethylene glycol (20ml) made up to 1 litre with dioxan. Heterogeneous samples (e.g. tissue homogenates) were counted for radioactivity in the dioxan system after gelling (5%, w/v) (Bridges *et al.*, 1967) with Cab-O-Sil (Packard Instrument Co., Wembley, Middx., U.K.).

Preparation of samples for counting. Urine from excretion studies was made up to 250ml with sodium citrate buffer (0.01M, pH4.5). Faeces were homogenized with citrate buffer in a Waring blender and were made up to 250ml. Portions (1ml) of the above samples were counted for radioactivity in the dioxan system. Tissues (0.1–1.0g) were homogenized with citrate buffer (30ml) and a sample was counted for radioactivity in the Cab-O-Sil–dioxan system. Carcasses from excretion studies of [2′-^{14}C]streptozotocin and [1-^{14}C]streptozotocin were dissolved in NaOH (150ml; 40%, w/v) and ethanol (50ml) by heating at 40°C for 24h. The digest was acidified with excess of conc. HCl (135ml) and the pH adjusted to 7.0 by the addition of $NaHCO_3$. Carcasses from [3′-*methyl*-^{14}C]streptozotocin experiments were frozen in liquid N_2 and powdered in a percussion mortar. The resultant fine powder was mixed and portions (10×2g) were homogenized in citrate buffer (40ml). Samples from all carcass preparations were counted in the Cab-O-Sil–dioxan system. In a control experiment the recovery of ^{14}C label after the intraperitoneal administration of labelled streptozotocin was approx. 90%. The efficiency of ^{14}C counting in toluene and dioxan systems, as determined by the channels ratio method, was approx. 85%.

Experimental animals

Sprague–Dawley rats maintained on a standard diet were used in all studies. For investigations of the diabetogenic activity of synthetic streptozotocin, male rats (300±50g body wt.) were used. For excretion experiments, both male and female rats (200±10g body wt.) were used. Whole-body radioautography and tissue-distribution studies were performed on male rats (200±10g body wt.).

Administration and dose of drugs

In all studies except for dose–response curves, streptozotocin (70mg/kg) was administered intravenously (via a lateral tail vein) while the animal was under light ether anaesthesia. In dose–response studies, doses administered were 0, 20, 40, 80 and 100mg/kg body wt. Owing to the instability of streptozotocin in aqueous media, all solutions for injection were made up (70mg/ml) in citrate buffer immediately before administration.

Diabetogenic studies. The synthetic streptozotocin and authentic streptozotocin (batch no. 9681 GGS-119 F_1; Upjohn Co., Kalamazoo, Mich., U.S.A.) were administered as described above. The concentration of blood glucose and plasma immunoreactive insulin at critical time-intervals after injection of the drug were measured. In addition the daily variation of body weight was recorded. Blood glucose was assayed by the glucose oxidase method of Huggett & Nixon (1957) and plasma immunoreactive insulin by the procedure described by Hales & Randle (1963).

Excretion studies

Eight animals (four male and four female) were used for studies with [2′-^{14}C]streptozotocin. With [3′-*methyl*-^{14}C]streptozotocin and [1-^{14}C]streptozotocin, six animals (three male and three female) were used. After injection of the appropriate labelled streptozotocin, animals were housed in individual metabolism cages, where urine and faeces were collected separately after 6h, 12h, 24h and 48h, and were assayed for radioactivity as described previously. At the end of 48h, animals were killed by a blow on the back of the head and the residual radioactivity in the carcass was assayed.

Whole-body radioautography

The technique adopted in the present study was based on that described by Martin *et al.* (1962). At suitable time-intervals after injection (ranging from 30s to 24h) the animals were killed and rapidly frozen by immersion in an acetone–solid CO_2 mixture at −78°C. Each carcass was embedded in aqueous acacia gum (5%, w/v) and machined in the ventrolateral plane to a suitable level (so as to expose liver, pancreas and kidney clearly). At all times the carcass was maintained in the frozen state by intermittent cooling with liquid N_2. The frozen animal surface was thoroughly cleaned and placed in apposition to Kodirex A 54 T X-ray film (Kodak Ltd., Kirkby, Liverpool, U.K.). The preparation was then stored in the dark at −20°C for periods of 2–5 weeks. After the appropriate exposure period, the film was developed in Kodak D 19 developer. A colour photograph of the animal surface was taken and by comparing the photograph together with the radioautogram, the distribution and localization of the administered label was apparent.

Tissue-distribution studies

Rats were separately injected with each of the labelled forms of streptozotocin. Animals were killed at 1min, 10min, 20min, 30min, 40min, 50min, 60min, 1.5h, 2h, 3h, 4h and 6h after the injection of the labelled drug. Tissues were rapidly excised, dried

on filter paper, weighed and immediately frozen in liquid N_2. Tissues were then powdered in a percussion mortar and radioactivity was assayed by the Cab-O-Sil method as described above.

Results and Discussion

Diabetogenic activity of synthetic streptozotocin

The diabetogenic activity of synthetic streptozotocin was characterized and compared with that of authentic streptozotocin. The triphasic pattern of blood glucose variation after a diabetogenic dose (70mg/kg) of streptozotocin is shown in Fig. 2. It is evident that the three characteristic phases, namely the initial hyperglycaemia, hypoglycaemia and the final permanent hypergylcaemia, appeared at identical times and were of the same degree of severity for both synthetic and authentic streptozotocin. It was also found (E. H. Karunanayake, D. J. Hearse & G. Mellows, unpublished work) that the plasma immunoreactive insulin was high during the hypoglycaemic phase and although similar to controls after the development of permanent diabetes, was abnormally low when related to the co-existing hyperglycaemia. These results, in addition to confirming the biological activity of synthetic streptozotocin, are also in accordance with studies reported by Rakienten *et al.* (1963), Rerup & Tarding (1969) and Junod *et al.* (1969).

In addition to the induction of hyperglycaemia, the administration of streptozotocin has been associated (Junod *et al.*, 1969; Rerup & Tarding, 1969) with significant loss of body weight. In order to characterize this loss at a dosage of 70mg/kg body wt., and also to substantiate further the authenticity of the synthetic streptozotocin, weight-loss curves were determined. Rats ($n = 10$) were separately given synthetic and authentic streptozotocin (70mg/kg body wt.) at 9:30a.m. and the body weight was recorded daily at 9:30a.m. for 7 days. The results (Fig. 3) indicate no significant difference between synthetic and authentic streptozotocin and indicate a mean weight loss of approx. 10.5g/day over the first 5 days.

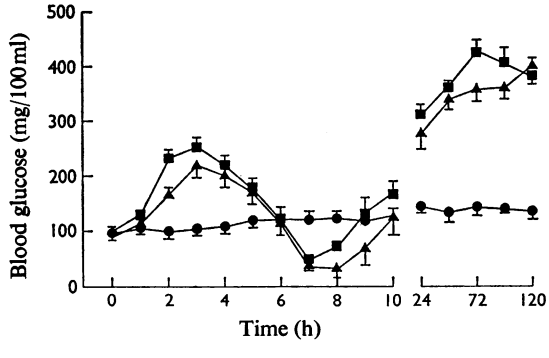


Fig. 2. *Streptozotocin-induced changes in blood glucose*

Streptozotocin was administered (70mg/kg) intravenously to rats and the blood glucose (mg/100ml) determined periodically over 5 days. ▲, Authentic streptozotocin; ■, synthetic streptozotocin; ●, control. Each point represents the mean of eight animals; S.E.M. values are indicated by the bars.

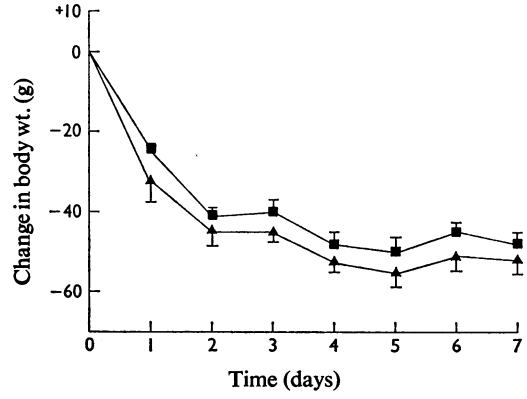


Fig. 3. *Streptozotocin-induced loss in body weight*

Streptozotocin was administered (70mg/kg) intravenously to rats and body weight was recorded daily for 7 days. ■, Synthetic streptozotocin; ▲, authentic streptozotocin. Each point represents the mean of 10 rats and S.E.M. values are indicated by the bars.

Dose–response studies

The biological activity of the synthetic streptozotocin was further confirmed and characterized in a dose–response study. In comparison with a control group of rats injected with citrate buffer (1ml/kg body wt.), it was found (Figs. 4*a* and 4*b*) that any dose of synthetic streptozotocin between 60 and 100mg/kg body wt. induced a characteristic triphasic pattern for blood glucose and a sustained weight loss over 7 days. A dose of 40mg/kg or less resulted in a less diabetic state, with an ascending weight curve after 6 days. These findings are in agreement with those of Junod *et al.* (1969).

Excretion studies

Initially eight animals (four male and four female) were used for experiments with [2′-^{14}C]streptozotocin. After the administration of labelled drug, the urine and the faeces were continuously collected. These were taken after 6h, 12h, 24h and 48h for the analysis of radioactivity. At the end of 48h, the animals were killed by a blow on the back of the head and the residual radioactivity in the carcass was measured. The results for each sex are shown in Table 1. No significant sex difference was apparent for the excretion

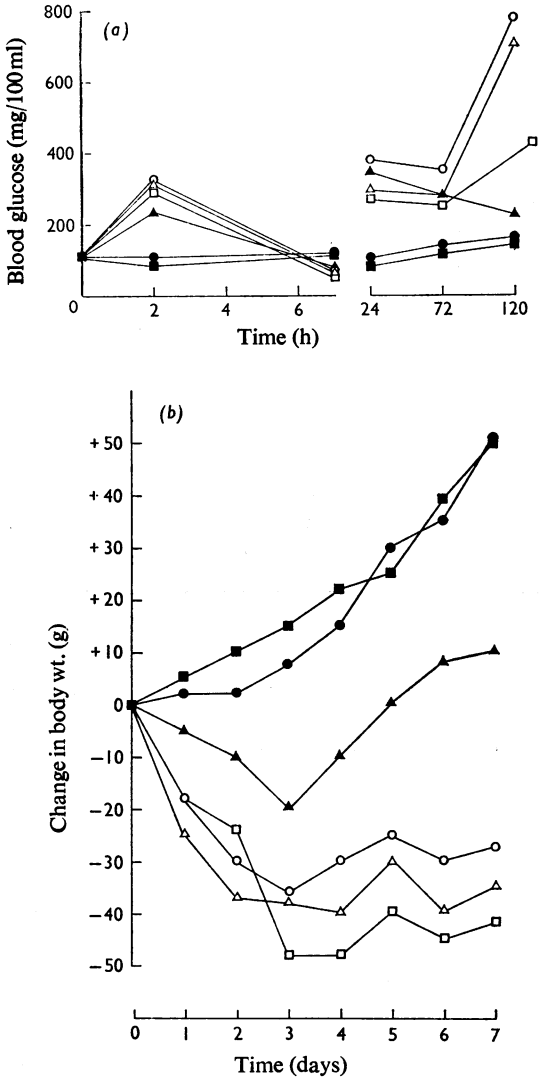


Fig. 4. *Dose–response curves for streptozotocin-induced changes in blood glucose (a) and body weight (b)*

Streptozotocin was administered intravenously to rats and the blood glucose (mg/100ml) and body weight were recorded periodically for up to 7 days. ●, Controls (citrate buffer-injected); streptozotocin: ■, 20mg/kg; ▲, 40mg/kg; ○, 60mg/kg; △, 80mg/kg; □, 100mg/kg.

pattern of streptozotocin. Thus in the subsequent experiments with [1-^{14}C]streptozotocin and [3′-*methyl*-^{14}C]streptozotocin six animals (three male and three female) were used, again no sex difference was apparent and the results were therefore combined.

In experiments with [2′-^{14}C]streptozotocin approx. 81% of the injected radioactivity appeared in the urine over the 48h experimental period, the major proportion (73%) appearing during the first 6h. Approx. 8% of the injected radioactivity appeared in the faeces. Although this may be interpreted as evidence for either biliary or intestinal excretion of injected radioactivity, the possibility of some contamination of the faeces by the urine cannot be ruled out. Analysis of residual radioactivity in the carcasses accounted for a further 10% of the injected radioactivity. Thus an overall recovery of 99% was recorded. In experiments with [1-^{14}C]streptozotocin (Table 2), approx. 71% of the injected radioactivity appeared in the urine over the 48h experimental period, once again the major proportion appearing during the first 6h. Faeces contained 10% and carcasses 18% of the injected radioactivity.

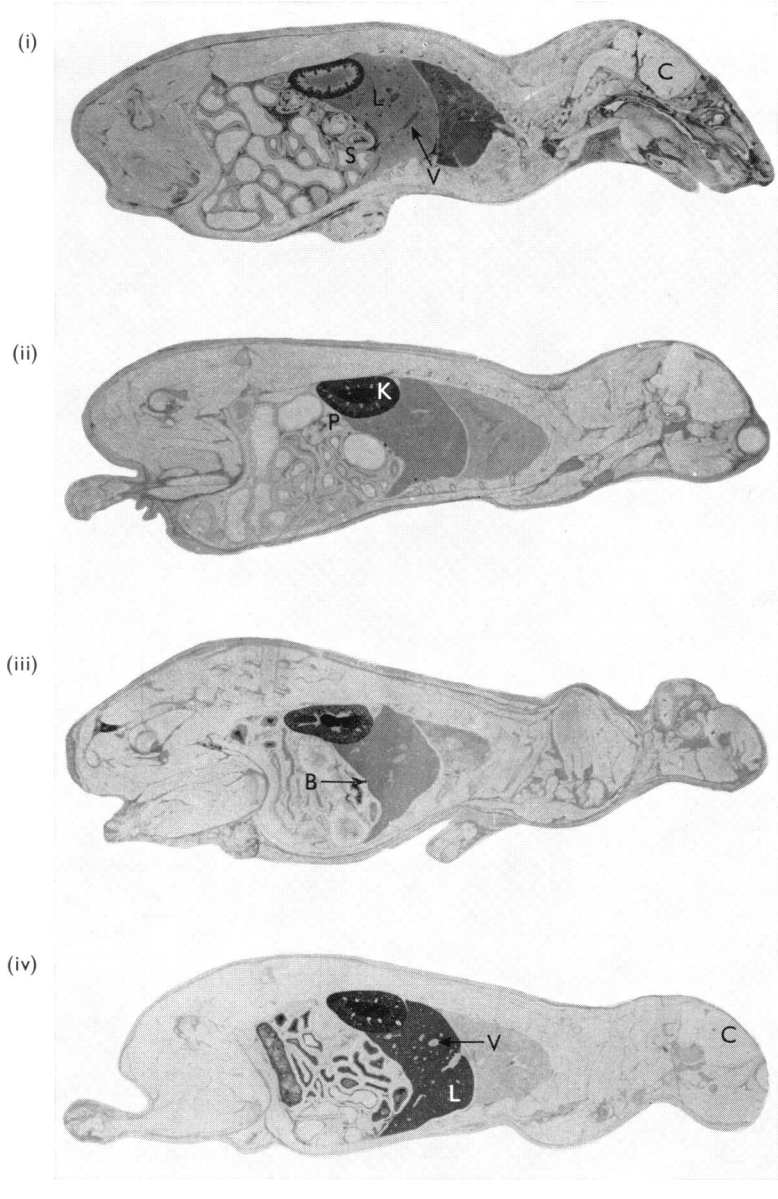
In contrast with [2′-^{14}C]streptozotocin and [1-^{14}C]-streptozotocin, the percentage recovery of injected radioactivity during the 48h experimental period after administration of [3′-*methyl*-^{14}C]streptozotocin was approx. 42%, the major portion appearing during the first 6h (Table 2). Only 4% was recovered in the faeces. However, approx. 54% of the injected radioactivity remained in association with the carcasses.

These collective findings may suggest a possible metabolic fate for streptozotocin. The differing but relatively high urinary clearance (70–80%) during 48h of the [1-^{14}C]streptozotocin and [2′-^{14}C]streptozotocin and the much lower clearance (42%) and very high residual carcass radioactivity of [3′-*methyl*-^{14}C]-streptozotocin would suggest metabolic degradation

Table 1. *Recovery of radioactivity after the administration of [2′-^{14}C]streptozotocin*

[2′-^{14}C]Streptozotocin was administered intravenously (70mg/kg) to rats. Urine and faeces were collected over 48h. After this period the radioactivity associated with urine, faeces and carcasses was determined. The results are given as means±S.E.M. for eight animals (four male and four female).

Time after dosing (h)	Percentage of injected radioactivity		
	Male	Female	Male+female
Urine			
0–6	70.8±4.1	75.5±2.2	73.2±2.3
6–12	4.4±0.4	4.3±0.4	4.3±0.3
12–24	2.7±0.4	2.1±0.1	2.4±0.2
24–48	0.7±0.2	1.0±0.1	0.9±0.1
Sub-total	78.6±3.5	82.9±2.0	80.7±2.0
Faeces			
0–6	1.7±0.6	0.8±0.3	1.3±0.3
6–12	2.7±0.3	0.9±0.8	1.8±0.5
12–24	3.3±0.9	1.4±0.8	2.5±0.7
24–48	1.0±0.1	4.0±1.9	2.5±1.1
Sub-total	8.7±1.4	7.1±1.2	7.9±0.9
Carcasses	11.1±2.7	9.3±0.9	10.2±1.4
Grand total	98.4±0.8	98.8±1.0	98.6±0.7



EXPLANATION OF PLATES 1 & 2

Whole-body radioautograms obtained at different time-intervals after the administration of labelled streptozotocin

Radioautograms obtained from rats after intravenous administration of [3′-*methyl*-^{14}C]streptozotocin (70mg/kg). Plate 1: (i) 10s; (ii) 2min; (iii) 5min; (iv) 15min. Plate 2: (v) 45min; (vi) 60min; (vii) 120min; (viii) 240min. K = kidney; L = liver; P = pancreas; S = small intestine; B = bile duct; V = blood vessel: C = brain.

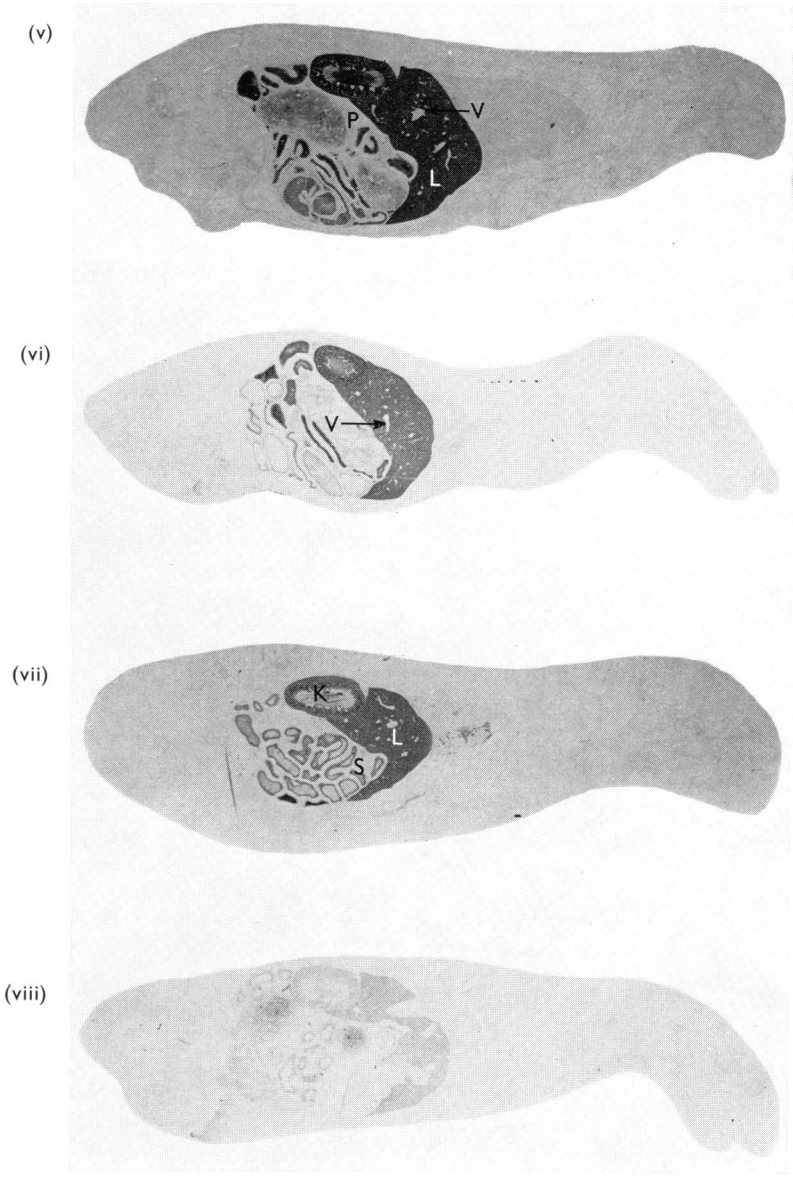

(v)
P
V
L
(vi)
V
(vii)
K
L
S
(viii)

Table 2. *Recovery of radioactivity after the administration of [1-^{14}C]streptozotocin and [3′-methyl-^{14}C]streptozotocin*

[1-^{14}C]Streptozotocin and [3′-*methyl*-^{14}C]streptozotocin were administered intravenously (70mg/kg) to rats. Urine and faeces were collected over 48h. After this period the radioactivity associated with urine, faeces and carcasses was determined. The results are given as means±S.E.M. for six animals.

Time after dosing (h)	Percentage of injected radioactivity: [1-^{14}C]-Streptozotocin	[3′-*methyl*-^{14}C]-Streptozotocin
Urine		
0–6	63.5±2.1	36.6±3.0
6–12	3.7±0.5	2.8±0.7
12–24	2.3±0.4	1.3±0.1
24–48	1.1±0.0	1.2±0.1
Sub total	70.5±1.2	41.8±2.7
Faeces		
0–6	1.9±0.8	1.4±0.6
6–12	3.0±1.4	0.7±0.2
12–24	2.7±0.5	0.4±0.1
24–48	2.9±0.7	1.5±1.1
Sub total	9.8±1.9	4.0±1.5
Carcasses	18.0±0.8	53.8±3.5
Grand total	98.3±1.1	99.7±0.8

leading to different fates for each radioactively labelled carbon atom. With specific reference to [3′-*methyl*-^{14}C]streptozotocin, the low recovery is not altogether surprising, because it has been suggested (Schein *et al.*, 1967; Fiscor *et al.*, 1971; Bhuyan, 1970; Reusser, 1971; Bhuyan *et al.*, 1972) that the reported anticancer (Mortel *et al.*, 1971) and antileukaemic properties (Evans *et al.*, 1965) depend on the *N*-methylnitroso moiety of the streptozotocin. Further, it has been shown (Reusser, 1971) that streptozotocin induced DNA degradation in actively dividing *Bacillus subtilis* cells, and Bhuyan (1970) has reported that the drug inhibits the incorporation of precursors into mammalian cells *in vitro*. The latter workers have suggested alkylation of double-stranded DNA by streptozotocin as a possible mode of action. The high residual carcass radioactivity for the [3′-*methyl*-^{14}C]streptozotocin observed in the present study may provide support for the above hypothesis.

In view of the differing patterns of excretion for the three labelled forms of streptozotocin, and the possibility of some biliary or intestinal elimination, the distribution of streptozotocin was further studied by the technique of whole-body radioautography.

Whole-body radioautography

Other than a greater residual radioactivity for the [3′-*methyl*-^{14}C]streptozotocin no major differences were apparent in distribution patterns of injected radioactivity at any time between zero time and 24h following injection. However, the radioautograms revealed considerable information about the distribution and possible metabolism of streptozotocin. Plates 1 and 2 show, and is essentially representative of, radioautograms obtained from animals after the injection of diabetogenic doses (70mg/kg) of labelled streptozotocin.

In each instance, the radioactivity in the blood was very high for the first 1–3min after the injection, but there was evidence of rapid clearance from the blood stream, such that after 15min only relatively low radioactivity was apparent in the blood. During this 15min period rapid and intense accumulation of radioactivity was observed in the kidney. In as short a time as 2min after injection heavy labelling of the pelvis was apparent. Peak kidney radioactivity was clearly apparent for 15min and then, although steadily declining, was clearly apparent for 4h. A constant residual radioactivity appeared to remain associated with the kidney tissue for the period 4–24h. The pattern of renal labelling by each radioactive form of streptozotocin would be consistent with the very rapid and extensive renal clearance of radioactivity observed in the excretion studies. The high kidney uptake and accumulation of injected radioactivity may also explain the extensive renal toxicity of streptozotocin reported by Schreibman *et al.* (1971) and Arison & Feudale (1967).

In addition to renal labelling, marked accumulation of radioactivity was observed in the hepatic tissue. In less than 5min the radioactivity associated with the liver was greater than that of the bloodstream. This uptake reached a maximum at about 30min after injection and thereafter steadily declined. There was some evidence of radioactivity accumulation in the bile as revealed by occasional sections through the bile duct. These observations would suggest either hepatic metabolism or active transport of the streptozotocin into the liver with the possibility of some biliary excretion. As with the kidney, a constant residual radioactivity appeared to remain associated with the liver up to 24h; there was also some accumulation of radioactivity in the small-intestinal mucosa. The observations of residual radioactivity in liver, kidney and intestinal mucosa was particularly evident with [3′-*methyl*-^{14}C]streptozotocin. The observation of residual labelling of the intestinal mucosa is noteworthy in the light of a report (Caspary *et al.*, 1972) in which it has been shown that the activity of intestinal-mucosa brush-border hydrolases in streptozotocin-diabetic rats was significantly elevated. Whether this increased enzyme activity was directly caused by the drug or a secondary effect of the diabetic syndrome would be worthy of further investigation.

In general the distribution of each radioactive form of streptozotocin was restricted to the blood, the urinary excretory system, the liver and intestine. There was little or no radioactivity in muscle tissue and there was no evidence for the passage of label across the

blood–brain barrier. It has been shown (Schein, 1969) that streptozotocin, unlike *N*-methyl-*N*-nitrosourea, does not cause neurological effects in the rat and that streptozotocin was not detectable in the brain. The present finding, though confirming the above report, does not, however, contradict the neurological effects of streptozotocin in the cat (Weinstein & Gertner, 1971).

It is important to note that in relation to the general amount of detectable radioactivity no accumulation could be associated with pancreatic tissue.

These collective findings suggest that after its administration to the rat, streptozotocin is rapidly metabolized and excreted. The very early accumulation of radioactivity in the kidney before significant hepatic labelling had occurred may be suggestive of the renal clearance of unchanged streptozotocin. The subsequent accumulation of radioactivity by the liver would suggest a possible site for metabolism of streptozotocin. However, the relatively low amount of radioactivity detected in the gut and biliary system, though indicating some biliary clearance, would suggest that any liver metabolites are cleared via the renal system. The absence of any major pancreatic labelling, though not precluding either the direct action of minute amounts of streptozotocin or one of its metabolites, may also suggest the possibility of the existence of a secondary factor in the diabetogenic action of streptozotocin.

Tissue-distribution studies

In an attempt to quantify, on a time-related basis, the relative distribution of radioactivity in major tissues and also to ascertain whether there was any detectable accumulation of radioactivity in the pancreas, a tissue-distribution study was undertaken. In preliminary studies, the tissue content of radioactivity 30min and 60min after the separate administration of each of the three labelled forms of streptozotocin was determined. Liver, kidney, small intestine, pancreas, large intestine, duodenum, blood, lungs, brain, heart, stomach, thymus, testes, oesophagus and skeletal muscle were studied, and it was found that only in the liver, kidney and pancreas did the amount of radioactivity in the tissue exceed that in the blood. Consequently in a more detailed study using 12 rats for each of the three labelled forms of streptozotocin, the tissue distribution was determined 1, 10, 20, 30, 40, 50, 60, 90, 120, 180, 240 and 360min after administration of the drug. The tissues studied were kidney, liver, pancreas and blood. All contents were compared with that in the blood at the time of sampling. The results are illustrated in Figs. 5, 6, and 7, and are expressed as percentage of injected radioactivity/g wet wt. of tissue.

Several important points are apparent from an examination of Figs. 5, 6 and 7. First, in agreement with our findings described above, the majority of the injected radioactivity was associated with the liver and the kidneys. Further, the high radioactivity observed soon after the administration declines steadily after 1–2h. In each instance, but to a different extent, the distribtuion profiles for hepatic and renal radioactivity are biphasic during the first 2h period.

There was also evidence of a biphasic distribution pattern in the blood. In agreement with radioautographic studies the specific radioactivity of the blood rapidly declined after the administration of each labelled form of streptozotocin and within 5min the specific radioactivity of the blood was less than that observed in the liver and kidney. After 60–90min the specific radioactivity in the blood remained essentially constant for 5h.

It is apparent that small but detectable amounts of radioactivity were associated with pancreatic tissue. However, it is important to dissociate any contribu-

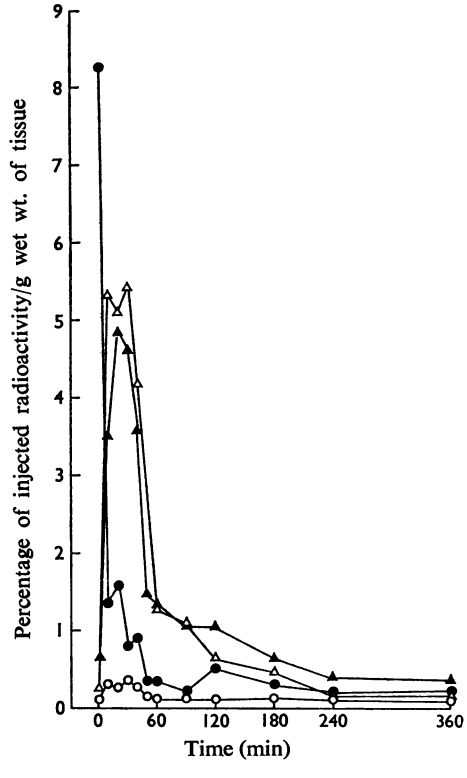


Fig. 5. *Tissue radioactivity after the administration of [1-^{14}C]streptozotocin*

Rats ($n = 12$) were given [1-^{14}C]streptozotocin (70mg/kg intravenously). Tissues were removed after various time-intervals and assayed for radioactivity (% of injected radioactivity/g wet wt. of tissue). ●, Blood; △, kidney; ▲, liver; ○, pancreas.

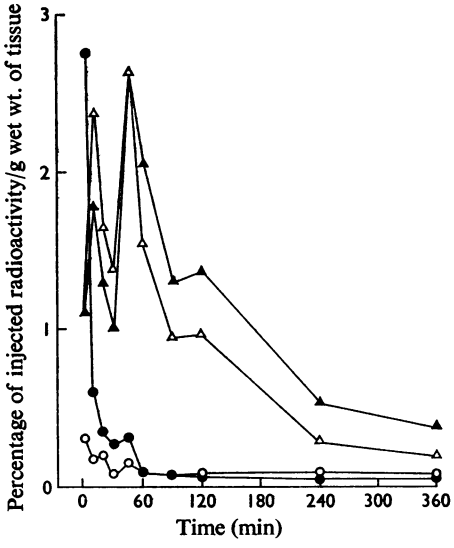


Fig. 6. *Tissue radioactivity after the administration of [2′-^{14}C]streptozotocin*

Rats ($n = 10$) were given [2′-^{14}C]streptozotocin (70mg/kg) intravenously. Tissues were removed after various time-intervals and assayed for radioactivity (% of injected radioactivity/g wet wt. of tissue). ●, Blood; △, kidney; ▲, liver; ○, pancreas.

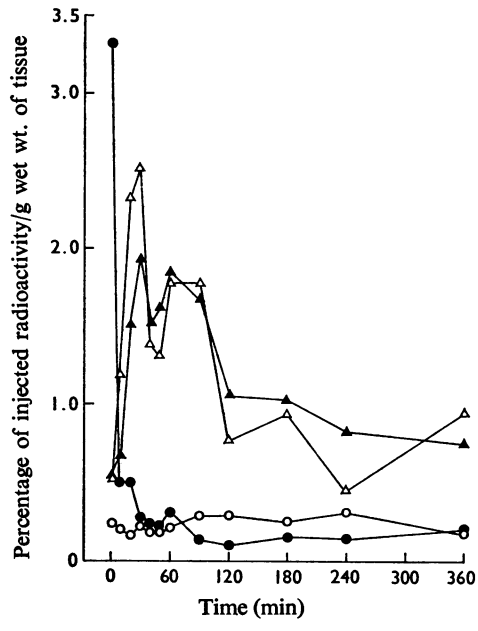


Fig. 7. *Tissue radioactivity after the administration of [3′-methyl-^{14}C]streptozotocin*

Rats ($n = 12$) were given [3′-*methyl*-^{14}C]streptozotocin (70mg/kg) intravenously. Tissues were removed after various time-intervals and assayed for radioactivity (% of injected radioactivity/g wet wt. of tissue). ●, Blood; △, kidney; ▲, liver; ○, pancreas.

tion made by labelled blood perfusing the pancreas from that of the pancreatic tissue. Thus a prerequisite for evidence of pancreatic accumulation must be the occurrence of a greater specific radioactivity in the pancreas than in the blood. With each labelled form of streptozotocin the specific radioactivity of the pancreas remained well below that of the blood during the first 60min after administration. However, with [3′-*methyl*-^{14}C]streptozotocin, there was a clear cross-over (Fig. 7) with a significantly higher specific radioactivity in the pancreas than in blood for the remainder of the experiment. There was also evidence (Fig. 6) of some pancreatic accumulation of [2′-^{14}C]-streptozotocin. However, with [1-^{14}C]streptozotocin the amount of radioactivity remained well below that of the blood.

The results indicate several significant differences in the distribution between each of the three labelled forms of streptozotocin. These observations, in comparison with the findings described above, may provide evidence for the metabolic fate of streptozotocin. The appearance in the kidney and blood of an early peak of radioactivity of short duration may be consistent with the rapid renal clearance of unchanged streptozotocin. The low biphasic tissue concentration (Fig. 7) of [3′-*methyl*-^{14}C]streptozotocin would agree with the short half-life of streptozotocin in blood as reported by Schein & Loftus (1968), where they used the chemical assay of Forist (1964), based on the *N*-methylnitroso moiety, to measure blood concentrations of streptozotocin.

The biphasic pattern of distribution during the first 60–120min with a second peak of activity appearing in the liver, kidney and blood may suggest the accumulation and hepatic metabolism of streptozotocin followed by the renal clearance of the metabolite. The differences in the duration, magnitude and maxima of these peaks may indicate different metabolic fates for each of the labelled forms of the drug.

In comparison with [2-^{14}C]streptozotocin and [3′-*methyl*-^{14}C]streptozotocin the sugar-labelled streptozotocin showed a great accumulation in liver and kidney but was cleared more rapidly. In addition, the failure to show any accumulation in the pancreas would suggest that the sugar moiety (70% recovered in the urine after 6h in excretion studies) may be cleaved from the rest of the molecule and therefore may not be involved directly in the diabetogenic activity of streptozotocin. This may contradict the hypothesis advanced by Schein & Loftus (1968) to explain the diabetogenic action of streptozotocin.

Their hypothesis was based on the finding that the aglycone of streptozotocin (*N*-methyl-*N*-nitrosourea) was non-diabetogenic in rats. Thus they have postulated that the attachment of the sugar moiety to *N*-methyl-*N*-nitrosourea conferred on the resulting streptozotocin molecule the ability to enter and destroy the pancreatic cell. However, the suggestion advanced by Dixit *et al.* (1972) that streptozotocin may affect the permeability of the β-cell membrane cannot be ruled out.

It has been shown (Bannister, 1972) that analogues and isomers of streptozotocin either with altered stereochemistry in the sugar moiety or with the side chain bonded to different positions of the sugar moiety are not diabetogenic in the rat. Thus the possibility remains that the stereochemistry associated with the sugar moiety may either be essential for enzymic degradation of the drug (yielding the diabetogenic metabolite) or it may facilitate the transport of streptozotocin into liver for metabolic transformation (which may yield a metabolite with potent diabetogenic properties).

In connexion with the possible loss of the carbohydrate moiety and the diabetogenic activity of the ureido side chain or its metabolite, it is interesting to compare the accumulation of radioactivity in the pancreas with the reported time-course for the pathogenesis of streptozotocin-induced β-cell necrosis. In their studies, Howell & Whitfield (1972) observed some degree of hypertrophy of the β-cell golgi complex after 1h, marked hypertrophy after 3h, vesiculation of β-cell endoplasmic reticulum after 4.5h and complete destruction at 6h. Thus the similarity between the time-course of β-cell necrosis and the pancreatic accumulation of radioactivity after the administration of [3′-*methyl*-^{14}C]streptozotocin may suggest that a metabolite formed from the ureido side chain may be involved in the induction of diabetes.

It is noteworthy that the second peak of hepatic radioactivity after the administration of [3′-*methyl*-^{14}C]streptozotocin coincides with the initial hyperglycaemic peak after a diabetogenic dose of streptozotocin. Since Schein & Bates (1968) have conclusively shown that this primary hyperglycaemia is not dependent on the adrenal gland but on the availability of liver glycogen it would be of interest to investigate whether glycogenolysis is influenced directly or indirectly by streptozotocin.

In conclusion, the results presented in this paper, in addition to the description of distribution and excretion of streptozotocin provide some preliminary evidence of the metabolic fate and possible mode of action of the drug. The variation in distribution and excretion patterns between each of the three labelled forms of streptozotocin would suggest that major metabolic degradation occurs and this results in different fates for each of the labelled forms of the drug. In each instance a large proportion of the injected radioactivity was rapidly cleared from the bloodstream by the kidneys and liver. The initial rapid renal clearance probably involves unchanged streptozotocin, although renal metabolism may occur, and the rapid hepatic uptake may point to the possible site of major metabolism. Although some hepatic radioactivity may be lost via the bile, the bulk appears to be cleared via the kidneys.

The failure to associate large quantities of radioactivity with the pancreas may reflect the rapid clearance from the body. The diabetogenic effect of streptozotocin may therefore result either from the relatively small amount of the drug reaching the pancreatic tissue or from one of the metabolites of streptozotocin. In addition the possibility cannot be excluded that either streptozotocin or one of its metabolites may affect some other tissue and that pancreatic damage is secondary to this effect.

The retention of a large proportion of the radioactive dose only with [3′-*methyl*-^{14}C]streptozotocin, together with its observed pancreatic accumulation and the non-accumulation of the sugar moiety in the pancreas, may suggest that a metabolite containing the 3′-[^{14}C]methyl group may be directly involved in the pancreatic damage, whereas the sugar moiety may be required, possibly at another site, for the formation of this metabolite. A detailed metabolic study, including identification of metabolites should clarify the above possibilities.

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