with a mixture of DNA fragments that cause nonspecific priming.

The experimental approach we describe is not limited to Fok I. The procedure probably could be adapted to over 15 other class-IIS enzymes, including Bbv I, Bbv II, Bin I, Bsp MI, Eco 31I, Eco 57I, Gsu I, Hga I, Hph I, Mbo II, Mme I, Mnl I, Sfa NI, Taq II, Tth 111II, and others (*1, 2*). In the experiments of Podhajska and Szybalski (*3*) the class-IIS restriction enzymes had to be free of endonucleolytic activity directed toward ss DNA; this requirement is of much less importance for the ds DNA digestion experiments described here. Our method created one predetermined end, whereas the other terminus could be produced by a cut with any other enzyme, not only by Fok I, as described here.

Theoretically, two more improvements could have been made in our method. Replacement of deoxycytidine 5′-triphosphate (dCTP) by 5-methyl-dCTP during the PolIk-mediated DNA synthesis (Fig. 1) might have eliminated all other Fok I cuts on the RF molecule (Fig. 2). However, methylation of the C residues has little or no effect on Fok I recognition or cleavage functions (*9*). Alternatively, the Fok I sites originally present in M13mp7 DNA could perhaps be inactivated by M·Fok I methylase (*8*); however, this might not be practical because our original target is an ss DNA molecule and, moreover, Pósfai and Szybalski (*9*) have discovered that M·Fok I methylates in an asymmetric manner only the unique A located on one of the strands (GGATG) of the Fok I recognition site.

In conclusion, we have designed and described a four-component system which, by the use of a specially designed adapter-primer oligodeoxynucleotide, permits one to produce DNA fragments with a predetermined end located between any two base pairs of the target DNA. This approach is especially useful for DNA fragments cloned in ss DNA-generating vectors (*10*), and it could also complement our other preprogrammed DNA trimming method (*11*). The present experiments should also be important for dissecting the mechanisms of enzyme recognition, endonucleolytic cleavage, and methylation (*9, 12*).

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Stimulation of RNA and Protein Synthesis by Intracellular Insulin

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Like insulin-sensitive somatic cells, stage IV oocytes from *Xenopus laevis* increase their synthesis of RNA, protein, and glycogen in response to extracellular insulin. Synthesis of RNA and protein are also increased when oocytes are maintained under paraffin oil and insulin is microinjected into the cytoplasm. The effects of external and intracellular insulin are additive, suggesting separate mechanisms of action. Experiments with nuclei isolated under oil show that RNA synthesis can be stimulated by applying insulin to the nucleus directly. Thus, the nucleus appears to be one intracellular site of hormone action.

IN SENSITIVE CELLS, INSULIN REGULATES many activities, including membrane transport, energy metabolism, transcription, and translation. Evidence points to the plasma membrane as a primary site of action for the rapid effects of insulin. However, the detailed mechanism (or mechanisms) by which insulin alters cytoplasmic and nuclear function over longer intervals remains to be established. Researchers have focused on potential second messengers (*1*) and signaling through protein phosphorylation (*2*), but other mechanisms could be involved. One possibility is that the insulin molecule plays a direct role in intracellular regulation, but evidence for this is circumstantial. (i) Cells take up insulin from the extracellular medium (*3*); the fate of internalized hormone (with or without receptor) appears to be dependent on cell type (*4*). (ii) Insulin receptors are found inside many cells (*5*); the function of these receptors is unknown. (iii) Experiments with isolated nuclei suggest that the nuclear envelope is one site of insulin action. The envelope contains specific receptors, and insulin exposure increases envelope phosphorylation, RNA release to the medium, and envelope pore permeability to dextrans (*6, 7*).

Testing the hypothesis that intracellular insulin controls metabolic activity requires an experimental system in which hormone can be introduced into a cell with minimal disruption and metabolic flux can be measured in the same cell. Technical problems related to small cell size make such experiments impossible with most insulin-sensitive cells. My approach was to use a giant cell, the *Xenopus laevis* oocyte, which can be studied by the use of intracellular microinjection techniques and single cell analysis.

Research on mechanisms of insulin action

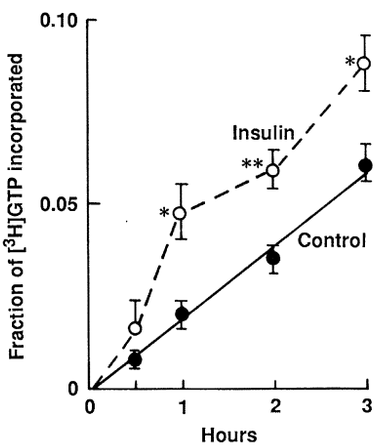

Fig. 1. Cells were transferred to oil and microinjected with 25 nl of intracellular buffer containing 0.01% BSA, 450 fmol of ^{3}H-labeled GTP, and 0 or 18 ± 2 fmol of insulin. After 0.5 to 3 hours, cells were processed to determine GTP incorporation, as described in Table 1. Each point represents the mean fraction incorporated from 7 to 12 (control) or 4 to 6 (insulin) oocytes; variability is given as SE bars. Statistical comparisons by protected *t* test (*$P < 0.05$; **$P < 0.01$).

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in *Xenopus* oocytes has focused primarily on events surrounding the reinitiation of meiosis in full-grown, stage V to stage VI oocytes (*8*). Insulin cannot induce smaller oocytes to mature (*9*). Although these cells are growing rapidly, little is known about their metabolic responses to insulin and growth factors. My initial experiments were designed to determine whether external insulin stimulates macromolecule biosynthesis in stage IV *Xenopus* oocytes. Oocytes were incubated for 2 hours in Ringer solution containing 0, 7, or 700 n*M* insulin, transferred to paraffin oil (*10*), and then microinjected with radiolabeled guanosine triphosphate (GTP), methionine, or glucose. After a 1-hour labeling period, each cell was processed to determine the fraction of injected precursor that was incorporated into RNA, protein, or glycogen. Treating oocytes with 7 or 700 n*M* insulin stimulated GTP incorporation by 27% and 73%, respectively, and methionine incorporation by 82% and 132%; 700 n*M* insulin stimulated glucose incorporation into glycogen by 300% (Table 1). In other experiments (*8*) external insulin concentrations as low as 3 n*M* caused significant stimulation of GTP incorporation into RNA and methionine or leucine incorporation into protein. Dose-response curves varied with the donor. Maximal stimulation occurred with external insulin concentrations exceeding 200 n*M*.

I then determined the effects of intracellular insulin on rates of RNA synthesis. Oocytes under oil were microinjected in the cytoplasm with ^{3}H-labeled GTP or ^{3}H-labeled GTP plus insulin. After 0.5 to 3 hours, injected cells were processed to measure label incorporation. In control oocytes (*11*), GTP incorporation into RNA was linear with time after injection for at least 3 hours (Fig. 1). Control incorporation rates varied little among individual cells from the same animal. However, animal-to-animal variation was greater; mean rates for oocytes from ten toads varied from 0.005 to 0.035 per hour (mean ± SEM, 0.019 ± 0.005 per hour).

Microinjection of 18 fmol of insulin into oocytes caused a doubling of the rate of GTP incorporation into RNA (Fig. 1). Statistically significant increases occurred after 1, 2, and 3 hours. At shorter times, insulin generally caused small increases in GTP incorporation; in some experiments these were statistically significant. It would be advantageous to define the early time course of insulin action. However, the large size of the oocyte makes diffusion times for both precursor and hormone relatively long (minutes rather than seconds in somatic cells). Thus, in microinjection experiments, early events and rapid biochemical responses tend to be obscured.

Experiments with cells from other animals showed dose-dependent stimulation of RNA synthesis 1 to 2 hours after insulin microinjection. Pooled dose-response data for four to six animals show significant stimulation with doses of 5 to 10 fmol and maximal stimulation with 21 to 40 fmol (*12*) (Table 2). Although the magnitude of maximal stimulation varied with donor animal, there was no simple relation between control rate of incorporation and the maximal insulin effect.

Table 2. Dose response for stimulation of RNA synthesis by microinjected insulin. Experiments were conducted as described in Fig. 1. Data were expressed as the percentage of increase in incorporation over a period of 1 or 2 hours for each animal and then pooled to give the means ± SE; the number of animals is given in parentheses.

Insulin injected (fmol per cell)	Stimulation (%)
5 to 10	26 ± 5 (5)
11 to 20	55 ± 13 (6)
21 to 40	73 ± 29 (6)
41 to 60	61 ± 6 (4)

Further intracellular dosing experiments with epidermal growth factor and each of the two polypeptides that constitute insulin failed to demonstrate any effects on GTP incorporation. In these experiments, polypeptide dose levels as high as 100 fmol per cell were injected and incubation times were 1 to 2 hours.

Possible interactions of external and internal insulin were investigated by exposing oocytes to 0, 140, or 700 n*M* external insulin for 2 hours, after which the oocytes were transferred to paraffin oil and microinjected with ^{3}H-labeled GTP or ^{3}H-labeled GTP plus insulin. After 1 hour under oil, cells were processed to measure GTP incorporation into RNA. External insulin increased incorporation in a dose-dependent manner (Table 3). Microinjection of 19 ± 2 fmol of insulin into the cytoplasm increased GTP incorporation, whether or not cells had been exposed to external insulin. The magnitude of the increase was dependent on the concentration of insulin in the medium to which the oocytes had been exposed. For both dose levels of Ringer insulin, the combined effect of extracellular and intracellular hormone was additive.

Since the amount of external hormone that was internalized during the 3-hour experiment is unknown, these data should be interpreted cautiously. Preliminary experiments in which oocytes were incubated in medium with labeled insulin showed detectable radioactivity in the nucleus and cytoplasm within 3 to 5 hours (*9*); whether the radioactivity represents undegraded hormone or hormone bound to receptor has not been determined. From these initial data, one would expect that at most a few femtomoles of hormone had entered the cytoplasm of the cells exposed to a high external dose of insulin. The unknown amount of internalized insulin complicates detailed interpretation of the data; however, it does not change the basic conclusion that

Table 1. Stimulation of RNA, protein, and glycogen synthesis in stage IV *Xenopus* oocytes by external insulin. Stage IV (diameter, 800 ± 50 μm) oocytes in follicles were isolated from unstimulated *Xenopus laevis* (Nasco) into an amphibian oocyte Ringer solution by using watchmaker's forceps and fine scissors. Oocytes were incubated in Ringer containing 0.01% bovine serum albumin (BSA) and 0, 7, or 700 n*M* bovine pancreatic insulin (24 IU/mg) (Sigma). After 2 hours, cells were removed from the Ringer solution, blotted, and transferred to paraffin oil (*16*). In oil, each cell was microinjected in the vegetal hemisphere with 30 nl of intracellular buffer (22 m*M* NaCl, 125 m*M* KCl, 1 m*M* KH_2PO_4, and 2 m*M* $NaHCO_3$, *p*H 7.2) containing 300 fmol of ^{3}H-labeled GTP (33.9 Ci/mmol), 35 fmol of [^{35}S]methionine (1072 Ci/mmol), or 300 fmol of ^{3}H-labeled D-glucose (18 Ci/mmol) (New England Nuclear). One hour later, each cell was processed to determine both the amount of label injected and the fraction of label incorporated into macromolecules. For GTP and methionine injection experiments, each cell was transferred to a vial containing 1 ml of 10% TCA, and the unincorporated label was washed out overnight in the cold. The TCA-fixed cell was transferred through two additional TCA washes and solubilized, and radioactivity was counted to determine the amount of incorporated label. Portions of the TCA washes were also counted to determine unincorporated label. For the glucose injection experiments, each cell was dissolved in hot 30% KOH, and the glycogen was precipitated in cold 60% ethanol. For GTP and methionine incorporation, preliminary experiments established that (i) the fraction of label incorporated was independent of the amount microinjected over the range from 100 to 800 fmol and (ii) incorporation of label into RNA and protein was reduced 80% to 90% by 1-hour exposure of cells in Ringer solution containing 50 μg of actinomycin D per milliliter or 1 μ*M* cyclohexamide, respectively.

External insulin (n*M*)	Fraction of labeled precursor incorporated		
	GTP into RNA	Methionine into protein	Glucose into glycogen
0	0.0145 ± 0.0010	0.0336 ± 0.0036	0.002 ± 0.001
7	0.0184 ± 0.0012*	0.0613 ± 0.0035**	
700	0.0251 ± 0.0008**	0.0780 ± 0.0043**	0.008 ± 0.002**

*Significantly greater than controls, $P < 0.05$ (protected *t* test). **Significantly greater than controls, $P < 0.01$.

Table 3. Effects of external and microinjected insulin on ^{3}H-labeled GTP incorporation into RNA and [^{35}S]methionine incorporation into protein. Oocytes were incubated for 2 hours in amphibian Ringer solution with 0.01% BSA and 0, 140, or 700 n*M* insulin. After incubation, oocytes were transferred to paraffin oil for microinjection with (i) 300 fmol of ^{3}H-labeled GTP or 300 fmol of ^{3}H-labeled GTP plus 19 ± 2 fmol of insulin or (ii) 33 fmol of [^{35}S]methionine or 33 fmol of [^{35}S]methionine plus 17 ± 1 fmol of insulin. The labeling period was 1 hour. Data are given as the means ± SE of the fraction of injected label incorporated into RNA or protein; the number of cells is given in parentheses.

External insulin (n*M*)	Microinjection		Difference
	Control	Insulin	
	GTP incorporation into RNA		
0	0.024 ± 0.002 (10)	0.034 ± 0.004 (10)	0.010 ± 0.004
140	0.031 ± 0.002 (10)*	0.055 ± 0.009 (3)	0.024 ± 0.010
700	0.040 ± 0.003 (9)**	0.077 ± 0.007 (10)**	0.037 ± 0.008
	Methionine incorporation into protein		
0	0.055 ± 0.002 (9)	0.066 ± 0.003 (8)	0.011 ± 0.004
700	0.093 ± 0.005 (10)**	0.117 ± 0.002 (10)**	0.024 ± 0.005

*Significantly greater than control, $P < 0.05$ (protected t test). **Significantly greater than control, $P < 0.01$.

Table 4. Stimulation of RNA synthesis in isolated nuclei by insulin. Oocytes were isolated in amphibian oocyte Ringer solution and transferred to paraffin oil. Under oil, individual nuclei were isolated manually by extrusion through a hole poked in the animal hemisphere (*13*). RNA labeling was initiated by bringing a 5-nl microdrop containing 400 fmol of ^{3}H-labeled GTP (control) or 400 fmol of ^{3}H-labeled GTP plus insulin into contact with a nucleus; the microdrop was rapidly incorporated into the nuclear mass. After 1 or 3 hours the nucleus was picked up on a small piece of filter paper and immediately placed in 1 ml of 10% TCA. The fixed nucleus adhered to the filter paper and after 1 hour the two were transferred through two more TCA washes. The nucleus (on paper) was placed in a scintillation vial, solubilized, and counted, as were portions from the TCA wash solutions. Data were expressed as the fraction of label incorporated into RNA and then converted to a percentage of paired control values. Shown are means ± SE for 6 to 12 nuclei from three animals. The mean control fraction incorporated was 0.019 ± 0.004 for 1 hour and 0.031 ± 0.007 for 3 hours.

Dose (fmol)	Stimulation of GTP incorporation (%)	
	1 Hour	3 Hours
0.07	21 ± 18	42 ± 16*
0.3		50 ± 19*
0.7	70 ± 26*	98 ± 24**

*Significantly higher than controls, $P < 0.05$ (protected t test). **Significantly higher than controls, $P < 0.01$.

external and internal insulin effects on RNA synthesis are additive.

The increase in RNA synthesis when insulin is microinjected suggests that the rate of protein synthesis might also be altered. This possibility was tested by measuring the amount of [^{35}S]methionine incorporated into protein (Table 3). A 2-hour exposure to 700 n*M* external insulin significantly increased incorporation of microinjected methionine. Microinjection of 17 fmol of insulin into control oocytes also increased methionine incorporation. Microinjection of 17 fmol of insulin into oocytes exposed to Ringer insulin increased methionine incorporation more than that in controls and in cells exposed to external insulin alone. Two conclusions may be drawn from these data. (i) Microinjected hormone alone stimulates protein synthesis. (ii) The stimulatory effects of extracellular and intracellular insulin on protein synthesis appear to be additive. These findings parallel those presented for RNA synthesis; therefore, the effects of intracellular insulin on transcription and translation may represent a simple cause and effect relation.

The present microinjection experiments demonstrate that in an intact cell, microinjected intracellular insulin stimulates transcription and translation. Other experiments show that this is also the case for glycogenesis (*9*). Thus, three metabolic pathways under control of external insulin in sensitive cells are also sensitive to intracellular hormone.

The additive effects of external and internal insulin suggest that the activating mechanisms triggered by surface and intracellular hormone are spatially separate and possibly of a different nature. Two types of mechanisms could explain the microinjection data. (i) Internal insulin causes generation of a second messenger at the plasma membrane or in the cytoplasm; the messenger then diffuses to regulatory sites, which could be ribosomal or nuclear. (ii) Insulin or an insulin fragment interacts directly with regulatory elements associated with the metabolic pathways. These possibilities are not mutually exclusive, since a single mechanism does not have to mediate all the effects of internal insulin.

Experiments with isolated nuclei distinguished between the two mechanisms with respect to insulin effects on transcription. Several aspects of nuclear envelope function appear to be altered when isolated nuclei are exposed directly to insulin (*6, 7*). To test whether insulin directly stimulates GTP incorporation into nuclear RNA, I used the oocyte nucleus, manually isolated under oil (*13*). The pooled results of three experiments in which GTP incorporation into RNA was measured in nuclei isolated from stage IV *Xenopus* oocytes are shown in Table 4. Treating nuclei directly with insulin increased incorporation in a dose-dependent manner at both 1 and 3 hours. At 3 hours, even a dose as low as 0.07 fmol stimulated incorporation significantly. When the dose was increased to 0.7 fmol, GTP incorporation was twice that of the control value. This stimulation is roughly the same percentage of increase in GTP incorporation as that seen when 21 to 40 fmol of insulin were microinjected into intact oocytes (Table 2). However, with isolated nuclei, no elements of the plasma membrane were present in the test system, and the amount of contaminating cytoplasm was minimal. Most likely, insulin interacts directly with some nuclear component; at present the intranuclear location of this component is unknown. However, insulin receptors have been found to be associated with the envelope of isolated nuclei (*6*), and there is evidence for the binding of ferritin-labeled insulin to chromatin (*14*).

How insulin controls cellular function is one of the major unsolved problems of endocrinology. The clinical importance of the problem relates directly to the phenomenon of cellular insulin resistance, one of the most prominent manifestations of non–insulin-sensitive diabetes mellitus (*15*). It appears that no single integrated mechanism can accommodate all of the known effects of insulin on target cells and that several coordinated biochemical events may be involved. The data presented here show that intracellular insulin can alter metabolism in an intact cell. This finding may be important with regard to insulin's long-term effects and it also indicates that research must focus on the intracellular hormone.

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11. Data presented here are for animals used during the winter, spring, and fall of 1987. In June, July, and August, *Xenopus* ovaries spontaneously enter a rapid phase of oogenesis. During the summer, stage IV oocytes generally exhibited average GTP incorporation rates exceeding 0.04 per hour. In addition, the variability among cells from the same ovary increased substantially, and biochemical responses to insulin were greatly reduced or abolished.
12. The average insulin concentration in cell water (in nanomoles per liter) can be estimated by multiplying by 4 the number of femtomoles injected (calculation assumes: 0.5-mg cell mass, 50% water content, and unit cell density). However, my initial microinjection experiments with [^{125}I]insulin show that within 2 to 3 hours most of the injected hormone had been degraded; that is, it was not longer precipitable with trichloroacetic acid (TCA). Insulin degradation complicates attempts to relate the dose levels used here to receptor-binding characteristics, since neither the identity nor the local concentration of the active ligand is known.
13. Under oil, an oocyte is punctured at the animal pole with a 29-gauge hypodermic needle, and the nucleus is extruded through the hole by gently squeezing the cell at the equator. Nuclei isolated in this manner are essentially free of cytoplasmic contamination, as judged by light and electron microscopy and by the absence of yolk proteins in two-dimensional polyacrylamide gels. A comparison of two-dimensional gels from oocyte nuclei isolated under oil with those from nuclei isolated by cryomicrodissection shows that nuclear proteins are not lost during isolation in oil. Moreover, oocyte nuclei isolated in oil retain the ability to synthesize RNA from labeled GTP or uridine triphosphate added in microdrops. Synthesis continues for many hours and is sensitive to inhibition by actinomycin and Sarkosyl [S. B. Horowitz *et al.*, *J. Cell Biol.* **101**, 211a (1985); S. B. Horowitz *et al.*, in preparation]. Use of paraffin oil as an isolation medium avoids the following problems associated with conventional water-based procedures: (i) physical damage caused by tissue homogenization and (ii) massive loss of endogenous proteins, cofactors, ions, and metabolites through the porous nuclear envelope to the aqueous medium [P. L. Paine, C. F. Austerberry, L. J. Desjarlais, S. B. Horowitz, *J. Cell Biol.* **97**, 1240 (1983); I. Lang and R. Peters, in *Information and Energy Transduction in Biological Membranes*, H. J. Helmreich, Ed. (Liss, New York, 1984), pp. 377–386].
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Phosphatidylinositol-Glycan Anchors of Membrane Proteins: Potential Precursors of Insulin Mediators

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BC_3H1 myocytes release membrane-bound alkaline phosphatase to the incubation medium upon stimulation with insulin, following a time course that is consistent with the generation of dimyristoylglycerol and the appearance of a putative insulin mediator in the extracellular medium. The use of specific blocking agents shows, however, that alkaline phosphatase release and dimyristoylglycerol production are independent processes and that the blockade of either event inhibits the production of insulin mediator. These experiments suggest a new model of insulin action.

EARLY WORK ON INSULIN MEDIATORS suggested that they contained amino acids and carbohydrates and thus were termed glycopeptides (*1*). It was then suggested that they might arise from glycoproteins by proteolysis (*1*). More recent work has suggested that some of the putative mediators of insulin action may originate from membrane lipids containing inositol, sugars, phosphate, and saturated fatty acids (*2–4*). These precursors have been found in various cell membranes (*2–4*) and bear a remarkable resemblance to the glycophospholipid anchor of a number of exofacial cell membrane proteins, among which alkaline phosphatase (AP) was the first to be described (*5–9*). As suggested by this similarity, we propose that the glycophospholipid anchor of membrane proteins may serve as a precursor of insulin mediators. According to this hypothesis, (i) insulin should stimulate the release of glycophospholipid-anchored proteins from cell membranes; (ii) this release must be kinetically coupled with the production of insulin mediator; (iii) mediator production should occur, at least in part, on the outside of the cell; and (iv) at least two independent hydrolytic activities must be present in order to generate insulin mediator. We now report the results of our observations on the effects of insulin and insulin action blockers on the release of AP and insulin mediator and on the generation of [^{3}H]myristate-labeled diacylglycerol.

Treatment of BC_3H1 myocytes with insulin rapidly increases the activity of AP in the culture medium (Fig. 1a), reaching a maximum within the first 2 minutes of incubation. This process is accompanied by a significant loss (up to 80%) of the cell-associated AP activity (*10*). The AP released to the medium disappears rapidly and decreases to control levels after 5 minutes. This suggests that the enzyme is (i) rapidly inactivated after solubilization, (ii) taken up by the myocytes after its release, or (iii) rapidly bound to some specific sites in the cell membrane such as those described by Ishihara *et al.* with heparan sulfate proteoglycan (*11*). Inactivation of the enzyme was suggested as the most likely explanation on the basis of similar observations made with purified liver membranes (*10*). This alternative is also consistent with a significant decrease in the activity of AP associated with brain microvessels within 5 minutes of exposure to insulin (*12*).

The activity of insulin mediator in the incubation medium was examined with a pyruvate dehydrogenase (PDH) activation assay (*13*). These experiments were based on the following rationale. If insulin mediator originates from the glycophospholipid anchor region of membrane proteins, then, because these proteins face the extracellular medium, insulin mediator production should occur primarily at the outer layer of the cell membrane and mediator should be released rapidly to the extracellular medium. To investigate this possibility, we collected culture medium at specific times after stimulation of cells with insulin and purified the mediator by chromatography on AG-1 ion-exchange columns eluted with dilute HCl (*14*). Fractions were collected, concentrated

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