248–250 °C; $[\alpha]_D$ +88.7°; UV ϵ_{234} = 15 800; IR 1775, 1690, 1660 cm^{-1}; NMR 5.53 (C-4), 4.17 (m, C-1), 2.38 (s, $SCOCH_3$), 1.35 (C-19), 1.02 (C-18) ppm. Anal. ($C_{26}H_{32}O_4S$) C, H, O, S.

7α-(Acetylthio)-1α,2α:15α,16α-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone (20). A solution of 1 g (0.0027 mol) of **18** in 20 mL of MeOH, 4 mL of water, and 1.5 mL of thioacetic acid was stirred at room temperature for 16 h. After dilution with ether, the organic phase was washed for aqueous $NaHCO_3$ and water. The crude material was purified by column chromatography on silica gel. Recrystallization from diisopropyl ether provided 460 mg (38%) of **20**: mp 246.2 °C; $[\alpha]_D$ +120°; UV ϵ_{235} = 16 500; IR 1775, 1690, 1660, 1620 cm^{-1}; NMR 5.52 (C-4), 4.2 (m, C-7), 2.35 (s, $SCOCH_3$), 1.25 (C-19), 1.18 (C-18) ppm. Anal. ($C_{26}H_{32}O_4S$) C, H, O, S.

Synthesis of Two Glucagon Antagonists: Receptor Binding, Adenylate Cyclase, and Effects on Blood Plasma Glucose Levels

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In diabetes mellitus, hyperglycemia is often associated with elevated levels of glucagon in the blood. This suggests that glucagon (1) is a contributing factor in the metabolic abnormalities of diabetes mellitus. A glucagon-receptor antagonist would provide direct evidence for glucagon's role in diabetes mellitus. On the basis of careful consideration of conformational, amphiphilic, and structural factors, we have synthesized two new glucagon analogues with antagonist biological activities by using solid-phase methodology. These two new analogues, [Asp^3,D-Phe^4,Ser^5,$Lys^{17,18}$,Glu^{21}]glucagon (**2**) and [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Arg^{12},$Lys^{17,18}$,Glu^{21}]glucagon (**3**), had IC_{50} values 5.4% and 50% those of glucagon, respectively, and showed no measurable adenylate cyclase activity. When tested in normal rats, **2** lowered plasma glucose levels and suppressed glucagon-mediated hyperglycemia 105 ± 8%, back to basal levels. Analogue **3**, which lowered the basal adenylate cyclase activity in rat liver plasma membranes, increased plasma glucose levels at very high concentration in vivo and inhibited glucagon-mediated hyperglycemia in normal rats by 50%. However, neither of the new glucagon antagonists lowered the plasma glucose levels of diabetic animals. The data would suggest these new glucagon-receptor antagonists may have two actions: (a) in normal rats they can act as standard glucagon-receptor inhibitors of glucagon-mediated glycogenolysis; (b) in diabetic rats, however, because of the low levels of glycogen in the liver, the antagonists apparently have little or no antagonist effect or enhancement on glucagon-mediated glucose production.

Glucagon,[1] a 29 amino acid peptide hormone, is produced in the α-cells of the pancreas. Its main target is the liver cell, where it increases glycogenolysis and gluconeogenesis.[2] There is evidence for the existence of high- and low-affinity receptors for glucagon in rat and canine liver,[3,4] but only one receptor protein could be isolated by photoaffinity labeling.[5] The glucagon–receptor complex activates adenylate cyclase activity,[6] and the cAMP thus produced stimulates an enzyme cascade that leads to activation of phosphorylase and the inhibition of glycogen synthesis.[7] Additionally, glucagon stimulates amino acid and lipid catabolism which results in gluconeogenesis and ketosis[2] and lowers the intracellular Ca^{2+} concentration by inhibiting the Ca^{2+} pump in rat liver plasma membranes.[8] In addition to the classical cAMP-mediated stimulation of glycogenolysis by glucagon, there is evidence for a cAMP-independent pathway.[9]

In diabetes mellitus, hyperglycemia and ketosis are generally associated with elevated levels of glucagon in the blood. This suggests that excessive glucagon could be a contributing factor in the metabolic abnormalities of diabetes mellitus.[10] According to the bihormonal hypothesis,[11] insulin deficiency causes impairment of glucose utilization, but overproduction of glucose and ketones by the liver is primarily mediated by glucagon. Therefore, several investigators have tried to develop glucagon-receptor antagonists that would provide direct evidence for glucagon's role in diabetes mellitus.[12–14] The understanding of structure–activity relationships that are necessary for the development of an antagonist have been primarily based on studies utilizing receptor-binding and adenylate cyclase activity with rat liver plasma membranes. In this approach, an ideal antagonist should bind to the glucagon receptors in plasma membranes with high affinity, but without having any intrinsic adenylate cyclase activity.

Utilizing this approach, we recently have reported design principles and structural considerations that have led to the synthesis of a highly potent agonist,[15] to a better un-

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature. Other abbreviations include the following: cAMP, 3′,5′-cyclic adenosine monophosphate; THG, [1-N^{α}-trinitrophenylhistidine,12-homoarginine]glucagon; TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; HPLC, high-performance liquid chromatography.

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Table I. Amino Acid Analysis of New Glucagon Analogues[a]

amino acid	glucagon analogue 2			glucagon analogue 3		
	A	B	T	A	B	T
Trp	0.8	1.0	(1)	0.9	1.0	(1)
Lys	2.9	3.2	(3)	2.0	1.8	(2)
His	0.9	1.0	(1)	1.0	1.0	(1)
Arg			(–)	1.0	1.0	(1)
Asx	3.9		(4)	2.9		(3)
Asp		1.0	(1)		1.1	(1)
Thr	2.0		(2)	2.0		(2)
Ser	4.9	8.0[b]	(5)	3.6	8.1[b]	(4)
Glx	3.0		(3)	3.9		(4)
Glu		1.0	(1)		1.0	(1)
Gly	1.0	1.1	(1)	1.1	1.0	(1)
Ala	1.0	1.1	(1)	1.1	1.0	(1)
Val	1.1	1.0	(1)	1.0	1.1	(1)
Met	0.9	1.0	(1)	1.1	1.1	(1)
Leu	2.1	2.1	(2)	2.0	1.9	(2)
Tyr	2.1	2.0	(2)	3.4[c]	1.4[d]	(3)
Phe	3.0	2.1[d]	(3)	3.0	1.7[d]	(3)

[a] A: peptides were hydrolyzed in 3 N mercaptoethanesulfonic acid at 120 °C for 24 h. B: peptides were hydrolyzed first with leucine-aminopeptidase and then with carboxypeptidase Y. T: theoretical. [b] Leucine-aminopeptidase converts Gln into pyrrolidonecarboxylic acid; therefore this is the approximate value for the total of the overlapping peaks of Thr, Ser, and Gln. [c] Includes the value for 3,5-I_2-Tyr. [d] Enzymatic digestion left the dipeptide -D-Phe-X- undigested. **2**: [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon. **3**: [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon.

derstanding of the role of the glucagon 10–13 region in glucagon biological activity,[16,17] and to the design and synthesis of several totally synthetic glucagon analogues with partial agonist and antagonist activities.[18] Increasing the α-helical potential as calculated by Chou and Fasman method[19] in the 17–29 sequence of glucagon produced a highly potent agonist.[15] It has been reported that iodination of glucagon results in a hormone with increased adenylate cyclase activity and increased stability in vivo.[20] In this work, we have combined information available from previous studies to design and synthesize two new glucagon antagonist analogues by using solid-phase synthetic methods. We have examined their receptor-binding and adenylate cyclase activities with liver plasma membranes from normal rats and determined their plasma blood glucose levels and their ability to inhibit glucagon action in normal and diabetic rats. By utilizing this approach, we have tried to address the following questions: (1) Does a glucagon antagonist with respect to adenylate cyclase necessarily inhibit glucagon mediated glucose production in normal rats? (2) Do the structurally different new antagonists have different biological activity profiles than previous antagonist analogues? (3) Is there evidence for the existance of a cAMP-independent pathway for glucose production? (4) Does a glucagon-receptor antagonist necesssarily lower blood glucose levels of diabetic rats?

Results

Synthesis and Assessment of Purity of Glucagon Analogues. On the basis of a variety of considerations (see Discussion), we designed and synthesized [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon (**2**) and [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Lys^{17,18},Glu^{21}]glucagon (**3**). The preparations were accomplished by using established solid-phase methodologies for synthesis (see Experimental Section) and reverse-phase high-performance liquid chromatography (RP-HPLC) for final purification to obtain **2** in 2.5% and **3** in 5.1% yield based on the initial resin substitution.[16,18,21] RP-HPLC analysis gave a k' value of 3.72 for **2**, whereas glucagon had a value of 3.44 in the same system. The overall hydrophobicity of **3** was higher as expected, with a k' value of 5.06 (see Experimental Section). In order to make it more convenient and more accurate to determine the hormone concentration for bioassays, we determined the extinction coefficients of the new analogues and found 279 max was 8300 cm^{-1} mol^{-1} for **2** and 12 000 cm^{-1} mol^{-1} for **3**. Amino acid analysis after total hydrolysis with mercaptoethanesulfonic acid gave the correct amino acid composition (Table I). Additionally, both analogues were hydrolyzed with leucine-aminopeptidase (LAP) and carboxypeptidase Y. LAP converts glutamine into pyrrolidonecarboxylic acid, which overlapped in our system with the threonine and serine peaks in the amino acid analysis. Enzymatic hydrolysis also left the dipeptide -D-Phe-X- undigested. With the exception of these restrictions, enzymatic hydrolysis also gave the correct amino acid composition (Table I). These results show that less than 3% racemization occurred during the synthesis and that no modified amino acids are present in the purified analogues. Finally, both analogues were analyzed for their purity and sequence fidelity by peptide mapping. For this purpose, we digested both peptides by chymotrypsin and analyzed the fragments obtained on RP-HPLC. The patterns obtained were compared with those obtained with glucagon (Figure 1). Differences from glucagon occurred only in those peaks in which modifications were intentionally introduced during total synthesis. Amino acid analysis of the new HPLC peaks showed the expected composition.

Receptor-Binding and Adenylate Cyclase Activity. Receptor-binding and adenylate cyclase activities of glucagon and the two new analogues were determined with liver plasma membranes from normal rats. The EC_{50} of

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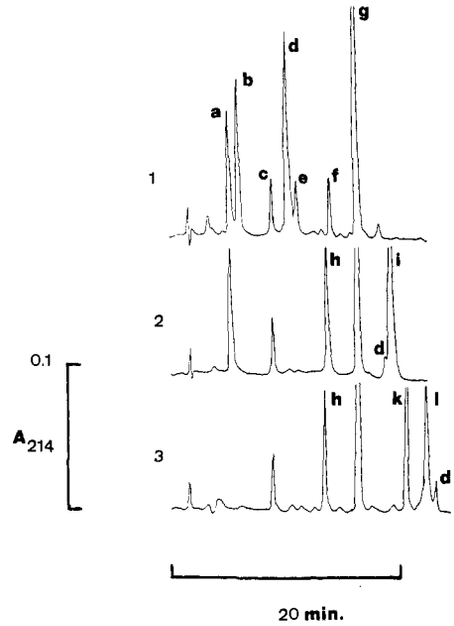

Figure 1. Peptide mapping: 1 mg of glucagon (1), [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon (2), or [D-Phe^4,Tyr^5,3,5-I_2Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon (3) was digested with chymotrypsin (100:1, w/w) three times. Then one-third of the sample was analyzed by RP-HPLC (trifluoroacetic acid 0.1%, acetonitrile gradient 5–30% in 25 min). Individual peaks were collected, hydrolyzed with 3 N mercaptoethanesulfonic acid, and subjected to amino acid analysis. Peaks represent the following partial sequences: a, 11–13; b, 7–10; c, 26–29; d, 1–6; e, 18–22; f, 14–17; g, 23–25; h, 14–22; i, 1–10; k, 6–13; l, 1–5.

glucagon was 9.6 nM (95% confidence limits: 4.5–16 nM) in the adenylate cyclase assay; analogue **2** showed no detectable adenylate cyclase activity at concentrations as high as 10 μM, and analogue **3** did not significantly effect basal activity (-5 ± 3%) (Figure 2, bottom). Both analogues can inhibit glucagon-mediated activity as previously shown for other glucagon antagonists[12,18] (data not shown). The IC_{50} values for binding of the analogues to glucagon receptors in liver plasma membranes was determined (see Experimental Section) by examining the ability of the analogues to displace [^{125}I]glucagon. Analogue **2** (IC_{50} = 130 nM) was about $^1/_{20}$ as potent as glucagon (which has an IC_{50} = 6.7 nM, 3.1–11 nM) in its ability to displace [^{125}I]glucagon in the binding assay, and analogue **3** (IC_{50} = 13 nM) was $^1/_2$ as potent as glucagon (Figure 2, top).

Effects on Plasma Glucose Levels. If glucagon enhances plasma glucose levels in vivo only through cAMP as a second messenger, then both analogues **2** and **3** should be unable to elevate the blood sugar level in normal rats. In order to test this, we injected both analogues into a femoral vein of normal rats and measured the blood glucose level as a function of time. Figure 3 shows how glucagon, in a dose of 1 μg/kg, elevates the blood glucose level by 95.1 ± 10 mg/dL within 10 min. Compound **3**, which lowered the cAMP level in the plasma membrane assay, was inactive at doses of 5 (data not shown) and 50 μg/kg (Figure 3), but unexpectedly increased the glucose level at a dose of 500 μg/kg (Figure 3). The half-life time of this latter effect by compound **3** was 16 min as opposed to 8 min for glucagon. Compound **2**, at a dose of 100 μg/kg, lowered the plasma glucose level by 45 mg/dL (Figure 3), and similar results were seen at 500 μg/kg (data not shown).

Competition of Antagonists with Glucagon-Mediated Increase in Plasma Glucose Levels in Normal Rats. In a second experiment we injected each analogue

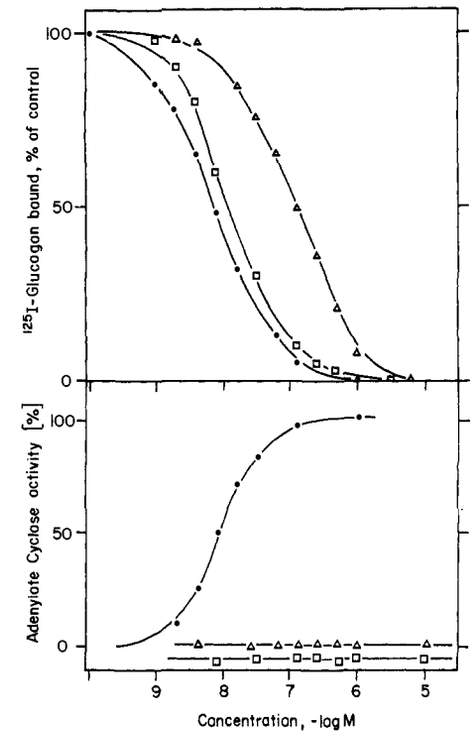

Figure 2. Receptor binding and adenylate cyclase activity. (Top) Receptor binding: Rat liver plasma membranes (50 μg of protein/0.5 mL) were incubated in Tris-buffer (pH 7.5) as described in the Experimental Section (detailed methodology have been previously reported[12,32]). Each 0.5 mL of suspension contained 100 000 cpm (25 fmol) of ^{125}I-labeled glucagon and unlabeled peptide at the concentration shown. The incubation time was 10 min at 30 °C. Shown are the curves for displacement of [^{125}I]glucagon by the concentration of glucagon or glucagon analogue after correction for nonspecific binding (see Experimental Section). Each point represents the mean of three determinations and has been corrected for nonspecific binding which remained in the presence of excess (1 μM) unlabeled glucagon. (Bottom) Adenylate cyclase activity. The procedure is described in the Experimental Section. The assay volume was 0.1 mL containing rat liver plasma membrane with 30 μg of membrane protein in Tris–buffer (pH 7.5) and 2 × 10^5 cpm [α-^{32}P]ATP. cAMP was isolated by chromatography. Assays were run in triplicates and repeated three times. The regression lines of the linear region of the dose–response curves were calculated from this data. (●) Glucagon (1); (△) [Asp^3,D-Phe^4,Tyr^5,Lys^{17,18},Glu^{21}]glucagon (**2**); (□) [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon (**3**).

together with glucagon in order to test if they were able to inhibit the glucagon-mediated increase of blood glucose (Figure 4). Compound **2**, in a 100:1 excess over glucagon, was able to inhibit the glucagon-mediated effect back to basal levels (105 ± 8% inhibition) (Figure 4A) as determined by the area under the curve. Compound **3** was an effective inhibitor in a 5:1 excess, but lowered the glucagon effect by only about 50% (Figure 4B) as determined by the area under the curve. At a higher excess (50:1), compound **3** was less effective and apparently started to show agonist effects (Figure 4B).

Effects in Diabetic Rats. On the basis of our experience with [N^{α}-trinitrophenyl-His^1,HomoArg^{12}]glucagon (THG), which has little or no adenylate cyclase activity in vitro[12,22] and which lowers blood glucose in diabetic animals,[13] it would be expected that compound **2** should be able to lower the blood glucose levels of these animals. Therefore, we injected both compounds **2** and **3** into rats made diabetic with streptozotocin, in a dose that was ef-

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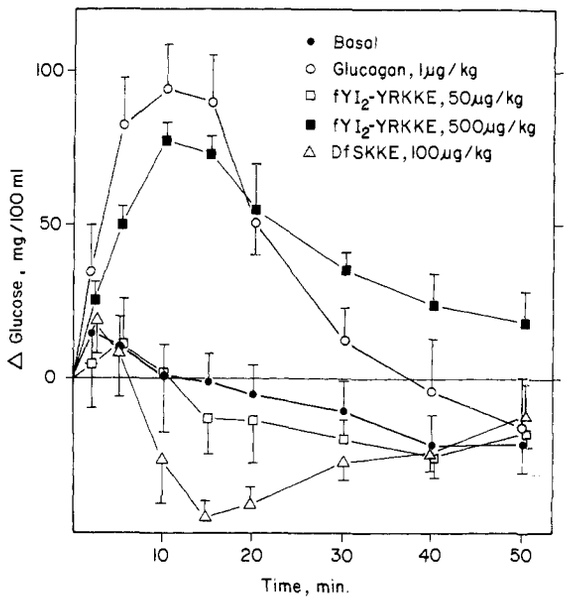

Figure 3. Effect of glucagon antagonist on the blood sugar level of normal rats. Male Wistar rats were anasthetized with pentobarbital. A femoral vein was catheterized for infusion of glucagon or the analogue and a femoral artery was catheterized for reptitive blood sampling. The glucose concentration of blood samples was determined with a Beckman glucose analyzer. Immediately before infusion a sample was taken to analyze the basal glucose level. After infusion of the compounds at time 0, samples were taken after 2, 5, 10, 20, 30, 40, and 50 min. Changes in the glucose level are given as difference from the basal level in each individual rat. Data points are the mean of four to six animals; standard errors are shown for all experiments. (○) Glucagon (1), (●) basal, (□) 50 μg/kg, and (■) 500 μg/kg, [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon (3), and (△) 100 μg/kg [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon (2).

fective in counteracting the effects of exogenous glucagon in normal rats. As Figure 5 shows, 100 μg/kg of compound **2** had no effect on the glucose level of diabetic rats. Interestingly, 50 μg/kg of compound **3** significantly increased glucose levels.

Discussion

Design of New Glucagon Analogues. The basic approach to design antagonists is to dissociate the structural, conformational, and dynamic properties necessary for binding from those necessary for transducing the message into the cell.[23] It is well established that most of the residues 1–29 in glucagon contribute to binding, whereas single modifications in positions 1–5 and 11, 12 can result in partial agonists.[12,16,17,23] In our earlier studies, we synthesized [Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon and [D-Phe^4,Tyr^5,Arg^{12}]glucagon, which were found to be glucagon-receptor antagonists with respect to adenylate cyclase.[18] These two compounds were inactive in the adenylate cyclase assay and had receptor-binding potencies of 1.3% and 1%, respectively, that of glucagon. In the studies reported here, we have sought to improve the binding potency of these analogues. Chou–Fasman calculations indicate a nearly equal probability for β- and α-helical structure in the 19–27 region of glucagon. Replacement of Arg^{17,18} by Lys^{17,18} and Asp^{21} by Glu^{21} leads to an increased probability for α-helical structure and a decreased probability for a β-structure in this region,[15] and the resulting analogue [Lys^{17,18},Glu^{21}]glucagon was 5 times more potent than glucagon in the glucagon receptor binding assay and 7 times more potent in the adenylate

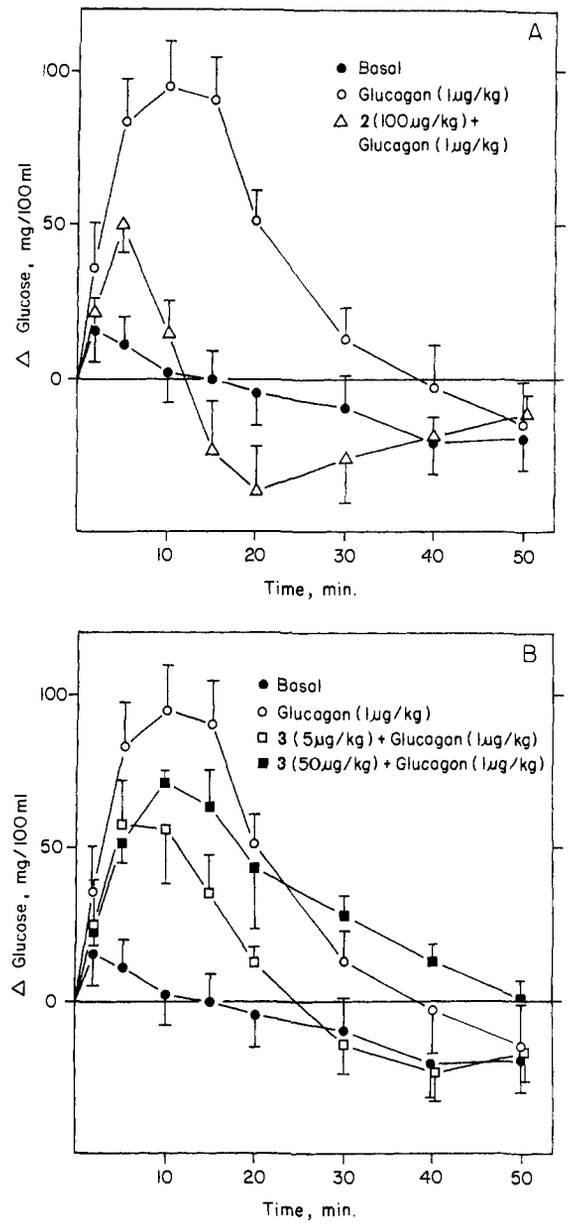

Figure 4. Inhibition of glucagon action by glucagon antagonists in normal rats. The experiments were carried out as described in Figure 3. At time 0 1 μg/kg glucagon was injected with the indicated excess of analogue. It can be seen that both compounds (2) (A) and (3) (B) inhibit the glucagon-mediated rise of blood glucose but in differing amounts (see Discussion). Points are the mean of four to six animals. Standard errors are shown for all experiments. (A) (○) Glucagon (1) only (1 μg/kg); (●) basal (without any peptide); (△) [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon (100 μg/kg) + glucagon (1 μg/kg). (B) (○) Glucagon (1) only (1 μg/kg); (●) basal (without any peptide); (□) [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon (3) (5 μg/kg) + glucagon (1 μg/kg); and (■) 3 (50 μg/kg) + glucagon (1 μg/kg).

cyclase assay.[15] Therefore, we decided to combine these latter modifications with our antagonist modifications and synthesized [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon (**2**). Our second approach utilized slightly different thinking. In micelle-bound glucagon, the Phe^6, Tyr^{10} and Leu^{14} residues form a hydrophobic patch which is important for the binding of glucagon to the micelles.[24] By introducing 3,5-diiodo-Tyr in position 10 we tried to make this patch more hydrophobic. Therefore, we synthesized [D-

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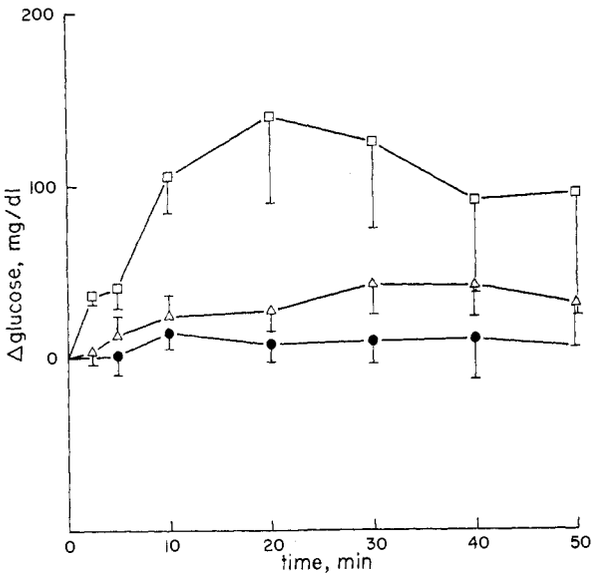

Figure 5. Effect of glucagon antagonists on the blood glucose level of diabetic rats. Male Wistar rats (200–250 g) were made diabetic by injecting 70 mg/kg streptozotocin. After the amount of glucosuria had stabilized (about 5 days), they were anaesthetized and a femoral vein and artery were catheterized. After measuring the basal glucose level, glucagon antagonists were injected as a bolus of the indicated amount. Values are given as difference from the basal level (400–700 mg/dL) of each individual rat. Points are the mean of four to five animals. (●) Basal; (□) [D-Phe^4,Tyr^5,3,5-I$_2$-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon (**3**), 50 μg/kg, (△) [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon (**2**), 100 μg/kg.

Phe^4,Tyr^5,3,5-I$_2$-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon (**3**). Both of the new analogues were obtained in a highly purified form.

Bioassay Results in Vitro in Binding and Adenylate Cyclase Assays and in Vivo in Normal and Diabetic Animals. The new glucagon analogues were examined for their adenylate cyclase activity in rat hepatic plasma membranes and for their ability to bind to glucagon receptor in rat hepatic plasma membranes as measured by their specific displacement of [^{125}I]glucagon (Figure 2). Though both of the two new glucagon analogues **2** and **3** were shown to bind well in the binding assay, neither had any measurable adenylate cyclase activity. Both analogues could block glucagon-stimulated adenylate cyclase activity (data not shown).

When tested in normal animals in vivo, however, their effects were different from each other (Figures 3 and 4). Compound **2** lowered the plasma glucose levels in normal rats considerably and was able to suppress glucagon-mediated increases in plasma glucose levels completely. Compound **3**, on the other hand, lowered basal cAMP production of plasma liver membranes up to a concentration 500 times higher than its IC$_{50}$ in the binding assay. Nonetheless, **3** slightly elevated plasma glucose levels at this concentration and lowered the glucagon-mediated increase in plasma glucose by only 50%. Both compounds are antagonists with respect to adenylate cyclase, but they apparently affect glucose production, as measured by plasma glucose levels, by two different mechanisms.

In recent studies done in collaboration with Houslay and co-workers,[22] it was demonstrated that glucagon can activate two different signal transduction systems in hepatocytes, one which is mediated via adenylate cyclase, the other via a phosphatidyl inositol pathway. Our results with normal rats in vivo is consistent with this observation. Thus, analogue **2** appears to be an antagonist for both the cAMP-dependent and the cAMP-independent pathways, whereas **3** appears to be an antagonist only for the cAMP-mediated production of glucose, but perhaps a partial agonist for the cAMP-independent pathway leading to production of glucose.

The explanation for the effects of the glucagon antagonists **2** and **3** in diabetic rats is more difficult to explain. Glucagon-stimulatable adenylate cyclase activity in the liver of animals having streptozotocin-induced diabetes mellitus has been reported to be both increased[25] and decreased.[26,27] If hyperglucagonemia contributes to the plasma glucose pool in diabetic animals, then a suitable glucagon-receptor antagonist should lower the plasma glucose levels in these animals. The previous results of Johnson et al.,[13] who found that an in vitro glucagon receptor antagonist THG was able to lower plasma glucose levels in diabetic animals, would seem to be consistent with this possibility. However, in diabetic animals, glycogen levels are greatly reduced relative to normal animals,[28] and hence the present glucagon antagonist **2** has little effect on the glucose levels of these animals in vivo while compound **3** appears to slightly increase blood glucose levels.

Conclusions

With respect to the four questions that these studies have sought to address (see introduction), the following tentative conclusions can be made: (1) A glucagon antagonist with respect to adenylate cyclase generally will inhibit glucagon-mediated glucose production in normal rats at an appropriate concentration. However, interestingly, at high concentrations an antagonist such as **3** can have little or no effect on glucagon-mediated glucose production and may even stimulate glucose production above basal levels. This result might suggest a cAMP-independent pathway for glucose production by **3** (see below). (2) The new glucagon antagonist analogues **2** and **3** have no measurable adenylate cyclase activity at any concentration and yet they do not decrease glucose levels in diabetic rats. (3) Though the evidence is circumstantial the effects of **3** at high concentrations would suggest the possibility of a cAMP-independent pathway for glucose production. (4) Previously it was demonstrated that THG, a glucagon-receptor antagonist, could decrease blood glucose levels in diabetic animals[13] (which suggests antagonism of both cAMP-dependent- and, if any, cAMP-independent-mediated effects of glucagon on glucose production), the data in the present study suggest that in diabetic animals the antagonists such as **2** and **3** do not antagonize the cAMP-independent-mediated effects of glucagon on glucose production, and **3** actually may be an agonist. The actions of **2** and **3** are complex and clearly are different in normal and diabetic rats. Further studies are needed to determine why some glucagon-receptor antagonists do not necessarily inhibit glucagon-stimulated glucose production or lower blood glucose levels in diabetic rats.

Experimental Section

Materials. N^{α}-Boc-amino acids were purchased from Vega Biochemicals (Tucson, AZ) and Peninsula Laboratories (San Carlos, CA) or were prepared by standard literature procedures. Boc-Asp(Chx) was obtained from peptides International (Louisville, KY). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA) (Halocarbon

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Products, NJ); anisole, 1,2-ethanedithiol, 1-hydroxybenzotriazole (HOBt), *N*-acetylimidazole, diisopropylethylamine (Aldrich, Milwaukee, WI); 3 N mercaptoethanesulfonic acid, ninhydrin, dicyclohexylcarbodiimide (DCC) (Pierce, Rockford, IL); chloromethylated polystyrene-resin 1% cross-linked with divinylbenzene (Lab Systems, San Meteo, CA); purified acetonitrile (Burdick and Jackson, Muskegon, MI); bovine serum albumin, chromographic alumina (type WN_3, neutral), cAMP, ATP, GTP, all enzymes (Sigma, St. Louis, MO); $Na^{125}I$, carrier free (Amersham, Arlington Heights, IL); [^{3}H]cAMP (New England Nuclear, Boston, MA); [α-^{32}P]ATP (ICN Radiochemicals, Irvine, CA); crystalline porcine glucagon (Elanco, Indianapolis, IN); dialysis membranes (Spectrum Medical Ind., Los Angeles, CA); Dowex AG 50 W4 cation exchange resin (Bio-Rad, San Diego, CA); streptozotocin (Upjohn, Kalamazoo, MI); sodium pentobarbital (Harvey, Philadelphia, PA); 3,5-diiodotyrosine (Aldrich, Milwaukee, WI).

A Vega-Coupler 1000 peptide synthesizer (Tucson, AZ) was used for peptide synthesis. Amino acid analyses were performed with a Beckman Model 120 C analyzer. A Perkin-Elmer HPLC system was used for analysis and purification of glucagon analogues. For binding experiments a Millipore (Bedford, MA) filtration apparatus and 0.45-μm cellulose acetate filters (Oxoid, Columbia, MD) were used. The glucose concentration was measured with a Beckman glucose analyzer. Surgery was performed with microsurgical instruments from Irex (Toronto, Canada). The male Sprague–Dawley and Wistar rats were obtained from Harlen Sprague–Dawley (Indianapolis, IN). Wistar rats were used in the in vivo studies because we (and others) have found they give more constant blood glucose responses to exogenous glucagon than Sprague–Dawley rats. Though liver plasma membranes from male Sprague–Dawley rats were used in our in vitro assays, studies of the hepatic membrane preparations from both animals have shown no differences in response to glucagon in the in vitro assays used here.

Synthesis. N^{α}-*t*-Boc-*O*-2-BrZ-3,5-I_2-L-Tyr·DCHA salt was synthesized as described and obtained in 10% yield as a pale yellow salt after crystallization from petroleum ether. Preparation of the resin, synthesis of peptides, cleavage, and deprotection was performed by general solid-phase procedures[21] as have been described in detail elsewhere.[16-18] Briefly, N^{α}-Boc-amino acids with side-chain protection were used. The resin substitution was 0.3 mmol/g, and the protected amino acids were coupled as preformed symmetrical anhydrides in 3-fold excess.[29] Double coupling was used throughout the synthesis; final cleavage and deprotection was performed in hydrofluoric acid, as previously reported.[16-18]

Purification and Assessment of Purity. The crude synthesis product was purified by dialysis, ion-exchange chromatography, and preparative reverse-phase high-performance liquid chromatography (RP-HPLC), with trifluoroacetic acid buffer (0.1%) and an acetonitrile gradient from 30% to 50% in 20 min, as described in detail previously.[18] For amino acid analysis pure peptides were hydrolyzed in 3 N mercaptoethanesulfonic acid and by carboxypeptidase Y and leucine-aminopeptidase as described previously.[16-18] The results are shown in Table I. Further purity was established by analytic reverse-phase high-pressure liquid chromatography and by thin-layer chromatography. The purified analogues were obtained as white powders after lyophilization: mol wt, [Asp^3,D-Phe^4,Ser^5,Lys^{17,18},Glu^{21}]glucagon tetrakis(trifluoroacetates), **2**, 3974; [D-Phe^4,Tyr^5,3,5-I_2-Tyr^{10},Arg^{12},Lys^{17,18},Glu^{21}]glucagon tetrakis(trifluoroacetates), **3**, 4331.

Peptide Mapping. Glucagon or glucagon analogue (0.3 mg) and chymotrypsin (100:1, w/w) were incubated in 1 mL of sodium bicarbonate buffer (10 mM, pH 8.2) for 15 h at room temperature. The same treatment was repeated two more times. After lyophilization, the sample was dissolved in 0.1% trifluoroacetic acid. The peptide mixtures were analyzed by RP-HPLC (0.1% TFA, acetonitrile gradient 5–30% in 25 min, flow rate 2 mL/min). Patterns for the glucagon analogues were compared with those obtained with glucagon in each case. Individual peaks were isolated and subjected to amino acid analysis.

Iodination of Glucagon. To 3 nM of glucagon (20 μL of a 0.15 mM solution in 2 mM Na_2CO_3, OD_{280} = 1.25) and 2.17 mCi [^{125}I]iodide (1 nM, 22 μL) in 20 μL of phosphate buffer (0.6 M, pH 7.2) was added 0.5 nM of chloramine T (2.8 mg/100 mL) three times at time intervals of 30 s. The reaction was terminated with 20 μL of $NaHSO_3$ (0.5%) and 20 μL of Trizma base (0.5 M); then 1 mL elution buffer was added. The purification was carried out with QAE-Sephadex A-25 as previously described.[29] The yield was 20 mL of solution with 4.5×10^8 dpm. A fraction was analyzed by RP-HPLC and the main peak contained 70% of the total radioactivity. The specific binding was 90%.

Receptor-Binding Assay. Rat liver membranes were prepared as described[30] and modified.[31] The assays were run as described in detail elsewhere.[12,32] The membrane preparation was stored in fractions in liquid nitrogen. Briefly, a mixture (400 μL of the membranes in Tris·HCl buffer (25 mM, pH 7.5, bovine serum albumin 1%), 50 μL of **1**, **2**, or **3** in the buffer at various concentrations, and 50 μL of [^{125}I]glucagon (100 000 cpm, 25 fmol)) was incubated for 10 min at 30 °C and then cooled in an ice bath for 10 min. The samples were then filtered through Oxoid filters and washed three times with 1 mL of Tris·HCl buffer. The radioactivity retained by the filter was counted. Assays were run in triplicate and repeated two or three times.

Adenylate Cyclase Assay. The assay was carried out as previously described,[33] and the cAMP was separated as previously reported.[34] The assay volume was 0.10 mL (Tris·HCl buffer 25 mM, pH 7.5, bovine serum albumin 1%, ATP 1 mM, $MgCl_2$ 5 mM, cAMP 1 mM, GTP 10 mM, phosphocreatinine 20 mM) with 25–30 μg of protein and 2×10^5 cpm [α-^{32}P]ATP and 0.72 mg/mL creatinine phosphokinase (100 units/mL). Assays were run in triplicate and repeated two or three times. The data are presented (Figure 2) as a percent of maximal stimulation. One-hundred percent adenylate cyclase activity corresponds to the maximum stimulation above basal (generally 3–5-fold) obtained by 10^{-6} M glucagon; 0% adenylate cyclase corresponds to the basal levels of adenylate cyclase activity of the liver plasma membranes as measured in the absence of glucagon or glucagon analogue.

Plasma Glucose Assays in Normal and Diabetic Rats. Male Wistar rats (250–400 g) were kept for at least 5 days after arrival in a room in the Department of Animal Resources under controlled conditions (light/dark cycle, temperature, humidity) with free access to rat chow and water. On the day before the experiment, the animals were transferred into the laboratory where they had free access to food and water during the day and night. All experiments were performed between 9:00 a.m. and 2:00 p.m. In these studies, nutritional status was stable and controlled. The effect of handling and surgery was kept minimal by good technique and appropriate anesthesia. Basal plasma glucose levels were determined at time 0 min in all cases. In normal animals basal levels of glucose are 148 ± 15 mg/100 mL (mean ± SD, n = 32).

For studies of diabetic animals, male Wistar rats (200–250 g) were made diabetic by intravenous injection of streptozotocin (70 mg/kg, in sodium citrate buffer, pH 4.5). These animals were placed in individual metabolic cages to permit measurement of daily urinary glucose excretion. After the amount of glucosuria had stabilized (usually 5 days), they were used for experiments. Again, nutritional status was stable and controlled, the effect of handling was minimal, and basal plasma glucose levels were determined at time 0 min in all cases. In diabetic rats, basal levels of glucose are 527 ± 102 mg/100 mL (mean ± SD, n = 14).

The following methodology was used for all experimental determinations of in vivo plasma glucose levels. Normal male Wistar rats (250–400 g) or diabetic rats, fed ad libitum, were anesthetized with pentobarbital (70 mg/kg). A femoral vein was catheterized for infusion of glucagon or analogue (0.5 mL, NaCl 145 mM, Na_2CO_3 2 mM, bovine serum albumin 0.25%). At the same time

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a femoral artery was also catheterized for repetitive blood sampling. The drugs were injected as a bolus 40–50 min after the injection of pentobarbital. The temperature of the animals was monitored throughout and automatically maintained at 36–37 °C. Before the injection of a compound, a blood sample was taken to determine the basal blood glucose level. The blood was collected in 60-μL microhematocrit capillary tubes and centrifuged. The plasma was directly analyzed for glucose with a Beckman glucose analyzer. Samples were taken after 2, 5, 10, 20, 30, 40, and 50 min. Each data point is the mean of four to six animals.

Control experiments (basal), to determine the level of blood glucose throughout the experiment, were performed in exactly the same manner. But only a buffer bolus (0.5 mL, NaCl 145 mM, Na_2CO_3 2 mM, BSA 0.25%) without glucagon or analogue was injected.

In the antagonist studies, both glucagon (1 μg/kg) and analogue were injected at time 0 min, dissolved in the same 0.5 mL buffer (Figure 4).

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Amplification of Bleomycin-Mediated Degradation of DNA

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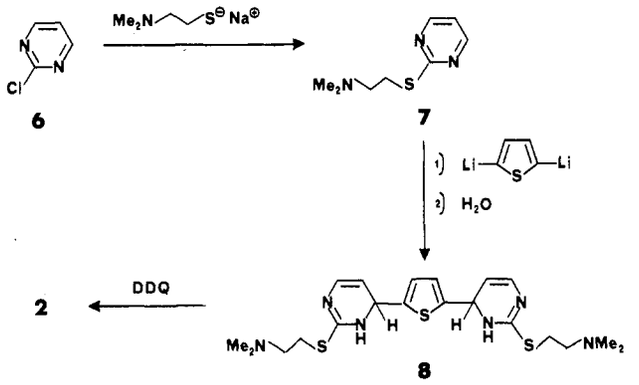
Three simple and independent tests have been introduced for studying the effect of DNA intercalating compounds on the bleomycin-mediated digestion of DNA in vitro. These methods are based on (i) hyperchromic changes of DNA solution, (ii) changes in viscosity of DNA solution, and (iii) HPLC quantitative analysis of the four bases released from digested DNA. All three tests give comparable results. However, the viscometric method is technically the simplest and at the same time the most sensitive. The amplification of the bleomycin-mediated degradation of DNA by three unfused heteropolyaromatic intercalator molecules, namely *N*-[2″-(dimethylamino)ethyl]-4-thien-2′-ylpyrimidin-2-amine (**1N**), *N*,*N*-dimethyl-2-[(4′-thien-2″-ylpyrimidin-2′-yl)thio]ethylamine (**1S**), and newly synthesized 2,5-bis[2′-[[2″-(dimethylamino)ethyl]thio]pyrimidin-4′-yl]thiophene (**2**) correlates well with the respective DNA binding constants for these compounds and is concentration dependent. The amplification activity of these compounds increases with increasing concentrations. The strongly binding compound **2** is the best amplifier of bleomycin in vitro found so far. Fused heteropolyaromatic systems, like ethidium bromide, are modest amplifiers of bleomycin at low concentrations but strongly inhibit the bleomycin chemistry at high concentrations.

The anticancer drug bleomycin is a family of enzyme-like molecules that bind with and cause degradation of DNA in the presence of ferrous ion and molecular oxygen[1] (Figure 1). The drug continues to interest oncologists because of its remarkable lack of bone marrow toxicity and preferential accumulation in cancerous cells. Because of pulmonary toxicity, bleomycin is used clinically in low doses, mainly in synergistic combinations with other anticancer agents, e.g., cisplatin, mytomycin, methotrexate, or doxurubicin.[2] Another approach to improving bleomycin chemotherapy is to find compounds (amplifiers) that alone do not have significant activity or toxicity but that enhance the activity of the drug. Amplifiers of the cytotoxic effect of bleomycin may, in general, (i) increase the intracellular level of the drug,[3] (ii) inhibit the DNA repair and/or synthesis,[4] or (iii) bind with DNA and by so doing stimulate directly drug-induced DNA fragmentation.[5] Modification of the bleomycin-mediated digestion of DNA by ethidium bromide and similar fused-ring intercalator molecules was noticed a decade ago.[5c] In general, enhancement[5c] of the digestion rate for low concentrations and inhibition[5c,6] for high concentrations of the intercalator were observed. More recent studies with mouse L1210 leukemia cells have suggested that combination chemotherapy of bleomycin with low levels of DNA intercalating agents may represent a novel and rational approach to the treatment of cancer.[5a] Recently we have also reported that

Scheme I



two unfused-ring compounds, **1N** and **1S**, intercalate with DNA and their binding constants correlate well with the

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