Studies of Insulin Secretion in the Perfused Rat Pancreas

Effect of Diazoxide and A025

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SUMMARY

Continuous glucose stimulation produces two phases of insulin secretion in the isolated perfused rat pancreas. Puromycin has no effect on the first phase but partially inhibits the second. The second phase has been reported previously to be associated in part with insulin synthesis.

In acute experiments, diazoxide and its derivative—A025—inhibit insulin secretion in both phases. This inhibition corresponds with an increase of the pancreatic tissue insulin content. These results suggest that both A025 and diazoxide decrease insulin release but have no effect on its synthesis.

In pancreatic perfusion of rats which had been chronically treated with diazoxide and A025 (per os), and who had been fasted and without drug for sixteen hours before the experiment, a greater insulin secretion was demonstrated in both phases compared with normal controls; however, if the perfusion is done three to four hours after the last dose, the amount of insulin secreted is smaller than the respective controls. This again suggests that during the period of action of the drug, pancreatic insulin is not released, but synthesis is not inhibited. The result, therefore, is an accumulation of insulin within the beta cells.

The acute and chronic experiments done under our experimental conditions in the perfused rat pancreas suggest that diazoxide and A025 inhibit insulin release, but have no effect on insulinogenesis. DIABETES *19*:271-81, April, 1970.

Diazoxide (3-methyl-7-chloro-1,2,4-benzothiadiazine-1,1-dioxide) is a benzothiadiazine with hypotensive and antidiuretic activity.[1] Apart from this, diazoxide was found to produce hyperglycemia in man[2,3] and animals.[4,5] The mechanism of diazoxide hyperglycemia had been a matter of intensive research. Although this drug has been shown to inhibit insulin secretion both in vivo[5,6] and in vitro,[7,8] many investigators support the concept of an extrapancreatic mechanism related to the adrenal glands.[9-11] Peripheral and hepatic mechanisms have also been discussed by some investigators.[12-14]

Diazoxide is used in the treatment of some hyperinsulinemic states but side effects, such as water retention, limit its use.[15,16] Several compounds derived from diazoxide were studied in our laboratory to determine the effect of structural modifications of the diazoxide molecule on its hyperglycemic, hypotensive and antidiuretic effects.[17] In these studies, one of the analogues, coded as A025, proved to have marked hyperglycemic activity, some effect on arterial pressure and no effect on diuresis in the rat and monkey.[18,19] The structural formula of A025 and diazoxide is shown in figure 1; they differ only by one methyl group in the 3-position. Although both diazoxide and A025 were shown to inhibit insulin secretion, little has been done to elucidate their action on insulin release and synthesis. This paper reports studies of the effects of diazoxide and A025 on insulin synthesis and release, using the isolated perfused rat pancreas.

DIAZOXIDE A025

FIG. 1. Chemical structure of diazoxide and A025.

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MATERIAL AND METHODS

Sprague-Dawley rats, 350 to 400 gm. body weight, were used in the pancreatic perfusion. Animals used for chronic force-feeding experiments weighed from 100 to 150 gm. at the beginning of the study. The animals were force-fed in order to observe the effect of chronic administration of A025 on the rate of weight gain. There was no difference in the increase of weight between the A025-treated and control animals. All animals, except the force-fed ones, received standard cube diet ad libitum. The animals in chronic force-feeding experiments were given 20 to 30 ml. of a 30 per cent suspension of a commercial powdered diet (Diet 4370, General Biochemicals, Chagrin Falls, Ohio), by stomach tube twice a day. In all cases, except when specified, animals fasted for sixteen hours prior to experiment were used for pancreatic perfusions.

The pancreatic perfusion was performed by the method of Penhos,[20] which will be published in detail elsewhere. After anesthesia with sodium pentobarbital and castration, all abdominal vessels, except the pancreaticoduodenal, the superior mesenteric and the splenic branches irrigating the pancreas, were tied between two ligatures and cut. The spleen, both kidneys and the large intestine were removed. All connections with the rectum, esophagus and the adrenals were tied off, so that all were completely separated from the systemic circulation. A polyethylene cannula placed in the aorta allowed the perfusate to go only through the intestine and pancreas. By means of another cannula placed into the portal vein, all the perfusate was collected in graduated tubes. A schematic drawing of the apparatus used is shown in figure 2.

A pump [1]* circulates the perfusate from the reservoir [6] to a filter [2]. After reaching the filter, the perfusate enters a tube [5], which is connected by a gauge 18 needle to a cannula inserted into the aorta [14]. A bypass [4] attached to the filter delivers the excess of perfusate back to the reservoir and helps to maintain proper oxygenation of the perfusate. By moving the filter up or down, the perfusion pressure was equilibrated with the blood pressure of each rat. The perfusate used was a mixture of Krebs-Ringer bicarbonate buffer containing 4 per cent of beef serum albumin and 50 mg./100 ml. glucose. This buffer was equilibrated with 95 per cent oxygen and 5 per cent CO_2, resulting in a pH ranging from 7.28 to 7.40. The effluent flow rate was between 6 to 12 ml./min., under a pressure of 45 to 58 mm. Hg. Occasional adjustments within the mentioned range were necessary to maintain the flow constant. None of the substances tested, including glucose, alter the flow rates. The arterial pH_2 was 350 to 450 mm. Hg and the portal pO_2 was between 160 to 220 mm. Hg.

The buffer was recycled for ten minutes to attain temperature stabilization; after this stabilization period all the buffer from the portal vein was collected in graduated tubes. Samples were collected at sixty-second intervals during the first twenty minutes, and every five minutes between twenty to sixty minutes. The samples from zero to three minutes were collected as controls. Glucose at a concentration of 300 mg./100 ml. was perfused from three minutes until the end of the experiment. When puromycin was used, it was recirculated through the perfusion thirty minutes before the experiment. Diazoxide and A025 were added to the perfusion in solution adjusted approximately to pH 10. The concentration of both drugs (15 and 30 mg. %) in the perfusate was based on our previous in vivo experiments. A hyperglycemic dose of the drug produces 15 to 20 mg./100 ml. blood drug levels in rats.

In another series of experiments, samples of the pancreas (80 to 150 mg.) were taken at 0, 12, 20, 40, and 60 min. of the perfusion. The pancreatic insulin was extracted with acid ethanol, as described by Taylor.[21] The insulin concentration of the portal effluent and of the pancreatic extracts was determined simultaneously in the control and experimental groups by the immunoassay method of Hales and Randle[22] with the use of pork insulin as standard. The results were expressed at μU./min. in the portal effluent and as mU./100 mg. tissue in the pancreatic extracts.

RESULTS

The rate of insulin secretion in μU./min. produced by buffer alone, buffer containing 300 mg./100 ml. glucose and buffer with 300 mg./100 ml. glucose plus 80 μg./ml. of puromycin, can be seen in figure 3. Buffer alone did not influence the insulin secretion in any significant way during the whole perfusion. In the second set of six perfusions, after the first three minutes, a glucose solution producing a final concentration of 300 mg./100 ml. was added to the perfusate. This solution reached the pancreas between 1.45 to 2.15 minutes after it was added. As can be observed in figure 3, glucose first produced a rapid increase of insulin secretion of about a two to three-minute duration; after this first peak the rate of insulin secretion sharply decreases within two to three minutes, in spite

*Figures in brackets refer to points within figure 2.

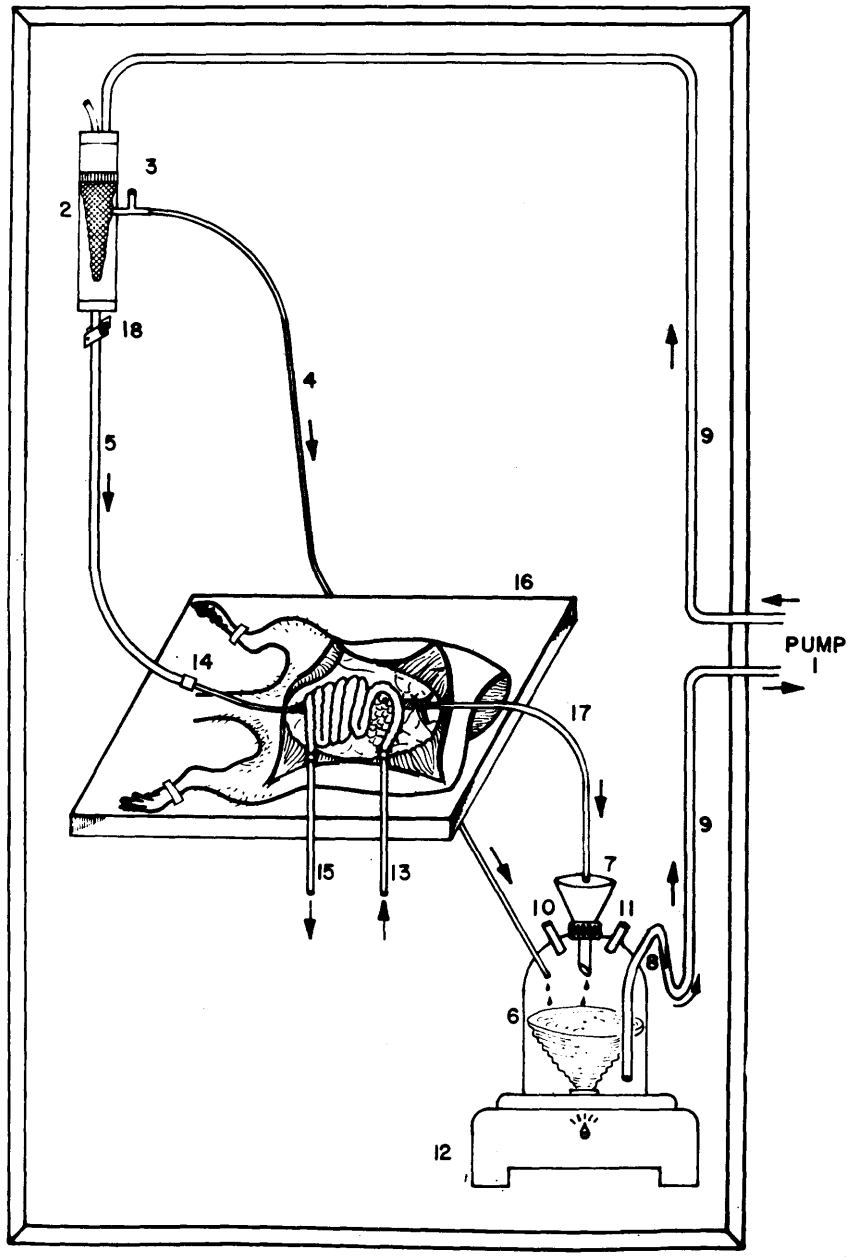

FIG. 2. Schematic drawing of the set-up used for pancreatic perfusion.

of the fact that glucose concentration (300 mg./100 ml.) remains constant. After this period, the levels showed a steady increase until the end of the perfusion. The amount of insulin secreted at seven and eight, and from ten to sixty minutes was significantly different at the $p < 0.001$ level, compared with the corresponding controls.

In another group of five perfusions, puromycin (80 μg./ml.) was added to the perfusate and recirculated for thirty minutes before zero time. At three minutes, 300 mg./100 ml. glucose was added to the buffer. Figure 3 shows that in the first twenty-five minutes of perfusion there is no difference in the amount of insulin secreted, compared to the pancreas perfused with glucose alone. After thirty minutes of perfusion, the group perfused with glucose plus puromycin showed a marked decrease in the amount of secreted insulin, when compared with the group perfused with glucose alone. The statistical significance between the two groups was $p < 0.005$ from thirty to forty minutes and $p < 0.001$ from fifty to sixty minutes.

The effect of diazoxide on both peaks of insulin

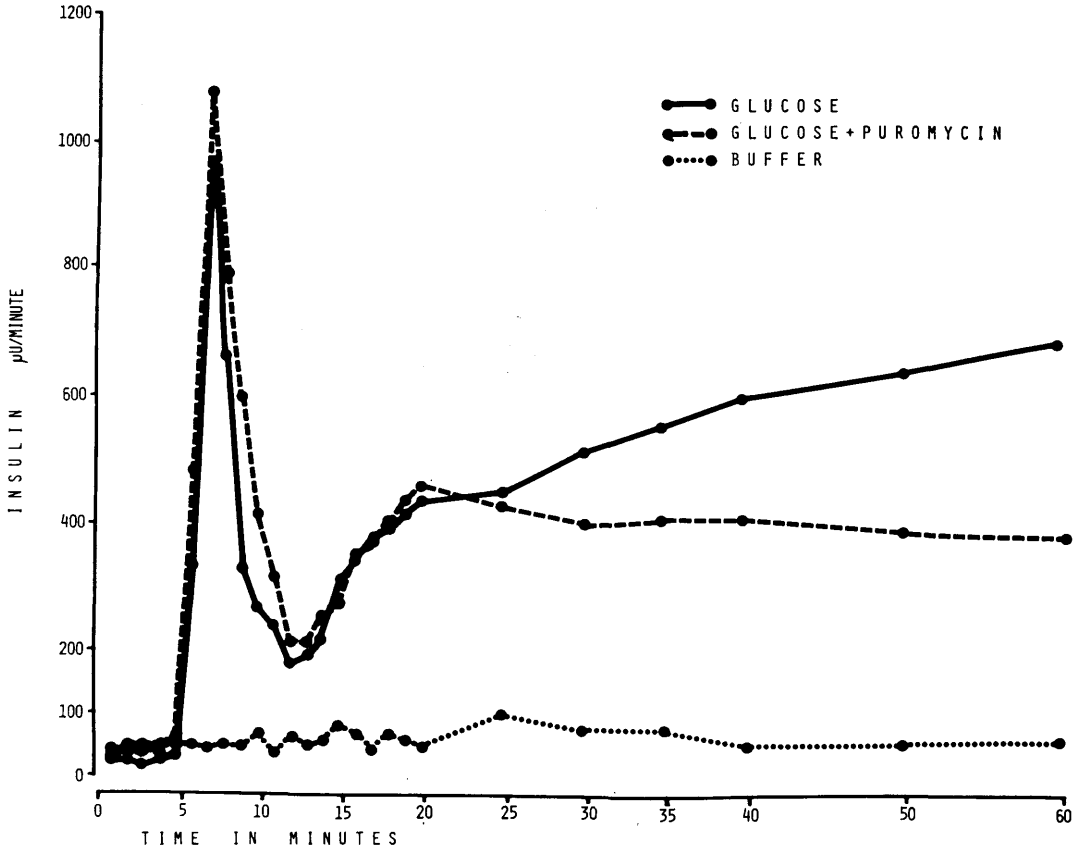

FIG. 3. Effect of buffer (n:3), glucose (n:6) and glucose plus puromycin (n:5), on the rate of insulin secreted by the perfused rat pancreas.

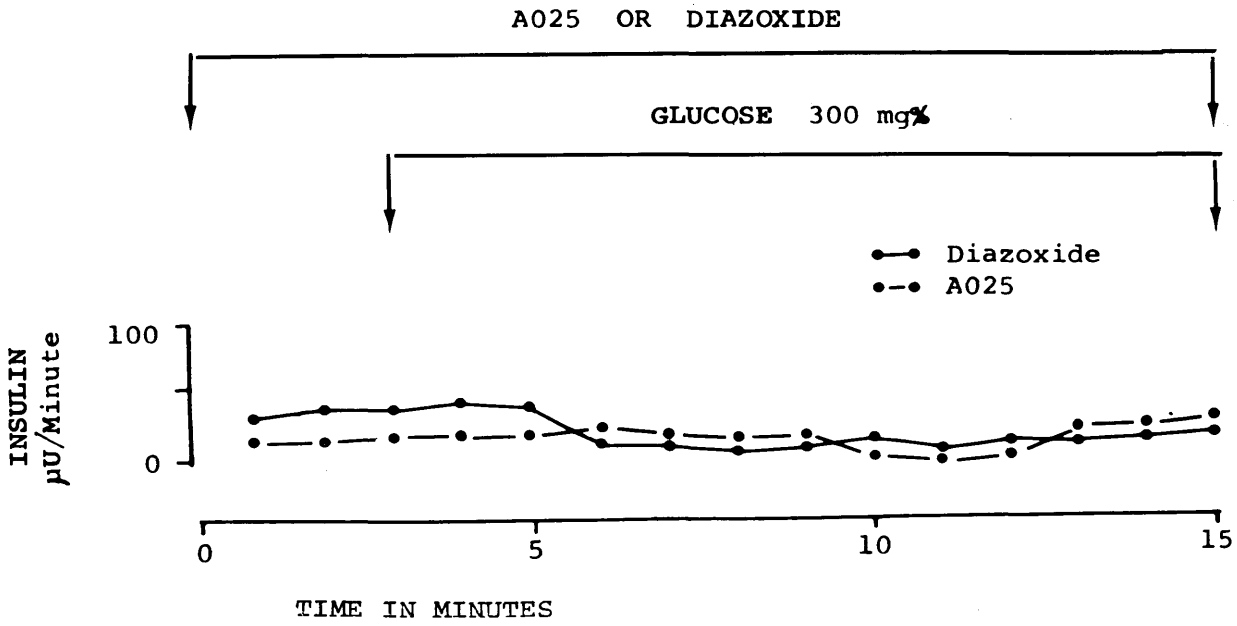

FIG. 4. Inhibitory effect of diazoxide and A025 on the first phase (3-15 min.) of insulin secretion produced by glucose stimulus; six perfusions in each group.

secretion produced by glucose stimulation is illustrated in figures 4 and 5: Diazoxide (15 mg./100 ml.) inhibited the first peak of insulin secretion in six perfusions (figure 4). When diazoxide was added after nine minutes of perfusion, it inhibited the second peak of insulin (figure 5). A similar inhibitory effect on both peaks of insulin could be observed when A025 (30 mg./100 ml.) was added to the respective group of perfusions (figures 4 and 5).

In another group of experiments, each consisting of five perfusions, pancreatic slices of 80 to 150 mg. weight were taken at 0, 12, 20, 40, and 60 min. of the perfusion and the insulin extracted. The following groups were studied: Group I—pancreas perfused with buffer alone. Group II—buffer plus glucose (300 mg./100 ml.) added at a constant rate from three minutes until the end of the perfusion. Groups III and IV—glucose added as in Group II, but diazoxide (15 mg./100 ml.) or A025 (30 mg./100 ml.) respectively, was added nine minutes after the perfusion was started. In the last two groups, the first peak of insulin secretion produced by glucose stimulation appeared, but the second peak was inhibited. The amount of pancreatic insulin in the four groups is illustrated in figure 6. It can be observed that there is no significant change in the groups perfused with buffer only or buffer with added glucose.

Groups III and IV showed no difference during the first forty minutes of perfusion, compared with Groups I and II. After sixty minutes of perfusion, they showed an increase in pancreatic insulin content when compared with Groups I and II. The difference was statistically significant at the $p < 0.01$ level.

Other pancreatic perfusions were carried out in a group of four force-fed rats chronically treated (nine weeks) with A025 at a dose of 100 mg./kg./day prior to perfusion. Another group of four force-fed rats was identically treated with A025, and the perfusion was carried out ten days after discontinuation of treatment. The control group received the excipient for the same period of time. All perfusions in the first and the control group were performed sixteen hours after the last dose of A025 or excipient and sixteen hours after the last force-feeding. In all cases 300 mg./100 ml. glucose was added to the perfusate at three minutes. Figure 7 shows that the first peak of glucose-stimulated insulin secretion in the chronically A025-treated rats was nearly twice as high as the control values ($p < 0.05$ at four minutes and $p < 0.001$ at five and six minutes). Furthermore, after the subsequent fall of the insulin levels, A025-treated rats showed a higher second peak of insulin secretion, compared with controls ($p < 0.005$ from twelve to forty minutes and $p < 0.001$ from fifty to sixty minutes). At sixty minutes of the perfusion, the magnitude of the second peak was six times greater than in the controls. A025-treated rats had also higher initial insulin secretion rates. The group of rats in which the perfusion was carried out ten days after discontinuation of A025 treatment showed the same magnitude of insulin secretion as the controls.

Diazoxide (200 mg./kg./day) and A025 (100 mg./kg./day) were administered by stomach tube for five to seven days to another two groups of rats. All animals had free access to food until sixteen hours before the experiment. Diazoxide-treated animals were subdivided into two groups: In one group, pancreatic perfusion was done three to four hours after the last administration of the drug, and in the other, the perfusion was carried out sixteen hours after the last dose of diazoxide. The same procedure was followed in rats treated with A025. Figure 8 shows that there was a higher insulin secretion in rats treated chronically with diazoxide or A025 than in controls, when the last dose of the drug was administered sixteen hours before the perfusion. On the other hand, when the last dose of A025 or diazoxide was given three to four hours before the perfusion, the output of insulin was lower than the respective controls; the amounts of insulin secreted were even lower than those observed in acute experiments (figure 3).

In the last set of experiments, rats with free access to food were given diazoxide (200 mg./kg./day) during seven days. Sixteen hours after the last dose, the isolated pancreas was perfused. Puromycin (80 μg./ml.) was added thirty minutes before zero time in one group; glucose added at three minutes of the perfusion was used as stimulus in both groups. Figure 9 illustrates that there was no difference in the amount of insulin secreted by the pancreas when puromycin was present or absent up to the first thirty minutes of perfusion. However, after thirty minutes, the preparations with added puromycin showed a marked decrease in the insulin output ($p < 0.01$ from thirty-five to sixty minutes and $p < 0.02$ at thirty minutes), compared with the other group.

DISCUSSION

Several in vitro methods have been developed to study the insulin secretion from pancreas, eliminating secondary effects resulting from changes in other or-

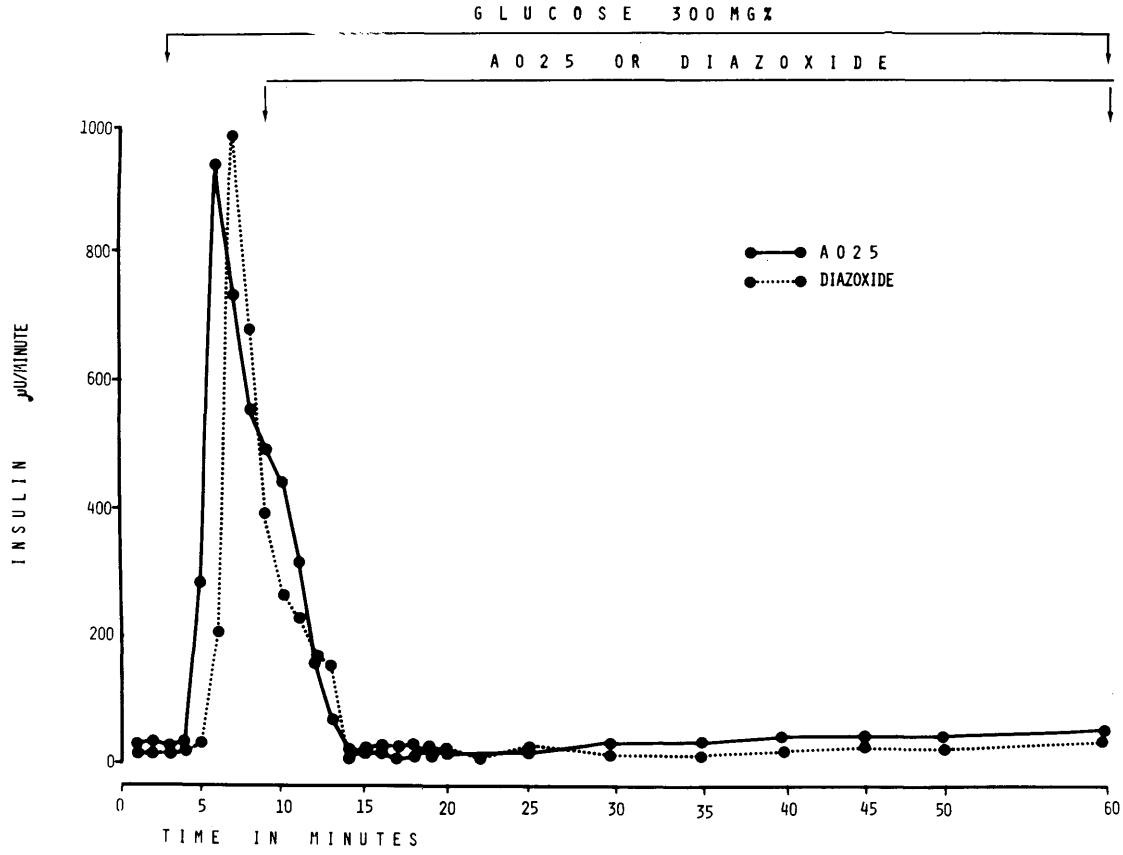

FIG. 5. Effect of diazoxide and A025 on the second phase of insulin secretion (15-60 min.); six perfusions in each group.

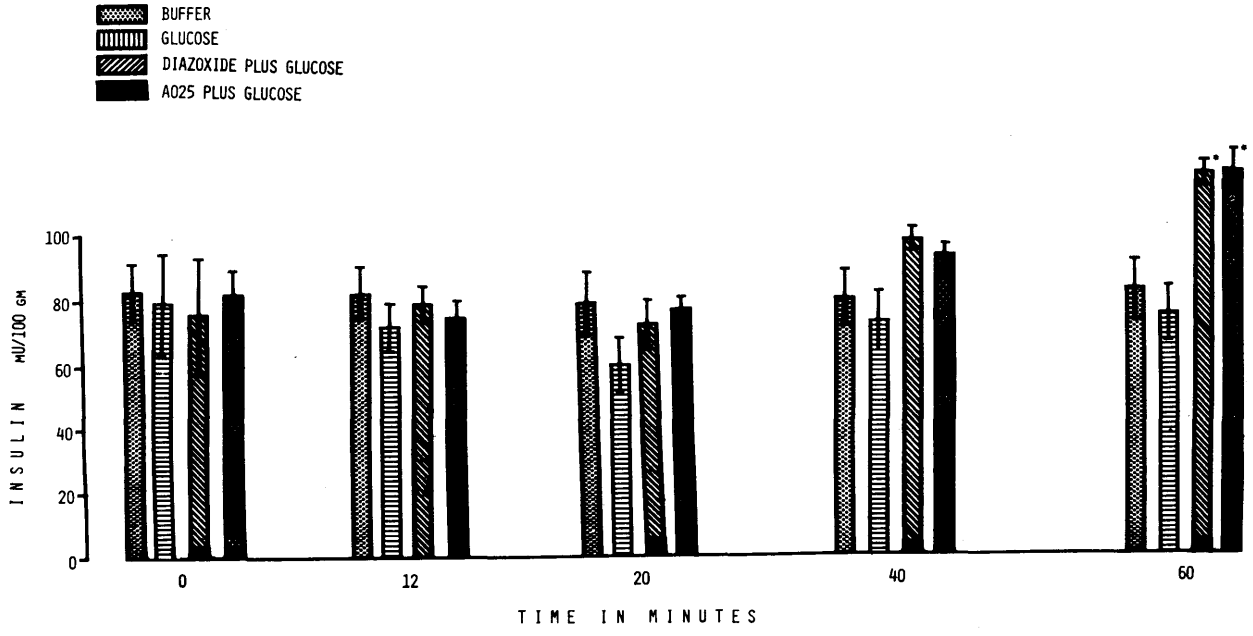

FIG. 6. *Content of pancreatic insulin, measured throughout the perfusion. Each value is an average of five measurements. Bars marked* ± $p < 0.01$.

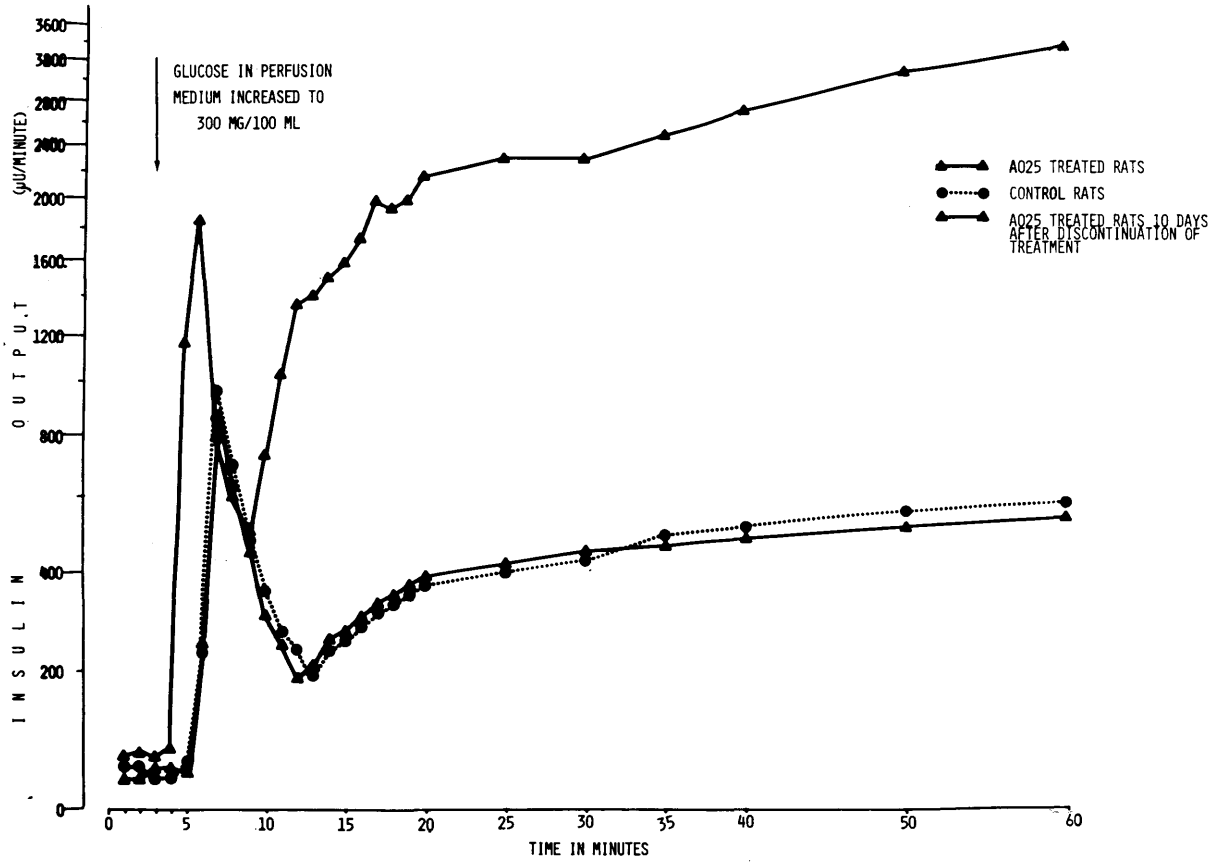

FIG. 7 Pattern of insulin secretion by the pancreas of rats treated with A025 for nine weeks prior to the experiment; four perfusions in each group.

gans.[20,23-28] Compared to all these methods, the isolated perfused pancreas has the advantage that even small changes can be detected on a minute to minute basis, giving a dynamic view of the action and interaction of different drugs, nutrients and hormones on the mechanism of insulin secretion.

In our perfusions, glucose, a substance known to produce secretion of stored and newly synthesized insulin,[29,30] was used as stimulus. We confirmed previous work published by Curry et al.[31] showing that glucose in the isolated perfused pancreas produces a diphasic pattern of insulin secretion. Also in a series of in vivo experiments, Kanazawa et al.[32] found two phases of insulin secreted into the pancreatic duodenal vein, after intravenous glucose stimulation. As shown in figure 3, after a first quick response of approximately two to three minutes, there was a second phase of insulin secretion, steadily increasing until the end of the perfusion. We would like to call attention to the sharp drop of insulin levels between the two peaks of insulin secretion. This decline in the rate of insulin secretion lasts about two to three minutes, and was also seen in all other experiments, using glucose as stimulus (see figures 3, 7, and 8).

The addition of puromycin had no effect on the first peak of insulin secretion and there was no effect during the first ten to twelve minutes of the second phase. However, after thirty minutes of the perfusion, i.e., after thirty-five minutes of continuous glucose stimulation, the pancreas perfused with glucose and puromycin showed a marked decrease in the rate of insulin secretion as compared with the pancreas perfused with glucose alone (figure 3). Puromycin is known to block the rate of incorporation of C-14 L-valine into total pancreatic proteins[31]; therefore, the amount of insulin secreted during the second peak, blocked by puromycin, was thought by Curry et al.[31] to be connected in some way with newly synthesized insulin. These authors postulate that stored insulin can be "pushed out" by the newly synthesized insulin. In other words, the in-

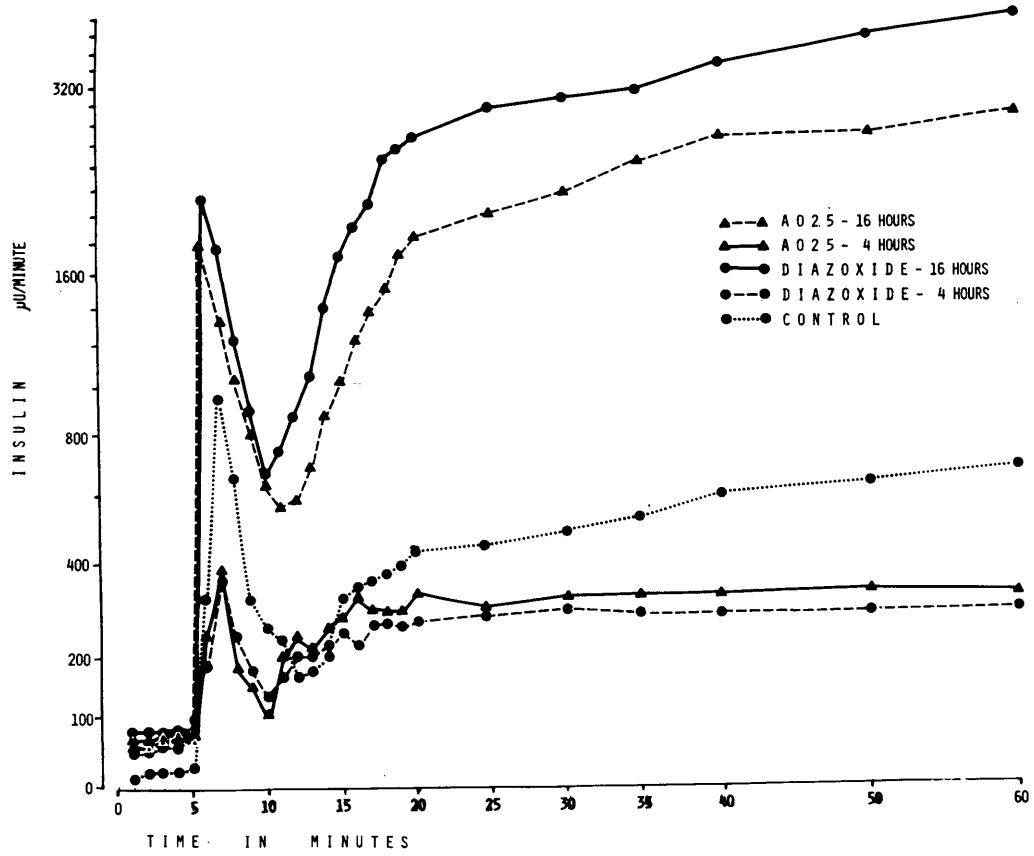

FIG. 8. Insulin secreted by the pancreas of rats treated with diazoxide or A025 for a period of five to seven days.
(a) Last dose of diazoxide (200 mg./kg./day) or A025 (100 mg./kg./day) was administered 16 hrs. before the perfusion: five perfusions in each group. Statistical analysis shows: For A025-treated animals—$p < 0.001$ (6, 9, 10 min., and 13 to 60 min.); $p < 0.005$ (11 and 12 min.). For diazoxide-treated animals—$p < 0.001$ (6 min. and 10 to 60 min.) $p < 0.005$ (3 and 7 min.); $p < 0.05$ (5, 8, and 9 min.).
(b) Last dose administered three to four hours before the perfusion; three perfusions in each group. Statistical analysis: A025-treated—$p < 0.001$ (7-10 min.); $p < 0.005$ (35 and 60 min.); $p < 0.01$ (30, 40, and 50 min.) Diazoxide treated—$p < 0.001$ (6, 7, and 60 min.); $p < 0.005$ (8, 10, 20, and 50 min.); $p < 0.05$ (19, 25-40 min.).
(c) Six perfusions were performed in the control group.

sulin secreted during part of the second phase represents the release of prestored insulin very closely related or promoted by insulinogenesis. Lacy et al.[33] described some mechanisms related with granule transport from the Golgi apparatus and/or from inner parts of the beta cells to the basement membrane. The action of puromycin could perhaps result in an impairment of the above-mentioned transport, and not of the insulin synthesis.

Diazoxide and A025 proved to be inhibitory to both phases of insulin secretion produced by glucose, depending on the time of introduction into the perfusion system. The depression of the rate of insulin secretion is in both cases accompanied by a corresponding increase of the pancreatic tissue insulin content, which seems to indicate that both diazoxide and A025 inhibit insulin release, but have no effect on its synthesis. In a recent paper, Creutzfeld et al.[34] reported that diazoxide inhibits pancreatic beta cell degranulation observed after infusion of anti-insulin serum in vivo. This was observed by electron microscopy. These results agree with our experiments, suggesting again that diazoxide inhibits insulin release, but not synthesis, in an acute experiment.

In the first group of chronic experiments, A025 was administered to a group of force-fed rats and sixteen hours after the last dose of the drug and the last force-feeding, the pancreas was perfused using glucose as stimulus. These pancreases secreted greater amounts of insulin than the respective controls. A possible inter-

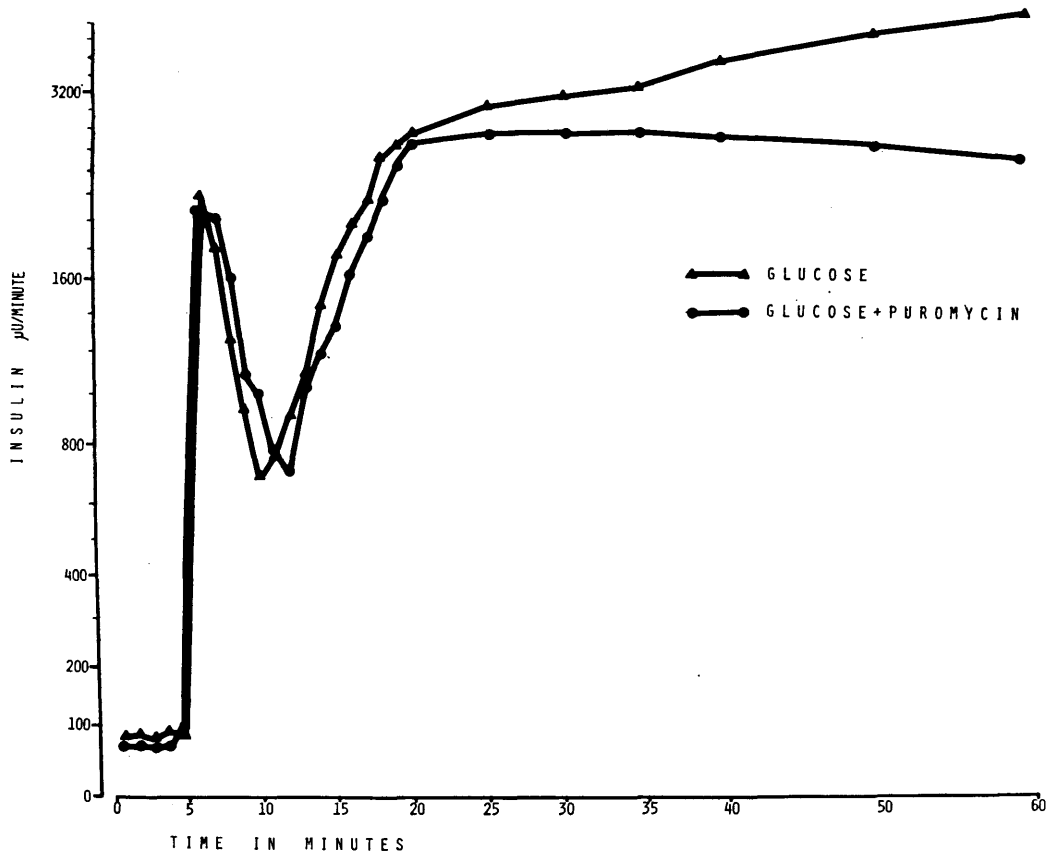

FIG. 9. Rate of insulin secretion by the pancreas from rats treated with diazoxide for five to seven days; effect of glucose (n:6) and glucose plus puromycin (n:5).

pretation of these experiments is the following: Force-feeding produces stimulation of pancreatic insulin release and synthesis, A025 inhibited release but not the synthesis, and insulin is accumulated in the beta cells. This accumulated insulin could account for the amount of insulin secreted when the pancreas was perfused sixteen hours after the last A025 administration, at a time when the effect of the drug was over. Basal levels were also higher in the pancreas of the A025-treated rats than in the controls. The normal insulin response, after discontinuation of A025 for ten days, confirms previous reports about the reversibility of the drug effect.[19]

Trying to find a possible interpretation of the above-described chronic experiment, two groups of rats were given diazoxide and A025 respectively. These experiments differ from the previous in that the time of the drug administration was only five to seven days rather than nine weeks and the rats had free access to food. All perfusions were performed on animals fasted for sixteen hours. The group in which the last dose of diazoxide or A025 was administered sixteen hours before the perfusion showed a higher rate of insulin secretion than controls, producing a pattern comparable to that seen before in rats treated for nine weeks with A025. However, when the pancreas was perfused three to four hours after the last dose, the rate of insulin secretion was markedly decreased in comparison with the control group.

This second set of chronic experiments seems to agree with the previously described interpretation of accumulation of insulin within the beta cells during chronic diazoxide and A025 treatment. Our results agree with the study published by Creutzfeld et al.,[34] who reported an electron microscopic picture of large amounts of stored insulin after treatment with diazoxide for twenty-four hours. This author also reported a further increase in the amount of insulin accumulated within the beta cell after three to four days of treatment with diazoxide.

The effect of puromycin on both phases of insulin secreted by the pancreas of rats treated with diazoxide

for five to seven days was studied in the last series of experiments. Puromycin markedly decreased the amount of insulin in the effluent, after thirty minutes of perfusion. It is interesting to note that the decrease in the insulin secretion produced by puromycin is more pronounced in the drug-treated rats than in animals without diazoxide treatment. This can be observed comparing the decrease in insulin levels produced by puromycin in figures 3 and 9. The real meaning of this difference is not easy to explain and is at present under study.

This communication reports studies of the effect of diazoxide and its derivative A025 on the pancreatic insulin release and synthesis. In conclusion, the results of our experiments in the perfused rat pancreas suggest that diazoxide and A025 inhibit insulin release, having no effects on insulinogenesis, and confirm that both drugs are useful substances to study the mechanism by which nutrients, hormones, and drugs influence insulin secretion.

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BOOK REVIEWS

HYPERLIPIDEMIA & HYPERLIPOPROTEINEMIA, *Shafeek S. Sanbar, M.D., $8.95, 153 pages, Boston, Little, Brown and Co., 1969.*

This small volume adequately reviews the entire subject of the hyperlipidemias. While somewhat sparse in references and depending greatly on the author's own impressions and comments, the book seems accurate in its content. Charts, definitions and case examples aid the reader in his appreciation of a somewhat confused field of clinical and investigative medicine. The volume presents a concise review of the subject matter and will provide an excellent reference for the student. Most of the material presented by Dr. Sanbar is readily obtainable in original articles; however, he has provided a service by placing it in a single reference book.

GALACTOSEMIA, *David Yi Yung Hsia, M.D. (Ed.); $18.50 trade edition, 318 pages, Springfield, Illinois, Charles C Thomas, 1969.*

This book represents the composite efforts of twenty-nine knowledgeable contributors who presented a series of papers at an international conference in November, 1967. Because of the expertise of these contributors, the investigations and commentaries are presented with clarity and authority. Dr. Hsia has added greatly to the volume by compiling and grouping the papers into a logical and understandable sequence.

The subject of this volume will be of intense interest to only a relatively small group of physicians. In addition, the volume does suffer from a problem that is often encountered when a conference is converted to a book—considerable difficulty in using the book unless one already has a working knowledge of the subject. Despite this objection, however, this volume does suffer from a problem that is often encountered in an "in-depth" study of the subject. The volume is helped immensely by the excellent discussions which are interspersed throughout. The final section on "Clinical Management" is particularly well done and the comments and discussions are useful even to the treatment of the nephrotic.

THE NEW MANAGEMENT OF STABLE ADULT DIABETES: A Compendium of Recent Research Findings, New Metabolic Insights and Improved Clinical Management. *Charles Weller, Ed., $6.75, 105 pages, Index, Springfield, Illinois, Charles C Thomas, 1969.*

This ambitious little book lives up to the billing in its subtitle moderately well. The chapter on diagnosis by Keen and Jarrett is concise and lucid. Poucher describes the "clinical paradox" of excess circulating insulin in the obese patient with diabetes and warns against the production of hypoglycemia. Evidence demonstrating insensitivity to insulin in these patients, presented by Berger and Tzagournis, summarizes data relating to the development of vascular complications.

Duncan's chapter on practical diet prescription includes a section on "no calorie" diets. In his hands, this method was safe and effective in a series of obese patients with stable diabetes. Over-all clinical management including special situations is discussed by DeLawter. This reviewer would have mentioned photocoagulation as an additional approach to the treatment of retinopathy. Faludi outlines oral hypoglycemic therapy and describes her findings with the use of phenformin. Although the publication date was too early to permit consideration of the FDA action on cyclamates, this is of minor importance in view of its wide publicity in the lay press.

In some instances the papers are diffuse and suffer from less than optimal organization. The inevitable overlap in a book written by multiple authors is not excessive. The bibliographies list a large number of recent key publications, and the author and subject indices facilitate reference to them. Many clinicians will find the book useful in the management of most of their patients with diabetes.