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11. In Ringer solution the isolated fiber exhibited vigorous all-or-none twitch responses when stimulated with a 40-V, 10-msec pulse across platinum point electrodes separated by a 1-cm gap. The fiber was examined with a Zeiss compound microscope (×430) for sarcomere uniformity along its length.
12. Sarcomere length was adjusted by displaying the first-order laser diffractions of the fiber, near the two ends and in the middle, on a screen 15 cm above the fiber. Length was adjusted to 2.2 to 2.3 μm at 1.0 T and checked in each test solution. The overall intensity of the diffraction pattern decreased reversibly in hypertonic solutions contuining NaCl and sucrose (*13*), but the width of the pattern appeared unchanged in different solutions.
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15. In these experiments, the fiber was equilibrated in standard Ringer solution with 1 m*M* caffeine for 30 minutes at 25°C and then subjected to the first temperature step. The fiber was allowed to rest for 15 minutes at 25°C before the next temperature step was applied. Three such activation-relaxation cycles were made at 1.0 T. Next, the fiber was equilibrated in NaCl or sucrose (with caffeine) for 30 minutes and then activated. Two additional activations were made in the same solution, with 12- to 15-minute rest periods at 25°C between activations. The fiber was then returned to the control solution. If the force response at 1.0 T was reversible to within 5 percent, the experiment was continued; otherwise, the fiber was discarded. The mean of force levels at 1.0 T was compared with the average value in the test solution, and the ratio was taken as a data point. Reversibility became a problem in NaCl above 1.4 T. This was avoided with sucrose, so the data point at 1.8 T is shown only with sucrose. As an additional precaution, preequilibration at 1.8 T (25°C) was reduced to 2 to 3 minutes; after activation the fiber was returned to the solution at 1.4 T instead of 1.8 T and the cycling was continued as described above. There was an appearance of resting "contracture" tension, lasting 60 to 80 seconds, each time the fiber was placed in sucrose solution at 1.8 T and 25°C [J. Lannergren and J. Noth, *J. Gen. Physiol.* **61**, 158 (1973)]. Fibrillation [T. G. Sato, *Annot. Zool. Jpn.* **27**, 157 (1954)] was always observed in sucrose solution at 1.8 T and only occasionally at 1.4 T; the onset of fibrillation was associated with the resting contracture tension. The fibers that fibrillated at 1.4 T survived less well during further experimentation. With KCl, reversibility may take 2 to 5 hours [R. H. Adrian in (*18*)] and was not attempted; the datum was recorded only if the force level was reproduced to within 5 percent in three consecutive cycles. The successful fiber was subjected to higher tonicity KCl and the same procedure was followed. Every solution in this series contained 1 m*M* caffeine, producing maximal activation. This was checked by measuring contraction force in 2 m*M* caffeine. The results were within 10 percent of those in 1 m*M* caffeine.
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Different Synaptic Location of Mianserin and Imipramine Binding Sites

Abstract. *The high-affinity binding sites for mianserin and imipramine appear to be located in different neurons of rat brain. Studies in which lesions were produced with 5,7-dihydroxytryptamine and other studies in which the 5-hydroxytryptamine content was decreased with* p*-chlorophenylalanine indicate that some of the imipramine binding sites are on serotonin axon terminals and others are on nonserotonergic synapses. The sites that bind mianserin are on postsynaptic serotonin sites as well as on synapses of other neuronal systems.*

Several clinically active antidepressants, with differing chemical structures, reduce the number of synaptic recognition sites for catecholamines (*1–3*), serotonin (*4*), or other putative neurotransmitters in the rat brain. Because both the down-regulation of recognition sites and the beneficial effects on symptoms of depression are apparent only after about 2 weeks of treatment with antidepressants, a relation between these two drug effects has been proposed (*1, 2, 5, 6*). Some antidepressants block the reuptake of putative neurotransmitters, thereby enhancing the content of neurotransmitters in the synaptic cleft (*7–10*); this increase could participate in the down-regulation of transmitter recognition sites (*3, 11*) because it prolongs the occupancy of neurotransmitter recognition sites by endogenous agonists.

Inhibition of transmitter uptake, however, is not essential to elicit down-regulation or to exert beneficial effects in endogenous depression, since atypical antidepressants, such as iprindole, bupropion, and mianserin, relieve depression and down-regulate recognition sites for neurotransmitters without blocking the reuptake of monoamines by nerve terminals (*12, 13*). Cocaine blocks monoamine uptake (*14*), but does not regulate transmitter recognition sites or elicit beneficial effects in depression. Whatever their action on monoamine uptake, antidepressants bind to crude synaptic membranes stereospecifically and with high affinity (*15–19*).

We investigated the relation between specific synaptic mechanisms and the high-affinity binding of [^{3}H]imipramine and [^{3}H]mianserin. We induced lesions pharmacologically in neurons storing a specific neurotransmitter (serotonin) and compared the binding characteristics of imipramine and mianserin before and after production of the lesion. We confirmed that imipramine binding sites are located in serotonergic synapses (*20, 21*) and demonstrated that there are differences in the brain location of imipramine and mianserin binding sites. Moreover, the binding sites of imipramine and mianserin are not limited to synapses that can be labeled with a specific putative neurotransmitter as a biochemical marker.

Male Sprague-Dawley rats weighing 180 to 220 g (Zivic Miller) were anesthetized with pentobarbital; lesions were produced by injecting 5,7-dihydroxytryptamine (5,7-DHT) stereotaxically either in the lateral ventricle or in the nucleus raphe medianus (A = 0.6; L = 0.0; V = 3.2) (*22*). Sham-lesioned animals were given injections of the vehicle solution, 0.01 percent ascorbic acid in saline. Animals in both groups were injected intraperitoneally with desmethylimipramine (DMI) (25 mg/kg) 40 minutes before the injection of neurotoxin (or vehicle) to prevent the uptake of the neurotoxin into noradrenergic axons and thus minimize the degeneration of these terminals (*23*). Lesioned and sham-lesioned rats were killed 10, 20, and 30 days after operation. In other studies, rats were given intraperitoneal injections of *p*-chlorophenylalanine (PCPA), 300 mg/kg daily for 3 days, then 100 mg/kg every other day for 15 days. At the end of each treatment, rats were killed by decapitation, and the brains were removed, dissected over ice, and stored at −70°C until assayed. Serotonin (5HT)

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content in all the brain areas tested was determined by high-performance liquid chromatography (HPLC) (*24*). [^{3}H]Imipramine binding assays were carried out at 0°C by incubating the radioactive ligand for 60 minutes with a crude synaptic membrane preparation (*15, 16*). [^{3}H]Mianserin binding assays were carried out at 37°C for 30 minutes with the same membrane preparation (*18*). Protein concentrations were determined as in (*25*).

Using 5HT content as a measure of the abundance of a specific neuronal population indicated that extensive destruction of the neurons by intracerebral injection of 5,7-DHT led to a significant decrease in the maximum number of binding sites (B_{max}) of [^{3}H]imipramine binding to membranes prepared from various brain areas (Fig. 1, A and B). The extent of the lesion and the impairment of [^{3}H]imipramine binding varied independently (Table 1). The decrease in specific binding (expressed as percent of binding) and the extent of the lesion (expressed as percent of decrease in 5HT content) were reduced to the same extent only in the hypothalamus; in all the other brain areas tested, the extent of decrease in 5HT content was greater than that in [^{3}H]imipramine binding. Although, in some regions, such as the hypothalamus, all of the imipramine binding sites may be on serotonergic axon terminals, this cannot be true for every brain area (Table 1). In the same brains, the lesion of serotonergic neurons was associated with an average increase of almost 40 percent in the specific binding of [^{3}H]mianserin (Table 1), an indication that [^{3}H]mianserin binds to sites different from those for imipramine. The decrease in imipramine binding was apparent 10 days after production of the lesion, but was significantly decreased by this time only in the hypothalamus.

Scatchard analysis of the specific [^{3}H]imipramine binding to membranes from the cortex and hypothalamus showed that B_{max} was significantly decreased in the animals with 5,7-DHT lesions; the affinity of the ^{3}H-labeled ligand for its recognition sites was unchanged (Fig. 1, A and B). In contrast, the B_{max} of the specific binding of [^{3}H]mianserin to cortical membranes was increased in the animals with 5,7-DHT lesions (Fig. 1C). Since mianserin binds to both serotonin ($5HT_2$) and histamine (H_1) receptors (*26*), we measured [^{3}H]mianserin binding in the presence of the specific H_1 antagonist mepyramine to determine the binding of [^{3}H]mianserin to each of these two synaptic sites. There were fewer binding sites related to the H_1 receptor than to the other receptors, and the binding of [^{3}H]mianserin to the H_1 receptor did not differ in lesioned and sham-lesioned animals (Fig. 1D) (*27*). Therefore, the effect of the 5,7-DHT lesion on the specific binding of [^{3}H]mianserin was mainly an increased sensitivity of serotonergic receptors. The specific binding of [^{3}H]mianserin was determined in lesioned animals ei-

Table 1. Serotonin content and specific [^{3}H]imipramine and [^{3}H]mianserin binding to membranes after selective destruction of serotonergic neurons by intracerebral administration of 5,7-DHT. The 5,7-DHT (8 μg of free base in 1 μl) was injected into the nucleus raphe medianus over a 4-minute period, and the rats were killed 1 month after the injection. Sham-lesioned animals were given injections of the vehicle (0.01 percent ascorbic acid in saline). Serotonin (5HT) content is expressed as nanograms per gram and was measured by HPLC with an electrochemical detector (*24*) after the rats were killed by microwave irradiation; values are means ± standard error (S.E.) of five determinations. Specific [^{3}H]imipramine binding is defined as the difference between total binding at 5 n*M* [^{3}H]imipramine and nonspecific binding measured in presence of 10 μ*M* DMI. Specific [^{3}H]mianserin binding is defined as the difference between the binding at 1 n*M* [^{3}H]mianserin measured in the absence and presence of 1 μ*M* mianserin. Binding results are expressed as femtomoles per milligram of protein, and the values are the mean ± S.E. for five animals, with each value measured in triplicate. Student's *t*-test was used to analyze the data.

Measure	Sham-lesioned rats	5.7-DHT-lesioned rats
Hippocampus		
5HT	208 ± 15	90 ± 8*
[^{3}H]Imipramine	262 ± 25	175 ± 20†
[^{3}H]Mianserin	230 ± 14	402 ± 35†
Striatum		
5HT	281 ± 20	131 ± 12*
[^{3}H]Imipramine	362 ± 28	280 ± 15†
[^{3}H]Mianserin	200 ± 25	258 ± 21
Hypothalamus		
5HT	532 ± 35	140 ± 13*
[^{3}H]Imipramine	614 ± 42	120 ± 15*
[^{3}H]Mianserin	221 ± 18	282 ± 15
Cortex		
5HT	140 ± 8	65 ± 10*
[^{3}H]Imipramine	396 ± 33	163 ± 17*
[^{3}H]Mianserin	295 ± 23	413 ± 35†

*$P < .01$; †$P < .05$, compared to sham-lesioned animals.

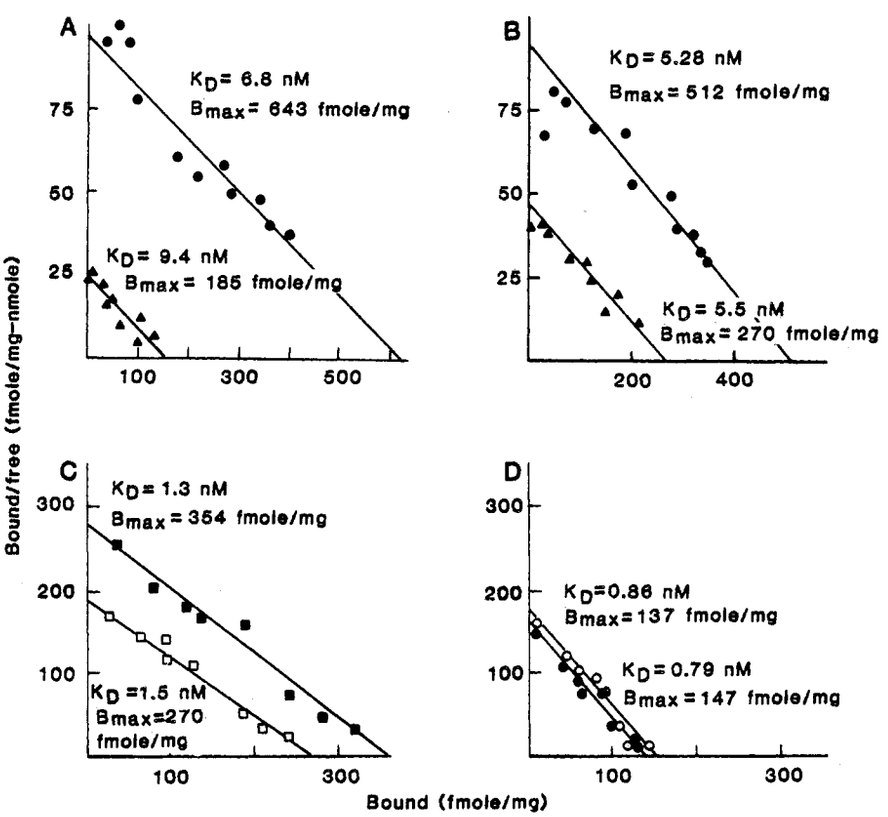

Fig. 1. Scatchard analysis of the specific binding of [^{3}H]imipramine and [^{3}H]mianserin to brain membranes derived from sham-lesioned and 5,7-DHT-lesioned rats (1 month after the operation). (A and B) Specific [^{3}H]imipramine binding in the hypothalamus and the cortex of sham-lesioned (●) and 5,7-DHT-lesioned (▲) rats. [^{3}H]Imipramine (0.2 to 10 n*M*) was incubated in duplicate with or without DMI (10 μ*M*). (C and D) Specific [^{3}H]mianserin binding to the cortex of sham-lesioned rats (open symbols) and 5,7-DHT-lesioned rats (closed symbols). [^{3}H]Mianserin (0.05 to 10 n*M*) was incubated in duplicate, and the specific binding was measured with an excess of mianserin (1 μ*M*) (C) or an excess of mepyramine (300 n*M*) (D). In all cases, the correlation coefficient to linear regression exceeded .93.

ther in the presence of 300 n*M* mepyramine (to block the histamine H_1 receptor component) or in the presence of 100 n*M* D-lysergic acid diethylamide (LSD) (to block the serotonin $5HT_2$ receptor component); in the cortex and in the hippocampus of the lesioned animals, an increase in the number of binding sites could be detected only when the histaminergic component was blocked, whereas the number of binding sites was the same when the serotonergic component was blocked (Table 2). We measured $5HT_2$ binding in the cortex of both groups by using [^{3}H]spiroperidol displaced by LSD; in the lesioned rats, the number of specific binding sites was increased by 40 percent (data not shown). These data support the hypothesis that mianserin recognition sites are linked to the serotonergic $5HT_2$ postsynaptic system.

Long-term treatment of rats with PCPA, which depletes the 5HT content without damaging the serotonergic nerve endings, did not decrease the B_{max} and the affinity (K_D) of [^{3}H]imipramine binding to cortical membranes, but increased the B_{max} without changing the K_D of [^{3}H]mianserin binding (Fig. 2). The increase in the number of [^{3}H]mianserin binding sites after long-term treatment with PCPA was related to an increased sensitivity of the postsynaptic 5HT recognition sites; the B_{max} of [^{3}H]mianserin binding was still increased by 50 percent in the presence of 300 n*M* mepyramine, but when the $5HT_2$ binding sites were blocked with 100 n*M* LSD, the increase in the number of mianserin binding sites was completely masked (data not shown). Thus, in the cortex, the [^{3}H]imipramine binding sites that are not on 5HT axons appear not to be on postsynaptic sites of serotonergic synapses, since, if this were the case, the number of binding sites for imipramine, as for mianserin, should have increased because of the long-lasting depletion of cortical 5HT content.

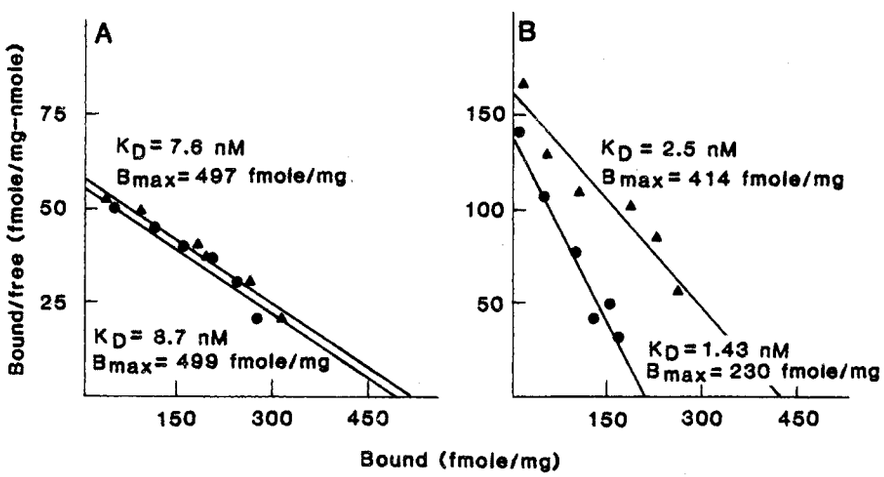

Fig. 2. Effect of long-term treatment with *p*-chlorophenylalanine on the binding of [^{3}H]imipramine and [^{3}H]mianserin. Serotonin content was 95 percent depleted in the cortex of the treated animals. (A) Specific [^{3}H]imipramine binding to the cortical membranes of control (●) and PCPA-treated (▲) animals. (B) Specific [^{3}H]mianserin binding to the cortical membranes of control (●) and PCPA-treated (▲) animals. The slight increase in K_D was not significant.

Table 2. Kinetic constants of [^{3}H]mianserin binding to membranes prepared from the cortex and the hippocampus of sham-lesioned and 5,7-DHT-lesioned rats. Animals were treated as described (Table 1). The binding of [^{3}H]mianserin at 0.1 to 5.0 n*M* was carried out at 37°C for 30 minutes. Saturation curves were also determined in the presence of 300 n*M* mepyramine or 100 n*M* LSD, in the presence or absence of 1 μ*M* mianserin as a displacer. Values for K_D and B_{max} were obtained from Scatchard analysis of at least four isotherms, with each point measured in duplicate. Student's *t*-test was used to analyze the data.

Rats	Incubation condition	K_D (n*M*)	B_{max} (fmole/mg)
	Cortex		
Sham-lesioned	Buffer	1.43 ± 0.22	223 ± 29
	Mepyramine, 300 n*M*	1.7 ± 0.23	93 ± 12.1
	LSD, 100 n*M*	1.7 ± 0.21	151 ± 19.5
5,7-DHT-lesioned	Buffer	1.78 ± 0.22	333 ± 23*
	Mepyramine, 300 n*M*	2.1 ± 0.26	185 ± 12.7†
	LSD, 100 n*M*	2.5 ± 0.30	128 ± 8.8
	Hippocampus		
Sham-lesioned	Buffer	1.45 ± 0.3	160 ± 24
	Mepyramine, 300 n*M*	1.21 ± 0.25	41 ± 6.3
	LSD, 100 n*M*	1.8 ± 0.37	131 ± 19
5,7-DHT-lesioned	Buffer	1.18 ± 0.10	265 ± 31*
	Mepyramine, 300 n*M*	1.6 ± 0.13	116 ± 12.5*
	LSD, 100 n*M*	1.6 ± 0.11	139 ± 16.2

*$P < .01$; †$P < .05$, compared to sham-lesioned animals.

Both typical and atypical antidepressant drugs appear to bind with high affinity to sites that are not limited to serotonergic synapses. [^{3}H]Mianserin, which binds to sites on histaminergic synapses (*26*), appears to bind to other synapses as well. Since, in serotonergic synapses, imipramine localizes presynaptically and mianserin postsynaptically, the occupancy of uptake sites for 5HT cannot be the trigger for the down-regulation of 5HT receptors. Imipramine may not bind to the uptake site on the 5HT axon terminal, because different amounts of [^{3}H]imipramine were bound to 5HT terminals in different brain areas having an equal extent of degeneration of 5HT axons (Table 1); although imipramine binding was prominent in the 5HT axons of hypothalamus, it was less important in the hippocampus, striatum, and cortex. [^{3}H]Mianserin does not bind to 5HT axons and, like [^{3}H]imipramine, may also bind to sites not located on serotonergic synapses. In the hippocampus and the striatum, the imipramine binding after impairment of 5HT nerves is probably not in postsynaptic sites of 5HT synapses, which were not modified by depletion of 5HT induced by long-term treatment with PCPA (Fig. 2A).

Imipramine and mianserin appear to bind to sites on different synapses relative to a particular transmitter. This relation between various neurotransmitters and the high affinity binding sites for antidepressants suggests that the binding sites may be occupied by an endogenous ligand that functions as a modulator of synaptic events in a number of synapses that have different transmitters. The sites where the antidepressants bind may be the binding sites for neuropeptides that coexist in the same neurons with monoamine transmitters (*28, 29*) and function by modulating the number or other characteristics of the recognition sites of the monoamine neurotransmitters (*28*). Some of the [^{3}H]imipramine binding sites may be related to or interact with the [^{3}H]mianserin binding sites; this possibility is in line with the efficient displacement of [^{3}H]mianserin bound to rat brain membranes by imipramine (*19*).

Our data support the view that antidepressants occupy sites in synapses in which 5HT and other putative neurotransmitters regulate transaction of information. The antidepressant binding sites may be binding sites for cotransmitters involved in the modulation of the properties of the recognition sites of a number of different putative neurotransmitters. The occupancy of these sites by the antidepressants could result in the down-regulation of transmitter recognition sites and thereby relieve some symptoms of depression. If this is the case, certain depressive states may be due to a deficit in cotransmitter modulation of transmitter detectors.

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27. In lesioned animals, B_{max} = 147 fmole per milligram of protein and K_D = 0.79 n*M*; in sham-lesioned animals, B_{max} = 137.2 fmole per milligram of protein and K_D = 0.86 n*M*.
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8 September 1981

Hyperglycemia of Diabetic Rats Decreased by a Glucagon Receptor Antagonist

Abstract. *The glucagon analog [1-N$^{\alpha}$-trinitrophenylhistidine, 12-homoarginine]-glucagon (THG) was examined for its ability to lower blood glucose concentrations in rats made diabetic with streptozotocin. In vitro, THG is a potent antagonist of glucagon activation of the hepatic adenylate cyclase assay system. Intravenous bolus injections of THG caused rapid decreases (20 to 35 percent) of short duration in blood glucose. Continuous infusion of low concentrations of the inhibitor led to larger sustained decreases in blood glucose (30 to 65 percent). These studies demonstrate that a glucagon receptor antagonist can substantially reduce blood glucose levels in diabetic animals without addition of exogenous insulin.*

The central role of relative or absolute deficiency of insulin in the development of diabetes has been recognized since the experiments of von Mering and Minkowski in 1889 (*1*) and the identification of insulin by Banting and Best in 1922 (*2*). The development of radioimmunoassay techniques for measuring glucagon in the late 1960's revealed a relative or absolute excess of circulating glucagon in virtually all forms of human and animal diabetes (*3*). These findings led to the "bihormonal" concept of diabetes (*3*), the disorder being attributed to a lack of insulin effect and an excess of glucagon.

The discovery of somatostatin (*4*) and its ability to suppress both insulin and glucagon secretion (*5*, *6*) provided two more lines of evidence supporting the importance of glucagon in glucose homeostasis. When the secretion of insulin and glucagon was suppressed by infusion of somatostatin, there was a fall in the blood glucose concentration (*6*) that could be reversed by concomitant infusion of glucagon (*7*, *8*). Furthermore, infusion of somatostatin to inhibit secretion of endogenous glucagon reduced the hyperglycemia of diabetic patients (*9*, *10*).

In view of the evidence implicating excessive glucagon secretion as a contributing factor in the metabolic abnormalities of diabetes mellitus, several investigators have tried to develop analogs of glucagon that would be specific antagonists (inhibitors) of glucagon effects on target tissues (*11–13*) and thus provide direct evidence for glucagon's role in diabetes mellitus. Recently we have prepared the highly purified glucagon analog [1-N$^{\alpha}$-trinitrophenylhistidine, 12-homoarginine]glucagon (THG, Fig. 1) and several related analogs that are potent antagonists of the glucagon-stimulated adenylate cyclase system in rat liver membranes in vitro. The present study was undertaken to determine whether THG was effective in vivo in reducing the hyperglycemia of diabetic animals. We report that THG causes a marked reduction in glucose levels in streptozotocin-induced diabetic rats. These results provide evidence that blocking endogenous glucagon with a glucagon receptor antagonist can significantly lower glucose concentrations in diabetic animals without added exogenous insulin.

The synthesis and purification of THG was carried out as described (*12*) except that the scale of the synthesis was expanded five to ten times. This scale-up caused no significant changes in purity as assessed by previously reported methods (*12*).

Male Wistar rats weighing 310 to 360 g were made diabetic by intravenous infusion of streptozotocin (50 mg/kg) through a tail vein (*14*). The animals were placed in individual metabolic cages to permit measurement of daily urinary glucose excretion. After the amount of glycosuria had stabilized (1 to 5 weeks), individual rats were anesthetized with intraperitoneally administered pentobarbital (65 mg/kg). A jugular vein was catheterized for repetitive blood sampling and infusion of THG.

Infusion of a bolus of THG (1.0 mg/kg dissolved in 0.2 ml of saline) produced a rapid decrease in the mean concentration of blood glucose (Fig. 2). In this particular experiment, 5 minutes after administration of THG the mean blood glucose concentration decreased 28 percent below baseline levels. Glucose levels returned to normal in about 10 to 20 minutes and remained stable up to 1 hour (Fig. 2).

In a second group of experiments anesthetized rats were given a THG bolus of 1.0 mg/kg followed immediately by a continuous infusion of THG, 33 μg per

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