Production of Alloxan Diabetes and Ketoacidosis in the Laboratory Rat

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SUMMARY

The synergistic action of dehydroascorbic acid and alloxan in producing a diabetes mellitus-like state in the laboratory rat is utilized. Results of this method, in which the diabetogenic agents are administered subcutaneously, are compared with those in a series of rats made diabetic by intravenously administered alloxan. With experience it is possible to produce diabetes in greater than 90 per cent of the animals by either technic. Approximately 55 per cent of the animals died in the first month after alloxanization; the major causes of death were drug toxicity and iatrogenic hyperinsulinism, in that order. With improvement in technic and control, the mortality in the first month decreased to approximately 30 per cent.

Induction of ketoacidosis was attempted by simultaneous withdrawal of insulin and institution of a ketogenic (high fat) diet. By such a regimen, ketosis could be attained in 75 per cent or more of those diabetic animals tried, whereas the incidence of ketoacidosis was considerably lower. Insulin withdrawal and continuation of the routine (high carbohydrate) diet was followed by ketosis but only sporadic ketoacidosis. The behavior of populations of diabetic laboratory rats is presented in graphic form. DIABETES *14*:289-94, May 1965.

Experimentally induced diabetes mellitus in the laboratory rat has provided a tool to study aspects of the disease which might otherwise be beyond investigation. Despite the massive accumulation of literature, however, only sporadic reports on the induction of severe ketoacidosis in the alloxan diabetic rat have appeared. Foglia[1] and Scow[2] observed a high incidence of ketoacidosis upon insulin withdrawal in rats made diabetic by surgical excision of the pancreas. Unfortunately this approach, to be effective, requires almost total ablation of the functioning islets. In addition, the resultant preparation presents significant nutritional problems.

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Multiple organic compounds have been shown to produce morphological changes in the β cells of the pancreatic islets, accompanied by hyperglycemia and glycosuria. The most widely employed agent of this group has been the ureide of meso-oxalic acid, alloxan. Kaplan et al.[3] used the alloxan diabetic rat in severe ketoacidosis to investigate the effect of insulin on plasma and hepatic phosphates. In view of the known nephro- and hepatotoxicity[4,5] of the drug, their use of comatose animals only seventy to eighty hours after the alloxan administration is open to criticism. To obtain a group of thirty animals in severe ketoacidosis, Knowles and Guest[6] were forced to alloxanize a population of some 600 rats.[7] On the other hand, a recent report by Steiner et al.[8] has indicated relative ease in producing a state of severe ketoacidosis.

We have attempted to take advantage of a possible synergism of two known diabetogenic agents, dehydroascorbic acid (DHAA)[9] and alloxan, to produce a diabetic state. The results of such an approach in a large population of animals will be presented and compared to a parallel series made diabetic by the method described by Steiner.[8]

MATERIAL AND METHODS

The rats used were Sprague-Dawley strain males in groups composed of approximately 70 per cent Sprague-Dawley Rockland and 30 per cent Sprague-Dawley Pullman.* They were maintained on a routine Purina diet† ad libitum, supplemented with fresh greens.

The alloxan was recrystallized[10] from reagent grade material;‡ dehydroascorbic acid (DHAA) was prepared as needed by the oxidation of ascorbic acid with benzoquinone by the method described by Patterson.[9]

Rats in the weight range of 100 to 150 gm. were fasted for forty-eight hours. With the commencement of the fast, a 40-mg.-per-kilogram "desensitizing" dose

*Rockland Farm, New City, New York, and Northwest Animal Supply, Pullman, Washington.

†Ralston-Purina Company, St. Louis, Missouri.

‡Eastman Kodak Company, Rochester, New York.

of DHAA (vide infra) was given subcutaneously. Twelve hours later, and each successive twelve-hour period thereafter, DHAA was given subcutaneously in a dose of 200 mg. per kilogram for a total of four doses. The last was followed within five minutes by 80 mg. per kilogram of alloxan in phosphate-citrate buffer, *p*H 4.0,[20] given subcutaneously as a solution containing 250 mg. of alloxan in 3.0 ml. of buffer. Two hours after the injection of alloxan the fast was ended. A similar group of animals was made diabetic following the method described by Steiner et al.,[8] where after a forty-eight-hour fast, 35 mg. of alloxan monohydrate per kilogram of body weight was injected intravenously under ether anesthesia.

Forty-eight hours after the alloxan, the urine of all the animals was tested for glucose by means of the glucose oxidase strip technic (Uristix*), and those testing 3-plus or greater were started on a daily dose of 3 U. insulin (PZI or Lente). The urine was checked every two to four days, and the insulin dosage advanced or decreased by one unit increments until the urine glucose tested negative to 1-plus. Anuric animals were started on 3 U. insulin and maintained at that level until subsequent urinalysis indicated that an alteration in dosage should be made. To avoid hyperinsulinism, insulin dosage was adjusted to that level consistent with minimal but evident urinary spillage of glucose. The diabetic animals were weighed at least once a week.

No animal was used for further experiments until the renal damage had resolved, usually a period of four weeks. To attempt the induction of ketosis and acidosis, insulin was withheld and the Purina diet replaced by shelled, unsalted roasted peanuts ad libitum. Daily weights and diurnal urine checks for glucose and ketone bodies were instituted. The criteria for acidosis included hyperglycemia, ketonemia, and a serum CO_2 combining power of less than 9 mm. per liter.

Tail-vein blood was used for chemical analyses. Urea was determined by the diacetyl monoxime procedure.[11] Potassium ferricyanide oxidation was employed to quantitate blood glucose.[12] Serum CO_2 combining power was determined by the classical Van Slyke manometric procedure using the Natelson microgasometer. Serum acetone was diffused into 2,4-dinitrophenylhydrazine,[13] and the phenylhydrazone formed assayed colorimetrically. Urine ketones were followed by nitroprusside impregnated strips (Ketostix*).

*Ames Company, Inc., Elkhart, Indiana.

RESULTS

The animals were alloxanized in groups of ten, maintaining several litter mates as weight controls. Tables 1a and 1b illustrate the results of the intravenous alloxan and DHAA-subcutaneous alloxan technics, respectively. As can be seen, the first week after alloxanization is marked by a significant number of

TABLE 1a

Total experience in the use of intravenous alloxan in the production of diabetes in the laboratory rat. By "trials" we have indicated those animals in which induction of ketosis was attempted.

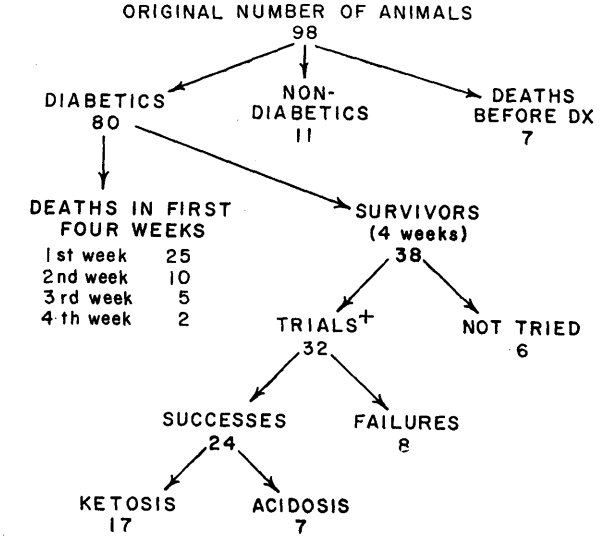
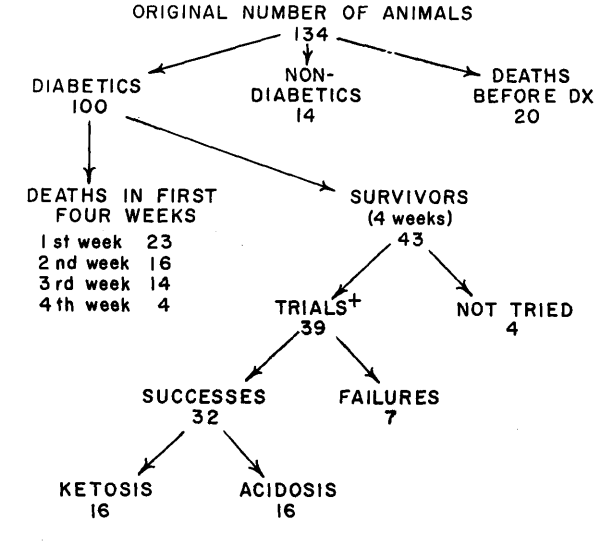


TABLE 1b

Total experience in the use of subcutaneous DHAA-alloxan in the production of diabetes in the laboratory rat.



deaths. These animals characteristically demonstrated anorexia, marked weight loss, oligo- or anuria, and blood urea nitrogens above 40 mg. per cent. Although the blood glucose levels were usually elevated, only trace plasma ketone bodies were detected. At autopsy histological examination revealed extensive renal damage, involving predominantly the tubular structures.

The observation of low serum *p*H and bicarbonate in conjunction with hyperglycemia in this early phase has occasionally been misinterpreted to represent acidosis of diabetic origin despite the impaired renal function and the presence of only small amounts of ketone bodies.

In many of these early deaths, the diagnosis of successfully induced diabetes mellitus could not be established unequivocally and, therefore, have been classified as *deaths before diagnosis.* Of ninety-eight rats treated with intravenous alloxan, thirty-two (33 per cent) died within the first week after alloxanization, twenty-five of which were definitely diabetic at the time of death. Using the combined DHAA-alloxan method there were forty-three (32 per cent) first-week deaths in 134 animals treated, with a diagnosis of diabetes in twenty-three of these at or before their demise. A not insignificant number of failures were also observed in the first week, totaling eleven (11 per cent) of the intravenous group, and fourteen (10 per cent) of the DHAA-alloxan group.

Before attempting to induce ketosis or acidosis, the diabetic animals were maintained on insulin for at least four weeks to allow complete recovery from the acute nephro- and hepatotoxic effects of the diabetogenic agents. In the second to fourth weeks additional deaths occurred, the most significant cause at this time being hyperinsulinism. Hyperinsulinism was suspected when many diabetic animals, which had appeared to be flourishing on insulin therapy, suddenly developed convulsive episodes, progressing to death. The weight gain, growth characteristics, and coat sheen of these animals were no different than in their nondiabetic litter mate controls. During the convulsive episodes, although the blood urea nitrogens and serum CO_2's were within normal limits, blood glucose levels were found to be depressed below 50 mg. per 100 ml., with some below 25 mg. per 100 ml. When detected in the early stages of the convulsive episodes, many animals responded to the administration of intraperitoneal glucose. Unfortunately, numerous such deaths occurred during the night. This difficulty was observed in animals made diabetic by both technics receiving an insulin dose greater than 2 units per 100 gm. of body weight for periods longer than ten days. Thus in our hands a sustained level of 2.5 units per 100 gm. of body weight[8] proved too high a dose for standardized use, although many animals achieved this level. By far the majority in both series demonstrated adequate control of glycosuria and weight gain on 1.5 units per 100 gm. body weight. It was soon concluded that no hard and fast rules could be established as to the insulin dose necessary to bring each individual into tolerable control.

At the conclusion of the four-week interval, thirty-eight (26 per cent) rats of the intravenous series were available for further studies, while forty-three (33 per cent) of those made diabetic by the combined DHAA-alloxan remained. Upon insulin withdrawal and replacement of the routine with a high fat diet, susceptible animals usually developed strongly positive ketonemia and ketonuria within forty-eight to seventy-two hours, many progressing rapidly into severe ketoacidosis and death. Normal controls, when transferred from a routine to a peanut diet, demonstrated no or sporadic "trace" ketonuria in the same period of time. Of thirty-two attempts on the thirty-eight available animals of the intravenous group, twenty-four (75 per cent) became ketotic, of which seven (29 per cent) progressed into severe ketoacidosis. The ketotic animals were found to have average blood glucose levels of 401 $\pm$ 29 mg. per 100 ml., serum CO_2 combining power values of 22 $\pm$ 4 mM. per liter, and serum acetone levels of 13 $\pm$ 6 mg. per 100 ml., while the respective values in the ketoacidotic animals of the group were 360 $\pm$ 66 mg. per 100 ml., 5.9 $\pm$ 2.6 mM. per liter, and 71 $\pm$ 19 mg. per 100 ml. In the series made diabetic by the DHAA-alloxan, thirty-nine attempts produced thirty-two cases (82 per cent) of ketosis, of which sixteen (50 per cent) progressed into severe ketoacidosis. In this group the ketotic animals had average blood glucose levels of 402 $\pm$ 64 mg. per 100 ml., serum CO_2 combining power values of 19 $\pm$ 3 mM. per liter, and serum acetone levels of 15 $\pm$ 10 mg. per 100 ml., while the respective values in the ketoacidotic animals were 476 $\pm$ 61 mg. per 100 ml., 5.9 $\pm$ 1.7 mM. per liter, and 74 $\pm$ 43 mg. per 100 ml.

As the series progressed, familiarity with technics led to distinct improvement in the results. This is amply demonstrated by tables 2a and 2b which illustrate several of the last series treated by both methods. Perhaps the most marked difference is in the number of dia-

TABLE 2a

The influence of proficiency on the production of diabetes by intravenous administration of alloxan.

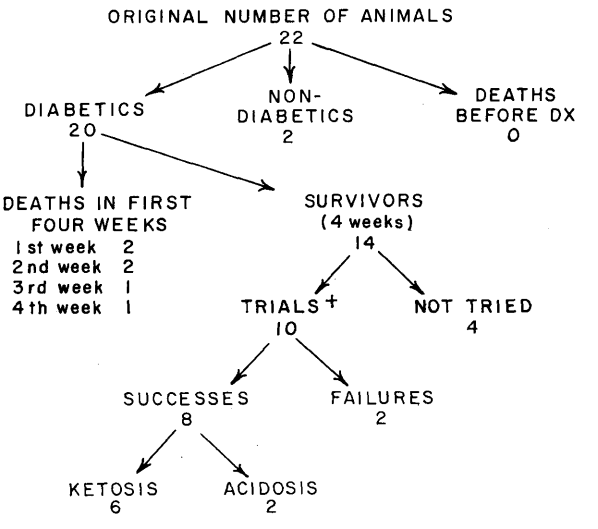
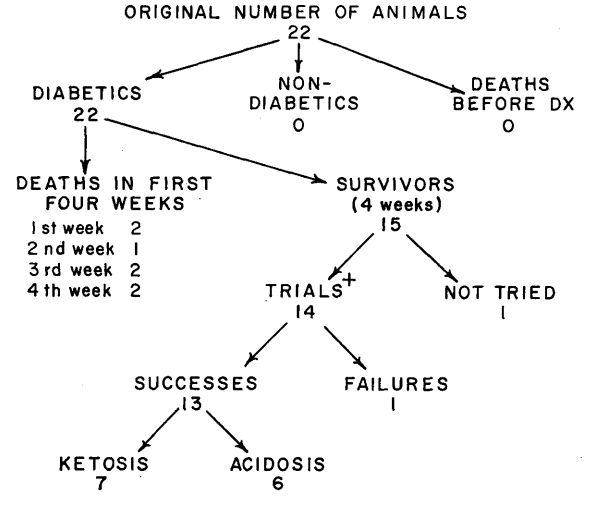


TABLE 2b

The influence of proficiency on the production of diabetes by the subcutaneous administration of DHAA-alloxan



betic animals carried to the "available" stage, representing fourteen (64 per cent) by the intravenous technic and fifteen (68 per cent) by the combined use of DHAA-alloxan, an improvement of 80 to 100 per cent. Of these, a very acceptable number developed ketosis or ketoacidosis upon insulin withdrawal and the institution of a high fat diet.

DISCUSSION

The production of a diabetes mellitus-like syndrome in the laboratory rat following alloxan administration was first recorded over twenty years ago.[14] Electron microscopy has permitted the observation of the toxic effects of the drug on the β cells of the pancreatic islets at a subcellular structural level.[15] Mitochondrial, endoplasmic reticular and cell membrane degeneration were observed within two hours, followed by nuclear and finally cell disintegration. Aside from the pancreatic islet damage, however, alloxan produces morphological changes in the convoluted tubules.[4] Clinically these renal lesions are accompanied by oliguria, proteinuria, and uremia. Hepatic necrosis of a focal type has also been reported following alloxan administration.[4,5]

The toxic effects of alloxan are intensified by fasting of the animal prior to the administration and, conversely, are ameliorated by concomitant administration of cysteine, glutathione, and BAL, as well as glucose. Although the mechanism of toxicity of this drug is not known, sulfhydryl involvement has been postulated[16] in view of alloxan's oxidative capabilities, observed decrease of circulating blood glutathione after administration of alloxan, and protection from the toxic effects of alloxan by exogenous sulfhydryl compounds. The monosubstituted amino alloxan homologues, glyoxal, dehydroascorbic acid and some homologues of ascorbic acid have been demonstrated capable of producing diabetes mellitus in the laboratory rat. While these compounds share chemical properties in common with alloxan, others of distinctly different structure exhibit diabetogenicity, e.g., styriloquinoline, 8-hydroxyquinoline, and diphenylthiocarbazone, implying the possibility of more than one mechanism of toxicity.

Brückmann and Wertheimer,[17] while investigating homologues of alloxan and dialuric acid, noted the persistence of renal toxicity despite the loss of diabetogenicity with some of the derivatives. This was interpreted as evidence of dissimilar mechanisms of diabetogenesis and renal toxicity. With such a potentiality in mind, we have attempted the consecutive use of two diabetogenic agents in hopes that we might obtain a more severe diabetes without increasing the mortality due to renal complications. We chose to supplement alloxan with dehydroascorbic acid (DHAA), the diabetogenic and synergistic properties of which have been reported by Patterson.[9]

It rapidly became apparent that the DHAA increased the renal sensitivity of the fasted animals to alloxan as well. Whereas a subcutaneous dose of 100 mg. of

alloxan per kilogram of body weight could be tolerated by animals untreated with DHAA, the same dose following a course of DHAA produced a mortality exceeding 70 per cent. Subsequently the dose of alloxan was reduced to 80 to 85 mg. of alloxan per kilogram of body weight.

Interestingly, Brückmann and Wertheimer report pulmonary edema and hemorrhage of a significant degree upon administration of alloxan homologues. DHAA also produces a severe "allergic" response, associated with rhinorrhea, lacrimation, and rhonchial, wheezing respiration. For this reason a small "desensitizing" first dose is employed. These patterns of behavior of the two groups of drugs (i.e., demonstrated synergistic renal toxicity and diabetogenicity, and "allergic" phenomena) imply similar rather than dissimilar mechanisms of action.

Both subcutaneous DHAA-alloxan and intravenous alloxan have been shown to give the same over-all results. Histological postmortem examinations reveal no morphological differences between the two technics at a tissue level. The early chemical changes following alloxan are complicated by renal involvement. To avoid loss of severe diabetics through ketoacidosis of diabetic origin during the period of this renal crisis, insulin was instituted within two days after the administration of the alloxan, based upon the appearance of glycosuria. In the case of the anuric animal, insulin (3 U. daily) was given despite the absence of the criterion of glycosuria and continued until urine flow resumed. The dosage was then predicted by the degree of glycosuria. Such an approach unfortunately precludes the use of blood glucose as an initial criterion of the severity of the diabetic state.

Glycosuria was employed as a convenient index of control to avoid mutilation of the tail veins by bleeding, as our subsequent experiments with the ketoacidotic animals required the patency of these vessels. In all cases the insulin dosage was adjusted to a level which permitted a "trace" of glycosuria as determined by the Uristix method. "Trace" glycosuria was found desirable to diminish the possibility of hyperinsulinism. The significance of this problem is emphasized by the observation that of the forty-five deaths in the second and third weeks after the alloxan administration (tables 1a and 1b), at least twenty-four (53 per cent) were considered attributable to hyperinsulinism, an impression supported by the clinical history and the finding of blood glucose levels below 50 mg. per 100 ml.

Steiner et al.[8] have reported a direct correlation between maintenance insulin dose and subsequent ketone level upon insulin withdrawal. Our attempts to employ doses of PZI or Lente Insulin above 1.0 to 1.5 U. per 100 gm. of body weight for any significant lengths of time resulted in marked increases in deaths in both groups due to hypoglycemia. In the later series (tables 2a and 2b), carefully maintaining the animals on an adequate but smaller dose, deaths due to hypoglycemia decreased. We were not able to establish that any deaths in these groups were due to hyperinsulinism. No detectable difference in weight gain was observed between control animals and diabetics controlled in this manner.

As described, ketosis was induced by simultaneous withdrawal of insulin and the institution of a diet of unsalted, roasted peanuts. Such a diet consists of 24 gm. of carbohydrate, 27 gm. of protein, and 44 gm. of fat per 100 gm. of peanuts.[18] This ketogenic diet was employed in an effort to augment the metabolic abnormalities accompanying insulin withdrawal in the diabetic, leading to the accumulation of ketone bodies. From this study no criteria (weight, duration of disease, insulin dosage, etc.) could be derived which would enable a prediction as to which animals would lapse into ketosis or acidosis on such a regimen. The withdrawal of insulin and food, withdrawal of insulin maintaining the routine diet, and the addition of cortisone to such programs were investigated as alternative methods of inducing ketosis and acidosis, with little success. These animals are not included in the present series. Although cortisone administration appeared to enhance the degree of glycosuria and ketosis, there was no marked increase in the incidence of subsequent acidosis. Fasting, while withholding insulin, usually produced only a transient ketosis of forty-eight to seventy-two hours' duration. Only an occasional animal would develop ketoacidosis.

Some animals exceeding 150 gm. in body weight were also alloxanized. A comparison of two weight groups (i.e., those over 150 gm. and those less than 150 gm.) revealed no significant difference in their response to the diabetogenic agents or to subsequent efforts to develop ketosis. Minor differences in strain susceptibility were experienced, but are difficult to evaluate. Our experience with the Long-Evans Hooded strain (not included in this series) substantiates other observations[5,19] that they are more resistant to the production of diabetes by alloxan than the white albino strains we used. Indeed, it was our impression that there were differences in susceptibility within the

two albino strains themselves. Our inability to produce ketoacidosis with the success reported by Steiner et al.[8] employing intravenous alloxan may well represent strain differences also.

Alloxan has been demonstrated to produce diabetes whether administered subcutaneously, intravenously, or intraperitoneally. The intravenous route has been preferred since it minimizes the uncertainty contributed by alloxan instability.[20] In our hands both the intravenous administration of alloxan and subcutaneous administration of DHAA-alloxan give similar results. (Ketoacidosis appeared to develop more frequently in the group made diabetic by the combined agents, but in view of the small population reported, the significance of this observation is uncertain.) The biochemical measurements of the ketotic and ketoacidotic animals made diabetic by either technic did not indicate any marked difference in the severity of the diabetes. The synergistic action of DHAA upon the diabetogenic action of alloxan has been verified. Interestingly, this synergism has also been found to pertain to the nephrotoxicity as well as the diabetogenicity of the compound.

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