QUANTITATIVE ANALYSIS OF PANCREATIC ISLET DEVELOPMENT AND INSULIN STORAGE IN THE FOETAL AND NEWBORN RAT

By H. M. P. Freie, A. Pasma and P. R. Bouman

ABSTRACT

Pancreatic islet development and insulin storage were studied in foetal rats during the last 4 days of gestation (day 19 to 22 post-coitum (p. c.)) and in 1 and 5 days old neonatal rats. Adult female virgin rats were also studied. The percentage of granulated B-cells per islet, the degree of B-cell granulation and the islet insulin concentration rose from low levels on day 19 to adult levels on day 22 and remained stable after birth. This indicates that the qualitative maturation of the pancreatic islets as insulin producing units is completed on the last day of gestation.

The percentage of islet tissue slowly rose from 0.7% at day 19 to 1.5% on day 22. A further and much more rapid rise occurred during the first day of birth. At the 5th postnatal day the islets comprised 3.6% of the pancreas versus 1.1% in adult rats. Likewise, the neonatal pancreatic insulin concentration was about 3 times higher than in the adult pancreas. The foetal pancreas as a whole showed rapid exponential growth between day 18 and 21 p. c., but a sudden decline in growth rate occurred from day 21 onward. The total mass of islet tissue, on the other hand, continued to expand at its high initial rate up to the first day after birth, whereafter this high rate also declined. The high concentration of insulin in the neonatal rat pancreas therefore appears to be due to differential growth rates of the endocrine and exocrine tissue during the last day of pregnancy and the first day after birth.

Pregnancy in the rat lasts for 22–22½ days. Embryonic islet-like structures appear in the pancreatic diverticulum around the 15th day of gestation and their size and number rapidly increase in the subsequent days (Hard 1944).
Granulated B-cells make their appearance between the 17th and 19th day (Hard 1944; McAlpine 1951; Frye 1957; Grillo 1964). In accordance with these findings, the foetal pancreatic insulin content increases rapidly to neonatal levels over the last 5 days of gestation (Dixit et al. 1964; Murell et al. 1966; Rishi et al. 1969; Pictet & Rutter 1972; Hegre et al. 1973).

During the initial 3–5 postnatal days, total pancreatic insulin continues to increase rapidly, causing peak concentrations of the hormone far in excess of adult rat values (Rishi et al. 1969; Lambert et al. 1970; Sodoyez-Goffaux et al. 1971; Lazarow et al. 1973). Likewise, the size and absolute number of the pancreatic islets appear to increase over this period (Hard 1944; Hellman 1959a,b, 1966).

The aim of the present study was to analyse further these perinatal changes in the rat by correlating the results of a morphometric analysis of the developing islet apparatus with the pancreatic insulin content. Our study covered the period of the last 4 days of gestation and the initial 5 postnatal days. Comparative data on adult female virgin rats were also obtained. Our results confirm and extend recent observations by Hegre et al. (1973) and by Leonard (cited by Lazarow et al. 1973), which were published when this study was in progress.

MATERIALS AND METHODS

Induction of pregnancy and tissue sampling

Female Wistar rats (170–200 g body weight) were selected for pro-oestrous by means of vaginal smears and housed with males over the subsequent night for a period of 14 h. The next day was designated as day 1 p.c. In case of pregnancy, spontaneous birth usually occurred in the late afternoon of day 22 p.c. The day following the day of birth was designated as day 1 post-partum (p.p.). Pregnant and newborn rats were always sacrificed between 11.00 a.m. and 2.00 p.m. Intake of food or suckling was prevented during the last 2 h preceding sacrifice.

At the specified stages of gestation the foetuses were removed from the pregnant animals by Caesarian section under light ether anaesthesia. The entire litters of foetal and newborn animals were killed by decapitation and the complete pancreas was excised and weighed. For each litter the tissue was pooled for either insulin extraction or histological examination. The pancreas of the adult female virgin rat of 180–190 g body weight was also studied.

Extraction and determination of pancreatic insulin

The frozen pancreatic tissue of complete litters was thawed, fragmented with scissors and homogenized for 2×30 seconds in 3 ml of cold acid ethanol with a Lourdes tissue homogenizer model MM. The acid ethanol mixture was made up of ethanol 75% (v/v), distilled water 23.5% and concentrated hydrochloric acid 1.5% (v/v). The homogenates were shaken for 90 min at 4°C and subsequently centrifuged for 20 min
at 6300 x g in a cooled Lourdes centrifuge. The residue was re-extracted with 2 ml of acid ethanol. The supernatants were combined and brought to a final volume of 5 ml. Samples of this extract were diluted at least 16-fold with veronal buffer pH 8.4 (ionic strength 0.1) containing 2.5 mg/ml of bovine serum albumin and stored at −40°C until assay.

Insulin was determined by radioimmunoassay according to Yalow & Berson (1960) with slight modifications as previously described (Konijnendijk & Bouman 1970). The assay system consisted of 131-labelled beef insulin, anti-rat insulin serum and crystalline rat insulin as a standard (Bosboom et al. 1973).

**Histological and morphometric procedures**

The complete pancreas of each litter was fixed in Bouin’s fluid and collectively embedded in paraffin. The entire blocks were cut with a Leitz-Minot microtome into sections of 5 µm thickness. At intervals of 250 µm one section was taken for further histological processing, which consisted of aldehyde-fuchsin staining (Gomori 1950) and counter-staining according to Halmi (1952). Tissue pools of each developmental stage were processed simultaneously.

The percentage of islet tissue in each tissue pool was determined by the method of Chalkley (1943) at a magnification of 15 x 40. The surface covered by one microscopic field was 61 400 µm². The ocular contained 10 randomly distributed points. The percentage of islet tissue was calculated from the number of randomly chosen microscopic fields which was needed to score 300 hits on islet tissue. An islet was defined as any circumspect collection of cells containing at least two adjacent aldehyde-fuchsin positive cells. Example: Assume that 2000 microscopic fields are needed to score 300 hits with the 10 ocular points. In the case of 100 % islet tissue the number of hits would have amounted to 2000 x 10. The true percentage of islet tissue is therefore (300:20 000) x 100 % = 1.5 %.

Each tissue pool was also counted for the number of islets and the number of solitary aldehyde-fuchsin positive cells per 1000 random microscopic fields. The actual number of fields counted varied between 1000 and 3000. Cell counts were furthermore made of each tenth islet to determine the percentage of granulated B-cells per islet. In addition, the degree of B-cell granulation in each of these islets was assessed in terms of an arbitrary scale of 0–5 and the mean was taken as the litter value.

**RESULTS**

Foetal rats were obtained on day 19, 20, 21 and 22 of pregnancy. Newborn rats were studied at 1 and 5 days after birth. At least 4 complete litters were studied at each of these different ages. The litter size varied between 6–9. The pancreatic tissue of each litter was pooled for quantitative histological examination. This resulted in single litter values for each of the various histological parameters, from which means of litters ± SEM were calculated. The results have been expressed in Tables 1 and 2.

As shown in Table 1 the percentage of islet tissue gradually increases over the last 4 days of gestation. The adult value is already reached at day

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Table 1.
Percentage of islet tissue, percentage of granulated B-cells per islet and degree of B-cell granulation per islet in foetal, neonatal and adult rat pancreas. Means of litter values ± SEM.

<table>
<thead>
<tr>
<th>Age days (litters)</th>
<th>/o Islet tissue</th>
<th>/o B-cells per islet</th>
<th>B-cell granulation(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 p.c. (4)</td>
<td>0.7 ± 0.04</td>
<td>22 ± 1.5</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>20 p.c. (4)</td>
<td>1.1 ± 0.04</td>
<td>37 ± 1.4</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>21 p.c. (4)</td>
<td>1.1 ± 0.09</td>
<td>60 ± 4.9</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>22 p.c. (5)</td>
<td>1.5 ± 0.24</td>
<td>71 ± 5.4</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>1 p.p. (4)</td>
<td>2.6 ± 0.25</td>
<td>57 ± 0.9</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>5 p.p. (4)</td>
<td>3.6 ± 0.47</td>
<td>58 ± 2.1</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>adult rats (3)</td>
<td>1.1 ± 0.10</td>
<td>61 ± 1.7</td>
<td>3.0 ± 0</td>
</tr>
</tbody>
</table>

\(^1\) Scale 0-5.  
NS = non-significant.  
* P ≤ 0.05 (t-test).  
** P < 0.01.  
*** P < 0.001.

20 p.c. A further and more rapid rise occurs during the initial days after birth. On the 5th postnatal day the percentage of islet tissue has increased to 3.6 ± 0.47%, this value being about three times higher than that seen in the adult rat pancreas (1.1 ± 0.10%). In contrast, the observed increase in the percentage of granulated B-cells per islet remains entirely confined to the foetal period, the adult level of 60% being reached on day 21 p.c. After an insignificant increase to 71% on day 22, the percentage of granulated B-cells stabilizes again at the adult level from day 1 p.p. onward. A roughly similar picture is displayed by the mean degree of B-cell granulation. The normal adult value is reached on day 21 p.c. There may be a tendency, however, to a small further increase during the initial postnatal days.

Data on the number of islets at the various developmental stages have been summarized in Table 2. The islet density per 1000 microscopic fields was found to increase gradually from 130 ± 4 on the 19th day of gestation to 170 ± 12 on day 22. One day after birth the islet density suddenly doubled to 342 ± 47. This high density was still present on the 5th postnatal day. At all stages studied the islet density was found to exceed the adult value of 95 ± 7 islets per 1000 fields.

The relative change in the total number of islets per pancreas was estimated by multiplying the islet density per 1000 fields with the mean pancreatic
weight at each developmental stage (Table 2). Thus it becomes evident that from the 19th day of gestation up to the first neonatal day the total number of islets increases exponentially by doubling each day. After day 1 p.p. this high rate of increase declines considerably. The course of the density and total number of islets bears a strong resemblance to that of the solitary aldehyde-fuchsin positive cells. However, during the initial postnatal days the density and total number of the latter appear to increase even more dramatically.

In further experiments, which involved 10–14 litters of the same developmental ages as in the previous series of experiments, the pancreatic tissue of each litter was pooled, weighed and extracted with acid alcohol. The results are shown in Table 3.

Total extractable insulin increased rapidly during the last 4 days of gestation and the first postnatal day. Between the 1st and 5th postnatal day the rate of increase declined. The pancreatic insulin concentration showed a similar pattern. The neonatal concentrations of pancreatic insulin varied between 6.3 and 7.8 mU/mg versus 2.7 mU/mg in adult rats. In the foetal pancreas the adult pancreatic insulin concentration was overtaken between day 21 and

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**Table 2.**

Islet density, number of islets and solitary aldehyde-fuchsin (AF) positive cells in foetal, neonatal and adult rat pancreas. Means of litter values ± SEM.

<table>
<thead>
<tr>
<th>Age days (litters)</th>
<th>Solitary AF-pos. cells per 1000 fields¹</th>
<th>Islet density islets per 1000 fields</th>
<th>Index for total number of islets per pancreas²</th>
<th>Index for number of sol. AF-pos.cells per pancreas³</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 p.c. (4)</td>
<td>8 ± 0.7</td>
<td>130 ± 4</td>
<td>NS</td>
<td>5</td>
</tr>
<tr>
<td>20 p.c. (4)</td>
<td>9.5 ± 1.5</td>
<td>148 ± 3</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>21 p.c. (4)</td>
<td>12 ± 1.1</td>
<td>135 ± 8</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>22 p.c. (5)</td>
<td>12 ± 1.4 (**)</td>
<td>170 ± 12</td>
<td>**</td>
<td>46</td>
</tr>
<tr>
<td>1 p.p. (4)</td>
<td>55 ± 8.3</td>
<td>342 ± 47</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>5 p.p. (4)</td>
<td>109 ± 29</td>
<td>434 ± 76</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>adult rats (3)</td>
<td>9 ± 0.7</td>
<td>95 ± 7</td>
<td></td>
<td>610</td>
</tr>
</tbody>
</table>

¹ 1000 microscopic fields = 61.4 mm².
² Islet density

\[
\text{Islet density} = \frac{\text{Index for total number of islets per pancreas}}{100} \times \text{pancreatic weight.}
\]

³ Density sol. AF-pos. cells

\[
\text{Density sol. AF-pos. cells} = \frac{\text{Index for number of sol. AF-pos.cells per pancreas}}{10} \times \text{pancreatic weight.}
\]

Statistical designations as in Table 1.
Table 3.
Insulin content of the pancreas and pancreatic islet tissue of foetal, neonatal and adult rats. Means of litter values ± SEM.

<table>
<thead>
<tr>
<th>Age days (litters)</th>
<th>Pancreatic weight mg/animal</th>
<th>Total insulin mU/pancreas</th>
<th>Pancreatic insulin conc. mU/mg</th>
<th>Islet mass(^1) mg/pancreas</th>
<th>Islet insulin conc. mU/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 p. c. (10)</td>
<td>3.7 ± 0.2</td>
<td>3 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.027</td>
<td>96 ± 7</td>
</tr>
<tr>
<td>20 p. c. (14)</td>
<td>8.6 ± 0.5</td>
<td>9 ± 1.4</td>
<td>1.0 ± 0.1</td>
<td>0.097</td>
<td>94 ± 14</td>
</tr>
<tr>
<td>21 p. c. (14)</td>
<td>19.2 ± 1.5</td>
<td>34 ± 4.2</td>
<td>1.7 ± 0.1</td>
<td>0.211</td>
<td>159 ± 20</td>
</tr>
<tr>
<td>22 p. c. (13)</td>
<td>27.1 ± 1.3</td>
<td>95 ± 6.9</td>
<td>3.5 ± 0.2</td>
<td>0.404</td>
<td>236 ± 17</td>
</tr>
<tr>
<td>1 p. p. (10)</td>
<td>28.0 ± 2.0</td>
<td>179 ± 29</td>
<td>6.3 ± 0.8</td>
<td>0.730</td>
<td>245 ± 39</td>
</tr>
<tr>
<td>5 p. p. (11)</td>
<td>34.1 ± 2.4</td>
<td>259 ± 22</td>
<td>7.8 ± 0.7</td>
<td>1.210</td>
<td>213 ± 18</td>
</tr>
<tr>
<td>Adult rats (10)</td>
<td>679 ± 56</td>
<td>1830 ± 160</td>
<td>2.7 ± 0.1</td>
<td>7.330</td>
<td>250 ± 22</td>
</tr>
</tbody>
</table>

\(^1\) Islet mass = \(\%\) islet tissue \(\times\) pancreatic weight; \(\%\) islet tissue taken from data in Table 1.

Statistical designations as in Table 1.
day 22 p.c. To analyse these phenomena the total mass of islet tissue was calculated from the islet/acinar ratio's (previously determined) and the pancreatic weights. The islet mass appeared to have increased continuously up to the 5th postnatal day. The insulin concentration per mg islet tissue, on the other hand, had reached the adult level on the last day of pregnancy and remained more or less stable after birth.

The various developmental patterns can be effectively illustrated by semi-logarithmic plotting of the relevant parameters (Fig. 1). Thus, it becomes evident that during the last day of pregnancy and the first day after birth the total islet mass continues to grow at its initial high rate, whereas the growth rate of the total pancreas declines considerably from the 21st day onward.

**DISCUSSION**

In accordance with observations by previous investigators (*Murell et al. 1966; Rishi et al. 1969; Lambert et al. 1970; Sodoyez-Goffaux et al. 1971; Pictet & Rutter 1972*), pancreatic insulin was found to increase rapidly during the last 4 days of pregnancy and the first postnatal day. The total insulin content increased exponentially over this period, the daily increase being in the order of a factor 3. From the 1st to the 5th postnatal day the rate of increase declined considerably (Table 3 and Fig. 1). Both the course and the absolute levels of the foetal and neonatal pancreatic insulin concentration almost duplicate the recent observations of *Hegre et al.* (1973) and Leonard (cited by *Lazarow et al.* 1973). Our investigation was intended to unravel this developmental pattern into some of its most obvious components. These include: 1. The total amount of islet tissue. 2. The percentage of granulated B-cells per islet. 3. The insulin content per B-cell. The latter factor can be indirectly assessed from the mean degree of B-cell granulation per islet.

Sparsely granulated B-cells were easily detectable on the 18th day of gestation, which is in keeping with observations by previous investigators in this field (*Hard 1944; McAlpine 1951; Frye 1957; Grillo 1964*). Between day 19 and day 22 the mean degree of granulation rose to levels closely approaching those seen in the adult pancreas. Likewise, the percentage of granulated B-cells per islet increased considerably to reach adult values between day 21 and day 22. In accordance with these changes, the insulin concentration per mg islet tissue reached the adult level on the last day of pregnancy. From these observations it can be concluded that the last day of pregnancy marks the end of a period of qualitative maturation of the pancreatic islets as insulin producing morphological entities.

The absolute and relative growth rate of the insular apparatus shows a quite different time pattern. During the period of qualitative islet maturation the total mass of islet tissue expands only slightly faster than the pancreatic weight.
as becomes evident from the slight change in the percentage of islet tissue over this period. However, after day 21 the growth rate of the pancreas as a whole declines considerably, which confirms observations of Rishi et al. (1969) and of Sodoyez-Goffaux et al. (1971). The total mass of islet tissue, on the other hand, continues to expand at a high rate for at least 2 more days, thereby causing the high neonatal islet/acinar ratios also observed by previous investigators (Hard 1944; Leonard cited by Lazarow et al. 1973). This also explains the high concentration of insulin in the neonatal rat pancreas.

The total mass of islet tissue is the sum of a volumetric distribution which represents the contribution of the various size classes of islets to the total islet volume (mass). The bell-shaped volumetric distribution curve has been shown to be symmetrical (Hellman 1959a). The numerical distribution of islets

Fig. 1.
Semilogarithmic plot of pancreatic immunoreactive insulin (IRI) and various morphological parameters for pancreatic islet development in the foetal and newborn rat.
over the various size classes, on the other hand, is highly asymmetrical in the sense that the classes of small sized islets contain the largest numbers of islets (Hellman 1959b). The smallest islets included in these studies of Hellman (1959a,b) had an approximate diameter of 47 μm, which is considerably larger than that of the smallest islets included in our study. With this restriction in mind, it can be calculated from his data that in newborn, 5 and 21 days old rats his two lowest classes of small sized islets (out of 11 size classes) comprise about 49% of the total number of islets. These two classes account for only 10.5% of the total islet volume.

On the basis of these considerations it will be clear that an increase in total islet mass over any given day will bear no immediate relation to the simultaneous increase in the total number of islets over that particular day. The latter phenomenon merely represents addition of newly formed islets of the smallest size to the existing islet population. The meticulous studies by Hellman (1959a,b) have also shown that during the first 3 weeks after birth, the number of islets increases within all size classes. However, the per cent contribution of the various size classes to the expanding total islet volume remains unchanged during this period. Growth of the islet mass, therefore, ultimately depends on the rate at which new small sized islets are being formed and the rate at which these islets will shift to higher size classes according to the constant distribution pattern associated with this period.

Our data do not allow a further analysis of these components, although some information on the rate of islet formation was obtained. In the present study an islet was defined as any circumspect collection of cells containing at least two adjacent aldehyde-fuchsin positive cells. Thus, even the youngest islets were included in our islet counts. The daily change in the index for the total number of islets (Table 2) will therefore reflect the rate at which new small sized islets are being formed. Apparently, the absolute rate of this process increases steadily during the last 4 days of gestation to reach a maximum on the first day after birth. In the subsequent postnatal days the rate of islet formation suddenly declines which coincides with a less rapid growth of the total islet mass. The latter phenomenon, however, is probably due to a reduced growth rate of previously formed islets.

The significance of the sudden and spectacular increase of the total number of solitary aldehyde-fuchsin positive cells during the early neonatal period remains unclear. Since these cells presumably originate from undifferentiated ductular and acinar elements (Hard 1944; Pictet & Rutter 1972), their number might be an index of the capacity or tendency to generate new B-cells from previously undifferentiated cells. However, their increased number could also be due to a reduced mitotic rate of differentiated B-cells following birth (Fehrmann et al. 1974), preventing solitary B-cells from forming miniature islets. This explanation would be in keeping with the reduced rate of islet
formation, the reduced growth rate of the total islet mass and the arrest of
the percentage of granulated B-cells per islet at a stable level from day 1 p. p.
onward.

rise is mainly caused by a progressively increasing concentration of insulin
concentration in the late foetal and early postnatal period is caused by a
number of entirely different phenomena. Between day 19 and day 22 p. c. this
rise is mainly caused by a progressively increasing concentration of insulin
within the pancreatic islets (qualitative islet maturation). The percentage of
islet tissue changes only slightly over this period. From day 22 onward the
qualitative maturation of the islets as units of production and storage of insulin
has been completed. The further rise in the pancreatic insulin concentration
appears to be due to an increasing concentration of islet tissue within the
pancreas. This is caused by a sudden decline in the growth rate of the acinar
tissue from day 21 onward, whereas the mass of islet tissue continues to grow
at its initial high rate for at least 2 more days, including the first day after
birth.

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