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DRUGS PRODUCING DIABETES THROUGH DAMAGE OF THE INSULIN SECRETING CELLS

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I. INTRODUCTION

In this review the word diabetes instead of diabetes mellitus will be used throughout. The contents constitute a part only of the research field called experimental diabetes and is concerned with those chemical agents which have been reported to exert an immediate, toxic effect on the β-cells of the islets of Langerhans, which is followed by a chronic though not necessarily life-long diabetic state. These substances have also been referred to as β-cytotoxic sub-

stances or simply β-cytotoxins with the implication that the actions are restricted to the β-cells of the islets of Langerhans. Accordingly growth hormone (GH) and glucose, which are capable of producing a permanent diabetes due to the chronic hyperglycemia accompanying long-term treatment, are not included. The absence of a direct toxic effect of the latter substances on the islet β-cells is shown by the fact that simultaneous insulin treatment prevents the occurrence of diabetes.

Among the agents to be discussed alloxan is by far the most important, partly because it has been investigated more thoroughly on account of its relatively early discovery, and partly because none of the diabetogenic drugs subsequently discovered until 1960 exerts an equally specific action. Streptozotocin, which was reported to be diabetogenic in 1963 (138), appears to be comparable to alloxan with regard to islet β-cell specificity and has received great attention during recent years.

The literature about alloxan alone is vast, thousands of papers having been published during the last 25 years. No attempt has been made to catalogue all papers concerning β-cytotoxic substances; the main aim is to give a picture of the present situation in the field with emphasis on the description of diabetogenesis and the mode of action of the diabetogenic substances. The reader is referred to the following excellent reviews: Duff, 1945 (37); Lukens, 1948 (117); Bailey, 1949 (5); Lazarow, 1949 (100); Lazarow, 1954 (102); Lazarus and Volk, 1962 (109); Lazarow, 1965 (103); Webb, 1966 (168); Frerichs and Creutzfeldt, 1969 (46).

Alloxan, the discovery of which introduced the term "chemical diabetes" and which probably is still most widely used for the induction of diabetes at the present time, will be discussed first and somewhat more extensively. Next will be mentioned a series of substances of different chemical structure, which have turned out to be less specific in their action on the islet β-cells: ascorbic acid derivatives, quinoline derivatives, and diphenylthiocarbazone, together with some substances of doubtful β-cytotoxic activity. Finally streptozotocin, a remarkable compound endowed with broad-spectrum antibiotic, antitumor, carcinogenic, and highly specific β-cytotoxic activities, will be discussed.

II. ALLOXAN

A. Chemistry

Alloxan may be called mesoxalylurea, mesoxalylcarbamide, 2,4,5,6-tetraoxohexahydropyrimidine, or pyrimidinetetrone. It crystallizes with four parts of water (tetrahydrate), three of which can be removed successively by drying over sulfuric acid, to yield the form usually employed, the monohydrate, which is most probably hydrated at position 5 of the molecule. Heating *in vacuo* yields the anyhydrous compound, which in contrast to the colorless hydrates shows a strong yellow color due to the presence of three adjacent oxo-groups. Structurally similar to alloxan are the following substances, which differ only with regard to the substitution at position 5 as shown in parentheses: barbituric acid  ;

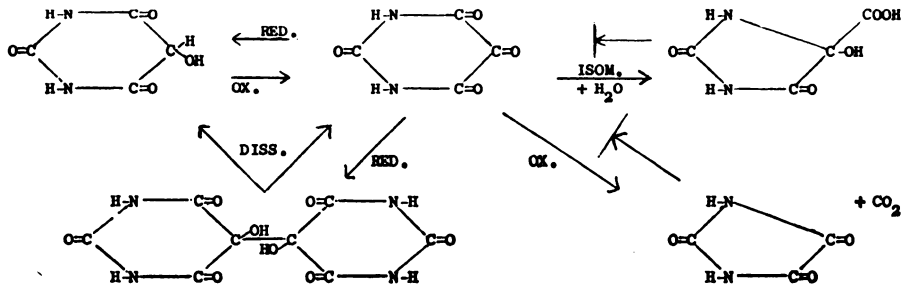

FIG. 1. Alloxan and its chemical transformation. The abbreviations used are: RED.: reduction; OX.: oxidation; DISS.: dissociation; ISOM.: isomeric transformation.

dialuric acid $\left(\begin{smallmatrix} H \\ OH \end{smallmatrix}\right)$; uramil $\left(\begin{smallmatrix} H \\ NH_2 \end{smallmatrix}\right)$; violuric acid (=N—OH). Alloxan has a slight oxidizing effect and yields alloxantin (uroxin), which may be regarded as a condensation product of two molecules of alloxan upon reduction. Further reduction yields dialuric acid (see above), which may be regarded as 5-hydroxy-barbituric acid. Dialuric acid and alloxantin may easily be reconverted into alloxan. In aqueous solution as well as in plasma (155) alloxan undergoes a spontaneous change into alloxanic acid, which is a structural isomer of alloxan monohydrate. Oxidation of alloxan yields parabanic acid and carbon dioxide. On long standing, particularly at room temperature, crystalline alloxan decomposes into alloxantin, oxalate, urea, CO_2, and murexide-like substances as indicated by the developing pink color. Structural formulae are given in figure 1.

Alloxan was made as early as in 1818 (22) by Brugnatelli, but a reproducible way of synthesis and a sufficient chemical characterization was given for the first time in 1838 by Wöhler and Liebig (173), who found the substance after direct oxidation of uric acid with nitric acid. Synthesis may be achieved in several ways (62, 128, 157, 173) with uric acid, alloxantin, or benzylidene-barbituric acid as starting material.

Alloxan is freely soluble in water and slightly acid with a pK_{a_1} of 6.63 according to Labes and Freisburger (92). The stability in aqueous solution is mainly dependent on pH and temperature. Below pH 3 alloxan is fairly stable at room temperature, whereas at pH 7 the solution has to be kept below 4°C ($t_{0.5} \geq 3$ hr), in order to avoid rapid formation of alloxanic acid. This indicates that in the body fluids of mammals and birds alloxan must decompose at a very high rate (see below). Certain chemical properties of alloxan have received particular attention in the past:

1) The substance mediates the so-called Strecker reaction, which means that α-amino acids are deaminated and decarboxylated to yield the corresponding aldehydes (159). Alloxan is not the only substance yielding a positive Strecker reaction; some other nondiabetogenic substances that share this property are ninhydrin, 1,8-mesoxalylnaphthalene, and isatin (139).

2) The substance has a high affinity for thiol groups, which become dehydrogenated (92, 107, 113, 114, 135). Even this reaction is not specific for alloxan.

3) The reported chelating property of alloxan (34, 36, 82, 95) has been questioned by Resnik and Cecil (144), who held that it is not alloxan but rather alloxanic acid which is the chelating agent.

B. Occurrence, determination and early reports

Liebig in 1862 (115) was the first to show the presence of alloxan in biological material. He treated a dialysate of mucus obtained from a patient having "intestinal catarrh" with ammonia and hydrogen cyanide and obtained oxaluramide, which he concluded was derived from alloxan. Lang in 1866 (94) gave evidence of the presence of alloxan in the urine of a patient with cardiac failure. As far as the author is aware there have been no further reports of the presence of alloxan in body fluids of man or laboratory animals. Alloxan has been fed to dogs (89, 120) without evoking a measurable change in urinary urea concentration or output, and alloxan itself could not be found. Wiener (171) reported that rabbits poisoned with alloxan died in a convulsive state. In the beginning of this century the substance and some of its derivatives were further investigated (15, 16, 145). Labes and Freisburger (92) showed that in frogs alloxan paralyzed gut capillaries and assumed that this was due to the reaction with thiol-groups. The same authors also reported the occurrence of convulsions and muscular rigidity after injection of high doses of alloxan (220 to 900 mg per kg of body weight) in these animals. Cerecedo (28) fed parabanic acid, alloxan, and alloxantin to dogs and investigated the metabolism of purine bodies without finding any change from normal. Jacobs in 1937 (75) injected alloxan into rabbits and observed several hours later a severe hypoglycemia accompanied by convulsions, which could be readily relieved by administration of glucose. He also observed a marked early hyperglycemia in the one nonfasted rabbit investigated. Jacobs was very close indeed to the discovery of the diabetogenic action of alloxan. After the discovery of alloxan diabetes in 1943 (6, 42, 43, 50, 53) more specific and sensitive methods for alloxan determination were elaborated. Among others (9, 19, 111, 173) Archibald (2) has given an excellent account of the determination of alloxan based on gasometric, titrimetric, colorimetric, or fluorometric methods. Fluorometry is the most sensitive and allows the detection of 20 μg of alloxan per 100 ml. In normal man and the dog alloxan has not been detected; this means that if present at all its level must be lower than 20 μg per 100 ml.

C. Diabetogenesis

1. Discovery. This happened quite unintentionally in 1943 and was triggered by findings of Dunn *et al.* (43), who by studying the crush syndrome in rabbits investigated the effect of a series of uric acid derivatives including alloxan with regard to kidney damage. The animals which recived alloxan became comatose after 12 hr, and showed hypothermia, high blood urea, and low blood sugar values. Shortly after the injection there was a marked hyperglycemia. Histological examination revealed early signs of the expected kidney damage, but the surprising finding was a partial or even total necrosis of the pancreatic islets.

Other tissues showed no damage. This and the following report (41) on a selective necrosis of the islets of Langerhans provoked by alloxan injection immediately led to investigations in several laboratories with the aim of keeping alloxan treated animals alive during the hypoglycemia in order to see whether a diabetic state would ensue. This was the case. In the same year alloxan injection was shown to result in a diabetic state in the rat by Dunn and McLetchie (42), in the rabbit by Bailey and Bailey (6) and Brunschwig *et al.* (24), and in the dog by Goldner and Gomori (50). The alloxan diabetes showed the classical signs of human diabetes, *i.e.*, hyperglycemia, glucosuria, polydipsia and polyuria, loss in body weight despite polyphagia, hyperlipemia, ketonuria, and acidosis. The discovery was the beginning of intense research all over the world concerned with the new "chemical diabetes," which supplemented the forms of experimental diabetes already known, *i.e.*, surgical diabetes (124) and growth-hormone diabetes (174). In 1948 it was known that alloxan produces diabetes in the rabbit, rat, dog, hamster, cat, sheep, monkey, man turtle, pigeon (117), and mouse (97). Hooded rats which, at first, appeared to be resistant to alloxan (53), were later shown to develop alloxan diabetes (38). Of the mammals investigated the only species resistant to alloxan diabetogenesis is the guinea pig (78, 79, 170).

2. Route of administration, distribution, dose, and sensitivity. Alloxan diabetes has been produced after intravenous (6, 7), intramuscular (42), intraperitoneal (53, 106), subcutaneous (42), oral (149), enteral (150), and intrapulmonary administration (48). In most papers no particular reason has been given for the route of administration, which then appears to have been dependent upon personal preference or skill. When a certain route was chosen deliberately it was either because no other route was successful (87) or in order to see whether still untried routes were effective (149, 150), or in order to perform a direct comparison of different routes (106). From the evidence available it appears safe to conclude that alloxan passes readily from most tissues into the circulation.

Alloxan has been reported to pass the placenta (47). At doses diabetogenic to the mother it did not cause diabetes in the offspring. This suggested that the alloxan never had reached a diabetogenic concentration in the embryo. It has been shown, in addition, that very young animals have a high resistance to the diabetogenic effect of alloxan (33).

In homeothermic animals the distribution of alloxan in the body is difficult to assess because of the extremely short half life of the substance at the prevailing pH and temperature. Earlier investigations based on alloxan determinations in rat pancreas or accumulation of radioactivity in various tissues after injection of ^{14}C-labelled or ^{15}N-labelled alloxan (21, 77, 110) did not indicate a selective concentration of the drug in the islets of Langerhans. A radioautographic study of tissues in rats treated with diabetogenic doses of ^{14}C-labelled alloxan led to the conclusion that alloxan is not selectively accumulated in pancreatic islet tissue (93). However, in mice treated with much lower doses, *i.e.*, 4 to 5 % of those used for production of diabetes, radioactivity accumulation in the islets far exceeded that in any other tissue except initially in the kidney (60). It is uncertain from the data whether alloxan entered the cells or was concentrated

at the cell membranes. In toadfish alloxan appears to be confined to the extracellular space (31, 32).

With regard to differences in sensitivity to the diabetogenic effect of alloxan, at least three sources of variation should be clearly distinguished: 1) the variation in mean sensitivity between species; 2) The variation in mean sensitivity within species between strains or laboratories; and 3) the variation in individual sensitivity within a given experiment under the prevailing laboratory conditions which may, as a first approximation, be assumed to follow a log normal distribution.

An exact comparison of the sensitivity to alloxan between and even within species would require dependable determinations of the ED50, the dose rendering half of the animals diabetic. Data of this type are scanty since the object of most studies has been to obtain as many diabetic animals as possible. Furthermore, the proper assessment of the diabetic ED50 in animals larger than the rat would normally face enormous practical difficulties. In the literature the diabetogenic dose has no strict definition. It has been cited as the dose rendering all (166), nearly all (105), a high percentage (121), or 80% (49) of the animals diabetic; these statements do not permit a quantitative expression of the ED50.

Factors influencing the size of the diabetogenic dose, but not the sensitivity to alloxan in a given species, are of course the route of administration and, in case of intravenous application, the speed of injection. The mean sensitivity of a group of animals, on the other hand, may depend upon the special laboratory conditions at hand, the animals' state of nutrition and the composition of the diet (section II C 5). In principle (55), in order for a drug with a short biological half life to reach a given minimal effective concentration during a given minimal time at a specific site, it should be given intravenously if it is desired to keep the general toxicity low. Alloxan has a very short half life in blood ($t_{0.5}$ <1 min) (21, 111, 134, 163). The ideal situation would be to inject or infuse it into the pancreatic artery. This would minimize the amount of alloxan and therefore yield a maximal protection against general toxic effects.

These theoretical considerations do agree well with the data from the literature showing that the doses for diabetogenesis usually are the lowest when given intravenously (5, 46, 117). In rats diabetes could be produced with 40 mg of alloxan per kg of body weight intravenously, whereas to obtain the same result after intraperitoneal injection the dose had to be increased at least 5-fold (106). Reports citing the diabetogenic dose to be 100 to 200 mg per kg intravenously or subcutaneously (46) have thus to be looked at with some reservation. The peroral route, which ordinarily would not be expected to be effective, was shown to yield diabetes (149) only under special conditions, *i.e.*, if high doses were given (0.5 to 1 g per kg), if the animals were starved, and if the alloxan-food mixture was eaten rapidly. This author agrees entirely with the view presented by Lazarow and Palay (106) that the intravenous route is the only rational one and the least toxic one. It is obvious that the production of diabetes also depends upon the speed of the intravenous injection at a given dose level, the slower injection rates causing less effect (137).

The comparison of diabetogenic doses of alloxan becomes even more difficult in view of the fact that no common rule has been followed to express alloxan concentration. Some authors give their doses as milligrams of alloxan monohydrate, others as milligrams of anhydrous alloxan, and many just give the term alloxan, which then may have been anhydrous, mono-, or tetrahydrate corresponding to 100, 89, and 66 % pure alloxan.

In view of these sources of variation in the diabetogenic doses of alloxan, it is not surprising to find controversial reports in the literature. For example the dog is usually listed as the most sensitive species (46, 139) becoming diabetic after 50 to 75 mg of alloxan per kg intravenously. On the other hand, the diabetogenic dose in rats is 30 to 70 (21) or 40 mg per kg. (106). It is the author's opinion that most of the minor quantitative differences in diabetogenic doses in mammals cited in the literature (46, 117, 139) cannot be regarded as proved, since they are based on comparions between laboratories. In studies especially aimed at this question and performed within one laboratory, however, the demonstration of quantitative differences in susceptibility to alloxan even within species, *e.g.*, mice (123) or rats (10), may be regarded as established. In addition, larger differences, *e.g.*, that between mammals and birds and the qualitative difference between the guinea pig and other mammals, are well documented.

3. Blood sugar, liver glycogen, and plasma insulin level changes. These three parameters will be treated together because of their interdependence and because marked fluctuations do occur after administration of alloxan. Even here the response to alloxan is subject to a wide inter- and intraspecies variation and depends upon several factors, *e.g.*, treatment with various substances, endocrine disturbances like hypophysectomy or adrenalectomy, the state of nutrition, including the composition of the diet, and unknown factors, which may be called the laboratory conditions at hand.

A. NORMAL ANIMALS. In normal nonfasted animals the blood glucose level fluctuates after a diabetogenic dose of alloxan in a characteristic way usually reported to be triphasic. The phases are: 1) an early, marked hyperglycemia of short duration (about 1 to 4 hr); 2) a more or less severe hypoglycemia lasting up to 48 hr and often resulting in convulsions and death, which may be prevented by treatment with glucose; 3) a chronic hyperglycemia of long, but not necessarily life-long duration, representing the alloxan diabetes. A slight fall in blood glucose immediately after injection of alloxan, and before the early hyperglycemia has been reported in the dog (156, 172), and hence for this species a tetraphasic blood sugar response to alloxan was postulated. This slight initial hypoglycemia in the dog has not been mentioned by others (49, 71), it has not been reported in other species, and in the mouse it has been shown to be absent (142). In most species, however, data on blood glucose level fluctuations during the first 30 min after administration of alloxan are lacking.

Liver glycogen is depleted during the early hyperglycemia (91, 125, 142); the lowest concentrations occur at about the time of peak hyperglycemia. During the hypoglycemic phase liver glycogen levels return to or increase above the preinjection level. During the state of alloxan diabetes liver glycogen levels

range from normal to about half of the normal values. Plasma insulin levels determined immunologically decrease to very low levels during the early hyperglycemia in mice and then increase markedly above normal levels, being highest at the time of most intense hypoglycemia or convulsive state (119). After the establishment of alloxan diabetes, plasma insulin levels have returned to below control levels and become very low or immeasurable in the course of a few weeks (119, 127).

In fasted animals the early hyperglycemia is attenuated or lacking (74, 75, 156, 172), whereas the hypoglycemia becomes more severe and leads to frequent attacks of convulsions and finally death in most animals if untreated. Since the manifest diabetic phase usually begins between 24 and 48 hr after alloxan injection, the effect of fasting on this phase cannot be properly assessed in view of the obvious need for food at this time.

The liver glycogen is already more or less depleted before alloxan treatment depending upon the duration of fasting, the metabolic rate of the animals, environmental temperature, *etc.* The fall in liver glycogen in fasted animals receiving alloxan will thus be smaller than in normals. It is reasonable to assume that it is the individual glycogen level in the liver at the time of alloxan injection that largely determines the extent of the early hyperglycemia and the severity of the hypoglycemic phase including the chance of survival (142).

B. ADRENALECTOMIZED ANIMALS. In these animals the early hyperglycemia after alloxan has been reported to be greatly reduced (142) or to be almost (74, 88) or totally absent (90). Mainly because of the lack of glucocorticosteroids, liver glycogen levels are lower in adrenalectomized than in normal animals. Animals lacking their adrenals experience a much more severe alloxan hypoglycemia than normals. Without repeated treatment with glucose at this stage all animals have convulsions and most of them die.

In order to elucidate the role of the adrenals in the mechanism of the early hyperglycemia Goldner and Gomori (51, 52) injected formalin into the adrenal medulla of rabbits. After injection of alloxan into these animals a pattern of blood sugar changes similar to that in adrenalectomized rabbits was seen. The authors therefore proposed that the adrenal medulla plays a major role in the development of the initial hyperglycemia. Kirschbaum *et al.* (88) and Iversen (74), however, showed that animals adrenodemedullated by means of enucleation or thermocautery as well as adrenalectomized animals treated with adrenal cortical extract all showed a marked hyperglycemia after alloxan. Their findings were later confirmed by Kosaka (90). The latter authors held that the adrenal cortex rather than the medulla is essential for the occurrence of the early hyperglycemia. Since Goldner and Gomori did not give evidence for a functioning adrenal cortex in their adrenodemedullated animals and since the early hyperglycemia is absent also in hypophysectomized animals (next section) with an intact adrenal medulla (74, 88, 142), more evidence should be obtained of the effect of the hormones of the adrenal medulla on the immediate rise in blood sugar after alloxan injection before an essential role of e.g. epinephrine can be generally accepted.

From what has been mentioned it is clear that in order to prepare alloxan diabetic adrenalectomized animals alloxanization should precede adrenalectomy. This procedure avoids the danger of fatal alloxan hypoglycemia and yields a maximal number of living preparations.

C. HYPOPHYSECTOMIZED ANIMALS. In these animals the early hyperglycemia after injection of alloxan is absent or quite insignificant as compared to normals (74, 88, 90, 142). This is in agreement with expectations from the results in adrenalectomized animals, since among other secondary deficiences no significant amounts of glucocorticosteroids are produced. The hypoglycemic phase is very pronounced, as in adrenalectomized animals, and, if untreated, all animals have convulsions and practically all die.

D. ANIMALS TREATED WITH INSULIN, TOLBUTAMIDE, OR GLUCOSE. The early hyperglycemia may be completely abolished by giving an appropriate dose of insulin together with alloxan. Higher doses of insulin cause an immediate drop in blood sugar level (49). In mice tolbutamide given 5 min *before* alloxan significantly attenuated but did not abolish the initial hperglycemia. Conversely tolbutamide given 5 min *after* alloxan did not diminish the early rise of blood sugar (119). Treatment with insulin does not influence the development of diabetes (49). The occurrence of the hypoglycemic phase may be prevented by glucose administration or by injection of anti-insulin serum (119), and the hypoglycemia and the convulsive state are readily overcome by administration of glucose (117).

The chronic hyperglycemia, *i.e.*, the alloxan diabetes, is sensitive to insulin, and fasting leads to a decrease of the elevated blood glucose level to normal or below (87, 117, 125). A second dose of alloxan, diabetogenic in normal animals, is not followed by the characteristic fluctuations of blood glucose level in alloxan diabetic mice (87, 142).

4. Potentiation and inhibition of diabetogenic effect. A. POTENTIATION. Highet and West (65) found a protective action of methylene blue given 30 min before alloxan. Both drugs were given intraperitoneally. The same authors also found that methylene blue lowered blood sugar in rats with established alloxan diabetes. Lazarow and Liambeis (104), on the other hand, showed that methylene blue, given immediately before alloxan (both drugs being given intravenously) increased the incidence of diabetes. The injection of methylene blue (35 mg per kg) followed immediately by alloxan 30 mg per kg rendered all rats diabetic (17/17), whereas the same dose of alloxan alone produced diabetes in only 52% of the rats (23/44). Stephens and Lazarow (158) also failed to observe any change of blood sugar level after methylene blue treatment in alloxan diabetic rats. These discrepancies are hard to explain and are given as an example of a large number of controversies in the field. This author's view appears from the heading of this chapter. Ammonium chloride given simultaneously with alloxan decreased the ED50 of diabetogenesis in rats to about 50% according to Brückmann and Wertheimer (21). No further study of this potentiating effect of ammonium salts on alloxan diabetogenesis appears to have been performed.

B. INHIBITION. Many factors are known that attenuate or inhibit the diabetogenic effect of alloxan.

i. Changes in blood circulation: Gomori and Goldner (54) clamped a portion of the pancreas in 13 dogs for different periods of time after intravenous injection of alloxan. The clamp was removed between 1 and 6 min after injection. It was shown histologically that clamping for less than 5 min yielded a complete protection against alloxan effects in the part of the pancreas that temporarily had been excluded from the general circulation. None of the dogs became diabetic.

Epinephrine has been reported to inhibit alloxan diabetogenesis (27, 86) and the interpretation of this effect is that the drug causes splanchnic vessel contraction which results in a diminished amount of alloxan reaching the pancreas (27, 105). Simultaneous treatment with dihydroergotamine, a competitive antagonist against epinephrine for smooth muscle receptors, abolished the inhibitory action of epinephrine on alloxan diabetogenesis (27).

ii. Inhibition by direct interaction with alloxan: Bernheim (11) observed that alloxan accelerated the metabolism of ethyl alcohol by liver tissue *in vitro* and that 3,4-diaminotoluene, phosphate buffer, pyrophosphate, bisulfite, or cyanide could inhibit this accelerating effect. The experiments of Jacobs (76) are indicative of a reaction of alloxan with reducing agents, *e.g.*, paraphenylenediamine, benzidine, pyrogallol, and iodides. This led Weinglass *et al.* (169) to test a series of substances, including the above, for an inhibitory action against alloxan diabetogenesis. Of the substances tested, 3,4-diaminotoluene, sodium bisulfite, and orthophenylenediamine showed definite inhibitory effects. Paraphenylenediamine, which was expected from the experiments *in vitro* to yield some protection, proved to be ineffective.

1,2-Dimethyl-4-amino-5(D-l'-ribitylamino)-benzene, which reacts with alloxan to form riboflavin, protected rats and rabbits against alloxan diabetes (8).

Lazarow and Patterson (108) obtained evidence that certain metal ions can protect against alloxan diabetes. They showed that zinc, cobaltous, and ferrous ions injected immediately before (but not after) alloxan, decreased the incidence of diabetes by 50% or more. Manganous, magnesium, cuprous, cupric, and ferric ions did not show this effect, It is generally believed that these protective compounds or ions interact chemically with alloxan.

iii. Inhibition by increase in level of physiological protective compounds: Alloxan injection results in a depletion of blood and tissue glutathione (21, 111). Lazarow (96, 98, 99, 107) showed that if glutathione, cysteine, or thioglycolic acid were given immediately before alloxan the diabetogenic effect was abolished; for this reason he called these substances natural protective compounds. Dimercaprol (BAL) also has a protective property (29, 98). These substances carry one or two free sulfhydryl groups and they are believed to protect by the reducing effect of the SH-group, which converts alloxan into dialuric acid (98, 99, 105, 107). Glutathione may also form an addition compound with alloxan called substance 305 (135). On a molar basis glutathione has the highest protective potency. The content of glutathione in the body may be increased by feeding thiouracil (70), the result being a protection against alloxan diabetes. Injection before alloxan of sodium nitrite or para-aminophenone, both of which raise the level of blood glutathione, also yield protection (165). Houssay (68) discussed

the action of sulfur compounds on carbohydrate metabolism and on diabetes. It appears that glutathione and cysteine interact with alloxan directly.

iv. Indirect inhibition: Banerjee (8) found that nicotinic acid, pyridinedicarboxylic acid, and 2-phenylquinoline-4-carboxylic acid also are protective against alloxan diabetes. The validity of the evidence regarding the effect of nicotinic acid (8) was questioned by Lazarow *et al.* (105), who later, however, confirmed that nicotinic acid and nicotinamide in fact protected if given 60 min before alloxan. Nicotinamide, but not nicotinic acid, was effective even if given immediately before alloxan, but only if the dose was increased by a factor of about three. Emerson *et al.* (44) found yeast pentnucleotide (N.N.R.) to protect rats against alloxan diabetes. Even in this case a better protection was obtained if the pentnucleotide was injected 1 hr or more before the alloxan. The fact that upon simultaneous administration of inhibitor and alloxan the necessary dose increased (or the effect of a given dose decreased) suggests that neither of these inhibiting substances takes part in a direct chemical reaction with alloxan. The mechanism of this protective action is not understood, but among the possibilities discussed by Lazarow *et al.* (105) the most probable seems to be that these compounds increase the free sulfhydryl groups in the blood, which in turn may react or combine directly with alloxan.

It has been shown recently that monoamine oxidase inhibitors, *i.e.*, nialamide and tranylcypromine, have a partially protective effect against alloxan diabetes (26, 126, 146). The drugs were effective when given 15 sec or 20 min before alloxan. The mechanism of their protective action appears so far to be unknown.

The inhibition of diabetogenesis by β-cell degranulation before alloxanization is beyond the scope of this review. Briefly, high doses of glucose, fructose, or mannose, but not galactose, given shortly before alloxan increase the resistance against alloxan diabetogenesis (13, 14). This increased resistance may be regarded as analogous to that following a high intake of fatty acids or a high protein diet (next section). The common situation is that the granule content of the β-cells is decreased below normal and this is associated with an increased resistance to the β-cytotoxic effect of alloxan. Treatment with either adrenocortical steroids or hypoglycemic sulfonylurea compounds resulting in β-cell degranulation may also lead to protection against alloxan diabetes (109).

5. Effect of diet composition and the state of nutrition on diabetogenesis. A. DIET COMPOSITION: Houssay and Martínez (70) investigated a series of diets with regard to mortality and diabetes incidence in rats receiving a standard dose of alloxan. They found that a low protein diet and a diet rich in lard or ox fat increased the incidence of alloxan diabetes (70). The same was found after feeding mixtures of high lard plus high protein content, high lard plus sulfanilamide, and high lard plus choline in the diet. On the other hand, the addition of coconut oil or methionine and in particular, thiouracil to a high lard diet prevented the increased incidence of alloxan diabetes. A diet with a high oleomargarine or high corn oil content partly protected and a high coconut oil diet completely protected against diabetogenesis. A high protein diet decreased the incidence of alloxan diabetes. The authors found that both the diabetogenic and the lethal

doses of alloxan were altered by diet and it is interesting to note that they described their results as changes in resistance rather than sensitivity which is the more usual expression in the literature. Their results have been confirmed (147).

B. STATE OF NUTRITION. During the first years after the discovery of alloxan diabetes it seems to have been difficult to obtain a satisfactorily high percentage of living diabetic animals. For this reason Kass and Waisbren (86) investigated the effect of fasting before injection of alloxan. Fasting increased the incidence of diabetes in rats after injections of 175 mg of alloxan per kg subcutaneously. It appears from their data that at a given dose level the incidence of diabetes inincreased with the time of starvation, but so did mortality, too. Their objective was achieved more conveniently by Lazarow and Palay (106), who showed that intravenous injection of alloxan (40 mg per kg) into fed rats yielded practically 100% diabetogenesis without fatalities. In rats starved for 48 hr 20 mg per kg of alloxan yielded 93% diabetic animals, whereas in fed rats this dose was followed by diabetes only sporadically (105). Manhoff and DeLoach (121) found that in the dog a period of 3 days starvation was necessary in order to obtain a sufficiently high percentage of diabetic animals. It appears, however, that less drastic fasting periods are equally efficient in the dog for the production of alloxan diabetes (33, 156, 172).

The early workers in the field used mostly fasted animals, but did not give a reason for doing so; this suggests the possibility that fasting of animals belonged to their laboratory routine. Jacobs (75) used fasted rabbits except one, which unfortunately received the largest dose of alloxan and convulsed. Had he used fed animals he would almost certainly have discovered the alloxan diabetes. The effect of fasting on the blood glucose response to alloxan has been dealt with above (section II C 3 a).

6. Histological changes in the islets of Langerhans. After a diabetogenic dose of alloxan a massive necrosis of the β-cells in the islets of Langerhans is observed in most mammals and certain other species (7, 37, 46, 109, 117). This effect of alloxan is so specific that besides slight and mostly reversible changes in the kidney and possibly the adrenal medulla (61) no other histological changes are encountered. As mentioned above it was the unexpected finding of this β-cell necrosis (43) that gave rise to the discovery of alloxan diabetes.

The histological changes in the islets after poisoning with alloxan have been investigated in most laboratory animals and reviewed excellently by several authors (37, 39, 41, 46, 109, 117), to whom the reader is referred for detailed descriptions. The changes appear to be similar though not always identical in various species. In brief, slight but definite changes consisting of diminished granule content have been reported to occur as early as 5 min after alloxan injection (7, 73) in rabbits and rats. Fifteen minutes after alloxan injection a marked reduction in the number of granules, shrinkage of the β-cells and an increase in pericapillary space are observed (109). One hour after alloxan poisoning nuclear pyknosis is evident and shrinkage of the cytoplasm more marked. The degenerative signs become more intense with time: the nucleus undergoes

karyolysis, the cytoplasm disintegrates, the cell boundaries disappear and finally a mass of debris containing fragments of nuclei witness the last phase of the necrotizing process, which is completed at about 24 hr after injection of alloxan in the rabbit. This time differs somewhat among species. A remarkable finding is the complete absence of a leukocytic response during and following necrosis.

Controversies with regard to the sequence of histological changes do exist, mostly between species, and the literature cited should be consulted for their study. As an example may serve the report that in the hamster the number of β-cell granules *increased* within 5 min after alloxan injection (67).

The important finding, which may be helpful for our understanding of the mechanism of action of alloxan, is that histological changes do occur *very rapidly* after administration of the poison.

7. Severity and duration. The severity of alloxan diabetes in an animal depends mainly upon the degree to which the β-cells have been damaged and the species in question. The duration of the diabetic condition is, in turn, related to the severity. After a high dose of alloxan resulting in an immediate destruction of nearly all β-cells the ensuing diabetes is very severe and death in diabetic coma within a few weeks is the usual consequence. Under these conditions reversible kidney damage is usually present. Insulin treatment is necessary to keep the animals alive for longer periods. Doses causing subtotal destruction of the β-cell population yield different pictures according to species. In carnivorous animals, *e.g.*, the dog or cat, the usual development is a permanent diabetes with ketoacidosis, which necessitates insulin treatment for survival. In rodents the diabetic condition may be permanent in some individuals, whereas in others a spontaneous remission occurs. This spontaneous recovery is particularly evident in rats and mice (25, 101, 140, 166), in which the majority of animals become normoglycemic, even though recovery may take many months in some individuals. The recovery from diabetes is believed to be the consequence of either a multiplication of the β-cells that survived the alloxan poisoning or a new formation of β-cells from the duct epithelium of the exocrine portion of the pancreas (25). In mice with manifest alloxan diabetes, provocation of insulin release by corticotropin is without a measurable response, whereas in mice that have partly recovered a marked increase in plasma immunoreactive insulin followed ACTH injection (140). Details of the recovery process are beyond the scope of this paper.

D. Effects on other organs

If more than the diabetogenic dose of alloxan is given to an animal the organ mostly reported to be seriously affected is the kidney. In the first studies on alloxan diabetes, in which higher than diabetogenic doses were often used, signs of kidney damage were usually present and consisted of vacuolation or hydropic changes, necrosis and desquamation of the tubular cells, whereas the glomeruli appeared normal (7, 23, 37, 41, 49, 69, 136, 150). The renal lesions are usually not permanent. After a few days a beginning restoration of the tubular cells towards their original appearance is seen, and in alloxan diabetes of long standing the

kidneys may look entirely normal. At extremely high doses the renal tubules undergo acute necrosis analogous to that of the islet β-cells and death from uremia is the consequence. The work of Lazarow and Palay (106) gives a good example of the relationship between diabetogenic and nephrotoxic doses of alloxan after administration by different routes. Alloxan, 40 mg per kg, given intravenously to 18 rats yielded 17 diabetic animals, 2 of which showed slight kidney necrosis. By contrast 200 mg per kg given intraperitoneally to 8 rats yielded kidney injury in all animals (3 showed severe, 2 moderate, and 3 slight kidney tubule necrosis), whereas only 6 rats developed diabetes. The impaired ratio of nephrotoxic over diabetogenic doses after intraperitoneal injection may be partly explained by the possibility of a direct absorption of alloxan from the peritoneal cavity into the kidneys. The functional impairment of the kidney is reflected by the finding of high nonprotein nitrogen values in the blood during the first week after induction of diabetes.

Toxic liver changes after alloxan at diabetogenic doses have not been reported consistently, whereas at higher doses fatty infiltration and central lobular necrosis have been observed in dogs (69). In rats, on the other hand, focal necrosis has been observed peripherally in the lobule (53, 106). Even here a leukocytic response was slight or absent. High intraperitoneal alloxan doses may lead to subcapsular liver necrosis, which may reflect a direct absorptive effect of the poison.

1. Other organs. The adrenals have been described by some authors to be slightly damaged after administration of alloxan (39, 150). Others have not confirmed these changes and it does not appear that the adrenal gland is highly susceptible to a toxic effect of alloxan at diabetogenic doses. Hard and Carr (61) observed necrotic spots in the adrenal medulla of rabbits injected with alloxan. The necrosis reported to occur in the adrenal cortex and the anterior pituitary gland (150) cannot be regarded as an established direct effect of alloxan, since the tissues were obtained from animals 5 days and 3 to 64 days after treatment. Also, these findings have not been confirmed.

E. Mechanism of diabetogenic action

The diabetic condition following alloxan poisoning is due to lack of insulin. The hormone level in the plasma is very low or unmeasurable and does not rise on injection of insulin releasing substances (119, 127, 140). Alloxan diabetic animals show a normal sensitivity to exogenous insulin. These findings together with the histological evidence of a selective islet β-cell destruction have given further strong evidence that insulin in fact originates in the β-cell.

The mechanism of action of alloxan on the islet β-cell is not understood in detail. Several hypotheses have been put forward, but none of them may be regarded as proved.

1. Strecker reaction. The possibility that alloxan may be diabetogenic on grounds of the Strecker reaction (section II A) appears very remote. The Strecker reaction proceeds slowly and, to provide an appreciable amount of the products in the β-cell, this reaction should take place intracellularly. In view of the short

half life of alloxan in blood and the finding that it follows the distribution of mannitol (31, 32, 167), being confined to the extracellular space, the occurrence of the Strecker reaction to any significant extent after injection of alloxan appears highly improbable. Diabetogenesis due to the Strecker reaction is also at variance with the fact that several nondiabetogenic substances yield a positive Strecker reaction.

2. Reaction with sulfhydryl groups. The hypothesis that alloxan may act by occupation or inactivation of SH-groups was advocated by Lazarow (96, 98, 107), who showed that substances containing free sulfhydryl groups protect against alloxan diabetes. These substances reduce alloxan to dialuric acid, which is nondiabetogenic unless it is reoxidized to alloxan. Furthermore, glutathione may react with alloxan to form an addition compound (135). Free sulfhydryl groups in the islet β-cell are necessary for insulin synthesis, and they are normally supplied by cysteine and glutathione or other substrates. Many enzymes contain free SH-groups essential for insulin synthesis. The reaction of alloxan with these SH-groups could, at least in part, explain the mechanism of diabetogenesis. The depletion of glutathione in the blood after injection of alloxan (21, 111) is in keeping with this concept. However, the Lazarow group later gave evidence (31, 32, 167) that alloxan does not enter the cell in the toad fish, and until now the proof that alloxan enters the islet β-cell of homeothermic animals appears to be lacking. For this reason the hypothesis of an intracellular action of alloxan on free sulfhydryl groups of amino acids, peptides, or active sites of enzymes needs much more supporting evidence before it can be generally accepted.

3. Chelating action. Kadota (82) has suggested that alloxan diabetogenesis may be due to a combination of alloxan with zinc in the islet β-cell, which in turn may cause cell necrosis. This effect would be analogous to the mode of action of the chelating agents oxine and dithizone reported by the same author and discussed below (section III B). With regard to alloxan this hypothesis became untenable when it was shown (144) that alloxan itself is not a chelating agent. A chelating reaction is observed, however, after conversion of alloxan into alloxanic acid, the isomer of alloxan monohydrate. Alloxanic acid, on the other hand, is nondiabetogenic. It thus appears that alloxan diabetes as the result of a chelating reaction is highly improbable.

4. Other hypotheses assuming enzymatic or metabolic alterations. The possibilities of a selective islet β-cell damage by enzymatic or metabolic alterations within, at the membrane of, or outside the β-cell have recently been presented in an excellent review by Webb (168). Possible actions include the following: inhibition of hexokinase or other enzymes; inactivation of coenzyme A; reaction with β-cell membranes and release of trypsin from the exocrine tissue. Hexokinase inactivation would require the alloxan to enter the cell, and, as for the reaction of alloxan with sulfhydryl groups, the evidence for this is lacking. All these mechanisms, though highly interesting, are entirely speculative, and for their detailed study the reader is referred to the paper by Webb (168).

In spite of the lack of detailed knowledge as to the mode of diabetogenic action

of alloxan we may arrive at some conclusions with a reasonable degree of certainty.

1) The site of action of alloxan for diabetogenesis is the islet β-cell membrane.

2) After intravenous injection the binding of alloxan to its site of action is completed within a few minutes.

3) The histological and most biochemical changes observed later than 5 min after intravenous alloxan injection are secondary changes and are not due to a direct alloxan effect.

5. Mechanisms responsible for changes in blood glucose during induction of diabetes. A. EARLY HYPERGLYCEMIA. The mechanism of the early hyperglycemia is not generally agreed upon. Some authors (49, 51, 52, 73) suggested that a sudden cessation of insulin release together with liberation of epinephrine result in the high blood sugar values recorded. Supporting evidence for this is that the hyperglycemia could be imitated by injection of epinephrine into normal rabbits (73) and that adrenodemedullation attenuated or abolished the early hyperglycemia (51, 52). Evidence against this view is that hyperglycemia has been observed in adrenodemedullated animals (74, 88, 90) and in adrenalectomized animals treated with adrenal cortical extract (74). Houssay *et al.* (71) held that the decisive factor for the early increase in blood sugar is the liver.

The initial hyperglycemia after alloxan cannot be explained solely on grounds of a sudden decrease or cessation of insulin release. Binding of released insulin by means of intravenous overdoses of anti-insulin serum in mice was followed by a much slower rise of blood glucose than after alloxan (119). An anti-insulin serum overdose would, however, be expected to yield a faster rise of blood sugar than a sudden cessation of endogenous insulin release, since the antibody would even combine with circulating insulin and probably with some insulin in the tissues.

On the basis of the marked fall in liver glycogen level initially it is this author's opinion that, at least in the mouse, the early hyperglycemia represents the sum of at least two concomitant effects, *i.e.*, a sudden short-lasting decrease or cessation of insulin release and a direct glycogenolytic effect on the liver. Epinephrine, if injected intravenously into mice, does not evoke a marked increase in blood glucose level (141). The mechanism of the glycogenolytic effect of alloxan *in vivo* (91, 119, 125) is not understood, and experiments do not, in general, confirm such an action *in vitro* (168).

It should be added here that because of the lack of systematic investigations covering most alloxan sensitive species no valid generalizations as to the mechanism of the initial hyperglycemia can be made at present.

B. HYPOGLYCEMIC PHASE. There is general agreement that the hypoglycemic phase is brought about by insulin. Evidence in favor of this view is that 1) plasma immunoreactive insulin is high during this phase (72, 119), and 2) anti-insulin serum treatment abolishes the hypoglycemia (119).

The occurrence of the hypoglycemic phase shows that alloxan does not inactivate the insulin present in the β-cell, and it has been suggested that this phase is the consequence of an uncontrolled leakage of insulin from the damaged

cells (73). If the integrity of the islet β-cell should depend upon an intact insulin releasing mechanism located in the cell membrane, the destruction of this functional part of the cell by alloxan would fit most of the experimental evidence.

The question whether the damaged β-cells still synthesize insulin is difficult to answer, since even after relatively high alloxan doses the presence of a small proportion of intact β-cells cannot be excluded.

C. CHRONIC DIABETIC PHASE. The mechanism of the third phase is uniformly agreed to be the consequence of insulin lack (117). Histologically only a few β-cells if any are detectable in animals with fully developed alloxan diabetes. Plasma immunoreactive insulin is very low or unmeasurable (72, 119). Exogenous insulin readily restores normal blood glucose levels (117). Insulin sensitivity persists during the whole period of alloxan diabetes. If a spontaneous remission is observed, as shown by decrease of mean blood sugar values with time towards normal, immunoreactive plasma insulin is again detectable after injection of an insulin-releasing substance (140).

F. Structure-activity relationships

Some substituted alloxans and substances formed by reduction of alloxan have been found to be diabetogenic, but none of these has an affinity for the site of action on the islet β-cell higher than or even equal to that of alloxan on a molar basis.

1. N-Substituted alloxans. Only short chain mono-N-substitution retains diabetogenic acitivity. Substitution at both nitrogen atoms yields highly nephrotoxic substances. Increasing chain length of the mono-N-substituted alloxans decreases the ratio of acute lethal dose to diabetogenic dose (21), which may be interpreted as a decrease of the specific diabetogenic action. Diabetogenic and lethal doses of alloxan and some derivatives are given in table 1. N-Propylalloxan as such could not be demonstrated to be diabetogenic since the animals died. However, at a reduced dose level in combination with ammonium chloride, which was known to potentiate alloxan diabetogenesis, N-propylalloxan induced diabetes. The excellent papers of Brückmann and Wertheimer should be consulted for detailed information (20, 21).

TABLE 1

*Approximate LD50 and diabetogenic ED50 values for alloxan and its mono-N-substituted aliphatic derivatives in the rat**

Substance	LD50	ED50	Ratio
	mg/kg	*mg/kg*	
Alloxan	250	45	5.6
Methylalloxan	135	53	2.5
Ethylalloxan	90	50–130	1.8–0.7
Propylalloxan	60	60†	≤1

* From the data of Brückman and Wertheimer (21).

† Diabetes production achieved only by addition of a potentiating agent.

2. C-Substituted alloxans. The reduction of alloxan takes place at the most reactive central carbon atom in position 5 and yields alloxantin or dialuric acid, which have been reported to be diabetogenic (21). Evidence of a direct diabetogenic effect is, however, lacking (see below). Other substitutions at position 5 in alloxan have been found to yield nondiabetogenic compounds, *e.g.*, barbituric acid, violuric acid, uramil, and phenoldialuric acid (139).

3. Alloxanic acid and parabanic acid. These are, respectively, an isomer and an oxidation product of alloxan, respectively, and have not been found to be diabetogenic. Formation of alloxan from these compounds *in vivo* does not seem to proceed at any measurable rate.

4. Alloxantin and dialuric acid. A direct diabetogenic effect of dialuric acid was postulated (21), but Lazarow (96, 98, 107) held that the substance is nondiabetogenic as such and that the diabetogenesis is entirely due to oxidation of the molecule *in vivo* yielding alloxan. Patterson *et al.* (135) furnished strong evidence for this view by showing that cysteine, for example, protects against alloxan diabetes by converting alloxan into dialuric acid (135).

Alloxantin in solution dissociates into equal parts of alloxan and dialuric acid, the latter of which is oxidized *in vivo* to alloxan. The diabetogenic action may, therefore, be considered to be secondary to alloxan formation.

The two above substances may thus be called indirectly acting diabetogenic agents. From what has been mentioned earlier about N-substitution of alloxan it was to be expected that even short chain mono-N-substituted dialuric acids or alloxantins should retain indirect diabetogenic action. Accordingly it was shown that monomethyldialuric acid (but not dimethyldialuric acid) and both dimethyl- and diethylalloxantin were diabetogenic (20, 21).

From these data some conclusions may be arrived at with regard to structural necessities for diabetogenesis by means of the alloxans: 1) an intact hexahydropyrimidine nucleus; 2) one free imino group, at least; 3) aliphatic N-substitution limited to maximally three carbon aoms; and 4) an oxo group at carbon atom 5 (irrespective of hydration).

III. β-CYTOTOXIC SUBSTANCES OF LOWER SPECIFICITY

A. Ascorbic acid derivatives

In 1946 Levey and Suter (112) showed that ascorbic acid increased the diabetogenic action of alloxan. Ascorbic acid itself is nondiabetogenic, but Patterson (131) showed that a single dose of dehydroascorbic acid produced a hyperglycemia of a few days duration and that three daily injections were capable in rats of producing "what appeared to be a permanent diabetes." Patterson pointed to several similarities between dehydroascorbic acid and alloxan. Both substances are ring structures contaiing three adjacent carbonyl groups in the anhydrous state. Both are hydrated in solution. Dehydroascorbic acid is partly reduced reversibly to ascorbic acid (66) and partly transformed irreversibly into diketogulonic acid (17), which is isomeric with monohydrated dehydroascorbic acid. This is reminiscent of alloxan which undergoes a reversible reduction to dialuric acid or an irreversible transformation into alloxanic acid, the

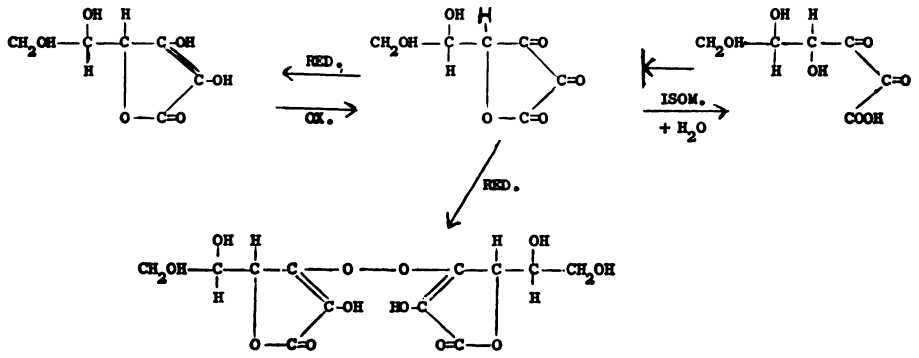

FIG. 2. Dehydroascorbic acid and its chemical transformation. The abbreviations used are: RED.: reduction; OX.: oxidation; ISOM.: isomeric transformation (mutarotation).

isomer of alloxan monohydrate. The transformations of dehydroascorbic acid appear in figure 2. The biological half life of dehydroascorbic acid is a few minutes (17). Reduction of dehydroascorbic acid may also yield an intermediary compound (12) analogous in structure to alloxantin upon reduction of alloxan. Both dehydroascorbic acid and alloxan form addition compounds with substance containing sulfhydryl groups (35, 132). Dehydroascorbic acid yields a positive Strecker reaction (132) (section II A).

The intravenous injection of dehydroascorbic acid into rats is followed by a characteristic reaction. About 200 mg per kg of body weight evoke excitation and aimless motor hyperactivity of about 2 min duration followed by respiratory arrest and collapse. Thereafter normal respiration is slowly resumed and the animals appear normal about 10 min after injection. A brownish red discharge around the eyes is usually observed and the state of prostration may be accompanied by a serous discharge from the nose and mouth. A higher dose results in death due to respiratory failure and the LD50 has been estimated to be about 320 mg per kg (132).

An interesting finding is that rats that have recovered from a sublethal dose of dehydroascorbic acid will survive a second dose up to 1 g per kg, which would be lethal in the untreated rat, with little or no hyperactive response, if given from 10 min to several hours after the priming dose. Permanent diabetes or diabetes of several weeks duration was not observed after a single dose of dehydroascorbic acid as high as 1.1 g per kg (achieved by means of a desensitizing priming dose), but with three daily doses of about 650 mg per kg 4 out of 5 rats were hyperglycemic for 3 weeks after the last injection. The use of repeated daily injections of dehydroascorbic acid was, however, accompanied by a high mortality rate. 25% of the animals died during the injection period, and of the surviving rats about 50% died within 3 weeks, with elevated blood glucose levels. It appears, therefore, that for the production of a diabetic state of longer duration in the rat dehydroascorbic acid is clearly very inferior to alloxan.

The mechanism of diabetogenic action of dehydroascorbic acid is not understood and according to Patterson (132) probably similar to that of alloxan, as

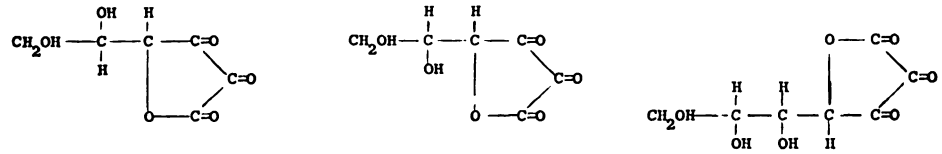

FIG. 3. Diabetogenic derivatives of dehydroascorbic acid.

indicated by a triphasic response of blood glucose following injection. The fact that animals diabetic from dehydroascorbic acid respond readily to exogenous insulin with a fall in blood sugar (132) indicates a decreased or abolished function of the insulin secreting cells.

Dehydroisoascorbic acid and dehydroglucoascorbic acid were also investigated for diabetogenic action (132, 133). The ring structure of dehydroisoascorbic acid is identical with that of dehydroascorbic acid, the difference being a D-configuration at carbon atom 5. Dehydroglucoascorbic acid, however, has the opposite configuration of the asymmetrical carbon atom involved in the ring structure and contains one more carbon atom in the side chain as shown in figure 3. Dehydroisoascorbic acid has a much weaker action, mole for mole, than dehydroascorbic acid in producing the hyperactive state, including lacrimation and salivation. Larger doses can thus be given and permanent diabetes has been reported to occur in rats given 1.5 g per kg. The compound is believed to have about the same diabetogenic potency as dehydroascorbic acid. After injection of dehydroglucoascorbic acid into rats the hyperactivity, lacrimation, and salivation were not observed. The substance was found to produce diabetes of at least 5 weeks duration after huge sublethal doses ranging from 3.5 to 3.9 g per kg. Doses larger than 4 g per kg were fatal. Diketogulonic acid, the mutarotation product of dehydroascorbic acid in which the lactone ring is opened, is inactive (133).

On account of the structural similarity of the above substances it is tempting to consider the mechanisms of their diabetogenic actions to be similar in nature. Much more experimental evidence is needed, however, in order to justify this assumption. In an attempt to elucidate the relation of chemical structure to diabetogenic action Patterson (133) suggested that a ring structure and three adjacent carbonyl groups are essential, whereas the configuration at the asymmetrical carbon atoms involved in and next to the ring structure is not a determining factor. It appears also that the antiscorbutic effect of dehydroascorbic acid is not in any known way linked to its diabetogenic effect.

B. Organic metal-binding compounds (chelating agents)

Styrylquinoline 90 was the very first compound observed to have a hyperglycemic action together with a toxic effect on the β-cells in the islets of Langerhans and on the kidneys. Chemically it is 2-para-acetylaminostyryl-6-dimethylaminoquinoline methochloride. The observations were made many years before the discovery of alloxan diabetes. Since the islet lesions were invariably fatal, however, the probability of achieving a permanent diabetic condition with this

compound may have appeared to be very low. The original account given by Sheehan from memory (43) was confirmed in part later (118). Because of the low ratio of mean lethal dose to mean diabetic dose the substance has not been used more widely.

The diabetogenic effects of 8-hydroxyquinoline (oxine), 8-hydroxyquinaldine, 8-hydroxy-5-aminoquinoline, and dithizone (diphenylthiocarbazone) were found in the following way. Okamoto (129, 130) reported on a histochemical method for demonstrating zinc by means of treating tissue with diphenylthiocarbazide or diphenylthiocarbazone. Applying his method Okamoto observed that zinc in the islets of Langerhans increased upon starvation and decreased markedly upon feeding with a diet rich in carbohydrate. He suggested that variations in the islet zinc content may reflect changes in carbohydrate metabolism. This, together with the earlier observation of Scott (154) that crystalline insulin contained zinc and the earlier belief that alloxan was a chelating agent capable of reacting with zinc (34, 36, 82, 95), led Kadota (82) to investigate organic compounds known to combine with metals for a possible diabetogenic effect. In the first series of 6 compounds oxine and dithizone were found to be diabetogenic in rabbits, yielding a diabetes of short duration. Diphenylthiocarbazide, which usually is applied for the histochemical demonstration of zinc, was inactive.

Later Kadota and Abe (1, 83) investigated further 13 quinoline derivatives and found 8-hydroxyquinaldine and 8-hydroxy-6-aminoquinoline to be diabetogenic. Chemically these two compounds may be looked at as 2-methyloxine and 5-amino-oxine, respectively, showing their close structural similarity to oxine. The structural formulae of diabetogenic quinoline derivatives and dithizone are given in figure 4.

The hydroxyl group in position 8, which is essential for the metal-binding property of the quinolines, appears also to be necessary for the diabetogenic action. Substitution with one methyl or one amino group at distinct positions retains the diabetogenic property; all other substitutions investigated led to loss of a specific β-cell toxic effect.

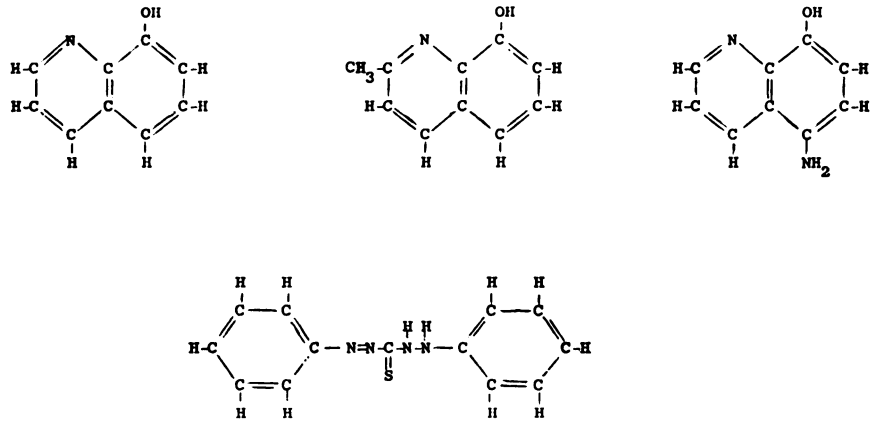

FIG. 4. Oxine and its diabetogenic derivatives, and dithizone.

After injection, and for dithizone even peroral application, of the above substances there is a triphasic blood sugar change that roughly parallels that after alloxan. Even histologically the similarity to the findings in alloxan injected animals is marked, including the absence of an inflammatory reaction in the necrotic islet tissue. Root and Chen (148) reinvestigated the diabetogenic effect of oxine in a series of species and made the interesting observation that oxine diabetes could not be produced in rats, guinea pigs, hamsters, cats, and dogs, but only in rabbits, the very species which Kadota (82) happened to use for his discovery. Root and Chen (148) also tried to induce diabetes by means of dithizone but had trouble in reproducing the results of Kadota because of difficulties in dissolving the compound.

It is apparent from the work cited that both oxine and dithizone diabetes are of much shorter duration than alloxan diabetes. After oxine less than 10% of the rabbits showed a hyperglycemia of more than 10 days duration, and reports on a long lasting diabetes after dithizone treatment appear to be lacking.

The dose of oxine necessary to produce diabetes was 50 mg per kg; the LD50 was estimated to be about 65 mg per kg. This means that oxine has about the same affinity, mole for mole, as alloxan for the islet β-cell, but that the former substance is far more toxic. The diabetogenic oxine derivatives have about the same diabetogenic potency and toxicity as oxine itself.

Very little can be said about the mechanism of β-cell damage and diabetogenesis by means of these substances. The original suggestion given by Kadota (82) that these agents may act primarily by combination with zinc is tempting but by no means proved. On the other hand, the similarity of the histological findings and the blood glucose fluctuations after oxine, dithizone, and alloxan treatment does not necessarily mean that the mechanism of diabetogenesis is identical.

In another study Kadota and Midorikawa (85) investigated a series of 24 organic metal-binding compounds including certain fatty acids, most of which proved to be nondiabetogenic. Two substances evoked an initial hyperglycemia followed by severe hypoglycemia in some animals and were studied in more detail.

Sodium diethyldithiocarbamate, 0.5 to 1 g per kg given intravenously to 15 rabbits, evoked in all animals an immediate, transient hyperglycemia, which was succeeded in 4 rabbits by an irreversible fatal hypoglycemia. There was no case of permanent diabetes. Degeneration of the α-cells and capillary lesions were observed in the islets of Langerhans, whereas no marked necrosis of the β-cells was recorded. It thus appears that diethyldithiocarbamate exerts a far more pronounced α-cytotoxic effect, which may be regarded as the opposite of a diabetogenic action. The mere occurrence of an initial hyperglycemia followed by a hypoglycemia together with histological islet damage, particularly in the α-cells, does not justify the expression "diabetogenic action" in the title of the paper (85), which may have been the reason for the statement in a recent review (46) that potassium or sodium diethylthiocarbamate have a very strong diabetogenic effect.

Potassium xanthate, also called potassium ethylxanthogenate or potassium

ethyldithiocarbonate, at doses between 200 and 350 mg per kg, evoked an initial hyperglycemia in all of 15 rabbits (85). Eleven animals were normoglycemic within 24 hr. Three rabbits became hypoglycemic and died despite attempts to keep them alive by administration of glucose. One rabbit became hyperglycemic for 2 months. The histological examination occasionally showed degenerative lesions in the islet β-cells, which were clearly less intense than those caused by alloxan, oxine, or dithizone. A more important finding was that degenerative β-cell changes always were accompanied by α-cell damage.

The evidence for a diabetogenic action of diethyldithiocarbamate is thus lacking, that of xanthate based on one out of 15 rabbits. No systematic study on many species has so far confirmed that these substances are diabetogenic by virtue of a specific β-cell damaging effect. Histologically their effect was either mainly in the α-cells or in both α- and β-cells, but not in the β-cells alone.

In a later study Kadota and Kawachi (84) investigated further 10 chelating substances with structural similarity to oxine. Three of these, *i.e.*, 6-hydroxy-*m*-phenanthroline, 1-hydroxyacridine, and 5-hydroxybenzo-f-quinoline produced an initial hyperglycemia, occasional convulsions, and a hyperglycemia of a few days duration in rabbits. No case of permanent or even subchronic diabetes was reported.

Therefore, until more convincing evidence has accumulated, one should be careful not to call the above cited 5 substances diabetogenic or specific β-cytotoxic agents.

C. Uric acid

Uric acid, which yields alloxan upon oxidation (173), has been reported by Griffiths (56, 57) to be diabetogenic in rabbits with artificially lowered blood glutathione levels. The incidence of diabetes was very low indeed since only one rabbit showed a hyperglycemia for as long as several weeks. The term transient hyperglycemia would seem more appropriate for the results cited, since only a low percentage of animals responded with a moderate hyperglycemia lasting for about 5 days. Collins-Williams and Bailey (30) tried to confirm the reported effect of uric acid. They did not obtain a single permanently diabetic rabbit and observed only one case of definite transient hyperglycemia for about 6 days in a group of 12 rabbits. Grunert and Phillips (59) were unable to produce uric acid diabetes in the rat. The doses used in rabbits were high: 1 g per kg was given intraperitoneally and the amount absorbed and the rate of absorption appear to have varied considerably (30). A more thorough histological investigation of the islets of Langerhans after treatment with uric acid seems to be lacking.

Griffiths (58) later put forward the view that an oxidation product of uric acid, *i.e.*, dehydrouric acid may also have a diabetogenic action. Since uric acid has not been found to be diabetogenic in normal animals of any species investigated, and only a low incidence of transient hyperglycemia has been reported in rabbits under marked dietary restrictions, this substance should not be listed among specific β-cytotoxic agents until more convincing evidence for its diabetogenic effect is forthcoming. Structural formulae of some of the substances with

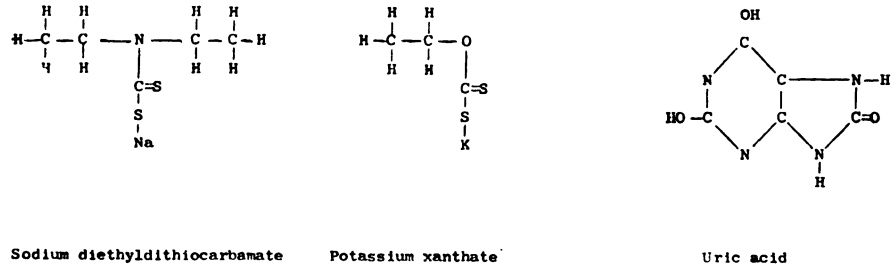

FIG. 5. Substances of doubtful diabetogenic action.

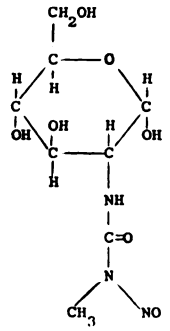

FIG. 6. Structural formula of α-streptozotocin.

doubtful specific β-cytotoxic action are given in figure 5. In conclusion it may be said that all substances dealt with in this chapter have not only a lower or, at best, equal molar affinity for the islet β-cell as compared to alloxan, but also a lower specificity of diabetogenic action as shown by the fact that their mean effective doses are much closer to their mean lethal doses than those of alloxan.

IV. STREPTOZOTOCIN

This substance is a broad-spectrum antibiotic. Its isolation from *Streptomyces achromogenes* was reported in 1959–1960 (63). Besides being an antibiotic it has been reported to be both an antileukemic agent (45) and a fairly effective carcinogen (4). A further unexpected property of streptozotocin was its highly specific diabetogenic effect, first reported by Rakieten *et al.* (138).

A. Chemistry

Streptozotocin consists, according to Herr *et al.* (64), of 1-methyl-1-nitrosourea linked to position C_2 of D-glucose as shown in figure 6. Its calculated molecular weight is 265. It is a colorless solid which decomposes at about 115°C with formation of gas. It is freely soluble in water and shows mutarotation to an equilibrium value of $[\alpha]_D^{25}$ 39°. The solid is usually a mixture of α- and β-isomers with regard to C_1 in the glucose moiety of the molecule. The substance is unstable at room and even refrigerator temperatures and has to be stored below 20°C. Upon solution in saline or distilled water at room temperature and neutral

pH it decomposes within a few minutes with visible formation of gas. Its stability in solution is optimal at pH 4 (138) and low temperature.

B. Diabetogenesis

1. Species, doses, and routes of administration. Streptozotocin induces diabetes in the rat, dog, hamster, monkey, mouse, and guinea pig (18, 116, 138, 152). Already the early reports show that the diabetes is of pancreatic origin (138, 160–162). Diabetogenic doses vary between species. In the dog a single intravenous dose of 50 mg per kg produced diabetes, but more than half of the animals died. The LD50 was estimated to lie between 25 and 50 mg per kg. In this species diabetes may conveniently be produced by repeated daily injections of lower doses; the minimum necessary was reported to be 15 mg per kg per day for 3 days (138).

In the rat the intravenous LD50 was estimated to be about 140 mg per kg. A single intravenous injection of 50 mg per kg was reported to yield 100% diabetes (138). Mice appear to be much less sensitive than rats and diabetes is usually produced with a dose of 175 to 200 mg per kg (143, 151, 152). Because of the low stability of streptozotocin the rapid intravenous injection appears to be the only dependable route of administration. The biological half life of streptozotocin was estimated to be about 5 min in mice (153).

2. Blood sugar, liver glycogen, and plasma insulin level changes. The triphasic pattern of blood glucose level fluctuations observed after diabetogenic doses of alloxan is also seen after streptozotocin (80, 81, 143, 151, 152). The effects of streptozotocin and alloxan have recently been compared under the same laboratory conditions in fed mice (143). It appeared that the initial hyperglycemia following streptozotocin had a characteristic delay in onset of about 45 to 60 min. This delay was not observed in fasted mice (151), but is also evident from experiments in rats (80, 81). After this delay the changes of blood glucose level in streptozotocin-treated animals roughly replicated those in alloxanized mice as seen in figure 7.

Hypoglycemia was more severe with streptozotocin than with alloxan, and accordingly fatal convulsions were more frequent in the former groups of mice. Tolbutamide did not attenuate the degree of initial hyperglycemia if given 5 min after alloxan (119), but evoked a clear reduction in the extent of the early hyperglycemia even if it was given as late as 60 min after streptozotocin injection (143).

Changes in plasma insulin and liver glycogen levels during induction of diabetes by streptozotocin resemble those seen after alloxan. Plasma immunoreactive insulin is high at the time of marked hypoglycemia (81, 143, 151). In mice a 7- to 9-fold increase in plasma insulin level over the normal was observed 7 to 9 hr after injection of the antibiotic (143, 151). When the diabetes has developed, plasma insulin was within the normal range in rats (81) though pancreatic insulin content had fallen to 2 to 5% of the normal. In mice, plasma insulin was not detected from 2 to 12 weeks after streptozotocin injection (141).

Initially liver glycogen depletion is roughly concomitant with blood sugar

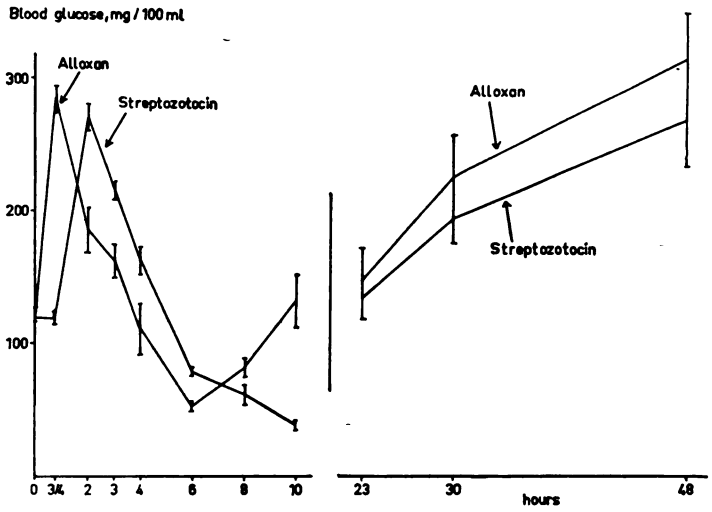

FIG. 7. Comparison of diabetogenic doses of streptozotocin and alloxan: changes of blood glucose in nonfasted female mice. The curves in the left part differ significantly ($P < .001$) except at 0 and 8 hr. The drugs were given at random to groups of 8 mice, and there were 11 blood samples (25 μl) per mouse. From Rerup and Tarding (143).

increase in the same way as after alloxan. Even here the delay in increase of blood glucose level is paralleled by a delay in liver glycogen fall. Liver glycogen levels were lowest about 90 min after streptozotocin, as opposed to about 30 min after alloxan. During the permanent diabetic state mice did not recover from the initial drop in body weight after streptozotocin injection, whereas alloxan-treated mice surpassed their preinjection body weight after 2 to 3 weeks. Streptozotocin diabetic mice, unlike alloxan diabetic animals, did not show spontaneous recovery from their chronic diabetic condition.

In rats, on the other hand, streptozotocin diabetes was much milder than alloxan diabetes as measured by ketosis and plasma free fatty acid concentration (122). Streptozotocin diabetes in the rat was described as a specific form of hyperglycemia with virtually no ketosis or elevation of plasma free fatty acids.

The above differences between species are given as an example of a variety of findings recently reported and to be expected about streptozotocin diabetes. It appears too early to try to arrive at generalizations with regard to the metabolic aspects of established streptozotocin diabetes.

3. Inhibition of streptozotocin diabetes. Nicotinamide (500 mg per kg) given intraperitoneally 10 min before streptozotocin, completely protected against diabetogenesis in mice and rats (152, 153). Nicotinic acid was ineffective, and glutathione, which protects against alloxan diabetes (96), did not show any inhibitory effect at doses up to 1.5 g per kg.

Recently Dulin and Wyse (40) found the following substances to be inactive in protecting against streptozotocin diabetes in the rat: mannoheptulose, glucosamine, diazoxide, NAD, epinephrine, 3,5-dimethylpyrazole, 3-carboxy-5-methylpyrazole, glutamic acid, glycine, asparagine, cysteine, tolbutamide,

guanidoacetic acid, glutathione, *p*-aminobenzoic acid, and ethyl alcohol. They confirmed the protecting effect of nicotinamide and found, in addition, pyrazinamide and 2-deoxyglucose to be effective. Structurally related compounds like nicotinic acid, glucose, and 2-carboxypyrazine, the latter of which is a metabolite of pyrazinamide, were inactive. The protecting effect of nicotinamide has been further confirmed (81).

4. Histology. In the early reports the main finding in the islets of Langerhans was a degranulation of the β-cells and disruption of the islets (116, 138, 164), but no conspicuous necrosis of the β-cells occurred. The α-cells appeared normal, though a possible simultaneous effect on these structures has been considered (3). Since the purity of the crystalline streptozotocin preparations used in the early investigations (45) was doubtful, the Renold group (80) reinvestigated the effects of pure streptozotocin in rats with both the light and electron microscope. These investigators clearly showed frank necrosis of the β-cells 7 hr after 65 mg of the antibiotic per kg given intravenously to fasted rats. The α-cells and the exocrine tissue appeared normal during the course of diabetes induction.

The question then arises whether the sequence of histological changes following streptozotocin treatment may be regarded as being like that after alloxan injection. There exist clear differences with regard to the time of occurrence of certain histological changes. After alloxan, changes in the number of β-cell granules have been reported to occur within 5 to 15 min, whereas after streptozotocin a comparable degranulation was not observed even after 1 hr. By 1 hr after alloxan treatment nuclear pyknosis is plainly apparent (109), whereas it was seen only occasionally 1 hr after streptozotocin injection.

Despite these differences with regard to the time of occurrence of certain phenomena the majority of histological findings point to a resemblance of the morphologic β-cytotoxic effects of streptozotocin and alloxan.

5. Diabetogenic and antitumor activity. Evidence of a complete independence of these two effects has been given (152). A single diabetogenic dose of streptozotocin markedly depletes liver NAD and NADH in mice for about 24 hr. Pretreatment with nicotinamide increases liver NAD and NADH for about 16 hr as a result of synthesis *de novo* of oxidized and reduced coenzyme. This pretreatment completely protects against streptozotocin diabetes (see above) but does not abolish antitumor activity. In fact, the combination of nicotinamide plus streptozotocin prolonged average life spans more than streptozotocin treatment alone in the L 1210 system (152). 1-Methyl-1-nitrosourea, which may be regarded as the aglycone of streptozotocin, acts as an antileukemic agent, but not as a diabetogenic agent (153). Like streptozotocin it depletes liver NAD and NADH. Alloxan does not lower the coenzyme level in the liver of mice. It thus appears that the presence of antitumor and diabetogenic activity in streptozotocin is due to different active sites in the molecule.

6. Metabolic differences as compared to alloxan diabetes. Streptozotocin diabetic rats, besides their pronounced hyperglycemia, have normal levels of blood ketones, plasma-free fatty acids and heart glycogen, whereas in alloxan diabetic rats all these parameters showed a marked elevation over nondiabetic

controls (122). In the perfused heart of alloxan diabetic rats glycogen, glucose-6-phosphate, fructose-6-phosphate, and citrate were significantly elevated over controls, whereas in perfused hearts from streptozotocin diabetic rats glycogen and citrate levels were lower than in normals and other glycolytic intermediates were within the normal range.

The above findings have been partly confirmed and partly extended by Junod *et al.* (81). Ketonuria was seen only after the largest dose of streptozotocin (100 mg per kg) in the rat. Furthermore, there was a quantitative relationship between the dose and the degree of islet β-cell damage. The best linear dependence observed was that between log dose and pancreatic immunoreactive insulin content 24 hr after injection of streptozotocin. In addition, 1 week after a low dose of streptozotocin (25 mg per kg), which did not yield a permanent hyperglycemia, the insulin response 30 min after a peroral glucose load was clearly decreased as compared to normal rats.

7. *Toxicity.* The ratio of acutely lethal doses to diabetogenic doses of streptozotocin is subject to a marked interspecies variation. It has been shown to be about 3 in the rat, about unity in the dog; for this reason single doses were not used in the latter species (138). In the mouse it has been estimated to be about 1.5 to 2 (141). It appears that the structures, apart from the islet β-cells, that are affected after overdoses of the drug are the kidney tubules, the liver, and the exocrine portion of the pancreas (3, 80).

Reports of spontaneous recovery from streptozotocin diabetes are conflicting. In mice streptozotocin diabetes showed no tendency to spontaneous remission (143), which is frequently seen in alloxan diabetic mice. On the other hand an apparent reversibility of islet β-cell damage in rats that received lower doses of the antibiotic has been repeatedly reported (3, 81). However, much more information is needed in order to settle the interesting question of spontaneous recovery from streptozotocin poisoning.

C. Mechanism of action

As for alloxan, the mechanism of action on the islet β-cell of streptozotocin is not understood. The specificity of the drug with regard to the effect on the β-cell is striking and has been claimed to be greater than that of alloxan (80). However, this appears to be true only for the rat. By contrast, in mice the margin between diabetogenic and generally toxic dose seems to be narrower for streptozotocin than for alloxan. Mole for mole, streptozotocin has generally no higher affinity for the site of action of the islet β-cell than alloxan, and again in mice the affinity for alloxan is higher: 0.42 millimoles of alloxan and 0.75 millimoles of streptozotocin are about equivalent with regard to diabetogenesis (119, 143).

It may be interesting in this connection to compare further streptozotocin and alloxan diabetes in order to try to judge possible differences in the diabetogenic mechanisms between these two highly β-cytotoxic compounds. From the evidence available it appears that the two substances do not act in an entirely identical way, though many observations show remarkable resemblance and sug-

gest great similarities of particular pathogenic reactions. Such similarities are the triphasic pattern of the blood glucose response, the depletion of liver glycogen at about the time of the peak of initial hyperglycemia, the elevated plasma insulin levels during the hypoglycemia, and the protecting effect of nicotinamide against diabetes induction by either drug. Conversely, clear discrepancies are the marked difference in time of the initial hyperglycemic peak response, time differences of histologically demonstrable changes in the islet β-cells, the ability of tolbutamide to influence the initial hyperglycemia if given after streptozotocin but not alloxan, and marked differences with regard to protection against diabetes as shown by the finding that glutathione, cysteine, nicotinic acid, epinephrine, and glucose protect against alloxan diabetes only. Even the permanent diabetic condition appears to be different especially with regard to the levels of plasma immunoreactive insulin (81) and, in addition, the observed differences are subject to interspecies variations as mentioned above.

Structure-activity studies of streptozotocin and related substances have to the author's knowledge not yet been presented. An interesting phenomenon, however, is the apparent necessity of the glucose moiety in the streptozotocin molecule for diabetogenic, but not antitumor activity as shown by Schein and Loftus (153). These authors considered the glucose moiety of streptozotocin to have a potential "carrier function" to bring about the contact with or the transport across the β-cell membrane.

Although the mechanism of the diabetogenic action of streptozotocin at present seems to be as poorly understood as that of alloxan, we may from the evidence available arrive at some general conclusions very similar to those applying for alloxan, *i.e.*, that the site of diabetogenic action of streptozotocin is the islet β-cell, that the binding of the drug to its site of action is completed within a very short time, and that the histological and biochemical changes observed later than about 15 min after intravenous injection are secondary changes and not due to a direct streptozotocin effect. It should be added here that a large number of questions pertaining to streptozotocin diabetogenesis and diabetes are currently under investigation. The outcome of this and future work will undoubtedly throw more light on the mechanism of diabetogenic action not only of streptozotocin, but also of alloxan.

V. CONCLUDING REMARKS

It may be remembered that the specific diabetogenic action of alloxan and streptozotocin represent an example of highest selective drug action on a single type of cell. The direct action may be assumed to be very rapid and practically all biochemical and histological changes observed after poisoning with these substances may safely be regarded as phenomena secondary to the triggering of the total sequence of events. This triggering effect may be the destruction or temporary inactivation in the β-cell membrane or within the β-cell of functional units, which are indispensable for the continuous life of the cell. The situation would thus be analogous to the effect of a temporary lack of oxygen or glucose for certain cells of vital importance in the central nervous system.

This view of a primary noxious effect on a vital enzyme system, a vital feedback system, or other vital islet β-cell structure, which in turn is followed by the consequences of the absence of these vital units as observed in experiments, is supported by the finding that irrespective of chemical structure, affinity, or specificity of known β-cytotoxic substances a typical triphasic blood glucose level fluctuation has been observed after poisoning.

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