Stimulation of H-3-Leucine Incorporation into the Proinsulin and Insulin Fraction of Isolated Pancreatic Mouse Islets in the Presence of Glucagon, Theophylline and Cyclic AMP

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SUMMARY

Incorporation of H-3-leucine into proinsulin and insulin and insulin secretion were determined in isolated pancreatic islets of mice. Batches of forty islets were incubated for three hours at 0, 100 or 300 mg. per cent glucose alone or in the presence of glucagon, theophylline or cyclic AMP. Proinsulin and insulin were separated by gel filtration on columns of Sephadex G50 fine in 1 M. acetic acid.

In the absence of glucose, no stimulation of insulin biosynthesis as judged from the incorporation of H-3-leucine was observed with glucagon, cyclic AMP or dibutyryl cyclic AMP; insulin secretion was not significantly stimulated. At 100 and 300 mg. per cent glucose, both leucine incorporation and insulin secretion were potentiated by the substances tested; the effect of glucagon was pronounced mainly at high concentrations of glucose. However, leucine incorporation and insulin secretion were not stimulated to the same extent in all cases.

Biosynthesis of insulin thus appears to be not always directly linked to insulin secretion. It is suggested that glucagon and the adenylcyclase system, besides regulating insulin secretion, are involved in insulin biosynthesis. DIABETES *22*:433-41, June, 1973.

Glucagon is a potent stimulator of insulin secretion.[16] It is known as an activator of adenylcyclase[23] and was found to increase levels of cyclic AMP within isolated pancreatic islets.[26] On the other hand, theophylline, which raises tissue concentrations of cyclic AMP by inhibiting phosphodiesterase,[7] as well as cyclic AMP itself may also increase insulin release, suggesting an involvement of the adenylcyclase system in the mechanism of insulin secretion.[12,22,27]

The purpose of this study is to examine the actions of glucagon, theophylline and cyclic AMP at various glucose concentrations on biosynthesis of insulin in comparison to insulin secretion. Incorporation of H-3-leucine into the proinsulin and insulin fraction of isolated pancreatic islets was employed as a parameter of insulin synthesis.

MATERIAL AND METHODS

Animals. Male Swiss albino mice of the strain NMRI weighing about 30 gm. were used as the source of pancreatic islets. The animals had free access to food (mouse pellets Altromin, Altrogge, Lippe, Germany) and water.

Isolation of pancreatic islets. Eight to ten mice were killed by decapitation, and the pancreases were removed and cut up with scissors in chilled Krebs-Ringer bicarbonate buffer, pH 7.4. Digestion using 40 mg. collagenase (Serva, Heidelberg, Germany) was carried out under constant stirring at 37° C. for seventeen to twenty-five minutes.[9] After three washings in chilled buffer and sedimentation procedures, intact and well separated islets of comparable size were collected under a stereomicroscope with a Pasteur pipette. About forty islets were obtained per mouse pancreas.

Incubations. Batches of forty islets were incubated in the presence of L-leucine-4,5-H-3 (50 μCi., 20 Ci./mmol, Radiochemical Center, Amersham) in 1 ml. of Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with bovine serum albumin (2 mg. per milliliter, Behringwerke, Marburg), a protease inhibitor (1,000 KIU. per milliliter Trasylol, Bayer, Leverkusen) and sev-

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Accepted for publication December 1, 1972.

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enteen naturally occurring amino acids (20 μg. per milliliter of each amino acid, leucine excluded). Glucagon (10 μg. per milliliter, Novo, Copenhagen), theophylline (10 mM., Merck, Darmstadt), cyclic AMP (cAMP, 10 mM., Serva, Heidelberg) or dibutyryl cyclic AMP (DAMP, 10 mM., Boehringer, Mannheim) was added. As for the porcine glucagon preparation used, a contamination of 0.9 mg. insulin per gram glucagon was determined in our laboratory. Glucose was present in the incubation media at concentrations of 0, 100 or 300 mg. per cent. Incubations were carried out under a constant atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide (v/v) for three hours, using a metabolic shaker at 37° C.

In three control experiments pancreatic acinar tissue without islets was incubated at 0, 100 or 300 mg. per cent glucose before fractionation on the Sephadex G 50 fine column.

Insulin secretion. Insulin secretion was estimated hourly in 2 x 0.01 ml. of the incubation media, using the radioimmunoassay method of Melani et al.[13] For this immunoassay, porcine insulin (obtained from Farbwerke Hoechst AG, Germany, 1 mg. ≙ 27 U.) was used as standard and for labeling as well as for production of antibodies in guinea pigs.

Insulin synthesis. After three hours of incubation, islets and incubation media were not separated from each other but examined together in order to estimate the total synthetic capacity. Ice-cold trichloroacetic acid was added to the samples, yielding a final concentration of 10 per cent. Afterward, ultrasonic disintegration was carried out for fifteen seconds. The precipitate obtained was washed with 5 per cent trichloroacetic acid and dissolved in 0.5 ml. of acetic acid (1 M). Quantitative solution of proinsulin and insulin in 1 M. acetic acid following treatment with trichloroacetic acid was established in five control experiments: Albumin, J-125-proinsulin, nonradioactive insulin and H-3-leucine were mixed in vitro and both incubated and treated exactly as the islet samples, including separation on the Sephadex column. In these experiments it was also shown that only negligible small amounts of H-3-leucine were attached to these proteins in vitro. Extraction procedures with acid ethanol were avoided in order to obtain information, at least limited, about protein synthesis in general. Separation of the islet proteins dissolved in 0.5 ml. of 1 M. acetic acid was carried out on a Sephadex G 50 fine column, 1.2 x 55 cm. The column had been equilibrated with 1 M. acetic acid and calibrated with albumin, proinsulin, insulin, glucagon and leucine. Elution was done with 1 M acetic acid at a flow rate of 10 ml. per hour; fractions of 1 ml. were collected. UV absorption was recorded continuously by an Uvicord apparatus (LKB-produkter, Sweden), and 0.1 ml. of the fractions was assayed for radioactivity in a liquid scintillation counter (Tricarb, Packard), using Bray's scintillator liquid.[2] Immunologically measurable insulin was determined in each fraction. The radioactivity peaks were pooled and lyophilized, and radioactivity as well as immunoassayable insulin were determined again in aliquots of the pooled peaks. In four experiments immunoreactivity for glucagon was determined in the fractions and in the pooled peaks, using a rabbit antibody against porcine glucagon and dextran-coated charcoal for the separation of the free and bound hormone. The proinsulin and insulin nature of peaks P and I, respectively, (see figures) was further secured by polyacrylamide gel electrophoresis, pH 8.6, 15 per cent gel.

The identity of peak P with proinsulin was also demonstrated, according to Steiner and Oyer,[21] by treatment of this material with trypsin (p-tosyl-L-phenylalanine chlormethylketone-trypsin, obtained from Serva, Heidelberg, Germany, 100 μg., ten minutes, 37° C.). After treatment of the peak P with trypsin and rechromatography on the Sephadex G 50 fine column, most of the radioactive material was then eluted in the position of insulin, giving strong reactions with antibodies to insulin. On the other hand, no tryptic conversion to insulin was observed when the first and the intermediate peaks (see tables 1 and 2) were studied in the same manner.

Biological activity. Norepinephrine-induced lipolysis in adipose tissue of rats was determined in the presence of the individual islet protein fractions according to the method of Schleyer et al.,[19] using rat insulin as reference substance for inhibition of lipolysis.

Calculations. Due to the large number and careful selection of the islets in each sample, no differences were found in the insulin content among the batches of forty islets. Thus, direct comparison was made between the amounts of radioactivity incorporated into the different peaks of the islet proteins as well as between the secretion data, based on equal insulin content and equal number of islets. For statistical evaluations, only data of identical experimental setups were taken for comparing the actions of the various test substances with those of glucose alone (table 2). Student's *t* test was used for carrying out the calculations of significance.

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TABLE 1

Radioactivity (total counts per minute, mean values ± S.E.M.) incorporated into four fractions of islet proteins at various glucose concentrations. Forty isolated pancreatic mouse islets were incubated for three hours in the presence of H-3-leucine at 0, 100 or 300 mg. per cent glucose. After incubation, trichloroacetic acid-precipitable islet proteins were separated on a Sephadex G 50 fine column. Total counts per minute were determined in the pooled peaks. n = number of experiments.

	First peak	Intermediate fraction	Proinsulin peak	Insulin peak	n
No Glucose	37,850±5,130	8,570±1,280	2,850±440	2,410±370	6
Significance 0 vs. 100 mg. per cent glucose			$p < 0.0025$	$p < 0.005$	
100 mg. per cent glucose	43,200±5,280	10,340±2,890	9,990±1,400	8,630±1,520	10
Significance 100 vs. 300 mg. per cent glucose	$p < 0.0025$		$p < 0.0005$	$p < 0.0005$	
300 mg. per cent glucose	82,180±8,600	11,830±3,530	29,620±2,460	30,760±2,990	12

TABLE 2

Radioactivity (total counts per minute, mean values ± S.E.M.) incorporated into four fractions of islet proteins. Forty isolated pancreatic mouse islets were incubated for three hours in the presence of H-3-leucine at glucose concentrations of 0, 100 and 300 mg. per cent. Glucagon (10 μg. per milliliter), theophylline (10 mM.), cAMP (10 mM.) or dibutyryl cyclic AMP (DAMP, 10 mM.) was added to the incubation media. After incubations, trichloroacetic acid-precipitable islet proteins were separated on a Sephadex G 50 fine column. Total counts per minute were determined in the pooled peaks. For statistical evaluations using Student's *t* test, only data of identical experimental setups were taken for comparing the actions of the individual test substances with those of glucose alone. Significant differences are indicated by asterisks: * $p < 0.05$; ** $p < 0.025$; *** $p < 0.01$. n = number of experiments.

	First peak	Intermediate fraction	Proinsulin peak	Insulin peak	n
No glucose	38,160 ± 6,120	9,020 ± 1,310	2,870 ± 470	2,320 ± 390	5
+ glucagon	31,540 ± 3,120	8,760 ± 930	2,220 ± 410	2,270 ± 420	
100 mg. per cent glucose	45,940 ± 5,010	12,860 ± 4,070	9,210 ± 1,490	8,280 ± 1,690	8
+ glucagon	48,980 ± 5,760	13,850 ± 5,040	12,320 ± 1,690*	9,190 ± 1,320	
300 mg. per cent glucose	79,170 ± 9,660	10,100 ± 2,650	26,790 ± 2,200	30,730 ± 2,590	8
+ glucagon	94,070 ± 10,180	12,260 ± 2,940	44,670 ± 7,490***	42,040 ± 7,440**	
No glucose	38,160 ± 6,120	9,020 ± 1,310	2,870 ± 470	2,320 ± 390	5
+ theophylline	32,050 ± 5,510	11,310 ± 1,280	3,760 ± 790	3,230 ± 720	
100 mg. per cent glucose	44,510 ± 5,130	7,600 ± 1,980	11,400 ± 2,740	9,150 ± 1,830	8
+ theophylline	52,860 ± 8,630	7,420 ± 3,930	24,130 ± 4,070***	17,090 ± 2,700***	
300 mg. per cent glucose	89,060 ± 14,220	13,070 ± 3,590	30,460 ± 2,920	32,960 ± 4,050	8
+ theophylline	73,380 ± 13,270	16,630 ± 2,290	41,360 ± 6,790*	31,730 ± 4,900	
No glucose	37,850 ± 5,130	8,570 ± 1,280	2,850 ± 440	2,410 ± 370	6
+ cAMP	21,340 ± 2,410***	6,750 ± 980	2,710 ± 360	2,480 ± 320	
100 mg. per cent glucose	43,110 ± 5,030	5,470 ± 1,630	10,490 ± 2,010	10,850 ± 1,910	7
+ cAMP	55,080 ± 7,670	8,670 ± 1,980	15,870 ± 2,860*	14,100 ± 2,630	
300 mg. per cent glucose	85,320 ± 13,120	5,290 ± 830	32,110 ± 3,030	30,570 ± 2,870	7
+ cAMP	97,540 ± 18,620	9,970 ± 1,790*	40,810 ± 3,150*	36,040 ± 2,760	
No glucose	37,850 ± 5,130	8,570 ± 1,280	2,850 ± 440	2,410 ± 370	6
+ DAMP	32,910 ± 5,260	6,430 ± 910	3,290 ± 480	2,210 ± 320	
100 mg. per cent glucose	46,430 ± 5,120	9,880 ± 1,060	11,420 ± 2,880	9,320 ± 1,990	6
+ DAMP	67,630 ± 18,710	14,440 ± 3,560	26,750 ± 4,640***	24,460 ± 4,310***	
300 mg. per cent glucose	77,100 ± 20,040	12,630 ± 3,916	30,590 ± 2,820	31,570 ± 2,480	6
+ DAMP	84,430 ± 15,010	10,430 ± 5,100	44,990 ± 3,630***	39,570 ± 3,010**	

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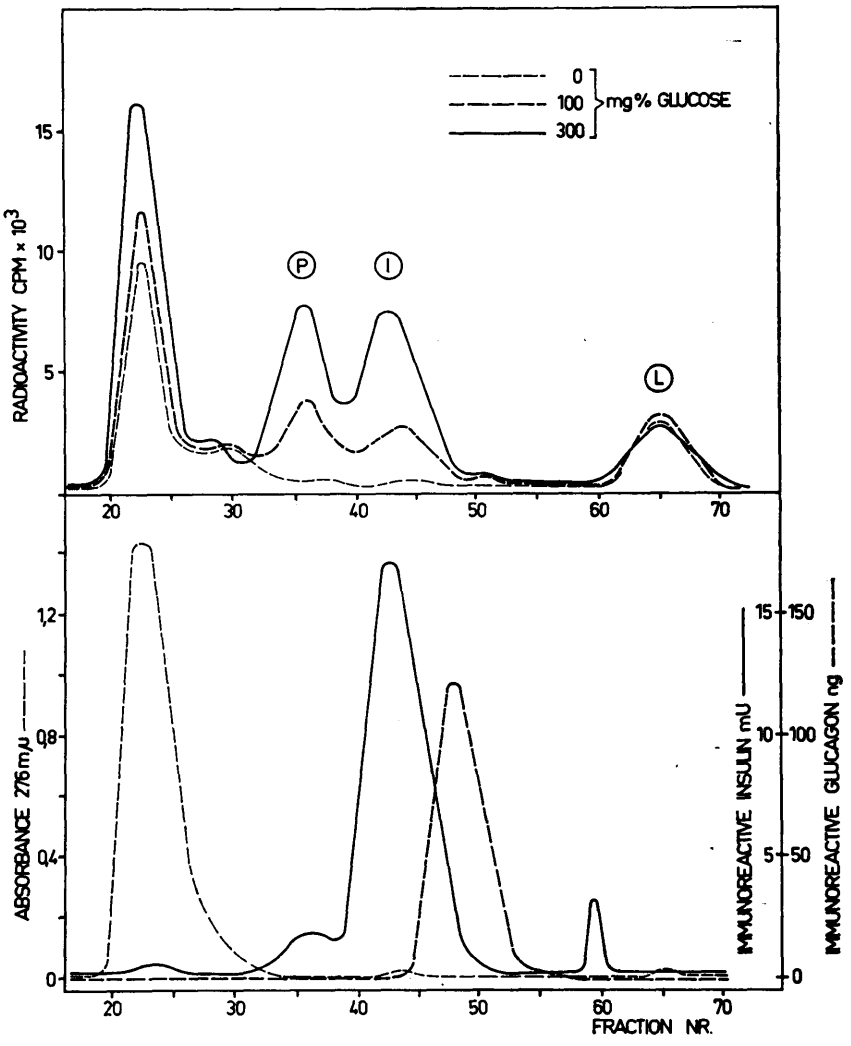

FIGURE 1

Effect of glucose concentration (0, 100 and 300 mg. per cent) on incorporation of H-3-leucine into the proteins of isolated pancreatic mouse islets. Batches of forty islets were incubated for three hours in the presence of H-3-leucine. After precipitation with trichloroacetic acid, the islet proteins were fractionated on a Sephadex G 50 fine column, using 1 M. acetic acid as eluent. UV absorption, radioactivity and immunoreactivity for insulin and glucagon were determined in the fractions. The first peak eluting with the void volume represents islet proteins excluded from the gel. P = proinsulin, I = insulin, L = free H-3-leucine. Mean curves are shown, derived from six to twelve experiments each. For statistics, see table 1.

RESULTS

Figure 1 shows elution patterns of islet proteins from the Sephadex column. The fractions of the insulin peaks (I) reacted strongly with insulin antibodies in our immunoassay system. Immunoreactivity for insulin was also established for the proinsulin peak (P). In the first peak eluting from the Sephadex column, on the other hand, no immunoreactive material was found in about two-thirds of our experiments; in the last third, very weak reactions were observed. In about 10 per cent of the experiments, weak immunoreactivity for insulin was also observed in fractions eluting between insulin and the salt peak. No differences in total insulin content were found between the individual batches of islets. It amounted to 64.7 ± 2.1 mU. per forty islets (mean ± S.E.M.). No definite immunoreactivity for glucagon was found in the first four radioactivity peaks. On the other hand, almost no radioactivity was incorporated into the glucagon fractions which were eluting after the insulin peak and identified by their strong reaction with glucagon antibodies.

With rising concentrations of glucose, *incorporation of H-3-leucine* into proinsulin and insulin was increased likewise (figure 1 and table 1). Addition of glucagon, cAMP and DAMP in the absence of glucose effected no stimulation of leucine incorporation into proinsulin and insulin; theophylline, however, induced small positive effects (figure 2). At 100 and 300 mg. per cent glucose (figures 3 and 4), leucine incorporation into proinsulin and insulin was increased in the presence of the agents tested as compared with glucose alone. With glucagon, this increase was more pronounced at 300 mg. per cent glucose than at 100 mg. per cent glucose. No major changes in the ratio of the amounts of radio-

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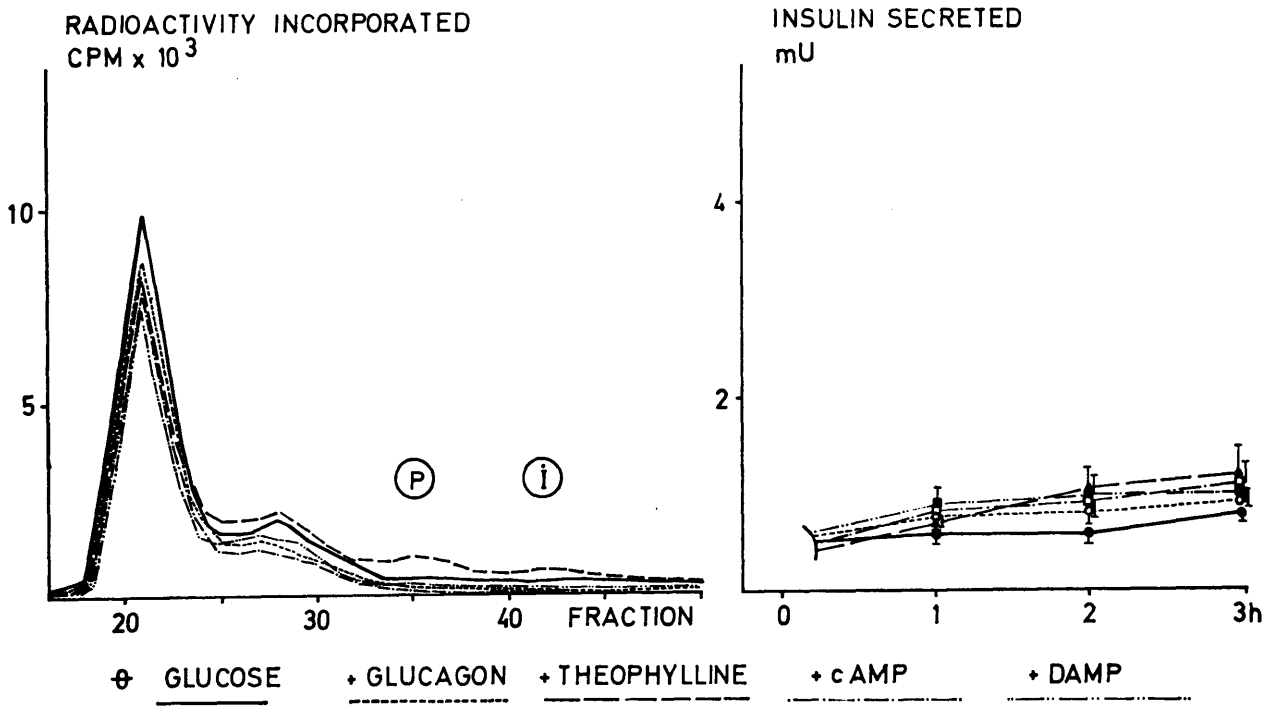

FIG. 2. Incorporation of H-3-leucine into the proinsulin (P) and insulin (I) fraction of forty isolated pancreatic mouse islets after incubation of the islets for three hours in the presence of H-3-leucine. Glucagon (10 μg. per milliliter), theophylline (10 mM.), cAMP (10 mM.) or dibutyryl cyclic AMP (DAMP, 10 mM.) was added to the incubation media. Figures 2, 3 and 4 show the results obtained at glucose concentrations of 0, 100 and 300 mg. per cent, respectively. After precipitation with trichloroacetic acid, the islet proteins were fractionated on a Sephadex G 50 fine column, using 1 M. acetic acid as eluent. Direct comparison between the individual radioactivity curves was based on the insulin content of the batches of forty islets. Each curve obtained in presence of the test substances represents the mean of five to eight experiments. The control curves obtained in absence of the test substances are mean curves derived from six to twelve experiments. As for the statistical evaluations of the incorporation data, see table 2. Insulin secretion into the media during the three hours of incubation is shown in the right part of the figures (mean ± S.E.M.). Significant differences between the insulin secretion occurring in the absence and presence of the test substances are indicated for the three hour-values.

activity incorporated into the proinsulin and insulin peaks were observed at different glucose concentrations. This ratio was also the same when glucagon, cAMP or DAMP had been added to the media (tables 1 and 2).

Incorporation of H-3-leucine into the other islet proteins excluded from the Sephadex gel and eluting with the first peak from the column also showed a dependence on the concentration of glucose. When the glucose concentration was raised from 100 to 300 mg. per cent, the radioactivity of this first peak was significantly increased (figure 1 and table 1). Glucagon, theophylline, cAMP and DAMP decreased leucine incorporation into the first peak when glucose was absent. At 100 and 300 mg. per cent glucose, on the other hand, radioactivity of the first peak was generally increased in the presence of the substances tested; these increases of the first peak, however, were relatively less marked than the concurrent increases of the proinsulin and insulin peaks and not significant (table 2).

An intermediate radioactivity fraction of variable magnitude eluting between the first protein peak and the proinsulin peak was observed in our experiments. It did not react with antibodies to either insulin or glucagon. No clear-cut dependence on experimental conditions was apparent.

In three control experiments, trichloroacetic acid-precipitable material stemming from exocrine pancreatic tissue without islets was fractionated on the Sephadex column following incubations in the presence of H-3-leucine at 0, 100 and 300 mg. per cent glucose. Radioactivity was found incorporated into material eluting in position of both the first peak and the intermediate peak, essentially irrespective of the glucose concentration present in the incubation media.

Regarding *biological activity,* in contrast to the in-

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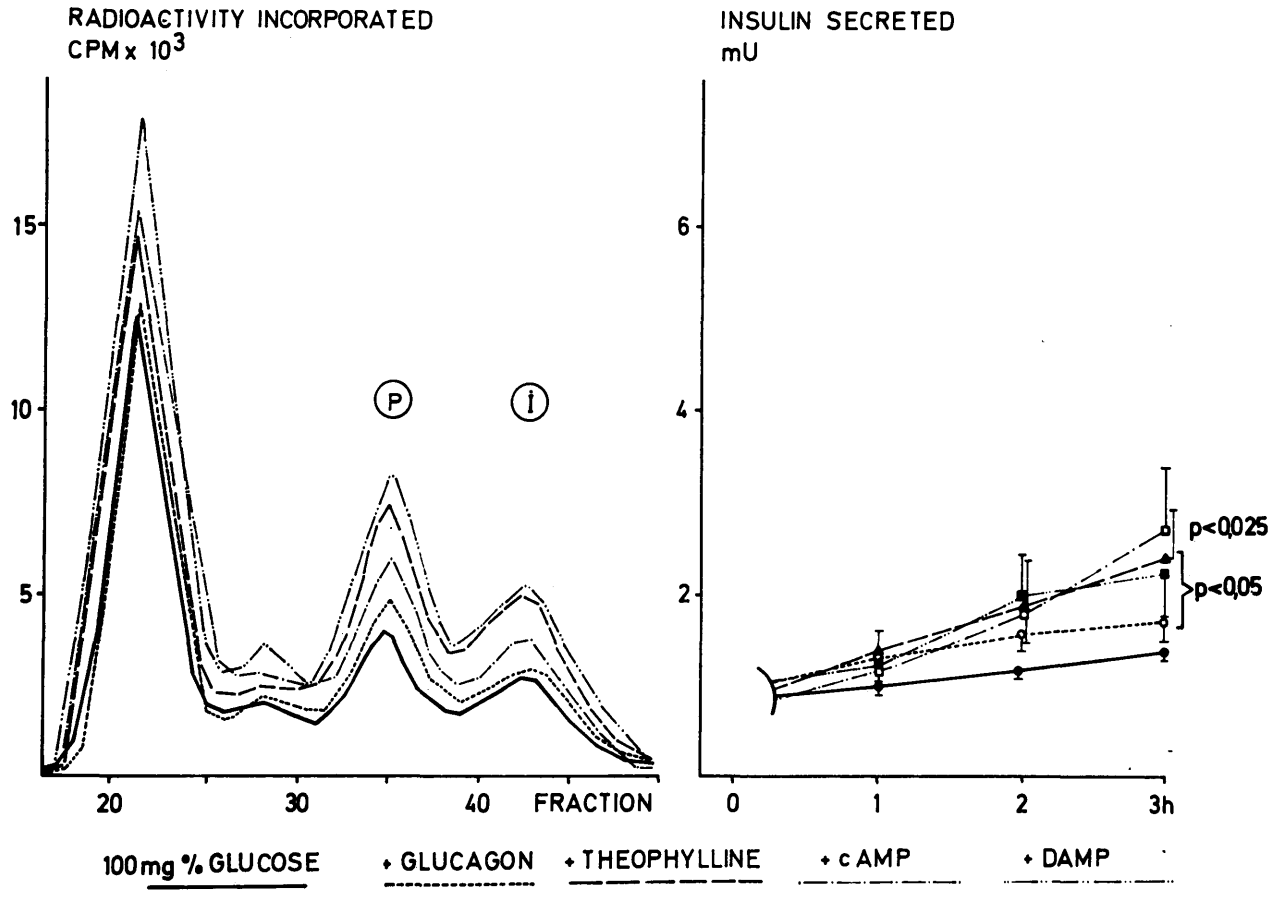

FIG. 3. Results of experiments conducted with a glucose concentration of 100 mg. per cent (see legend to figure 2).

hibitory action of standard insulin, peak I and peak P on norepinephrine-induced lipolysis in adipose tissue of rats,[5] no changes were observed in the presence of the first peak or the intermediate fraction.

Insulin secretion was increased by glucagon, theophylline, cAMP and DAMP (figures 2 to 4). In the absence of glucose, however, stimulation was small and non-significant. At 100 and 300 mg. per cent glucose, a potentiation of glucose-induced insulin secretion was observed; in the presence of theophylline, cAMP and DAMP, this potentiation was more than twice as high as in the presence of glucagon, unlike the findings concerning incorporation of H-3-leucine.

DISCUSSION

It was suggested by Samols et al. that glucagon is involved in the regulation of insulin secretion.[17] As shown in this paper, glucagon as well as the other substances tested stimulate not only secretion of insulin but also the incorporation of leucine into proinsulin and insulin. Increased incorporation of H-3-leucine into proteins in the presence of cyclic AMP was also demonstrated by Farese with adrenal sections of rats.[4] Whether enhanced transfer of amino acids from aminoacyl-tRNA to proteins and changes in calcium distribution (as discussed by Farese) may account for our results cannot be decided from this study. However, it was obvious that glucose played an important role in the stimulatory mechanism of the agents tested: Increases of leucine incorporation due to theophylline, cAMP and DAMP were found at both glucose concentrations employed (see also the study by Tanese et al.[24]); the potentiating action of glucagon was established predominantly at 300 mg. per cent glucose. On the other hand, no significant stimulatory actions of glucagon, theophylline, cAMP or DAMP were observed in the absence of glucose. Hence, it might be concluded that these substances can hardly act per se on insulin biosynthesis but require the presence of another substance, for example glucose. Similar conclusions have been offered previously with respect

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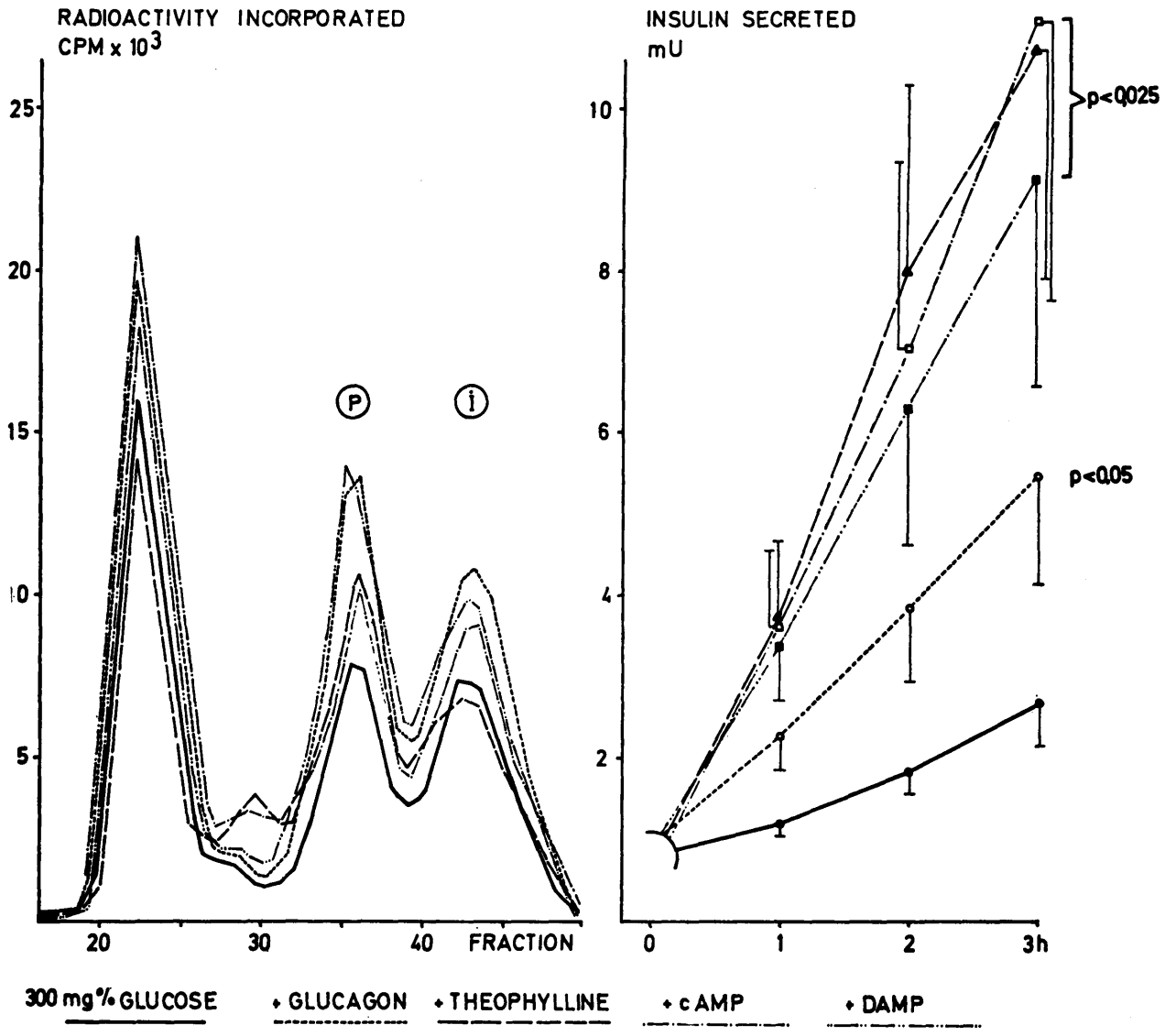

FIG. 4. Results of experiments conducted with a glucose concentration of 300 mg. per cent (see legend to figure 2).

to insulin secretion.[3,27] Baird reported that the stimulatory action of glucagon on proinsulin synthesis was more marked at low glucose than at high concentrations of glucose.[1] Differences exist, however, between the design of his experiments and ours.

An inhibitory action of cyclic AMP or glucagon on leucine incorporation, as described by Pryor and Berthet[15] and Hait et al.[6] in tissue of liver and myocardium, respectively, was observed in our studies performed with pancreatic islets, only in the absence of glucose. This inhibition was established mainly for the first protein peak. Procedures for extraction of insulin were not included in our experiments. Furthermore, it was shown that the peak eluting with the void volume of the Sephadex column contained essentially no material reacting with antibodies to insulin or glucagon. Proteins of this first peak were biologically inactive and not convertible to insulin by treatment with trypsin. Therefore, the amount of radioactivity incorporated into the first peak might serve—with restrictions—as a parameter of protein synthesis in general. Judged by the data listed in table 2 it appears that biosynthesis of proinsulin and insulin was stimulated by the substances tested much more than it was protein synthesis in general.

Concerning the intermediate fraction, no definite conclusions can be drawn from our studies. It was shown

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that the appearance of a peak in this position may result from contaminations of the islet cell preparation with exocrine pancreatic tissue. However, there was also some evidence that acinar tissue was not always the only source of radioactive material eluting in this position (see also the study by Poffenbarger et al.[14]).

The material which eluted from the Sephadex column in front of the salt peak reacting with insulin antibodies appeared to be of lower molecular weight and not identical with the "mini" insulin described by Lázaro.[10] Insulin fragments might have accounted for these inconsistent findings.

As regards absolute rates of insulin biosynthesis and protein synthesis in general, final interpretation of our labeling experiments must await determination of intracellular leucine pools. Possible changes in leucine oxidation as shown at different glucose concentrations by Hellman et al.[8] should be considered, too.

The essentially unchanged ratio of the amounts of radioactivity incorporated into proinsulin and insulin suggests that conversion of proinsulin to insulin per se is not altered by glucose or the substances concerned.[11]

Insulin secretion and incorporation of leucine were not stimulated to the same extent by the agents tested; compared with the actions of theophylline and cAMP as well as DAMP, glucagon only moderately potentiated insulin secretion due to 300 mg. per cent glucose. Incorporation of leucine, on the other hand, was markedly increased under the same conditions. Therefore, it seems unlikely that increased insulin biosynthesis was merely a consequence of enhanced insulin secretion. An indirect argument supporting this notion can be seen in the findings that sulfonylureas failed to increase[20,25] or inhibited[18] leucine incorporation in spite of stimulatory actions on insulin secretion. Obviously insulin secretion is not necessarily connected with insulin synthesis.

In summary it is concluded that glucagon and the adenylcyclase system, beside regulating insulin secretion, are involved in the regulation of insulin biosynthesis.

ACKNOWLEDGMENT

We wish to thank Dr. G. Hiller and Dr. J. D. Faulhaber, Center of Internal Medicine and Pediatrics, University of Ulm, for carrying out the biological tests of the protein fractions. Excellent technical assistance was provided by Miss E. Groner and Miss Ch. Unglert.

This study was supported by Deutsche Forschungsgemeinschaft Bonn-Bad Godesberg, SFB 87 Ulm, Proj. 11. From 1970 to 1972, Dr. Schatz was Fellow of the Alexander von Humboldt-Foundation, on leave from the 2nd Medical Department, University of Vienna, Austria.

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