JCI The Journal of Clinical Investigation

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J Clin Invest. 1976;57(1):230-243. https://doi.org/10.1172/JCI108264.

Research Article

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Biosynthesis of Proinsulin and Insulin in Newborn Rat Pancreas

INTERACTION OF GLUCOSE, CYCLIC AMP, SOMATOSTATIN, AND SULFONYLUREAS ON THE [^{3}H]LEUCINE INCORPORATION INTO IMMUNOREACTIVE INSULIN

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ABSTRACT The purpose of the present study was to investigate the regulation of insulin biosynthesis during the perinatal period. The incorporation of [^{3}H]leucine into total immunoreactive insulin (IRI) and into IRI fractions was measured by a specific immunoprecipitation procedure after incubation, extraction, and gel filtration in isolated 3-day-old rat pancreases without prior isolation of islets. IRI fractions were identified by their elution profile, their immunological properties, and their ability to compete with the binding of ^{125}I-insulin in rat liver plasma membranes. No specific incorporation of [^{3}H]leucine was found in the IRI eluted in the void volume, making it unlikely that this fraction behaves as a precursor of (pro)insulin in this system.

In all conditions tested, the incorporation of [^{3}H]leucine was linearly correlated with time. Optimal concentration of glucose (11 mM) activated six- to sevenfold the [^{3}H]leucine incorporation into IRI. Theophylline or N_6O_2-dibutyryl-(db)cAMP at 1.6 mM glucose significantly increased the [^{3}H]leucine incorporation. Glucose at 16.7 mM further enhanced the effect of both drugs. Contrarily, somatostatin (1–10 μg/ml) inhibits the rate of [^{3}H]leucine incorporation into IRI in the presence of 11 mM glucose; this effect was observed at 5.5 mM glucose and was not modified by any further increase in glucose concentrations up to 27.5 mM. Theophylline or dbcAMP at 10 mM concentration did not reverse the somatostatin inhibitory effect on either insulin biosynthesis or release. Somatostatin also inhibited both processes in isolated islets from the 3-day-old rat pancreas. High Ca^{++} concentration in the incubation medium reversed the inhibitory effect of somatostatin on glucose-induced insulin biosynthesis as well as release. In both systems the inhibitory effect of somatostatin on insulin biosynthesis and release correlated well. Glipizide (10-100 μM) and tolbutamide (400 μM) inhibited the stimulatory effect of glucose, dbcAMP, and theophylline on [^{3}H]leucine incorporation into IRI. The concentrations of glipizide that were effective in inhibiting [^{3}H]leucine incorporation into IRI were smaller than those required to inhibit the phosphodiesterase activity in isolated islets of 3-day-old rat pancreas.

These data suggest the following conclusions: (*a*) the role of the cAMP-phosphodiesterase system on insulin biosynthesis is likely to be greater in newborns than in adults; (*b*) the greater effectiveness of glucose and the cAMP system on insulin biosynthesis than on insulin release might possibly be related to the rapid accumulation of pancreatic IRI which is observed in the perinatal period; (*c*) somatostatin, by direct interaction with the endocrine tissue, can inhibit glucose and cAMP-induced insulin biosynthesis as well as release; calcium reverses this inhibition; (*d*) sulfonylureas inhibit insulin biosynthesis in newborn rat pancreas an effect which has

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Received for publication 2 June 1975 and in revised form 8 September 1975.

to be considered in the use of these agents in human disease.

INTRODUCTION

The capacity of the pancreatic beta cell to synthesize proinsulin and to convert it into insulin early in fetal life has been pointed out (1). During the first days of life, glucose stimulates the [^{3}H]leucine incorporation into insulin through proinsulin biosynthesis in isolated islets of rat pancreas (2–4). Several agents which naturally occur in pancreas such as cAMP (5–7) and somatostatin (8) have ben postulated to play an important role in the regulation of insulin secretion in neonates (9) and in adults (5–7, 10–18). However, there is currently no information available regarding the effect of somatostatin on insulin biosynthesis; moreover, the precise relationship between glucose, cAMP, and somatostatin on the beta cell function remains to be elucidated. On the other hand, drugs such as sulfonylureas (the use of which in diabetes is currently under question [19]) have been postulated to act on insulin release through the cAMP system (20–22). The effect of these drugs on insulin production is a matter of controversy because they have been reported to stimulate (23) or to inhibit (24–27) proinsulin biosynthesis in isolated islets of adult rodents.

The chief aims of the present work are: (*a*) to elucidate the interactions of glucose, cAMP, and somatostatin in the regulation of insulin biosynthesis and (*b*) to test the effect of sulfonylureas on this regulation. These studies were carried out in isolated newborn rat pancreas because it is of great physiological interest to investigate insulin biosynthesis in a period when the development and the function of the endocrine pancreas are considerable and to correlate the results obtained to the insulin release which has previously been reported using the same system (9).

METHODS

Materials. The rat and human insulins, 20 and 25 IU/mg, respectively, were kindly supplied by Dr. J. Schlichtkrull (Novo Research Institute, Copenhagen). The rat proinsulin was a generous gift of Dr. D. F. Steiner (University of Chicago, Chicago, Ill.). Synthetic somatostatin was provided by Dr. Raynaud (Laboratoires Roussel, Paris). ^{131}I-Human insulin, 150 μCi/μg (28); monoiodinated insulin, 380 μCi/μg (29); and monoiodinated-proinsulin, 250 μCi/μg (30) were labeled by the chloramine-T method (31). L-[4,5-^{3}H]leucine (40 Ci/mmol) and [8-^{3}H]3′5′-cyclic adenosine phosphate ammonium salt (27 Ci/mmol) were purchased from the Commissariat à l'Energie Atomique (C.E.A., Saclay, France), with a radiochemical purity of 99 and 97%, respectively. Cycloheximide, (N_6O_2-dibutyryl)-3′5′-cyclic adenosine monophosphoric acid monosodium salt (dbcAMP),[1] activated charcoal (Norit A), and snake venom were purchased from Sigma Chemical Co., St. Louis, Mo.). Actinomycin D, cAMP, and theophylline (B grade) were purchased from Calbiochem (San Diego, Calif.). Collagenase (200 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Bovine serum albumin (BSA, fraction V) was purchased from Pentex Biochemical (Kankakee, Ill.); human serum albumin (HSA) was from the Centre National de Transfusion Sanguine, (Orsay, France). Goat antiguinea pig serum (GAGP) was from Antibodies Incorporated, (Davis, Calif.). Analytical grade anion-exchange resin (AGI-X2) was from Bio-Rad, (Paris). Sephadex G-50 fine, dextran 250, and Ficoll 400 were from Pharmacia, (Le Chesnay, France). Other chemicals were of reagent grade.

Pancreas. The splenic lobes of the pancreas were removed from 3-day-old Wistar rats (9). The rats were suckled until time of death. The explants (8–10 per flask) were collected at 4°C in 40–50 ml of Krebs-Ringer bicarbonate buffer (KRB) containing 106 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM KCl, 40 mM $NaHCO_3$, 0.5% (wt/vol) of dialyzed BSA, pH 7.5. They were gassed (95% O_2, 5% CO_2) and preincubated in the same buffer for 30 min at 37°C. Incubation was done with shaking at 80 cpm in 0.8–1.0 ml of fresh medium containing the substances to be tested, 17 natural amino acids (Ala, Arg, Asp, Cys, Glu, Gly, His, Pro, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, Val) at 0.1 mM each, and [^{3}H]leucine at 500 μCi/ml instead of leucine. These additives replaced an equimolar quantity of NaCl to maintain the isoosmolality.

Immunoreactive insulin (IRI) was extracted from the whole pancreas according to Kenny (32) with the following modifications: 80–100 mg of pancreas was homogenized in 8–10 ml of acid-alcohol solution (ethanol 75% vol/vol, 12 N HCl 1.5% vol/vol) by a 2-min sonication at 50 W in a Branson sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). The homogenate was adjusted to pH 4 with 0.5–1.0 ml of sodium citrate and centrifuged at 1,500 *g* for 5 min. The supernate was supplemented with 100 μl/ml insulin-free human plasma, and dialyzed for 2 h in a 10-ml hollow fiber dialyzer (Bio-Fiber 50 Minibaker) against 10 mM Na_2HPO_4, 150 mM NaCl, and 0.3% BSA.[2] After centrifugation and concentration of the supernate to 3–5 ml by rotary vacuum evaporation, ice-cold TCA was added to obtain a 10% final concentration. After centrifugation, the precipitate was washed once with 5% TCA and dried with a mixture of ether-acetone (1/1, vol/vol) then with ether. The IRI recovered in the TCA precipitate was 85.7±2.6% of the acid-alcohol IRI (10 extractions). The dried TCA precipitate was then dissolved in 1 ml of 1 M acetic acid and applied to a 1 × 130-cm column of Sephadex G-50 fine. The column was equilibrated and eluted at 4°C with 1 M acetic acid at a flow rate of 10 ml/h. 1-ml fractions were collected in tubes containing 1 mg BSA in 100 μl H_2O, to minimize the adsorption of IRI to the plastic wall. The fractions were pooled according to the distribution of IRI and to the calibration as indicated in Fig. 1 and then lyophilized. Recovery from the column procedure was 81 ±2.3% for IRI and 85±6.5% for [^{3}H]leucine (10 fractionations). Pool I contained the IRI eluted in the void volume, pool II contained the IRI eluted with ^{125}I-rat pro-

[1] *Abbreviations used in this paper:* AIS, anti-insulin serum; BSA, bovine serum albumin; dbcAMP, N_6O_2-dibutyryl-3′,5′-cyclic AMP; GAGP, goat anti-guinea pig serum; HSA, human serum albumin; IRI, immunoreactive insulin; KRB, Krebs-Ringer bicarbonate buffer; PLM, proinsulin-like material.

[2] Pratt, P., and M. A. Permutt. Personal communication.

TABLE I

Effect of Dextran-Coated Charcoal on the Precipitation of [^3H]Leucine

IRI pools	[^{3}H]Leucine precipitated, *cpm/tube**			
	Without dextran charcoal		With dextran charcoal	
	AIS	NGPS	AIS	NGPS
Void volume	8,950	10,615	2,695	2,722
PLM	2,026	1,060	1,600	200
Insulin	940	680	550	60

* Samples of each lyophilized IRI pool, 100 μl of 1 mg/ml solution in 25 mM veronal buffer, pH 8.6, were diluted in 1 ml of the same buffer and incubated 2 h at room temperature with 2 μl AIS. 1 ml dextran-coated charcoal was then added. The solution was mixed thoroughly immediately centrifuged at 2,500 *g* for 20 min and the supernate was recovered. Immunoprecipitation was carried out with GAGP serum (15 μl/ml) for 18 h at 4°C, either directly (left column) or on the supernate obtained after charcoal-dextran adsorption (right column). The precipitates were collected by centrifugation at 1,500 *g* for 45 min, washed with 2 ml cold 0.1 M veronal buffer, pH 8.6, centrifuged again under the same conditions, then dissolved in 100 μl of 0.1 N NaOH and counted in 10 ml Instagel in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with 40% efficiency.

insulin or proinsulin-like material (PLM), and pool III included the IRI eluted with the ^{131}I-rat insulin.

Islets. Islets of 3-day-old rat pancreases were isolated as described elsewhere for adult pancreas (33). 50 islets per tube were directly incubated for various times in 500 μl of the KRB medium described above containing 50 μCi of [^{3}H]leucine. At the end of the incubation, islets were centrifuged at 4°C for 5 min at 200 *g* and then washed in buffer containing 10 mM leucine. After centrifugation, islets were resuspended in 100 μl H_2O and then homogenized by 10-s sonication at 50 W with a Branson sonifier.

Analytical procedures. The [^{3}H]leucine incorporated into IRI ([^{3}H]IRI) was determined separately on the tissue and on the incubation medium by a modification of the antibody precipitation technique previously described (34). It was verified with ^{125}I-hormone as tracer that 10 mU IRI/ml was completely bound by 2 μl/ml of the guinea pig anti-insulin serum (AIS). Virtually all (98±0.3%, $n = 10$) of the insulin–anti-insulin complex was precipitated by the GAGP. The GAGP serum also precipitated the non-IRI materials labeled with [^{3}H]leucine, as indicated by the controls containing normal guinea pig serum (NGPS) instead of AIS. The amount of non-IRI-tritiated material precipitated by GAGP serum was considerably decreased by adsorption of each sample with dextran-coated charcoal (35) before addition of GAGP serum (Table I). In the conditions indicated in Table I, dextran-coated charcoal preferentially adsorbed the non-IRI-tritiated material and less than 10% of the antibody complex, as monitored by ^{125}I-proinsulin or ^{125}I-insulin added as tracer. This is in agreement with data observed with isolated islets of adult rat pancreas (36). The [^{3}H]IRI was obtained by subtracting the NGPS-precipitated ^{3}H-material from the AIS-precipitated ^{3}H-material. The efficiency of the purification procedure of [^{3}H]IRI is indicated in Table II. The immunoprecipitation step was the most effective in purifying [^{3}H]-IRI. Dialysis further increased this purification step by a threefold factor. In most of the experiments, each 30 cpm of radioactivity in IRI was the result of incorporation of 1 fmol leucine. The smallest amount of [^{3}H]IRI to be measured in any tube was 200 cpm. Results are expressed in femtomols of [^{3}H]leucine incorporated per microgram of total IRI or of insulin and proinsulin. The leucine incorporated in the C-peptide was not taken into account because the antibodies failed to precipitate it. Radioimmunoassay of C-peptide (37) shows that the quantity of C-peptide in the supernate is similar before and after immunoprecipitation performed in the same conditions as for insulin. The amount of [^{3}H]IRI released in the incubation medium was also measured after immunoprecipitation and was below 5% of the total pancreatic [^{3}H]IRI, even under conditions where IRI release into the medium was maximally stimulated.

TABLE II

Efficiency of the Purification Procedure of IRI from the Pancreas

	[IRI (μg/ml)/[^{3}H]Leucine (cpm/ml)] × 10^{-4}		
	Acid alcohol	TCA precipitate	Immunoprecipitate*
Without dialysis	1	5	422
With dialysis	1	27	1,165

* IRI was extracted from pancreas after 240 min of incubation at 37°C with [^{3}H]leucine as described in Methods. Aliquots of dried TCA precipitate (20 μl at 1 mg/ml of 1 M acetic acid) were diluted in 1 ml of 25 mM veronal buffer, pH 8.6 containing HSA 0.25%. Immunoprecipitation procedure was performed after charcoal-dextran adsorption as indicated in Table I.

Measurement of IRI was performed by radioimmunoassay (38) using ^{131}I-human insulin, guinea pig antihuman insulin serum, and rat insulin as standard, with separation of free hormone by adsorption to silicate (39). The rat proinsulin used as standard was 2.2 times less potent than insulin on a molar basis in inhibiting the binding of ^{131}I-insulin to antibody; thus the concentration of the extracted PLM has to be multiplied by 2.2 when compared to proinsulin instead of insulin standard. The IRI of void volume, PLM, and insulin was also characterized in a radioreceptor assay system by measuring their ability to compete with the binding of mono-^{125}I-insulin in rat liver plasma membranes (40).

For measurement of phosphodiesterase activity, 500 islets were sonicated in 1 ml Tris 40 mM buffer containing 5 mM $MgCl_2$ and 3.7 mM mercaptoethanol, pH 8. The procedure of Thompson and Appleman (41) was adapted for the measurement of enzyme activity in a final volume of 75 μl: 20 μl of islet extract, which contained the phosphodiesterase activity of about 25 islets, was incubated for 10 min at 37°C with [^{3}H]cAMP (25 nM); this first reaction was stopped by boiling for 3 min. 50 μl snake venom from 1 mg/ml in H_2O was then added, and the solution was incubated for 10 min at 30°C. The 5′-AMP produced in the first reaction was further hydrolyzed to adenosine by the nucleotidase of the snake venom. This second reaction was stopped by the addition of 500 μl AGI-X2 ion-exchange resin (1:2 wt/vol, in H_2O). After centrifugation for 15 min at 1,500 *g*, a 200-μl aliquot sample of the supernatant solution was counted in 10 ml Instagel; the unit of phosphodiesterase activity is 1 pmol cAMP hydrolyzed per min. Proteins were measured by the method of Lowry et al. (42).

RESULTS

Effect of glucose and different drugs on the distribution of IRI after gel chromatography fractionation. The IRI content of newborn rat pancreas after 4 h incubation with [^{3}H]leucine in different experimental conditions was measured and characterized in the pools that were collected after gel filtration on Sephadex G-50 fine (Fig. 1). The IRI that is eluted in the void volume (pool I) follows the same dilution curve as rat insulin used as standard (Fig. 2) and thus can be measured in terms of rat insulin. The elution pattern of this pool remains unchanged when it is rechromatographed under the same conditions. These properties are similar to those described for "big big" insulin in human plasma (43). In contrast to the observations made in a human insulinoma (44), no specific incorporation of [^{3}H]leucine is found in this fraction, making it unlikely that this "big big" insulin behaves as a precursor of (pro)insulin in the newborn rat pancreas. A quantity of this IRI equivalent to 50 ng/ml insulin did not produce a significant competition in the radioreceptor assay system used (40): $B/Bo \times 100 = 97.2 \pm 2.1\%$ ($n = 3$) as compared to $25.2 \pm 2.3\%$ ($n = 3$) observed with the same quantity of rat insulin. PLM has properties similar to those of rat proinsulin used as standard when identified by the elution profile (Fig. 1), the immunological properties (see Methods) and the ability to compete with the binding of ^{125}I-insulin to liver plasma membranes (Fig. 3): in this radioreceptor assay the y intercept obtained with PLM differs significantly ($P < 0.001$) from that obtained with rat insulin used as standard, whereas slopes

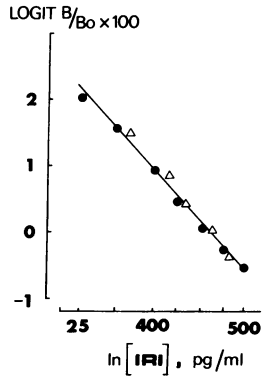

FIGURE 2 Effect of the IRI eluted in the void volume of the Sephadex G-50 fine in competing with a guinea pig AIS. Logit transformation of the percentage of initial binding ($B/Bo \times 100$) is plotted as a function of various concentrations of rat insulin. Each point is the mean of triplicate determinations. Individual values did not differ by more than 5%. (△), IRI of the void volume; (●), rat insulin.

are similar for both. Half-inhibition of the initial binding of ^{125}I-insulin is obtained with a quantity of PLM (80 neq IRI) 17.6 times higher than that of rat insulin on a molar basis. These results are in agreement with those observed with purified rat proinsulin in rat liver plasma membranes (30).

Glucose and the different drugs tested do not significantly modify the distribution of IRI in the three pools (Table III). However a slight decrease in the insulin pool ($P < 0.02$) mainly due to an increase in the IRI eluted before insulin (void volume + PLM) is observed at low glucose concentration with 0.4 mM tolbutamide

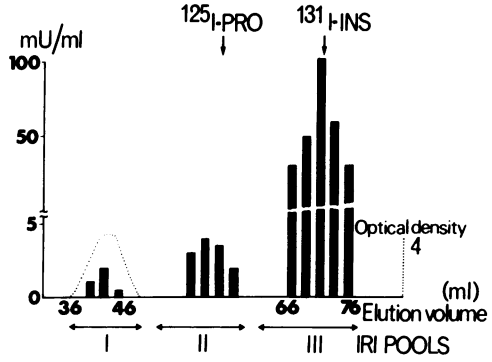

FIGURE 1 Elution profile of pancreatic IRI after gel filtration on Sephadex G-50 fine. 3-day-old rat pancreases were incubated for 4 h in a medium containing 16.7 mM glucose. After extraction, the TCA precipitate was fractionated in a 1 × 130-cm column and was eluted with 1 M acetic acid. Dotted lines show the optical density of proteins eluted in the void volume as measured at 280 nm in 1 to 10 diluted samples. Insulin content in each fraction was radioimmunoassayed using a pure rat insulin as standard. Details of the incubation, extraction, and gel chromatographic procedure are given in Methods.

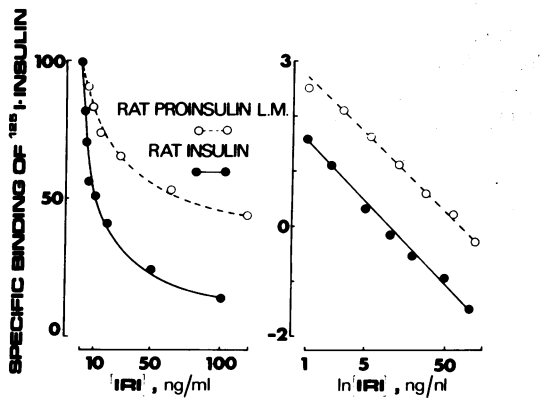

FIGURE 3 Effect of rat PLM and rat insulin on the binding of ^{125}I-insulin to rat liver plasma membranes. The percentage of initial binding of ^{125}I-insulin ($B/Bo \times 100$) to the left, and its logit transformation to the right, are plotted against the concentration of unlabeled insulin or rat PLM on an arithmetic (left) and on a logarithmic scale (right). Each point represents the mean of duplicate aliquots from each incubation tube (see Methods). Individual values did not differ by more than 5%.

TABLE III

Distribution of IRI after Incubation for 240 min with Theophylline (T), dbcAMP (D) and Sulfonylureas, Glipizide (G) and Tolbutamide (Tol)

Drugs	Percentage of total IRI					
	Glucose, 1.6 mM			Glucose, 16.7 mM		
	Void volume	PLM	Insulin	Void volume	PLM	Insulin
None (3)	0.3±0.2	3.0±0.1	96.5±0.3	0.8±0.2	4.4±1.2	94.2±1.2
T* (5)	0.4±0.1	3.8±0.7	95.7±0.7	0.3±0.07	4.1±1.9	95.5±2.0
D* (3)	0.9±0.5	2.0±0.8	96.0±1.3	0.6±0.2	3.6±1.2	95.7±1.9
T* + G‡ (5)	0.9±0.2	4.4±1.2	95.4±0.8	2.3±0.5	3.3±1.3	94.2±1.8
T* + Tol§ (4)	1.0±0.4	6.4±1.2	92.4±0.8‖	0.6±0.2	5.7±1.8	95.5±1.8

Numbers are means±SEM; the numbers of experiments are given in parentheses. Concentrations of the drugs: *10 mM; ‡10 μM; §0.4 mM. ‖ $P < 0.02$ vs. controls with theophylline. Each experiment was carried out with 8–10 explants.

and 10 mM theophylline (Table III). This modification is not due to a physical interaction of tolbutamide plus theophylline with insulin, as tested with ^{125}I-insulin in the same conditions of incubation and fractionation procedure.

Effect of glucose, theophylline, and dbcAMP on the specific incorporation of [^{3}H]leucine into IRI. In the presence of 1.6 mM glucose, [^{3}H]leucine is incorporated into IRI; after 240 min, the incorporation amounts to 125±16 fmol per explant.[3] Glucose activates the incorporation of [^{3}H]leucine into the IRI of the isolated newborn rat pancreas at concentrations ranging from 1.6 to 27.5 mM (Fig. 4, right). Glucose is maximally effective from 11 to 27.5 mM with a six- to sevenfold incorporation, as compared to the effect of 1.6 mM glucose. About 5 mM glucose is required to produce 50% stimulation of [^{3}H]leucine incorporation. The glucose dose effect is not dependent on the duration of the experiments within a 4-h period, since the incorporation of [^{3}H]leucine into IRI is linearly correlated with time (Fig. 4, left): at 11 mM glucose, the slope of the regression line is about 3.8 fmol [^{3}H]IRI/explant per min (r: 0.99, $P < 0.001$). This suggests that the transfer of the radioactivity from the precursor pool of amino acids into the IRI behaves as a zero-order process, in agreement with the model of the precursor-product relationship described by Steiner et al. (45). After 240 min, glucose at 16.7 mM enhances slightly (but not significantly) the percentage of [^{3}H]leucine incorporated into the insulin pool to 54.3±3.9 as compared to 37.9±8.7 at 1.6 mM glucose (Table IV).

Theophylline at 10 mM significantly increases the [^{3}H]IRI at both high and low glucose concentrations (Table IV). Kinetic studies (Fig. 5, left) show that this rise is observed at the earliest point tested (30 min) and remains a linear function of time. The slope of the regression line is about 2.3 (r: 0.98, $P < 0.01$) and 11.1 (r: 0.99, $P < 0.001$) fmol/explant per min, at 1.6 and 16.7 mM glucose, respectively. Theophylline plus glucose stimulated insulin biosynthesis synergistically (Table IV). The statistical significance ($P < 0.05$) was established after the calculation described elsewhere (9). After 240 min, theophylline at 10 mM significantly increases the percentage of [^{3}H]insulin to 80.6±1.6 and 80.3±3.8 at 1.6 and 16.7 mM glucose, respectively, as compared to the corresponding controls (Table IV).

dbcAMP causes a 6–24-fold increase in [^{3}H]leucine incorporation into IRI (Table IV). At 16.7 and at 1.6 mM glucose, 50% stimulation is observed at about 0.7 and 1.5 mM dbcAMP, and nearly maximal stimulation is achieved with 5 mM dbcAMP (Fig. 6) Each point of the dose-response curve of dbcAMP is higher at 16.7 than at 1.6 mM glucose, and the level attained with

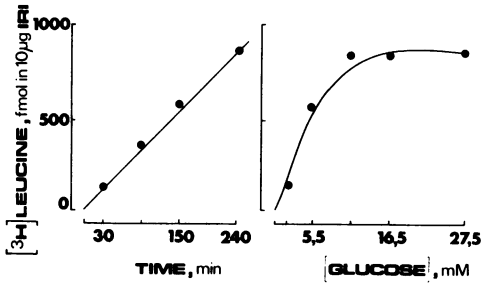

FIGURE 4 Effect of glucose on the [^{3}H]leucine incorporation into IRI in isolated 3-day-old rat pancreas. The time-course to the left was performed at 11 mM glucose. The dose response to the right was conducted after 240 min of incubation. After TCA precipitation, the immunoprecipitation was performed as indicated in Tables II and IV. Details of the incubation are given in the Methods. Each point is the mean of triplicate determinations. Individual values did not differ more than 10–15%.

[3] About 10 μg IRI.

TABLE IV

Effect of Glucose, Theophylline, 10 mM, (T) and dbcAMP, 10 mM, (D) on the [³H]Leucine Incorporation into Proinsulin and Insulin in the Isolated Newborn Rat Pancreas

Glucose	Drugs	Incorporation of [³H]leucine into the IRI of one explant	Sp act of IRI pools, fmol [³H]leucine/μg eq IRI		[³H]insulin in % of [³H]IRI
			Proinsulin	Insulin	
1.6 mM	None (3)	125±16	248±7	5.3±1.7	37.9±8.7
	T (5)	502±96§	252±35	45.0±9.0§	80.6±1.6§
	D (3)	3,379±631§	603±54§	332.0±63.0§	96.2±1.5§
16.7 mM	None (3)	791±135 (S)‡	983±109 (S)‡	45.0±8.0 (S)‡	54.3±3.9
	T (4)	2,661±501* (S)‡	739±89 (S)‡	247.0±50.0§ (S)‡	89.3±3.8§
	D (3)	6,108±450§ (S)*	1,740±384 (S)*	559.0±123.0*	87.8±0.8§

Results are given by mean±SEM. Numbers of experiments are given in parenthesis. Experimental conditions are described in Methods. When results after addition of drugs are different from those without drugs, at the same glucose concentration, the significance is given by $*P < 0.05$, $‡P < 0.02$ and $§P < 0.01$. Values with (S) refer to statistical comparison $*P < 0.05$, $§P < 0.01$ between the effect at 16.7 and 1.6 mM glucose. Each experiment was done with 8–10 explants. [³H]Leucine incorporated into insulin ([³H]I) in percent of [³H]IRI is ([³H]insulin/[³H]IRI)×100.

maximally stimulating doses of dbcAMP at 1.6 mM glucose is significantly lower ($P < 0.05$) than that achieved with dbcAMP at 16.7 mM glucose. The maximum percentage of increase due to dbcAMP as compared to the corresponding basal values is 24- and 6-fold higher at 1.6 and 16.7 mM glucose, respectively. In the same manner as theophylline, dbcAMP plus glucose stimulated insulin biosynthesis synergistically (Table IV, $P <$ 0.05). After 240 min, dbcAMP at 10 mM significantly increases the percentage of [³H]insulin to 96.2±1.5 and 87.8±0.8 at 1.6 and 16.7 mM glucose, respectively, as compared to the corresponding controls (Table IV).

Effect of somatostatin on the specific incorporation of [³H]leucine into IRI. When isolated newborn rat pancreas was incubated as previously described (9) for 90 min at 11 mM glucose, the addition of somatostatin (0.5–1.0 μg/ml) inhibited the glucose-induced insulin release (Table V). Theophylline or dbcAMP that synergistically stimulated the effect of glucose on insulin release (Table VI, [9]) failed to reverse this effect (Table VI). We have observed that in the same preparation, somatostatin inhibited the glucose-induced [³H]leucine incorporation into IRI. This inhibitory effect of somatostatin on insulin biosynthesis is dose dependent (Fig. 7, left). 5–10 μg/ml of somatostatin gave a maximal inhibition; 50% of the fall in the [³H]leucine incorporation into IRI is observed with a concentration of about 0.5 μg/ml. At the maximal inhibitory dose of somatostatin (5 μg/ml). the incorporation of [³H]leucine into IRI is reduced by half for concentrations of glucose ranging from 1.6 to 27.5 mM. However, somatostatin does not affect the concentration of glucose that is maximally (11.5 mM) or 50% (5.5 mM) effective

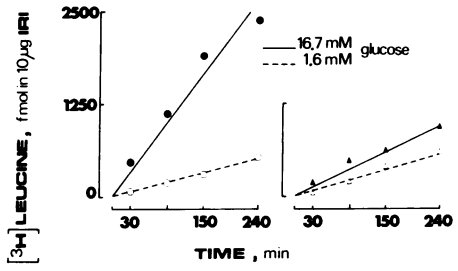

FIGURE 5 Effect of low (○) and high (●) glucose concentration on the time-course of [³H]leucine incorporation into IRI contained in one explant. [³H]IRI was measured after 240 min of incubation in presence of 10 mM theophylline without (left) and with (right) glipizide, 10 μM. Each point is the mean of triplicate determinations. Individual values did not differ by more than 10–15%.

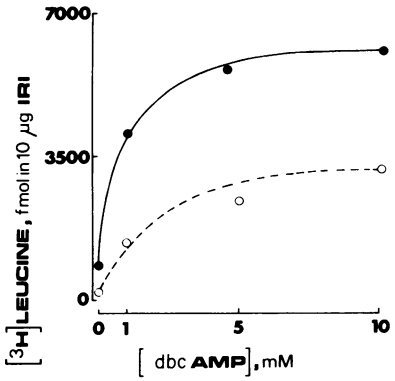

FIGURE 6 Dose response of dbcAMP on the [³H]leucine incorporation into IRI induced by 1.6 mM (○) and 16.7 mM (●) glucose incubated for 240 min with isolated 3-day-old rat pancreas. Each point is the mean of triplicate determinations. Individual values did not differ by more than 10–15%.

TABLE V

Somatostatin Effect on Glucose-Induced Insulin Release

Test solution		Somatostatin	IRI release % of pancreatic IRI
		μg/ml	
Glucose 11 mM	(3)	0	3.64±0.29
	(4)	0.5	1.53±0.08*
	(4)	1	1.25±0.05*
Glucose 11 mM and theophylline 10 mM	(4)	0	7.07±0.17
	(3)	0.5	5.42±0.28‡
	(3)	1	5.46±0.14*
Glucose 11 mM and dbcAMP 10 mM	(3)	0	8.28±0.21
	(3)	0.5	6.67±0.50§
	(4)	1	6.46±0.25‡

Each explant was incubated as previously described (9) for 90 min at 11 mM glucose. Values in parenthesis indicate the number of experiments. Statistical significance of the somatostatin effect for each experiment is given by comparison to the respective controls and indicated by $*P < 0.001$, $‡P < 0.01$, and $§P < 0.05$.

on insulin biosynthesis (Fig. 7, center). Time-course studies in the presence of somatostatin (Fig. 7, right) show that the decrease in [^{3}H]leucine incorporation is observed for the first experimental point measured (30 min); the incorporation of [^{3}H]leucine into IRI is linearly correlated with time, with a rate of about 1.9 (r: 0.98, $P < 0.01$) fmol/explant per min. Somatostatin also inhibits the stimulatory effect of theophylline or dbcAMP on the glucose-induced [^{3}H]leucine incorporation (Fig. 8). The effect of 10 mM theophylline or of 10 mM dbcAMP on the [^{3}H]leucine incorporation induced by 11 mM glucose after 150 min of incubation is decreased with a concentration of somatostatin of 1 μg/ml ($P < 0.05$ and $P < 0.01$, respectively).

The effect of somatostatin was also studied in isolated islets from newborn rat pancreas, since it had been reported that the effect of somatostatin on insulin release was no longer observed in isolated islets (14, 46). Somatostatin at 5 μg/ml decreases both insulin biosynthesis and release induced by 11 mM glucose in isolated islets (Fig. 9, Table VI): during the first 30 min of incubation, the rate of [^{3}H]leucine incorporation into IRI is similar with and without somatostatin. This rate remains constant at about 0.013 fmol/islet per min (r: 0.99, $P < 0.001$) in the controls to at least 150 min, and is decreased about 40% in the presence of 5 μg/ml somatostatin at 0.008 fmol/islet per min (r: 0.99, $P < 0.001$). Several authors using perfused rat pancreas (47) or isolated islet (48) have reported that increased Ca^{++} concentrations reverse somatostatin inhibition of glucose-induced insulin secretion. In the present experi-

TABLE VI

Effect of Ca^{++} Concentration on Somatostatin Inhibition of Glucose-Induced Insulin Biosynthesis and Release

Ca^{++}	Somatostatin	[^{3}H]Leucine	IRI release in % of islet IRI
meq/liter	*μg/ml*	*fmol/islet IRI*	
2	—	2.06±0.19	5.56±0.21
2	5	1.33±0.11*	2.84±0.04‡
10	—	2.70±0.33	6.28±0.82
10	5	3.45±0.30	7.52±0.66

50 islets were incubated for 150 min at 11 mM glucose as described in methods. Statistical significance of the somatostatin effect, as compared to the respective control, was indicated by $*P < 0.05$, $‡P < 0.001$. Each value is mean±SEM of triplicate determinations.

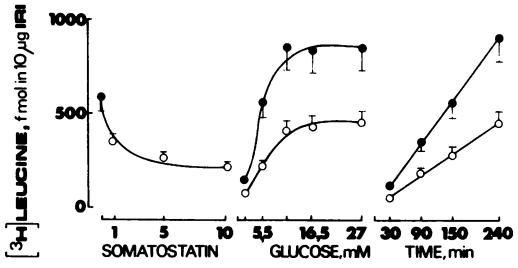

FIGURE 7 Effect of somatostatin on the [^{3}H]leucine incorporation into IRI induced by glucose in isolated 3-day-old rat pancreas. To the left, dose response to somatostatin (in micrograms per milliliter) at 11 mM glucose concentration, after 150 min of incubation. Center: dose response to glucose with and without somatostatin at 5 μg/ml after 240 min of incubation. To the right time-course studies of glucose (11 mM)-induced insulin biosynthesis with and without somatostatin at 5 μg/ml. Each point is the mean (±SEM) of triplicate determinations. ○, somatostatin present; ●, somatostatin absent.

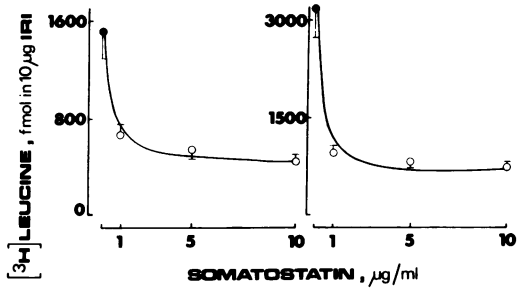

FIGURE 8 Effect of somatostatin (5 µg/ml) on the [^{3}H]-leucine incorporation into IRI induced by 11 mM glucose in isolated 3-day-old rat pancreas after 150 min of incubation in presence of 10 mM theophylline (left) or of 10 mM dbcAMP (right). Each point is the mean (±SEM) of triplicate determinations. ○, somatostatin present; ●, somatostatin absent.

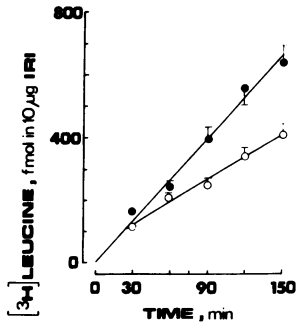

FIGURE 9 Time-course of [^{3}H]leucine incorporation into IRI induced by 11 mM glucose with isolated islets of 3-day-old rat pancreas. For details of incubation and immunoprecipitation procedure see Methods. The effects of 5 µg/ml of somatostatin (○) are shown. Each point is the mean (±SEM) of triplicate determinations. ○, somatostatin present; ● somatostatin absent.

ments, we also observed a reversal of the inhibitory effect of somatostatin on insulin biosynthesis by increasing the Ca^{++} concentration (Table VI). When Ca^{++} concentration of the incubation medium was raised from 2 to 10 meq/liter, somatostatin (5 µg/ml) failed to inhibit both insulin biosynthesis and release (Table VI).

Effect of sulfonylureas on the specific incorporation of [^{3}H]leucine into IRI. Glipizide inhibits the [^{3}H]leucine incorporation into IRI in the presence of 16.7 mM glucose (Fig. 10). Maximal inhibition is observed with glipizide at 100 µM, and half-maximal effect is seen at about 25 µM. No significant effect of glipizide is observed at 1.6 mM glucose under conditions where, in fact, the initial level of biosynthesis is too low to permit statistical evaluation of the difference.

The inhibitory effect of glipizide is still observed when theophylline is added to 16.7 mM glucose. In these conditions, the leucine incorporation into IRI remains linearly correlated with time (Fig. 5, right). The slope of the regression line is 2.4 (r: 0.99, $P < 0.01$) and 3.8 (r: 0.97, $P < 0.01$) fmol [^{3}H]leucine incorporated/explant per min at high and low glucose concentration, respectively. The persistence of this linear relationship when theophylline and glipizide are present in the medium indicates that the apparent steady state of equilibrium is maintained between the [^{3}H]leucine precursor pool and the IRI pool. In fact, the depletion of the IRI pool during the experiments is very low since even in the conditions of maximal stimulation of release (i.e. 10 µM glipizide, 10 mM theophylline, and 16.7 mM glucose), the IRI present in the medium after 4 h incubation does not exceed 8.7±1.2% ($n = 6$) of the pancreatic IRI, and the [^{3}H]IRI release is below 3.7±0.9% ($n = 6$) of the pancreatic [^{3}H]IRI. Regardless of the glucose concentration, the largest inhibitory effect of the sulfonylurea is observed with the dbcAMP-induced insulin biosynthesis: glipizide at 10 µM inhibits the [^{3}H]leucine incorporation into IRI by about 14-fold (437 instead of 6,108±450 fmol of [^{3}H]IRI per explant) and 3-fold (1,076±173 instead of 3,379±634 fmol of [^{3}H]IRI per explant), in the presence of 1.6 and 16.7 mM glucose, respectively. In all conditions the inhibitory effect of sulfonylureas is associated with a decrease in the percentage of [^{3}H]insulin (Table VII).

The inhibition due to glipizide was compared to that obtained with cycloheximide and actinomycin D on insulin biosynthesis. Optimal blockade of the 16.7 mM glucose-induced insulin biosynthesis is obtained with cycloheximide (50 µg/ml) after 90 as well as after 240 min of incubation with 85.8 and 93.2% inhibition, respectively (Table VIII). The effect of actinomycin D (10 µg/ml) is delayed with 5.5 and 86% inhibition at 90 and 240 min of incubation, respectively. In contrast to the effect of actinomycin D, the inhibitory effect of glipizide on insulin biosynthesis is observed earlier, as indicated in Fig. 5. The effect of glipizide on the phosphodiesterase activity in isolated islets of the 3-day-old rat was also

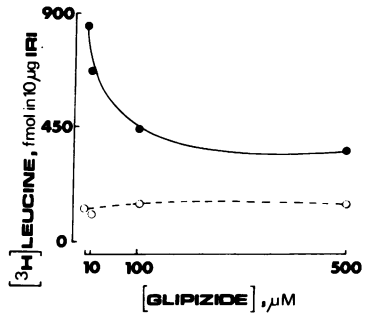

FIGURE 10 Dose response of glipizide on the [^{3}H]leucine incorporation into IRI induced by 1.6 (○) and 16.7 mM (●) glucose incubated for 240 min with isolated 3-day-old rat pancreas. Each point is the mean of triplicate determinations. Individual values did not differ by more than 10–15%.

TABLE VII

Effect of Glipizide, 10 μM, (G), and Tolbutamide, 0.4 mM, (Tol) on the [³H]Leucine Incorporation Induced by Glucose and Theophylline, 10 mM, (T) into the IRI of the Isolated Newborn Rat Pancreas

Glucose	Drugs	Incorporation of [³H]leucine into the IRI of one explant	Specific activity of IRI pools, fmol of [³H]leucine per μg eq IRI		[³H]Insulin in % of [³H]IRI
			Proinsulin	Insulin	
1.6 mM	T (5)	502±96	252±35	45.0±9	80.6±1.6
	T + G (5)	557±132	677±20§	27.0±8	42.6±8.2‡
	T + Tol (4)	927±173	969±151‡	33.5±9	38.8±4.0§
16.7 mM	T (4)	2,685±626	739±89	247.0±50	89.3±3.8
	T + G (3)	857±260*	498±67	80.0±20*	74.5±10.7
	T + Tol (3)	414±150‡	317±156	25.0±11‡	39.4±12.8‡

Results are given as mean±SEM. Numbers of experiments are given in parentheses. When results with sulfonylureas are different from those with theophylline alone, at the same glucose concentration, the significance is given by * $P < 0.05$, ‡ $P < 0.01$, § $P < 0.001$.
Each experiment was done with 8–10 explants.

examined. The rate of hydrolysis of cAMP due to islet phosphodiesterase at a linear dilution was studied with various concentrations of this substrate (Fig. 11, left). Estimates of the apparent K_m derived from the Lineweaver-Burk plot give a value of 3.2 μM cAMP. Glipizide (500 μM) decreases the V_{max} without altering the apparent K_m; thus the inhibitory effect of glipizide is noncompetitive. The apparent K_i for glipizide was studied with a low concentration of cAMP (25 nM). i.e., a concentration that is in the range of the cAMP concentration in the islets (Fig. 11, right); under these conditions, the apparent K_i is about 350 μM glipizide.

DISCUSSION

As a result of previous studies (23, 27, 34, 36, 49–52), it is known that glucose, theophylline, and dbcAMP stimulate insulin biosynthesis in isolated adult mammalian islets. In the present studies, we have been able to measure the specific incorporation of [³H]leucine into pancreatic IRI directly without prior isolation of islets and to indicate possible mechanism for the regulation of insulin biosynthesis in newborns. Our results demonstrate that glucose and cAMP are involved in insulin biosynthesis in newborn rats as in adults and,

TABLE VIII

Effect of Cycloheximide and Actinomycin D on the [³H]Leucine Incorporation into IRI Induced by 16.7 mM Glucose, in Isolated Newborn Rat Pancreas

Incubation	Cycloheximide	Actinomycin D	[³H]Leucine	Inhibition
min	*μg/ml*	*μg/ml*	*fmol in 10 μg IRI*	%
90			518	0
	50		73	85.8
	100		57	89
		10	489	5.5
240			800	0
	50		54	93.2
	100		34	95
		10	109	86

In each experiment 8–10 explants were incubated at 37°C as described in Methods. Cycloheximide and actinomycin D was added at 0 time of incubation. Each measure is mean of triplicate determinations; individual values did not differ by more than 5%.

furthermore, that somatostatin, a substance that naturally occurs in the pancreas (8), is able to oppose the effect of both these agents.

The comparison of the effects of glucose or of the cAMP showed that in newborns, unlike in adults, theophylline and dbcAMP appeared to be more efficient than glucose in inducing insulin biosynthesis. Another approach is to compare our results to relevant data reported by separate investigators: maximal stimulation in newborns was here achieved by 11 mM glucose and represented a sixfold increase. Pipeleers et al. (50) estimated that 16.7 mM glucose is further effective and determines a 10-fold increase of insulin biosynthesis in isolated islets of adult rats. 10 mM theophylline induced a stimulation which represents a 4-fold increase in newborns whereas in adults, the effect of this agent does not exceed the control value by more than 2-fold without theophylline (23, 51); the increase by dbcAMP of [^{3}H]leucine incorporation into IRI in newborn is considerable, 6–24-fold the corresponding control and has to be compared to the only 2-fold increase obtained in adults (23, 51, 52). These results indicate how well developed in neonates is the ability of the endogenous cAMP system to act on insulin biosynthesis and the marked response of beta cell to dbcAMP. Furthermore, the greater effectiveness of theophylline may be related to the inhibition of the phosphodiesterase that was found more active in islets of neonates than in adults (53). From these studies it can be postulated that the cAMP-phosphodiesterase system plays a more important role in neonatal islet function than in adults.

Under the conditions of this investigation, it was possible to obtain quantitative data on insulin biosynthesis in the same model as previously used for studying insulin release (9) such that quantitative responses to different agents could be obtained in neonates. This is of great physiological interest since in the 3-day-old rat pancreas regulation of insulin biosynthesis and release occurs in a period of islet and beta cell growth (54) and functional differentiation. Indeed, insulin content on the 3rd (55) day after birth rises to twice the insulin content of the 1st day of life (55). In this respect two points are noteworthy. First, the lower limit of glucose required to induce insulin biosynthesis was below that required to elicit insulin release: 50% of the effect was observed with 4–5 and 7–8 mM glucose, respectively for biosynthesis and for release (9). Second, theophylline and dbcAMP, which did not act on insulin release at low glucose concentration (9), stimulated insulin biosynthesis effectively. It can be suggested that the large accumulation of insulin observed in neonates was partly related to the exquisite sensitivity of insulin biosynthesis to glucose and cAMP. From a teleological point of view, the constitution of high insulin stores during the

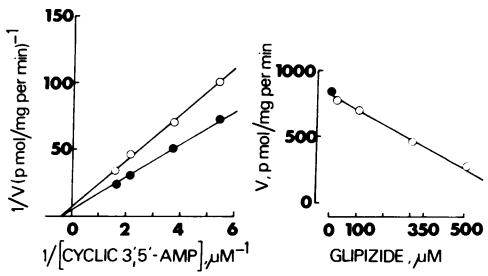

FIGURE 11 To the left, the cyclic nucleotide phosphodiesterase activity expressed from V (pmol of 3'5'-AMP split/mg protein per min), in isolated islets of 3-day-old rat pancreas is expressed as a function of cyclic 3'5'-AMP concentration with 500 μmol (●) or without (○) glipizide. Results are plotted in the double-reciprocal form of the Lineweaver-Burk plot. To the right, the effect of different concentrations of glipizide on the phosphodiesterase activity as measured by V (see above), are plotted as a function of glipizide concentration. The cAMP concentration was 25 nM. The enzyme preparation and the incubation procedure are described in Methods. Each point is the mean of triplicate determinations with an individual variability below 5%.

neonatal period is well fitted to the requirement of insulin for the disposal and storage of substrates when the organism is for the first time exposed to the challenge of discontinuous food supplies. The importance of normal insulin stores in neonates for normal growth is underlined by the fact that streptozotocin injection at birth, which depletes insulin stores, is still associated with a decrease of about 20% of the body weight at 21 days (56).

Although additional studies are required to determine the mechanism(s) of glucose and cAMP action on insulin biosynthesis, our experiments suggest that glucose did not only act on insulin biosynthesis solely through the cAMP system. The level of biosynthesis obtained with a high glucose concentration plus dbcAMP remained greater than that obtained with dbcAMP at low glucose concentration. This synergistic effect does not suggest a common pathway for glucose and cAMP in their action on insulin biosynthesis. Previous studies on the effect of glucose on insulin release demonstrated that glucose does not modify (11, 12, 57) or increase the cAMP levels of islets in the presence (58) or in the absence (33) of phosphodiesterase inhibitors. The fact that, in our experiments, glucose plus theophylline stimulated insulin biosynthesis synergistically would favor these hypotheses. However, up to now, the stimulatory effect of glucose on cAMP in islets has been described for 16.7–27.5 mM glucose, i.e., a higher concentration that is sufficient to induce insulin biosynthesis. On the other hand, the percentage of newly synthesized insulin is significantly different for glucose (54%) and for dbcAMP (89%) or theophylline (87%). This per-

centage was measured after a 4-h period because at that time the amount of radioactivity in proinsulin reaches a steady state between the formation of proinsulin and the conversion of proinsulin to insulin (see Table IV, [45]). The finding that dbcAMP and theophylline increased the percentage of [^{3}H]insulin suggests that these two drugs influence not only the proinsulin synthetic activity but also the process of conversion of proinsulin to insulin. However, although these agents did not modify the linearity of [^{3}H]leucine incorporation in total IRI, a relative increase in the incorporation of this amino acid into insulin pool might result from a decline in the biosynthetic rate for proinsulin during the latter half of the incubation period. Furthermore, dbcAMP and theophylline could also act on [^{3}H]leucine incorporation into total IRI (proinsulin + insulin) by modifyng the size of the amino acid precursor pool. Although our data did not allow us to localize precisely the mechanism(s) of glucose and cAMP action on insulin biosynthesis, the fact that actinomycin D inhibited glucose-induced insulin biosynthesis after a delay of at least 90 min is in agreement with the existence of glucose-dependent mechanisms at the transcriptional levels (36, 59) without excluding its effect at the translational levels (36, 60). On the other hand, time-course experiments of substances stimulating (theophylline, dbcAMP) or inhibiting (somatostatin, sulfonylureas) insulin biosynthesis demonstrated an early effect which suggests they are acting at the post-transcriptional levels.

Somatostatin is known as a potent inhibitor of glucagon release (61–62) and of both the acute and the chronic phase of insulin secretion (14–18). Because of its preferential action on glucagon (61–62), it has been found to be efficient in lowering blood glucose in experimental diabetes (15) and its use in human diabetes has been proposed. The present study shows that somatostatin was also able to inhibit glucose-induced insulin biosynthesis and release in isolated newborn rat pancreas. The doses that were effective (1–10 μg/ml) were similar to those used in the perfused rat pancreas to inhibit insulin release (14). Furthermore, unlike the contradictory results concerning the disappearance of the release-suppressing effect of somatostatin (14, 46, 48, 63) in isolated islets, the inhibition of insulin biosynthesis and the release-suppressing effect were still present in our isolated islets obtained after a brief exposure to low doses of collagenase. This observation suggests a direct effect of somatostatin on the beta cell. Previous studies on the rat pituitary have shown that somatostatin lowers cAMP levels (64). Even if similar effects on the endocrine pancreas could explain the inhibitory effect of somatostatin on glucose and theophylline action, it did not account for the inhibitory effect of this peptide on the dbcAMP-induced insulin biosynthesis and release. The fact that high Ca^{++} concentration reversed the inhibitory effect of somatostatin on glucose-induced insulin biosynthesis as well as release, favors the hypothesis that the mechanism of somatostatin inhibition may be involved with Ca^{++} inactivation or translocation at some step of the biosynthetic and secretory processes. However it has been shown that modifications in the extracellular concentrations of calcium which cause considerable changes in insulin release (65) did not modify insulin biosynthesis (60, 66). Whether the effect of somatostatin is coupled to intracellular modifications of Ca^{++} and/or cAMP remains to be determined. It is not possible on the basis of presently available data to conclude that somatostatin is a physiological regulator of insulin biosynthesis and release since it is likely that the concentrations of endogenous (pancreatic and/or plasmatic) somatostatin are much smaller than the doses used in the present experiments; however, such inhibitory effects on insulin biosynthesis have to be taken into consideration in the use of somatostatin in human diseases.

The inhibitory effect of glipizide and tolbutamide on the glucose-induced biosynthesis was in agreement with results obtained by others in isolated islets of adult rodents (24–27). This inhibitory effect was not reversed by an increase in the intracellular levels of cAMP obtained by theophylline or by dbcAMP. Moreover the stimulatory properties of sulfonylureas on insulin release (i.e. an inhibition of the phosphodiesterase activity which in turn should increase cAMP in isolated islets [20–22]) do not fit in with their inhibiting action on insulin biosynthesis, since a stimulation of the cAMP system causes a large increase of insulin biosynthesis (see above). An explanation for this discrepancy was that the doses of sulfonylureas that are required to be effective in inhibiting the phosphodiesterase activity in isolated islets were higher than the doses that are sufficient to inhibit the [^{3}H]leucine incorporation into IRI (Fig. 11). Therefore the action of these drugs on insulin biosynthesis must take place via non-cAMP mechanism or at a level beyond the cAMP system. Further investigations might suggest an interrelation between the inhibitory effect of sulfonylureas on insulin biosynthesis and the possible depression in the activity of beta cells which follows a long-term treatment of normal rats by these drugs (26). Indeed a previous report on human diabetics shows that the secondary failure after successful therapy with sulfonylureas is a relatively common occurrence (67). Moreover, the fact that sulfonylureas passed readily through the placenta (68) and were excreted by the mammary gland (69) provides additional reasons for avoiding their use during pregnancy and lactation for they may adversely affect the

islet function in inhibiting insulin biosynthesis during the perinatal period.

ACKNOWLEDGMENTS

We are indebted to Doctors D. F. Steiner (University of Chicago), J. Schlichtkrull (Novo Research Institut, Copenhagen) and J. E. Raynaud (Laboratoires Roussel, Paris) for supplying the rat proinsulin, the rat insulin and the synthetic somatostatin, to Dr. P. Freychet for his helpful comments concerning the manuscript and to Ms. N. Vauclin, C. Fessard, and F. Bagot for the skilled technical assistance. We thank D. Lhenry for her careful preparation of manuscript.

This work was supported by the Institut National de la Santé et de la Recherche Médicale, by the Centre National de la Recherche Scientifique (Equipe de Recherche Associée 494) and by grants from the délégation Générale à la Recherche Scientifique et Technique (74 7 0376) and the Fondation pour la Recherche Médicale Française.

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