Fishing in the Stream of Diabetes: From Measuring Insulin to the Control of Fetal Organogenesis

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The measurement of insulin
Non-insulin-dependent diabetes mellitus (NIDDM) is estimated to affect 100 million people worldwide. It has been observed to increase in prevalence when a population increases its plane of nutrition and decreases its energy expenditure. A striking example of this occurred when Ethiopian Jews were moved to Israel [1]. The Pacific population of the island of Nauru was described as suffering from an epidemic of diabetes when they became affluent after the Second World War following the expansion of phosphate mining [2]. Thus we can only anticipate that the numbers suffering from this type of diabetes will remorselessly increase in the future unless we discover the factors that cause it and take appropriate steps to prevent it. It is chastening to have to admit that it is still a matter of debate as to whether insulin deficiency, insulin resistance or a combination of the two is at the heart of the disease. A major problem in disentangling this issue is the close interlocking of the regulation of glucose and insulin metabolism: insulin deficiency leads to insulin resistance and conversely hyperglycaemia is detrimental to insulin secretion. Large prospective population studies should be capable of elucidating the time course of events, but it is only in recent years that it has become possible to measure insulin concentrations in human blood in sufficient numbers of individuals and with adequate specificity to have a real prospect of progress. Even now we have to admit that we can only study insulin production and secretion at such a distance from the β-cells of the islets of Langerhans that our conclusions may not be valid.

The introduction of the technique of radioimmunoassay by Yalow and Berson revolutionized our ability to measure insulin and indeed many other polypeptides at very low concentrations in plasma [3]. Our laboratory’s initial efforts were devoted mainly to making this method more robust and simple in application [4]. The technique also led to the commercialization of radioimmunoassays in the U.K. This must be one of the rare occasions when an invention in the United States was first commercialized in the U.K. It is also the cause of some nostalgia to recall the days when it did not even occur to the laboratory scientist to patent or profit from commercialization. Nevertheless it
was certainly profitable in terms of our modern obsession – the citation index [5]!

The theme in our research of assay development has been quite continuous over the years. Initially we explored the theoretical advantages that we predicted would arise from the use of labelled antibodies rather than antigens in immunoassays. We termed this assay, using an $^{125}$I label, 'immunoradiometric' and suggested also that using enzymes as alternative, amplifying labels might be advantageous [6]. This has indeed proved to be the case, and we now have a variety of other labels capable of delivering assays of greater sensitivity than radioisopes can. The generic term for such assays using labelled antibodies has come to be 'immunometric'. This approach was developed further by the introduction of the use of two antibodies – a technique that we described as a 'two-site' assay to reflect the fact that two epitopes on a single antigen were required to react [7,8]. From this additional structural requirement we predicted that assays of greater specificity would arise, and again this has proved to be the case, as illustrated by current assays of the insulin-related molecules (see below). The final manoeuvre in this series of experiments was to measure the two-site antigen–antibody complex indirectly by the use of a labelled anti-immunoglobulin (Figure 1). The advantage of this additional step was to be more economical in the use of the primary antibodies that were often in short supply and to have a labelled reagent that could be used in a variety of assays for different antigens [9]. At this stage of the work the technical problems of producing assays involving labelled antibodies were rendered trivial by the introduction of the technique for producing monoclonal antibodies [10].

Shortly afterwards the problems of raising antibodies to human insulin and its precursors were also greatly aided by the bioengineering of the production of human proinsulin [11].

The need to produce assays for insulin of greater specificity became apparent with the discovery of proinsulin [12], the demonstration of its presence in plasma [13,14] and the raised concentrations of proinsulin in the plasma of diabetics [15,16]. The close structural relationships between the proinsulin-derived molecules (Figure 2) illustrate that it would be extremely difficult, if not impossible, to measure insulin specifically in the presence of these related molecules by the use of a single antibody. An additional complication was discovered when we used bioengineered human proinsulin in our so-called 'proinsulin' assay [17] only to discover that it was unreactive until subjected to mild digestion with trypsin [18]! This led us to the conclusion that what we and probably others had been measuring up until then as 'proinsulin' was either entirely or in part incompletely processed proinsulin.

In order to resolve this situation we embarked on the production of a panel of monoclonal antibodies for use in two-site assays of the insulin-related molecules [19]. Our efforts were greatly assisted by the fact that there is very little of the 65,66-split proinsulin conversion intermediate either in human pancreas [20] or plasma [21]. More recently this has been explained by the kinetics of the subtilisin-like prohormone convertases that are involved in proinsulin processing [22,23].

Figure 1

Indirect two-site assay principle
Armed with these more specific assays, we have re-examined the insulin status of individuals with loss of glucose tolerance. The outcome of this research has been to sharpen the focus on loss of the ability to release insulin rapidly in response to oral glucose – a finding originally made by Yalow and Berson in 1960 [3] and reconfirmed by many of us over the subsequent years [24]. Despite these consistent findings there has been a tendency in studies of the aetiology of NIDDM in more recent years to put insulin resistance as the primary abnormality causing loss of glucose tolerance [25].

Insulin resistance has also gained in interest because of its association with ischaemic heart disease – an association Peters and Hales observed in 1965 [26].

Despite these swings in the pendulum of opinion over the years, most recently a consensus has developed that both insulin deficiency and insulin resistance are involved in the production of NIDDM. However, although this seems clear from population studies it is not yet clear at the individual level whether the condition is so heterogeneous that any person may become diabetic because of insulin deficiency, insulin resistance or a varying contribution of the two. Whatever the answer to this question, the fact remains that in seeking genetic and/or environmental causes of NIDDM one is looking for pathogenic processes that can lead to insulin deficiency, insulin resistance or a combination of the two.

**Early life and NIDDM**

We have come to the conclusion that events in fetal and possibly early postnatal life play a major role in determining susceptibility to NIDDM. At first sight, it seems highly improbable that such early changes could predispose to a condition that has its onset mainly after the age of 50 years. I will briefly review the epidemiological evidence that has led us to this hypothesis. I shall then summarize previous animal experimentation that is consistent with the hypothesis and review our own research on a new animal model.

Studies in Norway and the U.K. have revealed a relationship between infant mortality and subsequent death in the same cohort from ischaemic heart disease [27,28]. Although this was initially attributed to events in childhood or adolescence predisposing individuals to ischaemic heart disease, Barker and Osmond suggested that the critical window for damage might be fetal life [29]. In order to test this idea further, Barker's group sought archival records of early life measurements such as birth and placental weight, length, head and chest circumference, etc. to determine whether they were related to diseases such as ischaemic heart disease and hypertension in adults aged 50–70 years at the time of study. Both conditions were found to have strong statistical relationships to measurements made at birth or at the age of 1 year [30,31]. Thus relatively poorly grown infants are at greatly increased risk of these two late-onset diseases.

The realization that malnourished fetuses divert nutrients from the viscera to the brain in order to protect growth of the latter and that fetal $\beta$-cells have their most rapid growth in fetal life suggested that we should collaborate to study glucose tolerance in these populations. The outcome of these studies may be summarized as
follows. (i) Low birth weight or weight at 1 year is linked to glucose intolerance [32]. (ii) This relationship is observed in men and women [33]. (iii) The features of the insulin resistance syndrome (‘syndrome X’), namely glucose intolerance, hyperlipidaemia and hypertension, are even more strongly related to birth weight [34]. (iv) Insulin resistance is observed in adults who when new born were thin [35]. (v) Insulin secretion by adults in the age range 50–70 years is not related to parameters of early growth [36], but relationships may be seen in young men [37].

Many of these observations have subsequently been confirmed in widely divergent populations, such as the Pima Indians [38], Mexican Americans [39] and in Sweden [40]. There is therefore every reason to believe that they are correct and because of their statistical strength important. The question is what do they mean? Hales and Barker have hypothesized that they reflect poor maternal and fetal nutrition, which in turn have effects on the development of the structure and function of a variety of organs [41]. It is suggested that these changes are part of normal mammalian adaptation to adverse nutritional conditions and indeed aid survival when there is a shortage of nutrients. Thus metabolism in various organs is permanently changed (‘programmed’) [42]. These changes, however, may prove to be detrimental if the organism is exposed to normal or supranormal nutrition. This hypothesis may be tested by work on animal models.

Animal models of fetal and early life growth retardation
Studies in a variety of animals, predominantly rats, complement the conclusions drawn from studies in humans. It was clear from work carried out over 30 years ago that nutritional deprivation during fetal life that led to a reduction in tissue cell numbers, as measured by tissue content of DNA, could not be reversed by adequate nutrition postnatally [43,44]. Much of the development of the islets of Langerhans occurs in utero [45]. The exact timing of islet formation differs among species. In rats the number of islets increases rapidly in the last 4–6 days of intrauterine life. In humans β-cell mass increases more than 130-fold between the twelfth intrauterine week and the fifth postnatal month. There are few studies of the effects on β-cell development of under nutrition during early life.

In recent years the key role of protein in ensuring proper development of the islets of Langerhans has been demonstrated by Hoet, Remacle and colleagues in Louvain. It has been shown that pregnant rats fed a diet containing a little under half the normal protein content produced pups with reduced neonatal β-cell proliferative capacity and islet size. Particularly interesting in these studies was the observation that the most dramatic change was produced in the volume density of the blood vessels in the islets [46]. This observation may relate to an earlier study in which impairment of insulin secretion following protein-calorie deficiency was found to be more severe than would have been expected from the reduction in islet cells alone [47]. In other studies, rats weaned on a low-protein diet for only three weeks produced an insulin response to glucose that was permanently impaired, leading to the speculation ‘that early malnutrition may predispose to diabetes’ [48]. It was subsequently shown that offspring from protein-restricted mothers, maintained on low-protein diets to the adult age of 70 days, had reduced glucose tolerance, associated with reduced insulin secretion. A group of animals fed normally after birth had an intermediate response [49]. Thus this experimental model suggests that even a brief period of exposure to poor nutrition, followed by normal food intake, can lead to irreversible changes.

Taken together these studies suggest that the early development of the endocrine pancreas is sensitive to the availability of amino acids and that nutritional effects on early development may regulate the capacity of pancreatic endocrine function during later life.

Maternal low protein and hepatic enzyme development
We found that a maternal low protein diet during pregnancy profoundly altered the activities of the key insulin-sensitive enzymes of glycolysis (glucokinase) and gluconeogenesis (phosphoenolpyruvate carboxykinase) in the offspring. The glucokinase activity was permanently reduced (to 50% approximately) whereas that of phosphoenolpyruvate carboxykinase was permanently increased (100%) [50]. It is known that glucokinase and phosphoenolpyruvate carboxykinase are predominantly located in different metabolic zones of the liver, the former being expressed around the perivenous zone and the latter around the periportal zone [51]. We have therefore
hypothesized that during development different hepatic cells may have multiplied differentially according to the nutritional status of the fetus, resulting in the population of perivenous cells being contracted and that of periportal cells being expanded [50].

Maternal low protein and pancreatic glucokinase in the offspring

Consistent with the above findings regarding the dramatic effect that a low-protein diet has on fetal islet growth and development are our recent observations on pancreatic glucokinase. This enzyme plays a central role in the regulation of glucose-stimulated insulin release from the β-cell. Glucose enters the β-cell by facilitative transport via the GLUT2 transporter protein. It is then phosphorylated by glucokinase, which has a $K_m$ value higher than that of physiological concentrations of glucose and thus is the major glucose sensor in the β-cell [52]. Indeed mutations in this enzyme have been shown to be present in some pedigrees of the Maturity Onset Diabetes of the Young phenotype [53]. We measured glucokinase activity in whole pancreatic extracts of the offspring of ‘control’ (20%) and ‘low-protein’-diet- (8%) fed mothers. In addition, we studied two cross-over groups where the offspring of low protein and controls were cross-fostered. This gave us a ‘recuperated’ group where pups from a low-protein mother were nursed by a mother on a control diet. The reverse group, termed ‘postnatal low protein’ were offspring from control mothers who were nursed by low-protein mothers. The offspring from all groups were weaned on to a control (20% protein) diet at 21 days of age. We chose to use whole pancreatic extracts rather than isolated islets to avoid any bias towards selecting certain islets. It was observed that at 6 weeks of age the low-protein offspring had significantly ($P<0.001$) lower levels of pancreatic glucokinase compared with controls (Figure 3A). The recuperated and postnatal low-protein groups were not significantly different from the controls. At 3 months of age, the low-protein offspring still had significantly (Figure 3B) lower levels of pancreatic glucokinase activity compared with controls and the cross-over groups. Thus the change appears to be permanent and is not reversed by 9 weeks of feeding a control diet. It is striking that such changes can occur in animals who themselves have not been fed a low-protein diet.

Maternal low protein and glucose tolerance of the offspring

In the light of these observations we performed glucose-tolerance tests on the offspring of both control and low-protein animals at 3 and 15 months of age. The animals were fasted overnight, and blood samples were taken from the
Each animal was then injected intra-peritoneally with 1 mg of glucose per gram body weight using a 10% (w/v) glucose saline solution. Blood was sampled at timed intervals. Samples were assayed for glucose (using a B-glucose photometer supplied by Hemocue AB, Angelholm, Sweden) and insulin (using a Linco rat insulin radioimmunoassay kit supplied by Biogenesis, Poole, U.K.).

At 3 months of age in both the male and female rats, there were no significant differences in either fasting blood glucose (Figures 4A and 4B) or plasma insulin levels between the two groups (Figure 5). However, after intraperitoneal injection of glucose, the low-protein animals were more glucose tolerant than controls were. This is shown by the significantly \((P<0.05)\) lower blood glucose values observed at 15 and 30 min after glucose administration in the low-protein male rats (Figure 4A). In 15-month-old animals the pattern observed was quite different. Again there were no differences in the fasting blood glucose of the male (Figure 6A) or female (Figure 6C) low-protein and control animals. However, at this age the low-protein animals were less glucose tolerant than controls were. This is apparent from the significantly \((P<0.05)\) higher blood glucose values observed in the low-protein males at 15 and 30 min after glucose administration (Figure 6A). A similar trend is observed in the female rats (Figure 6C); however, the values do not reach statistical significance. This loss of glucose tolerance in low-protein animals can be seen more clearly when we compare loss of glucose tolerance between 3 and 15 months in the two groups (Figure 7). Both the male and female rats from the control and low-protein groups become less glucose tolerant with age. However, the worsening of glucose tolerance in low-protein animals is much more extensive than that of controls. This is reminiscent of the human situation. During the glucose-tolerance test, no significant differences in plasma insulin were seen between the two groups in either males (Figure 6B) or females (Figure 6D), due mainly to the large scatter. However, it can be seen that the low-protein male rats tended to have higher plasma insulin concentrations compared with controls. This suggests that their glucose intolerance may be due mainly to insulin resistance. Interestingly, in females the situation appears to be reversed, where the low-protein animals show a tendency.
towards lower plasma insulin. This suggests that their lower glucose tolerance may be due mainly to insulin deficiency. Again this pattern reflects the human situation, in which males are more insulin resistant than females are [35].

**Maternal low protein and longevity of the offspring**

The influence of maternal low-protein diet on the longevity in the offspring was investigated (Table 1). The findings showed a sexual disparity in lifespan. The female rats had a significantly increased lifespan compared with the male rats. This was true for the control, low-protein and recuperated rats but not the postnatal rats. The reason for this trend is not known, but it may be that a faster rate of growth and development of male rats renders them more vulnerable to adverse nutritional influences in early life.

Significant differences in lifespan between the four groups were evident only in the male rats. The maternal low-protein diet throughout gestation and lactation, as in the case of the low-protein offspring, had no significant effect on the longevity. However, in the male rats a change in the diet during the postnatal period from the one to which the fetus had originally adapted had an opposite effect on longevity. There was a beneficial effect in terms of increasing the lifespan of male offspring when the protein restriction was postnatal. However, when the maternal protein restriction occurred during pregnancy, it had a detrimental effect and the lifespan of the male offspring was significantly shorter. Thus a general pattern (seen also in the females although the differences did not reach statistical significance) was observed. (i) If the rate of growth established during fetal life was maintained during postnatal life, longevity was not affected. (ii) However, slowing of growth during postnatal life, induced by suckling when mothers were on a low-protein diet, tended to increase longevity. It has

**Figure 6**

Glucose and insulin concentrations during a glucose-tolerance test in 15-month-old rats

Blood glucose (A and C) and plasma insulin (B and D) concentrations during a 3 h glucose-tolerance test of male (A and B) and female (C and D) rats from the control and low-protein groups. Values shown are means ± S.E.M. of 15 males and 15 females from eight pregnancies per group; *, P < 0.05 versus control.
been known for several years that feeding rats a
low-protein diet after weaning has a beneficial effect
on longevity [55]. Our findings show that a brief
period of exposure to lactating dams that have been
protein deprived has a similar effect on offspring.
This brief exposure is also capable of permanently
reducing the growth trajectory of offspring [55a].
(iii) Conversely, a highly detrimental effect on lon-
gevity was observed when the pups’ growth was
retarded during pregnancy, but was then acceler-
ated as a result of good postnatal nutrition by
being suckled by protein-unrestricted mothers. The
rate of growth of such pups when expressed relative
to their initial body weight was faster than the con-
trols [55a]. One may speculate that under these
conditions fetal growth restriction has generally
reduced cell numbers and that subsequent ‘catch-
up’ growth is achieved by the over growth of a
limited cell mass. In some way yet to be discovered,
this overgrowth is detrimental to long-term survival.

Table I
Lifespan of offspring of protein-restricted dams

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Males</th>
<th>Females</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.1±3.7</td>
<td>18.2±4.1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Low protein</td>
<td>16.3±4.1</td>
<td>18.7±4.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Postnatal low protein</td>
<td>17.0±3.5*</td>
<td>18.8±3.7</td>
<td>NS</td>
</tr>
<tr>
<td>Recuperated</td>
<td>13.1±3.3*</td>
<td>17.4±2.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

It will be interesting and important to try to estab-
lish more precisely (i) the mechanisms and physio-
logical systems involved, and (ii) the reasons why
male rats are so much more vulnerable — again an
observation consistent with data on human longev-
ity. It is worth noting that an extra 2 months in
longevity of the male control over the male recuper-
ated offspring when translated into relative human
longevity could represent approximately 15 years of
additional life at age 60. This difference is very
similar to that seen in humans who die prematurely
of the so-called degenerative diseases. The observa-
tion of an apparent penalty associated with the
imposition of rapid ‘catch-up’ growth on a poorly
grown newborn, if applicable to humans, would
have important implications for the management of
growth-retarded human infants.
Conclusions

Advances in immunoassay methodology have led to major improvements in assay specificity and sensitivity. The ability to produce monoclonal antibodies has greatly facilitated the widespread adoption of labelled antibody ('immunometric') techniques as the methods of choice for assaying polypeptides at very low concentrations [56]. These approaches have been successfully applied to the problem of measuring specifically the insulin-related molecules in human plasma in subjects with impaired glucose tolerance and NIDDM. The loss of glucose tolerance in these two conditions is associated with insulin deficiency and insulin resistance. An explanation of the pathogenic mechanisms leading to NIDDM will involve accounting for the close linkage of these two changes in human metabolism.

Epidemiological studies using archival records of measurements related to human fetal growth have shown strong statistical links between this aspect of early development and NIDDM and the insulin-resistance syndrome in adult life. It has been hypothesized that the processes explaining these linkages involve adaptive alterations of fetal organogenesis in response to maternal and fetal nutrition. These adaptations may permanently alter adult metabolism in a way that is beneficial to survival under continued conditions of malnutrition but detrimental when nutrition is normal or supranormal.

This hypothesis is being tested in a rat model in which dams are fed an isocaloric protein-deficient diet during pregnancy and/or lactation and the consequences for the offspring studied. Thus far we have demonstrated (i) permanent changes in key hepatic enzymes of glycolysis and gluconeogenesis (glucokinase and phosphoenolpyruvate carboxykinase) in a direction that would bias the liver towards a 'starved' setting of increased gluconeogenesis. We have speculated that this may be part of an altered zonation of hepatic function and hence associated with other important changes in hepatic function. (ii) A parallel reduction in the β-cell glucose-sensing enzyme glucokinase. (iii) Increased age-dependent loss of glucose tolerance (but not frank diabetes). (iv) A lower male than female life span with, in the male, postnatal (lactation) protein restriction of dams increasing and postnatal restoration of protein decreasing the lifespan of the offspring.

We conclude that these studies show that organ metabolism and even longevity can be permanently altered by events during fetal life. The changes observed suggest that both insulin secretion and sensitivity may be affected. The relevance of these findings to the pathophysiology of human NIDDM warrants further investigation.

To turn all this into a summary appropriate to the Scottish setting. There is an ever-growing stream of diabetes flowing into the sea of the world population. It is not clear whether this derives from a perpetual genetic spring as many believe or, as we propose, is a spate that will subside if and when we can properly feed the deprived. We have fished in this stream and the blood stream, perhaps catching a few Grilse [for the uninitiated: young (hence small) salmon], but the record catch undoubtedly still eludes us!

We thank D. Hutt, A. Flack, A. Wayman and L. Smith of Dunn Nutritional Laboratory, Animal Unit, for their invaluable assistance. This work was supported by the British Diabetic Association, the MRC and the Parthenon Trust. Photograph of C. N. Hales reproduced by permission of the Royal Society.

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