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Effect of Alloxan on the Incorporation of D-Glucose into Cultured Pancreatic Endocrine Cells of the Rat

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The effect of alloxan on cultured rat pancreatic endocrine cells was investigated by following changes in the incorporation of D-[U-^{14}C]glucose into proteins(chloroform/methanol-insoluble fraction) and lipids (chloroform/methanol-soluble fraction).

Cultured cells exposed to either alloxan or alloxan plus D-glucose anomers (16.7 mM) at 37° for 5 min were incubated in a medium containing D-[U-^{14}C]glucose at 37° for 60 min. Alloxan at concentrations of 1.25 and 6.25 mM produced inhibitory effects on incorporation into proteins but had no effect on incorporation into lipids. Incorporation into both proteins and lipids was inhibited by 31.2 mM alloxan. The combination of 1.25 mM alloxan plus 16.7 mM D-glucose anomers synergistically reinforced the inhibitory effect of alloxan on the incorporation of labeled glucose into proteins and lipids. In addition, the action of alloxan was more effective in the presence of the β anomer than the α anomer. SDS-polyacrylamide gel electrophoresis of cells labeled with D-[U-^{14}C]glucose indicated the involvement of cell components having molecular weights of more than 200000, 120000, 52000 and 20000.

In conclusion, the present data indicate that alloxan inhibits the incorporation of D-[U-^{14}C]glucose into proteins and lipids in cultured pancreatic endocrine cells and that the β anomer of D-glucose reinforces the action of alloxan to a greater extent than the α anomer.

Keywords——alloxan; pancreatic endocrine cells; monolayer cell culture; D-glucose incorporation; UDP-glucose incorporation

Introduction

Alloxan has been widely used to induce experimental diabetes, but the exact mechanism by which alloxan produces a specific destruction of pancreatic B cells remains unclear. Recently, Orci *et al.*[2] demonstrated that alloxan at a relatively high concentration produces a significant decrease in the number of membrane-associated particles in rat islet of Langerhans, suggesting that the diabetogenic action of alloxan may be associated with the fluidity of the membrane structure. Moreover, D-glucose prevented the ultrastructural alteration of the plasma membrane caused by alloxan.

In our recent experiments using uridine diphospho(UDP)D-[U-^{14}C]galactose,[3] the activity of galactosyltransferase on the outer surface of rat islets was found to be severely inhibited by alloxan and this effect of alloxan was abolished to a considerable extent in the presence of the α anomer of D-glucose but not in that of the β anomer. These results seem to support the hypothesis that the α anomer and alloxan may compete for a common site possibly located on the plasma membrane, *i.e.*, the glucoreceptor.[4]

In the present study, we describe the incorporation of D-[U-^{14}C]glucose into cultured pancreatic endocrine cells exposed to either alloxan alone or alloxan plus D-glucose anomers.

1) Location: *1-1, Mukogawa-cho, Nishinomiya, 663, Japan.*
2) L. Orci, M. Amherdt, F. Malaisse-Lagae, M. Ravazzola, W.J. Malaisse, A. Perrelet, and A.E. Renold, *Lab. Invest.*, 34, 451 (1976).
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Materials and Methods

Reagents——All reagents used were of special grade. Collagenase (type 1), α-D-glucose, β-D-glucose and tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co., USA. Trypsin (1: 250) was from Difco, USA. Bovine serum albumin (Fraction V) was from the Armour Laboratories, USA. Medium 199 with Hanks' salt was from Grand Island Biological Co., USA. Fetal bovine serum was from Flow Laboratories, Australia. Penicillin G potassium-salt (10^5 U/vial) and streptomycin sulfate (titer, 1 g/vial) were from Meiji Seika Co., Japan. UDP-[U-^{14}C]glucose (199 mCi/mmol) and D-[U-^{14}C]glucose (336 mCi/mmol) were from the Radiochemical Centre Amersham, England. Insulin RIA kit was from Dainabot Co., Japan. The kit for amylase test was from Daiichi Chemical Co., Japan. PCS and NCS solubilizers were from Amersham Co., USA. Ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), alloxan, D-glucose and other reagents were from Wako Pure Chemical Industries, Japan.

Monolayer Cell Culture of Newborn Rat Pancreas——Pancreases were removed, using a sterile technique, from 20 to 30 of newborn Sprague-Dawley rats aged 2 to 4 days and kept in saline until dissection. Dissected pancreases were washed twice in saline and cut into small pieces. The fragments were washed for 10 min at 37° in saline with continuous stirring. The supernatant was discarded and the washing was repeated once again. Then, 10 ml of prewarmed trypsin solution and 2 ml of collagenase solution were simultaneously added to the remaining material. After incubation for 15 min with gentle magnetic stirring at 37°, the supernatant was discarded. This trypsin–collagenase treatment was repeated five times and each of the supernatants was transferred to 10 ml of cooled culture medium containing 10% fetal bovine serum, 91 mUof penicillin and 10 μg of streptomycin per ml at 4°. The pellets were collected and again washed and centrifuged. The washed pellets were resuspended in an appropriate volume of the culture medium to a concentration of approximately 10^6 cells per ml. Ten ml aliquots of the cell suspension were added to plastic culture dishes (Falcon plastics, code 3003, Becton Division, Dickinson and Company, USA). The primary cell culture was performed at 37° under an atmosphere of 95% air and 5% CO_2. After culture for 20 hr, each of the cell suspension was transferred to new culture dishes and the culture was allowed to continue for 24 to 48 hr. In the present experiment, trypsin was dissolved in Ca^{2+}- and Mg^{2+}-free phosphate buffer containing 137 mM NaCl, 2.7 mM KCl and 2.8 mM glucose (7.7 mM, pH 7.4) to a concentration of 0.2%. Thirty mg of collagenase was dissolved in 10 ml of 150 mM NaCl solution. The final concentration of glucose in the culture medium was 16.7 mM.

Preparation of Cultured Cell Suspension——Monolayer cultured cells were washed with 2 ml of Ca^{2+}- and Mg^{2+}-free phosphate buffer (7.7 mM, pH 7.4). One ml of the buffer supplemented with 10 mM EDTA was added to culture dishes, which were placed back in the incubator. After incubation for 10 min, the suspending cells were collected by centrifugation at 150 ***g*** for 5 min. The cell pellet was washed with Krebs-Henseleit bicarbonate buffer (KHB buffer) and resuspended in an appropriate volume of KHB buffer.

Incorporation of D-[U-^{14}C]Glucose into Proteins and Lipids——The medium used was buffer A, which was consisted of 5.0 mM Tris, 139 mM Na^+, 4.7 mM K^+, 2.6 mM Ca^{2+}, 1.2 mM Mg^{2+} and 153.3 mM Cl^-. The suspended cells were placed in microplastic tubes and collected by centrifugation at 150 g for 2 min. Each batch of cells (approximately 10^5) was equilibrated in 500 μl of KHB buffer containing 2.8 mM glucose and 0.5% bovine serum albumin at 37° for 30 min. The cells were treated with alloxan or alloxan plus D-glucose anomers in 500 μl (final volume) at 37° for 5 min and then washed twice with 2 ml of KHB buffer. Each batch of pretreated cells was incubated in 200 μl of buffer A supplemented with 1 mM $MnCl_2$ and D-[U-^{14}C]-glucose (14.4 μM, 9×10^5 dpm) at 37° for 60 min. Labeled cells were disrupted by sonication for 30 sec at setting 2 on a Sonifier (80 W) (Branson Industries, USA) in 250 μl of the medium. One hundred μl of each sonicated sample was transferred to 500 μl of 10% TCA and centrifuged at 10^4 ***g*** for 5 min. After adding 50 μl of 1 N NaOH and subsequently 1 ml of 5% TCA to the pellet, the suspension was centrifuged at 10^4 ***g*** for 5 min. Precipitated materials were extracted twice with 500 μl of chloroform/methanol (2: 1, v/v); the extract soluble in chloroform/methanol contained lipids and the residue contained proteins. Chloroform and methanol were removed from lipids by heating at 100° for 5 min in a scintillation vial. Proteins and lipids were each dissolved in 10 ml of PCS-xylene (2: 1) and their radioactivities were determined in a liquid scintillation counter (Packard 3385, Packard Instrument Inc., USA). The blank value of the reaction, which was obtained by incubating cells boiled at 100° for 3 min prior to incubation with labeled glucose, was subtracted from each measurement. Experiments with UDP-[U-^{14}C]glucose (4.05 μM, 3.8×10^5 dpm) were carried out according to the same procedures.

SDS-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was carried out by the method of Fairbanks *et al.*[5] The column gel contained 5.6% (w/v) acrylamide, 0.21% (w/v) bisacrylamide, 0.04 M Tris–CH_3CO_2H (pH 7.4), 0.1% (w/v) SDS and 0.002 M EDTA and was then chemically polymerized by the addition of 0.025% N,N,N′,N′-tetramethylethylenediamine and 0.15% ammonium persulfate. Forty μl of the sample, which was prepared by dissolving the labeled cell pellet in 0.1 ml of 10 mM Tris buffer (pH 8.0) containing 1% SDS, 10% sucrose, 1 mM EDTA and 1% mercaptoethanol, was applied to the gel.

5) G. Fairbanks, T.L. Steck and D.F.H. Wallach, *Biochemistry*, **10**, 2606 (1971).

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The gel was run at 6 mA per gel column for about 1 hr until the tracking dye (Coomassie brilliant blue R-250) had migrated to the end of the gel column (6—6.5 cm from the origin). Each gel was cut into 2.5 mm segments. Each segment was kept overnight at room temperature in 10 ml of a scintillation fluid, which contained 4 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 100 ml of NCS solubilizer per liter of toluene, in a scintillation vial and then the radioactivity was counted with a scintillation counter. In other experiments, the gel was stained for protein with 0.04% Coomassie blue and 25% isopropanol in 10% acetic acid and then destained with several changes of 10% acetic acid. Trypsin inhibitor from soybean (MW 21500), bovine serum albumin (MW 68000) and RNA-polymerase from *E. coli* (α-subunit MW 39000, β-subunit MW 155000, β'-subunit MW 165000) were used as protein standards.

Determination of the Number of Suspended Cells——The number of suspended cells was determined by light microscopy.

Measurement——The content of insulin (immunoreactive insulin, IRI) and the activity of amylase in the culture medium were measured by a double antibody radioimmunoassay and by the blue starch method, respectively.

Calculation——Student's "*t*" test was used to determine statistical significance.

Results

Characterization of Cultured Cells

The content of insulin and the activity for amylase release into the culture medium per day were 414±28 ng and 528±8 IU/l, respectively, at 24 hr after introduction into a fresh culture medium. The former did not change with time of culture, while the latter rapidly diminished, indicating that most of the cultured cells consist of endocrine cells at 3 days after commencing the culture (Fig. 1).

Effect of pH on the Incorporation of D-[U-^{14}C]Glucose

The pH was varied within the range of 6.7 to 7.4 in increments of 0.2 pH units. Incorporation of D-glucose into proteins and lipids reached a maximum at pH 6.8; the change of incorporation with pH for proteins was particularly sharp. With UDP-[U-^{14}C]glucose, incorporation into(glyco) proteins and (glyco)lipids reached a maximum at about pH 6.8, but the optimum pH was rather broad as compared with that for the incorporation of D-[U-^{14}C]glucose (Fig. 2).

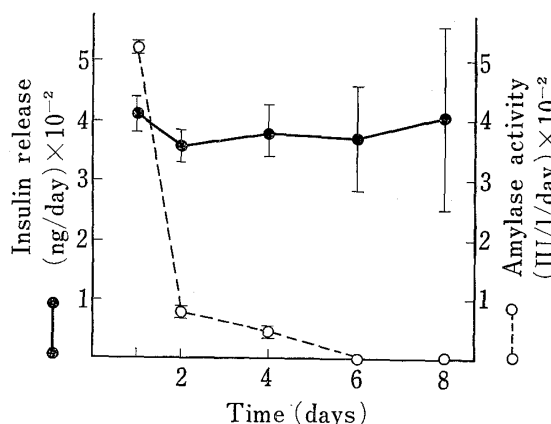

Fig. 1. Changes in the Content of Insulin and the Amylase Activity in the Culture Medium with Time

The content of insulin and the amylase activity per day released into 1 ml of the culture medium in the dish were determined for 8 days after transferring cultured pancreatic cells to new culture dishes at one- or two-day intervals as described in "Materials and Methods." Each dish contained approximately 10^5 cells 2 days after the transfer to the new culture dishes. The solid line represents the content of insulin (ng/day) and the dotted line the activity of amylase (IU/l/day; IU. international unit). Points are the mean (±SEM) values of five observations.

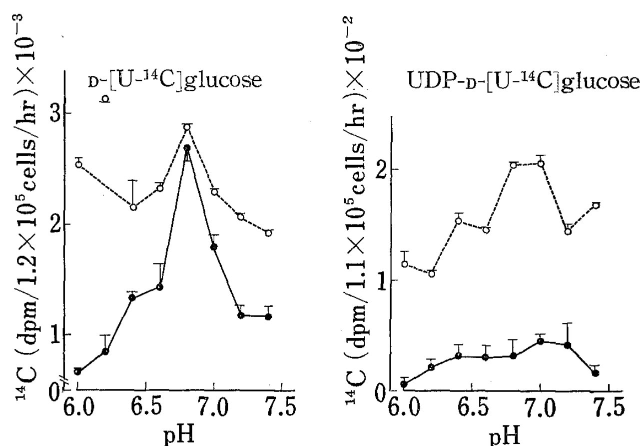

Fig. 2. Effect of pH on the Incorporation of D-[U-^{14}C]Glucose or UDP-[U-^{14}C]Glucose

Cultured pancreatic endocrine cells were incubated in buffer A containing 1 mM $MnCl_2$ with D-[U-^{14}C]glucose or UDP-[U-^{14}C]glucose for 60 min at 37°. Incorporation into proteins and lipids was determined as described in "Materials and Methods." Closed circles represent proteins and open circles lipids. Points are the mean (±SEM) values of four observations.

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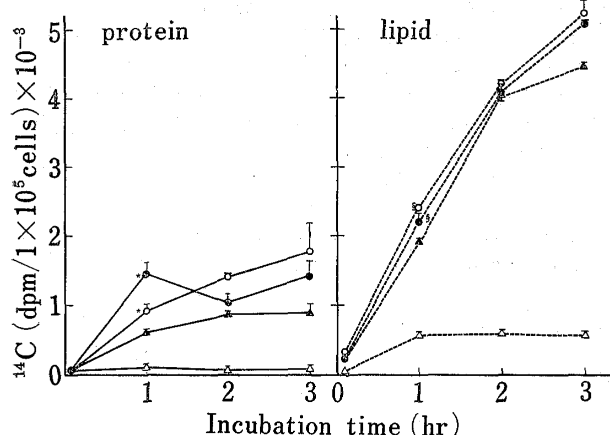

Fig. 3. Effect of Incubation Time on the Incorporation of D-[U-^{14}C]Glucose into Proteins and Lipids of Either Intact or Alloxan-treated Cells

Cultured pancreatic endocrine cells were pretreated with various concentrations of alloxan (mM) (0, ●: 1.25, ○: 6.25, ▲: 31.2, △) for 5 min at 37° and incubated with D-[U-^{14}C]-glucose for up to 3 hr. Points are the mean (±SEM) values of four observations. Statistical comparison was performed between the control and 1.25 mM alloxan-treated groups.
*$p<0.05$, ξ not significant.

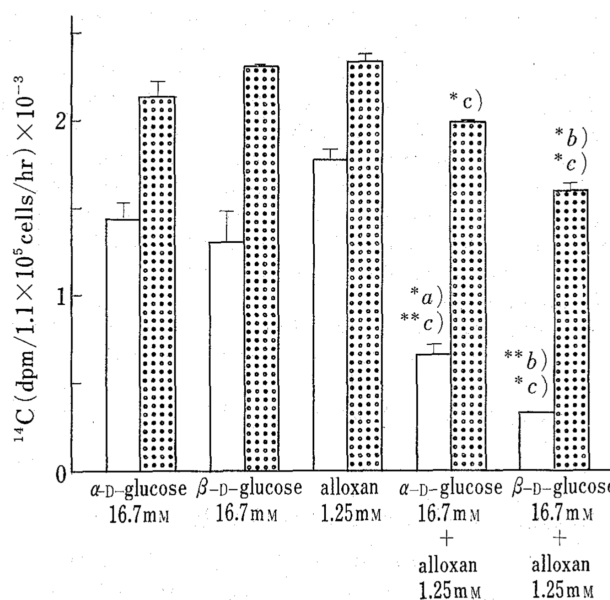

Fig. 4. Effect of Alloxan plus D-Glucose Anomers on the Incorporation of D-[U-^{14}C]Glucose

Cultured pancreatic endocrine cells were pretreated with 1.25 mM alloxan, 16.7 mM D-glucose anomers or a combination of them for 5 min at 37° and then incubated with D-[U-^{14}C]glucose for 60 min at 37°. Each value represents the mean (±SEM) of four observations. Statistical comparison was performed against α-D-glucose (*a*), β-D-glucose (*b*) and alloxan (*c*).
*$p<0.001$, **$p<0.01$.

Effect of Various Concentrations of Alloxan on the Incorporation of D-[U-^{14}C]Glucose

The incorporation of D-[U-^{14}C]glucose into proteins and lipids of either intact or alloxan-treated cells (exposed to either 1.25, 6.25 or 31.2 mM alloxan for 5 min prior to incubation) is shown as a function of incubation time in Fig. 3.

With intact cells, incorporation of D-[U-^{14}C]glucose into proteins can be divided into two components; a rapid one during the first 60 min and a slower one between 60 and 180 min. On the other hand, incorporation into lipids was nearly proportional to the incubation time. The ratio of proteins to lipids was 1.5 at 60 min and 3.3 at 180 min. Alloxan produced a concentration-dependent inhibitory effect on incorporation into proteins, and completely inhibited incorporation at 31.2 mM. However, alloxan at up to 6.25 mM concentration produced no significant effect on incorporation into lipids, while at 31.2 mM it markedly inhibited the incorporation.

Effect of Alloxan plus D-Glucose Anomers on the Incorporation of D-[U-^{14}C]Glucose

Cultured cells were treated with 1.25 mM alloxan alone, 16.7 mM D-glucose anomers alone or a combination of them for 5 min prior to incubation in the medium containing D-[U-^{14}C]glucose for 60 min at 37° (Fig. 4).

No remarkable differences in incorporation into proteins and lipids were observed among groups treated with alloxan or D-glucose anomers. The combination of alloxan and D-glucose anomers synergistically induced severe inhibition of incorporation into proteins and lipids, which was greater in the presence of the β anomer than the α anomer.

Using UDP-[U-^{14}C]glucose, which serves as a substrate for the outer surface glucosyltransferase of the cells, the activity of glucosyltransferase was very weak and glucose transfer was slightly reduced by 1.25 mM alloxan. This inhibitory effect of alloxan on the incorporation into (glyco)lipids was reduced by the concomitant presence of anomers (Table I).

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Table I. Effect of Alloxan plus D-Glucose Anomers on the Incorporation of UDP-[U-^{14}C]Glucose

	Concen. (mM)	^{14}C Incorporation (dpm/6.4 × 10^4 cells/hr)	
		Protein	Lipid
Control		56±5	143± 6
Alloxan	1.25	42±7	103±13*[a)]
Alloxan	1.25	33±7	123± 4
+α-D-glucose	16.7		
Alloxan	1.25	35±5	147±15**[b)]
+β-D-glucose	16.7		

Cultured pancreatic endocrine cells were pretreated with alloxan alone or alloxan plus D-glucose anomers for 5 min at 37° and then incubated with UDP-[U-^{14}C]glucose for 60 min at 37°. Each value represents the mean (±SEM) of four observations. Statistical comparison was performed against the control (*a*) and alloxan (*b*). *$p<0.02$, **$p<0.05$.

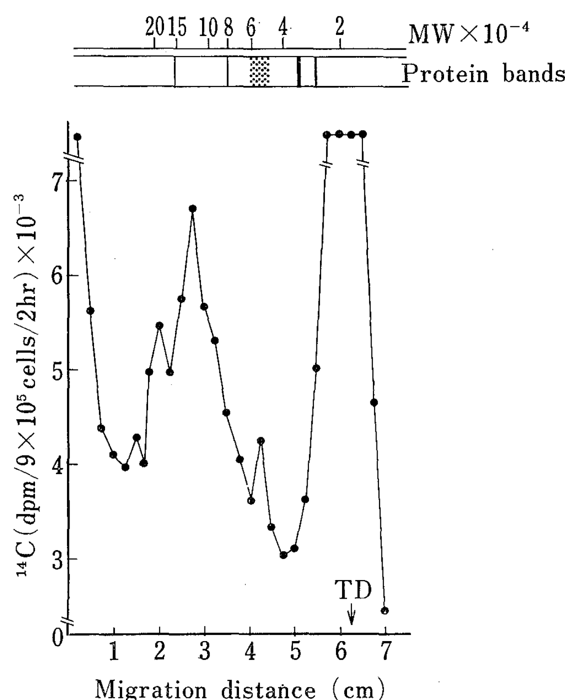

Fig. 5. Electrophoretic Analysis of Cultured Pancreatic Endocrine Cells labeled with D-[U-^{14}C]Glucose

This graph shows the distribution of cell components labeled with D-[U-^{14}C]glucose on SDS-polyacrylamide gel electrophoresis. Cultured pancreatic endocrine cells were incubated with D-[U-^{14}C]glucose at 37° for 2 hr.

Electrophoretic Analysis of Radioactive Substances labeled with D-[U-^{14}C]Glucose

Cultured cells (9×10^5) were incubated in the medium containing D-[U-^{14}C]glucose (7.3×10^6 dpm/300 μl) for 2 hr at 37°. The samples for electrophoretic analysis were prepared according to the method described earlier, without chloroform/methanol extraction (Fig. 5).

SDS–polyacrylamide gel electrophoresis of the sample labeled with D-[U-^{14}C]glucose indicated label distribution into cell components having molecular weights of more than 200000, 120000, 52000 and 20000.

Discussion

In the present study, we demonstrated that alloxan decreases the incorporation of D-[U-^{14}C]glucose into proteins and lipids of cultured rat pancreatic endocrine cells and that the inhibitory effect is synergistically enhanced by D-glucose anomers.

Recently, it has been shown that the α anomer of D-glucose provides more effective protection of rat islets of Langerhans against insulin release induced with glucose[6)] and glucose oxidation[7)] than the β anomer, suggesting that the site of alloxan action and the glucoreceptor may be involved in insulin release and glucose metabolism. Our recent experiments using UDP-[U-^{14}C]galactose support the hypothesis that the glucoreceptor may exist on the cell membrane of the islets.[3)] In the present experiments using UDP-[U-^{14}C]glucose, a slight decrease in glucosyltransferase activity caused by alloxan seemed to be reduced by the presence of anomers. However, it was not possible to compare exactly the incorporation of

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7) F. Ishibashi, T. Sato, K. Onari and R. Kawate, *Endocrinol. Jpn.*, **26**, 395 (1979).

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labeled glucose into (glyco)proteins and (glyco)lipids between groups treated with alloxan alone and with alloxan plus D-glucose anomers since the outer surface glucosyltransferase was too active in catalyzing glucose transfer from UDP-glucose.

In contrast to UDP-[U-^{14}C]glucose, incorporation of D-[U-^{14}C]glucose into proteins and lipids occurred to a greater extent, and was therefore convenient for observing the synergistic effects of alloxan plus D-glucose anomers. The present experiments showed that the presence of anomers synergistically reinforces the inhibitory effect of alloxan on the incorporation of D-[U-^{14}C]glucose. A similar effect of D-glucose anomers was observed in glucose-induced insulin release from islets pretreated with iodoacetic acid, where the β anomer reinforced the inhibitory effect of iodoacetic acid more markedly than the α anomer.[8] These effects of D-glucose anomers were in contrast to the specifically protective effects of the α anomer mentioned earlier. Weaver *et al.*[9] reported recently that D-glucose enhances the uptake of labeled alloxan into the islets. These findings may reflect the nonspecific action of alloxan; the presence of anomers may lead to greater alteration of the conformation of cell membranes, followed by changes in the fluidity of the cell membranes, than in their absence, giving alloxan molecules relatively free access to the cell membranes. Moreover, since alloxan is known to be converted to alloxanic acid, which has no diabetogenecity, immediately after entering the islets,[9] it seems possible that the synergistic action of alloxan plus D-glucose anomers may occur on the plasma membrane of cultured pancreatic endocrine cells.

In conclusion, we suggest that the action of alloxan might be associated not only with inhibition of the intracellular metabolism of glucose but also with alteration of the membrane structure of cultured pancreatic endocrine cells.

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