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On the Accumulation of Alloxan in the Pancreatic β-Cells

LARS HAMMARSTRÖM, BO HELLMAN and SVEN ULLBERG

Department of Pharmacology, Royal Veterinary College, Stockholm, and Department of Histology, University of Umeå, Umeå, Sweden

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Summary. Autoradiographic studies revealed that the radioactivity in the pancreatic islets was higher than in any other mouse tissue after intravenous injections of tracer doses of ^{14}C-2-alloxan. The concentration of radioactivity in the endocrine pancreas concerned a great majority of the cells indicating that at least β cells were involved. The uptake of the radioisotope in the pancreatic islets was considerably reduced when the small amounts of ^{14}C-2-alloxan were complemented with carrier to bring up the total dose to the diabetogenic level or were preceeded by higher doses of non-radioactive alloxan. There was no accumulation of radioactivity in the islets after injection of the non-diabetogenic conversion products of ^{14}C-2-alloxan obtained in an alkaline medium and only insignificant uptake was noted after exposure of the radioactive alloxan to the reactive SH-groups of glutathione. The absence of significant radioactivity in the islets of growing animals after tracer doses of ^{14}C-2-alloxan suggests that the ability of the β cells to concentrate alloxan is confined to the adult age.

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A propos de l'accumulation d'alloxane dans les cellules β du pancréas.

Résumé. Des études autoradiographiques ont révélé que la radioactivité était plus élevée dans les îlots du pancréas que dans les autres tissus de la souris, après des injections intraveineuses de doses traceuses d'alloxane-2-^{14}C. La concentration de la radioactivité dans le pancréas endocrine a pu être localisée dans une grande majorité des cellules, indiquant qu'au moins les cellules β étaient impliquées. La captation du radioisotope dans les îlots du pancréas était considérablement réduite, quand les petites quantités d'alloxane-2-^{14}C étaient administrées avec de l'alloxane non radioactif pour élever la dose totale au taux diabétogène, ou quand elles étaient précédées par de plus fortes doses d'alloxane non radioactif. Il n'y avait pas d'accumulation de la radioactivité dans les îlots après injection de produits de transformation non diabétogènes de l'alloxane-2-^{14}C obtenus dans un milieu alcalin, et on n'a observé qu'une captation non significative après exposition de l'alloxane radioactif aux groupements -SH réactifs du gluthation. L'absence de radioactivité significative dans les îlots des animaux en cours de croissance, après des doses traceuses d'alloxane-2-^{14}C, suggère que l'aptitude des cellules β à concentrer l'alloxane se limite à l'âge adulte.

Über die Anhäufung von Alloxan in Pankreas-Beta-Zellen.

Zusammenfassung. Nach i. v. Injektion geringer Mengen von ^{14}C-2-Alloxan fand sich bei autoradiographischen Untersuchungen wesentlich mehr Radioaktivität in den Langerhans'schen Inseln als in den übrigen Geweben von Mäusen. Die Radioaktivitäts-Anhäufung im endokrinen Pankreas betraf die meisten Zellen, ein Hinweis darauf, daß die Beta-Zellen zumindest mitbeteiligt sind. Die Aufnahme des Radioisotops in die Pankreas-Zellen sank beträchtlich, wenn die kleinen Mengen von ^{14}C-2-Alloxan mit nicht radioaktivem Alloxan als Trägersubstanz versetzt wurden, so daß die Gesamt-Dosis der zur Erzeugung eines Diabetes notwendigen Menge entsprach oder wenn vorher höhere Dosen von nicht-radioaktivem Alloxan verabreicht worden waren. Nach Injektion nicht-diabetogener Umwandlungsprodukte von ^{14}C-2-Alloxan aus alkalischem Milieu wurde keine Radioaktivität in den Inselzellen gespeichert und auch nach Behandlung des radioaktiven Alloxans mit reaktiven SH-Gruppen von Glutathion fand sich nur eine geringe Aufnahme. Nach Injektion kleiner Dosen von ^{14}C-2-Alloxan zeigten Tiere im Wachstum keine Radioaktivitätsansammlung in den Langerhans'schen Inseln, was darauf hindeutet, daß die Fähigkeit der Beta-Zellen, Alloxan zu konzentrieren, auf das Erwachsenen-Alter beschränkt ist.

Key-words: pancreatic islets, alloxan, alloxanic acid, diabetes, autoradiography, specific uptake, mice, rats.

Despite the extensive use of alloxan for the induction of experimental diabetes, the mechanism underlying the destruction of the insulin producing β cells is still controversial. While several studies (LANDAU and RENOLD, 1954; COOPERSTEIN et al., 1964; COOPERSTEIN and LAZAROW, 1964) have failed to demonstrate any accumulation of alloxan in the islets, HAMMARSTRÖM and ULLBERG (1966) observed a considerable concentration of radioactivity in the pancreatic islets of normal mice after intravenous injections of small amounts of ^{14}C-2-alloxan. The marked lability of alloxan above pH 6 raises the question, whether the accumulation of radioactivity in the latter studies was due solely to alloxan or also to non-diabetogenic conversion products.

In the present investigation autoradiography has been utilized to compare the radioactivity distribution patterns after injection of ^{14}C-2-alloxan with those obtained from different ^{14}C-2-alloxan derivatives. The concentrating ability of the pancreatic islets for alloxan was also studied by varying the dose and by including mice pretreated with non-radioactive alloxan. Young animals are known to be less sensitive to the diabetogenic action of alloxan (FERNER, 1952). The possibility that this is related to the alloxan-concentrating ability of the β cells was also checked.

Material and Methods

A. Whole body autoradiography. For the preparation of the different alloxan solutions both a radioactive sample (^{14}C-2-alloxan, lot MH 5518 from Volk Radiochemical Co., Chicago; specific activity 5 mC/mM) and a non-radioactive preparation (Eastman Organic Chemical, Rochester, N.Y.) were used. The stability of the alloxan was ensured by dissolving it in HCl at pH 3.8 and injecting it in this form when not otherwise stated.

Intravenous injections of radioactive solutions of alloxan or its conversion products were performed in a total of 13 adult male mice weighing about 20 g. The mice belonged to the N.M.R.I. strain and were allowed free access to food. 0.2 ml solution, corresponding to 5 μC radioactivity, was injected in the tail vein of each animal. Two animals were injected with a diabetogenic dose (107 mg/kg; specific activity 0.33 mC/mM) and 7 animals were given a subdiabetogenic dose of a radioactive alloxan solution (7 mg/kg body weight; specific activity 5 mC/mM). In the latter group 5 of the mice had been pretreated by the intravenous injection, 15 minutes earlier, of 0.2 ml non-radioactive alloxan, corresponding doses of 0 mg, 14 mg, 50 mg, 100 mg and 200 mg per kg body weight respectively. The distribution of some conversion products of alloxan was also studied after treating the ^{14}C-2-alloxan in a concentration (0.7 mg/ml) equivalent to the above mentioned subdiabetogenic dose (7 mg/kg) either with 10 times the molar concentration of reduced glutathione or temporarily increasing the pH to 10.4 for 15 minutes. The non-diabetogenic conversion products obtained in each case were injected in 4 animals.

The distribution of alloxan was also studied in growing animals by injection of 0.1 ml of radioactive alloxan (specific activity 5 mC/mM) intraperitoneally in a 6 days old mouse or intracardially in a 10 days old rat. The dose was equivalent to 5 mg/kg body weight in the rat and to 9 mg/kg body weight in the mouse.

Both the adult and growing animals were killed 60 minutes after the injection of the radioactive alloxan or its conversion products by immersion (after ether anesthesia) in a mixture of solid carbon dioxide and hexane at a temperature of about −75°C. 20 μ thick sagittal sections from the frozen animals were cut, dried in a cold room (−10°C) and pressed against Structurix X-ray film. The exposure time was about 6 months. For a detailed description of the technique of whole body autoradiography reference is made to ULLBERG (1954; 1958). For semiquantitative estimations of the autoradiograms a comparison was made with the blackening obtained by ^{14}C-standards containing known concentrations of the isotope (BERLIN and ULLBERG, 1963).

B. Microautoradiography. Four male adult mice, which had been injected intravenously with a subdiabetogenic dose of ^{14}C-2-alloxan (7 mg/kg body weight, specific activity 5 mC/mM) were killed by decapitation after 15, 30, 60 and 240 minutes. Specimens of pancreas were removed immediately, rapidly frozen in isopentane, cooled with liquid nitrogen, and then freeze-dried and embedded in paraffin. Scotch tape was fastened to the paraffin blocks, and 5μ thick sections, adhering to the tape, were taken and mounted in a dark room on glycerine treated nuclear emulsion plates (Ilford, type G 5). After exposure for 3—6 months at −10°C, the pieces of tape were removed chemically and the plates developed with the sections still attached to the photographic emulsion. The microautoradiographic technique, which has previously been described in detail (HAMMARSTRÖM et al., 1965), prevents the tissue from coming into contact with water before the development of the film.

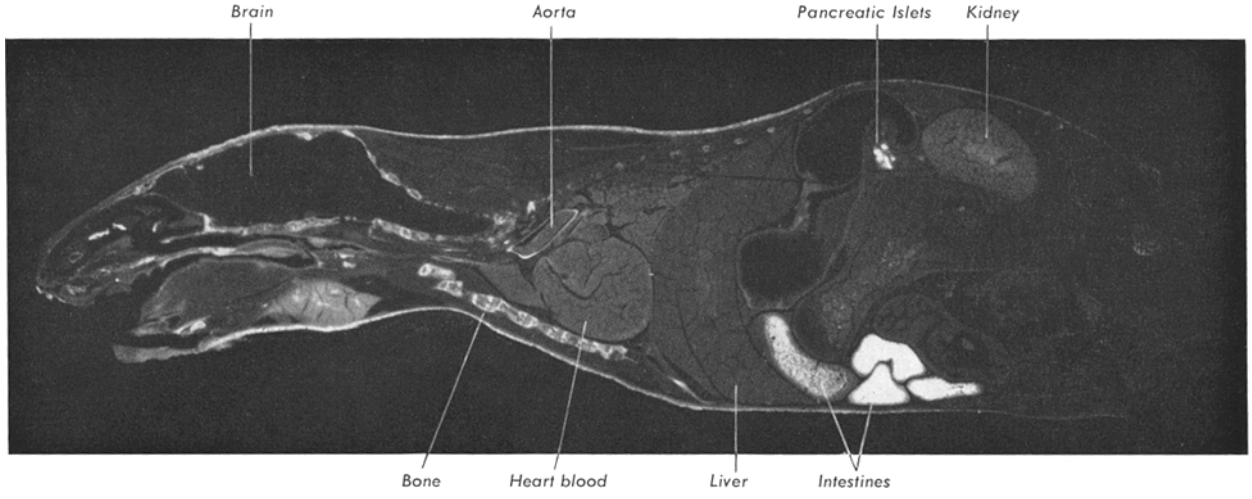

Fig. 1. Autoradiogram of a mouse 60 minutes after an intravenous injection of ^{14}C-2-alloxan. White areas correspond to high concentration of radioactivity. Note the uptake in the pancreatic islets. There is also a considerable radioactivity in the aortic wall, bone and intestinal lumen

Results

The whole body autoradiographic technique showed that the radioactivity in the pancreatic islets was higher than in any other tissue after the injection of

the subdiabetogenic dose of ^{14}C-2-alloxan (Fig. 1). While the radioactivity was insignificant in the exocrine pancreas, in the pancreatic islets it was more than 30 times higher than in the blood. The existence of a considerable accumulation of radioactivity in the pancreatic islets was confirmed by microautoradiography (Fig. 2) and found to persist nearly unchanged from 15 minutes after the injection during the following 4 hours. The higher resolution of the latter technique furthermore permitted the conclusion that the radioactivity was located in the majority of the islet cells, indicating that at least the β cells were involved. Administration of a subdiabetogenic dose of ^{14}C-2-alloxan also resulted in a higher radioactivity than in the blood for the arterial walls, bone and tendons (Fig. 1). The liver parenchyma displayed only moderate radioactivity and no radioisotope was taken up in the brain. Considerable urinary excretion was evident from the observation of a high radioactivity in the urinary bladder content. In the intestine radioactivity only appeared below the entrance of the common bile duct.

When equimolar amounts of the non-diabetogenic degradation products of ^{14}C-2-alloxan were injected, the pattern for the distribution of radioactivity among the tissues was modified in some important respects (Fig. 3 and 4). With the glutathione-reduced alloxan there was only insignificant uptake of radioactivity in the pancreatic islets, and the alkaline conversion product (alloxanic acid) did not accumulate in the endocrine pancreas at all. There appeared to be a higher radioactivity in the liver parenchyma when alloxanic acid was injected instead of alloxan or the reduced conversion products. The distribution pattern for the ^{14}C-2-alloxan inactivated with reduced glutathione was characterized by a rather high activity in the blood and a particularly pronounced affinity for bone.

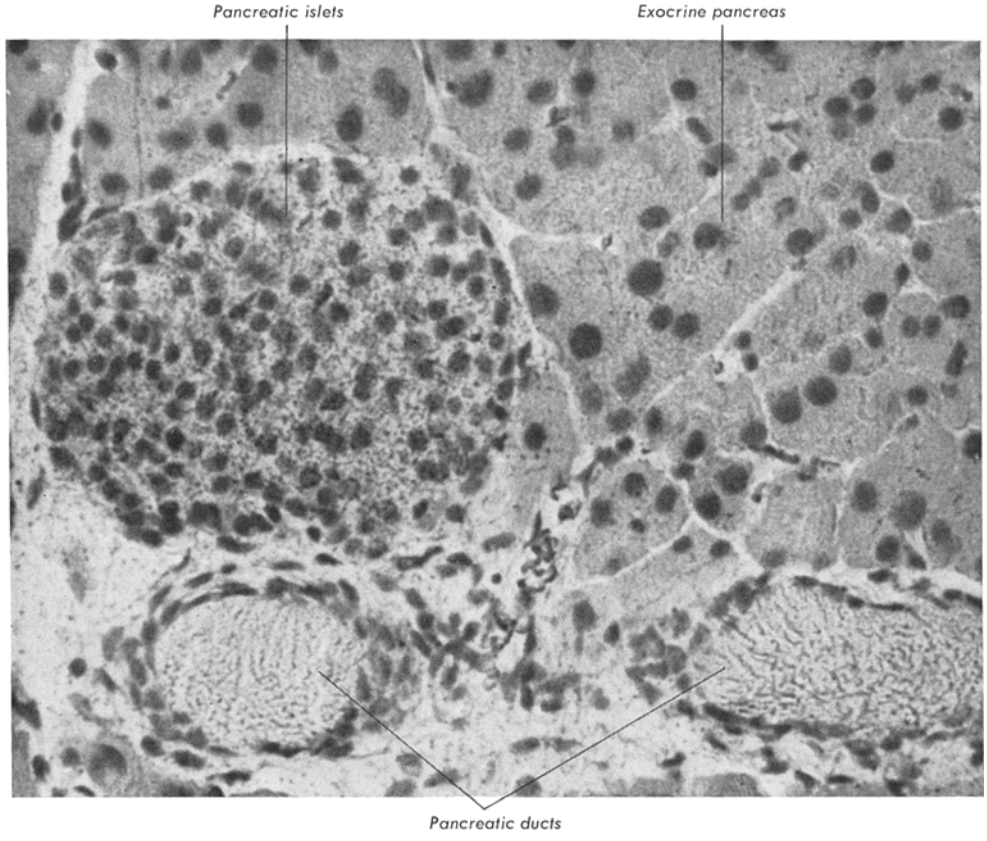

Fig. 2. Microautoradiogram of the pancreas of a mouse 60 minutes after an intravenous injection of ^{14}C-2-alloxan. Black grains show the localization of the radioactivity. Note the accumulation of radioactivity in most of the cells in the pancreatic islets

The accumulation of radioactivity was less pronounced in the pancreatic islets of the adult mice when ^{14}C-2-alloxan was administered together with carrier to bring up the total dose to the diabetogenic level. Injections of non-radioactive alloxan 15 minutes before the subdiabetogenic dose of ^{14}C-2-alloxan also diminshed the uptake of radioactivity in the pancreatic islets. While no radioactivity was concentrated in the islets after a preceeding dose of 50 mg/kg body weight or more, the islet radioactivity was reduced as compared with the buffer-injected controls when 15 mg/kg body weight was injected intravenously 15 minutes before the subdiabetogenic dose of ^{14}C-2-alloxan.

The growing mouse and rat injected with ^{14}C-2-alloxan were exceptional in not displaying accumu-

lation of radioactivity in the pancreatic islets. Considerable radioactivity was, however, noted in the kidney, bone, tendons and the arterial walls also in these animals.

Discussion

Since the important observation of DUNN et al. (1943) that injections of alloxan produce necrosis of the pancreatic β cells, this compound has been widely used for the induction of experimental diabetes.

After injections of ^{15}N- and ^{14}C- labelled alloxan the concentration was not higher in the whole pancreas than in other tissues such as kidney, liver, lung and spleen (LEE and STETTEN, 1952; JANES and WINNICK, 1952). Since the pancreatic islets only constitute a small fraction of the total weight of the mammalian pancreas, the distribution of labelled alloxan was studied also with autoradiographic techniques adapted for water soluble substances. Five minutes after the intravenous injections of diabetogenic doses of ^{14}C-2-

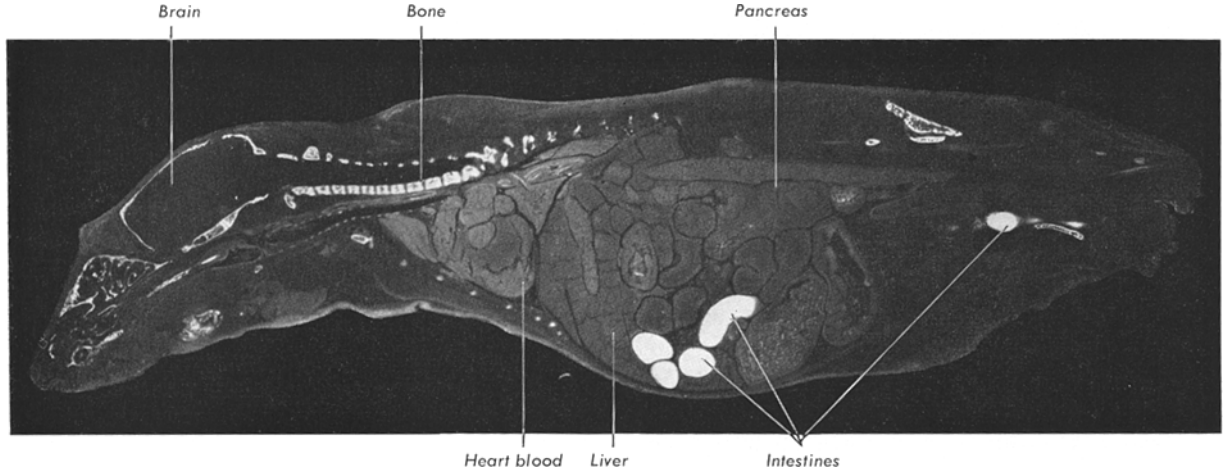

Fig. 3. Autoradiogram of a mouse 60 minutes after an intravenous injection of glutathione-reduced ^{14}C-2-alloxan. The very slight radioactivity in the pancreatic islets was too low to be visualized in the picture. There is, however, a high concentration in bone

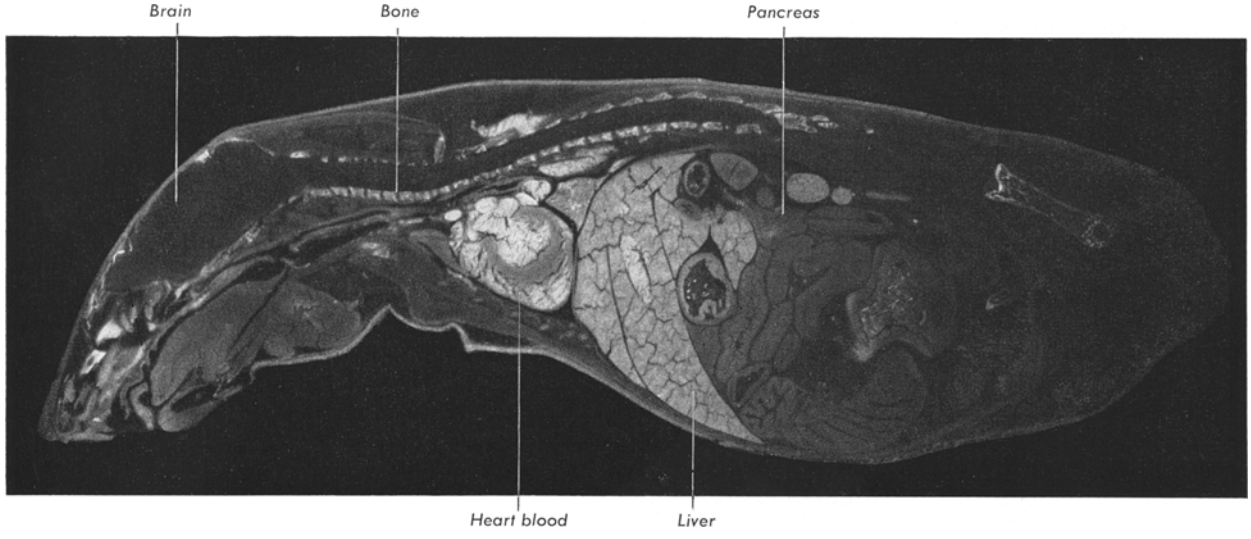

Fig. 4. Autoradiogram of a mouse 60 minutes after ani ntravenous injection of ^{14}C-2-alloxanic acid. While no radioactivity was accumulated in the pancreatic islets, there was a relatively high concentration in blood and a slight uptake in bone

HELLMAN and DIDERHOLM (1955) demonstrated that necrosis of the β cells could be obtained in the absence of extra-pancreatic influences by exposing the pancreatic parenchyma for a few minutes to moderate concentrations of alloxan. Whether this effect of alloxan was due to selective accumulation of the drug within the β cells or to a specific sensitivity of these cells to alloxan could, however, not be evaluated.

alloxan, LANDAU and RENOLD (1954) found that the radioactivity was not higher in the islets than in cells of other tissues. The latter authors concluded that the results tended to support the theory of a greater sensitivity of β cells to alloxan rather than that of a selective accumulation of this drug. It seems, however, possible that the high doses of alloxan used by LANDAU and RENOLD (1954) might be so destructive

as to render the β cells incapable of retaining any alloxan which had already been accumulated. Such a concept would be in agreement with the present observation that the accumulation of radioactivity in the β cells considerably decreased when the small amounts of ^{14}C-2-alloxan were complemented with carrier to bring up the total dose to the diabetogenic level or were preceeded by injections of higher doses of non-radioactive alloxan. The possibility of a saturation of possible alloxan receptor sites in the β cells after high or repeated doses of this compound should, however, also be considered.

Present ideas about the mechanisms of action of alloxan on the pancreatic β cells have, to a great extent, been based on experimental studies in bony fishes, where the islet tissue is segregated into a few discrete bodies. The fact that large amounts of islet tissue can easily be dissected in some fishes has, for example, made it possible to get more quantitative data about the distribution of radioactivity after injections of ^{14}C-2-alloxan than that obtained by autoradiography. After injection of tracer doses in a gill arch vessel of the toad fish COOPERSTEIN and LAZAROW (1964) reported that the radioisotope content of the islets never exceeded 50% of that in the blood. The latter authors therefore concluded that the injected alloxan was not selectively concentrated in the islet tissue of this species. From another series of experiments, mainly performed *in vitro*, it was suggested that small doses of alloxan did not penetrate the β cell membrane, which could be the primary site of the diabetogenic action of this compound (COOPERSTEIN et al., 1964; WATKINS et al., 1964a; b). The absence of alloxan accumulation in the fish islets is surprising in view of the present data and recent observations of a rapid and long-persisting concentration of radioactivity in the mouse islets after injection of ^{14}C-2-alloxan (HAMMARSTRÖM and ULLBERG, 1966). It should be borne in mind that it is possible to induce alloxan diabetes with necrosis of the β cells also in fish (FALKMER, 1961).

The absence of significant radioactivity in the islets of the growing animals after tracer doses of ^{14}C-2-alloxan suggests that the ability of the β cells to concentrate alloxan is confined to adult animals. The existence of such an age-dependent mechanism is in agreement with previous observations that young animals are resistant to the diabetogenic action of alloxan (FERNER, 1952). Some caution is, however, warranted in a more detailed comparison of the observations in the young rat and mouse with those in the adult animals in view of the different modes of injection, with resulting differences in the proportions of alloxan and its conversion products in the blood reaching the islets. It emerged from the present study that the non-diabetogenic conversion products of alloxan obtained in an alkaline medium or in the presence of reactive SH-groups did not accumulate in significant amounts in the pancreatic β cells. Alloxan is known to be transformed into alloxanic acid and water at alkaline pH (BILTZ et al., 1917), but its interaction with reduced glutathione is still not completely understood (PATTERSON et al., 1949; RESNIC and WOLFF, 1956). Since both types of conversion into non-diabetogenic products are known to occur rapidly *in vivo* (LAZAROW, 1949; SELIGSON and SELIGSON, 1951), an important fraction of radioactivity demonstrated after injections of ^{14}C-2-alloxan apparently reflects the distribution of the non-diabetogenic conversion products rather than of alloxan itself. This means that the accumulation of non-metabolised alloxan in the islets as compared to other tissues is still higher than indicated by the distribution of the radioactivity. The observation that alloxan, in contrast to its non-diabetogenic degradation products, significantly accumulates in the pancreatic β cells raises the question whether this also holds for other diabetogenic agents with similar structural formulae. Recent experiments by one of the present authors (HAMMARSTRÖM, 1966) have shown that dehydroascorbic acid also rapidly accumulates in the pancreatic islets. The latter substance has a chemical configuration almost identical with the mesoxalic portion of the alloxan molecule, and induces diabetes when administered in very large doses on several successive days (PATTERSON, 1950).

When injected in higher doses, the remarkable toxicity of alloxan for the β cells is frequently accompanied by damage to other organs. The observation that the β cells are particularly active in accumulating alloxan is, however, in itself a sufficient explanation for selective destruction of the β cells. A more than 30-fold concentration of alloxan in the β cells, as compared with the blood level, is for example consistent with an interaction with some intracellular components such as essential sulfhydryl enzymes. Even if it is not postulated that the β cells are deficient in sulfhydryl groups as a consequence of their specialization for the synthesis of the disulfide groups of insulin, the "sulfhydryl theory" of LAZAROW (1949; 1954) may well explain the alloxan destruction of the β cells. As a matter of fact, no deficiency of sulfhydryl groups has been found in the islet cells of either rats or fish, while the injection of alloxan selectively decreased the islet content of sulfhydryl groups in both species (MACDONALD, 1959; FALKMER, 1961).

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LARS HAMMARSTRÖM, Ph. D.
Department of Pharmacology
Royal Veterinary College
Stockholm, Sweden