J. Embryol. exp. Morph. Vol. 28, 3, pp. 607–614, 1972
Printed in Great Britain

Insulin and glucagon release from the isolated pancreas of foetal and newborn mice

By ÅKE LERNMARK[1] AND BRITT INGER WENNGREN[1]
From the Department of Histology, University of Umeå, Sweden

SUMMARY

The simultaneous release of insulin and glucagon was studied with isolated pancreas preparations from foetal and newborn mice. Glucose, alone or in combination with arginine, did not affect immunoreactive insulin (IRI) of glucagon-like immunoreactivity (GLI) release from the pancreases of 18-day-old foetal mice. However, on the first postnatal day, glucose stimulated the release of IRI and, in the presence of arginine, depressed that of GLI. It is concluded that the systems for recognition of glucose in mouse pancreatic α_2-cells as well as those in the β-cells do not mature until after birth.

INTRODUCTION

Maturation of the insulin-releasing mechanism in foetal and newborn animals has been studied in several species. It seems to be established that glucose is unable to promote insulin release in several foetal mammals although glucagon and leucine are effective insulin secretagogues (see Grodsky, 1970). The fact that glucagon is able to stimulate insulin release from foetal β-cells (Milner, 1969; Milner, Barson & Ashworth, 1971; Espinosa de Los Monteros, Driscoll & Steinke, 1970) raises the following question: when do the glucagon-producing α_2-cells acquire functional competence? Is endogenous glucagon of significance for the release of insulin in response to arginine in foetal and newborn mice?

MATERIALS AND METHODS

Adult mice were obtained from a local colony carrying the gene *ob* (Hellman, 1965). The homozygous occurrence of this gene gives rise to a syndrome of obesity and hyperglycemia (Ingalls, Dickie & Snell, 1950). However, in the present study only mice of normal phenotype were used. Female mice were caged with males for 2 days. The second day was counted as the first day of the pregnancies that resulted. The length of gestation in our colony is 22–23 days. All animals were fed *ad libitum* and were killed by decapitation. The pancreatic glands of 18- and 21-day-old foetuses and of 1- and 3-day-old mice were rapidly excised under a stereomicroscope.

[1] *Authors' address:* Department of Histology, University of Umeå, S-901 87 Umeå 6, Sweden.

Extraction of pancreas

The pancreases from all foetuses (5–6) of each pregnant mouse were immediately dropped into isopentane chilled to its freezing-point (−160 °C) by liquid nitrogen. After freeze-drying, the specimens were weighed and extracted by the acid-ethanol method of Kenny (1955). The proteins precipitated with ethanol-ether were freeze-dried. Appropriate dilutions of the freeze-dried precipitate were assayed for GLI (glucagon-like immunoreactivity). When [^{125}I]glucagon was added to samples of the mouse-pancreas extract, 20–30 % was recovered in the extraction procedure.

For determination of pancreatic IRI (immunoreactive insulin), aliquots of the above mentioned acid-ethanol extract were transferred to polyethylene microtubes and protein was precipitated by the addition of acetone. The precipitate was collected by centrifugation, washed with 2 ml of acetone and centrifuged once again. The supernatant was discarded and the precipitate freeze-dried. Dilutions of the freeze-dried extracts were assayed for IRI. When [^{125}I]insulin was added to samples of the mouse pancreatic extract, 60–70 % was recovered in the extraction procedure.

Incubation procedure

Whole pancreatic glands were incubated *in vitro* using the method of Coore & Randle (1964) and the amounts of GLI and IRI released into the medium were measured by radioimmunoassay (see below). The basal incubation medium consisted of Krebs–Ringer bicarbonate (KRB) medium (Umbreit, Burris & Stauffer, 1964) supplemented with 500 Kallikrein Inactivator Units (KIU) of Trasylol ® and 5 mg human serum albumin per ml. The KRB media were equilibrated with 95 % O_2 and 5 % CO_2. All incubations were performed at 37 °C with shaking (120 strokes/min with an amplitude of 1·25 cm). Each incubation vessel contained 5–8 pancreases from 18-day-old foetuses, 3–5 pancreases from 1-day-old mice or 2 pancreases from 3-day-old mice. The total wet weight of pancreas included in each vessel was 5–11 mg. A preincubation period of 30 min was followed by five successive incubation periods, each of 30 min duration: (1) medium without glucose, (2) medium with 17 mM glucose, (3) medium without glucose, (4) medium with 5 mM L-arginine and (5) medium with 5 mM L-arginine plus 17 mM glucose. After incubation, the pancreatic glands were quickly removed from the incubation vessels and weighed. The incubation media were immediately frozen and stored at −90 °C until assayed for GLI and IRI within 3 weeks.

Radioimmunological determinations

Glucagon-like immunoreactivity (GLI) was measured in triplicate for the standards and in duplicate for the unknown samples by the procedure of Heding (1970). Anti-glucagon serum (Batch K 37) and [^{125}I]glucagon were kindly donated by L. G. Heding, Novo Research Institute, Copenhagen, Denmark.

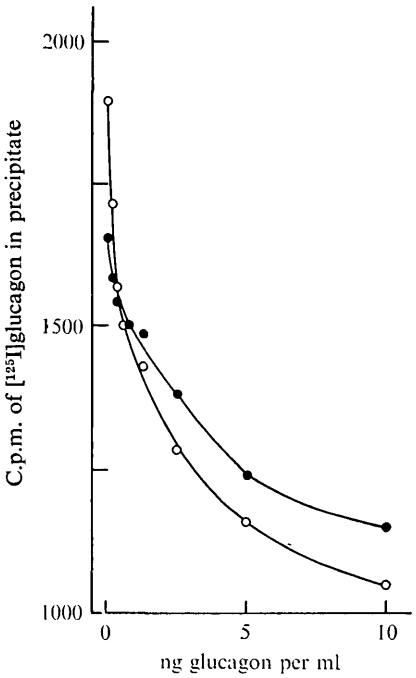

Fig. 1

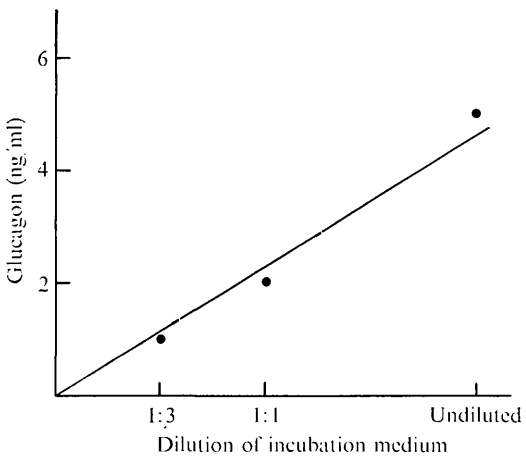

Fig. 2

Fig. 1. Difference between porcine glucagon standard curves prepared in 40 mM phosphate buffer, pH 7·4, containing 6 mg/ml NaCl (●—●) or in Krebs–Ringer bicarbonate buffer (○—○). In addition, all buffers were supplemented with 500 KIU/ml and 5 mg/ml human serum albumin.

Fig. 2. Effect of dilution of the incubation medium on the amounts of glucagon-like immunoreactivity (GLI) measured after incubating pancreatic glands of five 18-day-old foetal mice for 39 min in the presence of 17 mM glucose.

To determine the GLI release *in vitro*, all incubation media and the standards (crystalline porcine glucagon) had to be assayed directly in the KRB medium. The crystalline human serum albumin (AB Kabi, Stockholm, Sweden) used was found not to degrade the [^{125}I]glucagon (Heding, personal communication). The difference between standard curves for crystalline porcine glucagon prepared in 0·04 M phosphate buffer, pH 7·4 (Heding, 1970), or in basal KRB medium, both containing 500 KIU/ml Trasylol, is shown in Fig. 1. The amounts of GLI measured when the incubation media were serially diluted in the KRB medium were exactly proportional to the degree of dilution (Fig. 2).

Immunoreactive insulin (IRI) was determined in duplicate on each sample using the radioimmunoassay technique of Heding (1966). Crystalline mouse insulin was used as standard.

Table 1. *Immunoreactive insulin (IRI) and glucagon-like immunoreactivity (GLI) concentrations (ng/mg dry weight pancreas) in foetal and newborn mouse pancreas*

(Number of animals studied is given within parentheses. Mean values ± S.E.M.)

Age (days)	Pancreatic content (ng/mg dry weight) of IRI	GLI
Foetal age		
18	201 ± 40 (7)	0·8 ± 0·3 (5)
21	156 ± 90 (7)	2·5 ± 0·5 (6)
Postnatal age		
1	387 ± 39 (5)	5·6 ± 0·6 (6)

Table 2. *Effects of glucose on the amounts of immunoreactive insulin (IRI) and glucagon-like immunoreactivity (GLI) released (ng/mg wet weight pancreas and 30 min) from the pancreases of foetal and newborn mice*

(The figures within parentheses denote the number of experiments; each including several pancreatic glands (2–8) per incubation vessel. Mean values ± S.E.M.)

Age (days)		Glucose concentration (mM) 0	17	Mean difference
Foetal age				
18	GLI	0·38 ± 0·09 (7)	0·47 ± 0·08 (7)	0·09 ± 0·06
	IRI	0·74 ± 0·28 (7)	1·26 ± 0·33 (7)	0·52 ± 0·25
Postnatal age				
1	GLI	0·16 ± 0·02 (16)	0·18 ± 0·03 (16)	0·02 ± 0·02
	IRI	1·01 ± 0·18 (19)	1·58 ± 0·26 (19)	0·57 ± 0·14*
3	GLI	0·31 ± 0·06 (11)	0·37 ± 0·07 (11)	0·06 + 0·09
	IRI	1·03 ± 0·10 (11)	1·37 ± 0·14 (11)	0·34 ± 0·10†

* $P < 0{\cdot}001$. † $P < 0{\cdot}01$.

Statistical evaluation

Student's *t* test was used for comparison of the pancreatic contents of insulin and glucagon. Effects observed in the *in vitro* experiments were calculated from the mean difference between control and test incubations in each series of independent experiments.

RESULTS

Pancreatic concentrations of glucagon and insulin

The pancreatic concentrations of extractable immunoreactive glucagon and insulin in 16- to 21-day-old foetal and 1-day-old newborn mice are shown in Table 1. The concentration of IRI was almost twice as high in the 1-day-old mice

Table 3. *Effects of arginine and glucose on the amounts of immunoreactive insulin (IRI) and glucagon-like immunoreactivity (GLI) (ng/mg wet weight pancreas and 30 min) from the pancreases of foetal and newborn mice*

(The figures within parentheses denote the number of experiments; each including several pancreatic glands (2–8) per incubation vessel. Mean values ± S.E.M.)

Age (days)	Glucose (mM)... / Arginine (mM)...	0 / 5	17 / 5	Mean difference
Foetal age				
18	GLI	0·50 ± 0·18 (7)	0·28 ± 0·04 (7)	−0·22 ± 0·18
	IRI	0·55 ± 0·12 (7)	1·83 ± 0·64 (7)	1·28 ± 0·56
Postnatal age				
1	GLI	0·20 ± 0·03 (16)	0·13 ± 0·02 (16)	−0·07 ± 0·03*
	IRI	0·89 ± 0·15 (19)	1·50 ± 0·24 (19)	0·61 ± 0·16†
3	GLI	0·31 ± 0·04 (11)	0·25 ± 0·05 (11)	−0·06 ± 0·06
	IRI	1·09 ± 0·16 (11)	1·42 ± 0·12 (11)	0·33 ± 0·10‡

* $P < 0·05$. † $P < 0·005$. ‡ $P < 0·01$.

as in the foetal specimens. The amounts of GLI measured in the pancreatic extracts were 0·4–2 % of the insulin values. A significant increase in GLI content was noted between the 18- and 21-day old foetuses ($t = 2·76$; $P < 0·05$) as well as between the 21-day-old foetuses and the 1-day-old animals ($t = 3·97$; $P < 0·005$).

Release of glucagon and insulin from the isolated mouse pancreas

The effects of glucose on the release of GLI and IRI from isolated pancreases of foetal and newborn mice are shown in Tables 2 and 3. During 30 min of incubation 17 mM glucose had no significant effect on either IRI or GLI release from the pancreases of 18-day-old foetal mice regardless of whether 5 mM arginine was also present. Glucose (17 mM) stimulated the release of IRI from pancreases of 1- and 3-day-old mice but did not affect the release of GLI (Table 2). In the presence of 5 mM arginine the release of IRI was again markedly stimulated by 17 mM glucose whereas the simultaneous release of GLI from pancreases of 1-day-old mice was significantly depressed (Table 3).

DISCUSSION

Electron-microscopic studies on the development of the mouse pancreatic islets have disclosed granulated β-cells on the 11th–13th day of gestation (Munger, 1958; Wessels & Evans, 1968). The mouse β-cells do not stain with aldehyde-fuchsin until the 18th day of gestation (Munger, 1958; Denffer, 1969) and α-cells were not found until after birth (Munger, 1958). Data on the content and release of insulin or glucagon from the foetal mouse pancreas seem, how-

ever, to be lacking. In the rat, the amounts and synthesis of immunoreactive insulin have been studied in the pancreas from the 12th day of gestation onwards (Clark, 1968). In a recent report, typical α-cells were readily demonstrated in rats on the 18th day of gestation (Orci *et al.* 1969). Earlier gestational ages were not studied. Orci *et al.* also measured the pancreatic glucagon concentration in amounts comparable to those found for the mouse in the present study. It therefore is conceivable that glucagon-producing cells are also present in the late foetal mouse pancreas.

The results of the present study in the mouse are consistent with previous reports that glucose is unable to stimulate insulin release from foetal β-cells in various species (cf. Grodsky, 1970). A rapid maturation of the recognition system for glucose in the mouse is obvious from the stimulatory effect of glucose on insulin release from the pancreas of 1-day-old newborn mice. In this respect the mouse is similar to the rabbit (Milner, 1969) but somewhat different from the rat, whose β-cells do not respond to glucose until the second day after birth (Asplund, Westman & Hellerström, 1969).

Glucose had no effect on the release of GLI from foetal or 1- to 3-day-old animals. Similar results were obtained in a recent study on the release of glucagon from the isolated pancreas of newborn rats (Edwards, Asplund & Lundquist, 1972). In the present experiments, however, 17 mM glucose significantly depressed the release of GLI from pancreases of 1-day-old mice incubated in the presence of 5 mM arginine. This indicates that the α_2-cells of newborn mice may be sensitive to glucose. However, full glucose-sensitivity of the α_2-cells is apparently acquired later in postnatal life, since high concentrations of glucose alone inhibit the release of glucagon from isolated islets of adult mice (Chesney & Schofield, 1969) and rats (Vance, Buchanan, Challoner & Williams, 1968).

Since it was difficult to isolate islets from the foetal mouse pancreas, the present experiments had to be performed with whole pancreatic glands. This technique is handicapped by the fact that lytic factors from the exocrine parenchyma destroy insulin and glucagon released into the medium (Malaisse, Malaisse-Lagae & Wright, 1967). The degradation of insulin can be inhibited by adding either anti-insulin serum (Malaisse *et al.* 1967) or Trasylol® (Malaisse, Malaisse-Lagae & King, 1968) to the incubation medium. Since the latter proteolytic enzyme inhibitor also counteracts glucagon degradation *in vitro* (Vance *et al.* 1968; Unger, Ketterer, Dupré & Eisentraut, 1967; Hazzard *et al.* 1968; Heding, 1970) it was added to all incubation media and immunoassay buffers. In addition, a human serum albumin without [^{125}I]glucagon-degrading properties was used throughout. In spite of these precautions further experiments with pancreas taken from progressively older animals revealed an almost total degradation of released GLI and, with increasing age, a less significant increase in the amounts of IRI released after glucose stimulation. The fact that glucose apparently failed to significantly inhibit the release of GLI from pancreas of 3-day-old mice incubated with arginine (inhibition was noted in 8 of 11

experiments) might be explained by increased activity of the exocrine tissue cells after birth.

Glucagon is an effective stimulus for insulin release from the isolated foetal rabbit and human pancreas (Milner, 1969; Milner *et al.* 1971) as well as from isolated foetal human islets (Espinosa de Los Moteros *et al.* 1970) and cultivated pancreatic explants of foetal rats (Lambert, Jeanrenaud & Renold, 1967). These observations led Milner (1969) to suggest that glucagon plays a role in the regulation of insulin release *in utero* in analogy to what has previously been suggested for adult mammals (Hellman & Lernmark, 1970). Provided that the amount of GLI measured after incubating foetal mouse pancreatic glands reflect the intrainsular release of this hormone, the present failure to alter the release of GLI by arginine and glucose does not suggest that endogenously released glucagon is important for foetal β-cell function in our strain of mice.

This work was supported by the Swedish Research Council (12 ×-3923), the Swedish Diabetic Association and the Medical Faculty of Umeå. Thanks are due to Dr Lise Heding, the Novo Research Institute, Copenhagen, Denmark, for a generous supply of labelled hormones and antisera.

REFERENCES

ASPLUND, K., WESTMAN, S. & HELLERSTRÖM, C. (1969). Glucose stimulation of insulin secretion from the isolated pancreas of foetal and newborn rats. *Diabetologia* **5**, 260–262.

CHESNEY, T. McC. & SCHOFIELD, J. G. (1969). Studies on the secretion of pancreatic glucagon. *Diabetes* **18**, 627–632.

CLARK, W. R. (1968). The synthesis and accumulation of insulin in the rat embryo during development. *Diss. Abstr. int.* **30**, 483–484.

COORE, H. G. & RANDLE, P. J. (1964). Regulation of insulin secretion studied with pieces of rabbit pancreas incubated *in vitro*. *Biochem. J.* **93**, 66–78.

DENFFER, H. (1969). Autoradiographische und histochemische Untersuchungen über das Teilungsvermögen von β-Zellen der Langerhansschen Inseln im Pankreas fetaler und neugeborener Mäuse. *Histochemie* **21**, 338–352.

EDWARDS, J. C., ASPLUND, K. & LUNDQVIST, C. (1972). Glucagon release from the pancreas of the newborn rats. *J. Endocr.* (in the Press).

ESPINOSA DE LOS MONTEROS, A., DRISCOLL, S. G. & STEINKE, J. (1970). Insulin release from isolated human foetal pancreatic islets. *Science, N.Y.* **168**, 1111–1112.

GRODSKY, G. M. (1970). Insulin and the pancreas. *Vitams Horm.* **28**, 37–101.

HAZZARD, W. R., CROCKFORD, P. M., BUCHANAN, K. O., VANCE, J. E., CHEN, R. & WILLIAMS, R. H. (1968). A double antibody immunoassay for glucagon. *Diabetes* **17**, 179–186.

HEDING, L. G. (1966). A simplified insulin radioimmunoassay method. In *Labelled Protein in Tracer Studies* (ed. Donato *et al.*), pp. 345–350. Brussels: Euratom.

HEDING, L. G. (1970). Radioimmunological determination of pancreatic gut glucagon in plasma. *Diabetologia* **7**, 10–19.

HELLMAN, B. (1965). Studies in obese-hyperglycemic mice. *Ann. N.Y. Acad. Sci.* **131**, 541–558.

HELLMAN, B. & LERNMARK, Å. (1970). A possible role of pancreatic α_1- and α_2-cells as local regulators of insulin release. In *The Structure and Metabolism of the Pancreatic Islets* (ed. S. Falkmer, B. Hellman & I.-B. Täljedal), pp. 453–462. Oxford: Pergamon Press.

INGALLS, A. M., DICKIE, M. M. & SNELL, G. D. (1950). Obese, a new mutation in the mouse. *J. Hered.* **41**, 317–318.

KENNY, A. J. (1955). Extractable glucagon of the human pancreas. *J. clin. Endocr. Metab.* **15**, 1089–1105.

LAMBERT, A. E., JEANRENAUD, B. & RENOLD, A. E. (1967). Enhancement by caffeine of glucagon-induced and tolbutamide-induced insulin release from isolated foetal pancreatic tissue. *Lancet* i, 819–820.

MALAISSE, W., MALAISSE-LAGAE, F. & KING, S. (1968). Quantitative and qualitative aspects of islet function in the rat. *J. Lab. clin. Med.* **74**, 56–64.

MALAISSE, W., MALAISSE-LAGAE, F. & WRIGHT, P. H. (1967). A new method for the measurement in vitro of pancreatic insulin secretion. *Endocrinology* **80**, 99–108.

MILNER, R. D. G. (1969). The secretion of insulin from foetal and post-natal rabbit pancreas in vitro in response to various substances. *J. Endocr.* **44**, 267–272.

MILNER, R. D. G., BARSON, A. J. & ASHWORTH, M. A. (1971). Human foetal pancreatic insulin secretion in response to ionic and other stimuli. *J. Endocr.* **51**, 323–332.

MUNGER, B. L. (1958). A light and electron microscopic study of cellular differentiation in the pancreatic islets of the mouse. *Am. J. Anat.* **103**, 275–311.

ORCI, L., LAMBERT, A. E., ROUILLER, CH., RENOLD, A. E. & SAMOLS, E. (1969). Evidence for the presence of A-cells in the endocrine fetal pancreas of rat. *Horm. Metab. Res.* **1**, 108–110.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. P. (1964). *Manometric Techniques.* Minneapolis: Burgess.

UNGER, R. H., KETTERER, H., DUPRÉ, J. & EISENTRAUT, A. M. (1967). The effects of secretin, pancreozymin, and gastrin on insulin and glucagon secretion in anaesthetized dogs. *J. clin. Invest.* **46**, 630–645.

VANCE, J. E., BUCHANAN, K. D., CHALLONER, D. R. & WILLIAMS, R. H. (1968). Effect of glucose concentration on insulin and glucagon release from isolated islets of Langerhans of the rat. *Diabetes* **17**, 187–193.

WESSELS, N. & EVANS, S. J. (1968). Ultrastructural studies of early morphogenesis and cytodifferentiation in the embryonic mammalian pancreas. *Devl Biol.* **17**, 413–446.

(*Manuscript received 11 April 1972, revised 9 June 1972*)