STUDIES ON THE METABOLIC ADAPTATION TO PREGNANCY

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<tr>
<td>ADP</td>
<td>Adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Diaminoethanetetraacetic acid</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Haemoglobin A1c</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>125I insulin</td>
<td>$^{125}$I moniodinated insulin</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>NAD</td>
<td>$\beta$-nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>$\beta$-nicotinamide-adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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CHAPTER ONE

GENERAL INTRODUCTION
Metabolism in pregnancy and foetal nutrition

Pregnancy is known to have a profound effect on the maternal metabolism of most, if not all, foodstuffs (Freinkel 1980). The weight gained by the mother as pregnancy proceeds derives entirely from food ingested by her. In early pregnancy this is channelled into maternal stores (Hyttten 1971) which are subsequently mobilised in late pregnancy and used to provide energy and anabolic precursors for the dramatic weight gain of the foetus and placenta. The levels of blood metabolites in women during pregnancy have been found to differ from those of non-pregnant women, both in the fasted state and post-prandially. Thus, in the early stages of a fast in the pregnant woman, there are more marked changes in the plasma concentrations of alanine (Tyson et al 1971), free fatty acids and β-hydroxy butyrate than in the non-pregnant woman. The most notable difference after feeding in the pregnant woman is the rise in plasma glucose concentration which reaches a higher level than in the non-pregnant state (Freinkel 1980). Pregnancy therefore exaggerates the normal metabolic oscillations which occur between the fed and fasted state of the non-pregnant woman. Such exaggerated metabolic responses have been called "accelerated starvation" and "facilitated anabolism" (Metzger et al 1982). One of the major reasons for these changes in pregnancy is the rapid adaptation of maternal metabolism to the use of fat for energy, thus sparing glucose for passage to the foetus. Thus, even during relatively short periods of fasting, for example if breakfast is missed, accelerated starvation begins to occur. Maternal fat is then mobilised to provide energy; later in pregnancy, when the foetus makes increased demands on maternal glucose stores, the amount of energy produced by this mobilisation of fat becomes inadequate, and there is some catabolism of maternal muscle to provide amino acid substrates for gluconeogenesis. This situation is quickly
reversed on meal eating. Results of oral glucose tolerance tests have shown that the oral glucose dose causes a greater rise in plasma glucose in late pregnancy than in the non-pregnant state (O'Sullivan & Mahan 1964). This is paralleled by a greater rise in plasma insulin. Clearly there is a resistance to the effects of insulin, in the peripheral tissues, in late pregnancy.

Placental hormones

The major hormones produced by the placenta are human placental lactogen (hPL), human chorionic gonadotrophin (hCG), oestrogens and progesterone, all of which contribute to the maintenance of the foeto-placental unit. HCG is synthesised mainly by the syncytiotrophoblast, and its function in early pregnancy is to maintain the ovarian production of steroids until the placenta takes over. Later in pregnancy, hCG may be adrenocorticotrophic in the foetus and play a part in regulating oestrogen metabolism. HPL is produced in large amounts in late pregnancy and has lactogenic properties, helping the development of the breast tissue. Blood levels of hPL are measured to assess placental function. The placenta has a limited part to play in steroid metabolism since it lacks some of the enzymes required for the synthesis and metabolism of these hormones. Oestrone and 17-β oestradiol produced by the placenta are passed to the foetus, where they are hydroxylated to form oestriol in the foetal liver and adrenal glands. The oestriol passes back to the maternal circulation and is excreted in the urine. Urinary oestriol can be measured to assess foeto-placental unit function. Progesterone is synthesised by the placenta using pregnenolone sulphate from the foetus, and cholesterol and pregnenolone from maternal sources. The functions of these steroids include control of myometrial growth and
contractility, and the preparation of the breasts for lactation (Passmore & Robson 1976). One of the effects of these hormones, particularly hPL, is to raise blood glucose levels. The placental hormones therefore act in antagonism with insulin, which is the only hormone to effect a reduction in blood glucose levels, and are said to have contra-insulin properties. The increasing production of these hormones through pregnancy is one of the causes of the insulin resistance described above.

The function of insulin resistance is thought to be to prolong hyper-glycaemia in order to facilitate the passage of excess glucose to the foetus (Freinkel 1980). Thus, by releasing more insulin, the pancreas is able to maintain overall control of glucose homeostasis, albeit with a higher concentration of circulating metabolites than that which is observed in the non-pregnant state. This ensures that the growing foeto-placental unit has the nutrients it requires without an excessive increase in maternal nutrient levels. Maternal malnutrition may result in the foetus being deprived of essential nutrients. The foetus is usually regarded as a parasite, but it is possible that it is less successful than is widely believed, and that it may be more severely affected by malnutrition than the mother (Leader et al 1981).

**Metabolic derangements in pregnancy**

Metabolic derangements in pregnancy are thought to be connected with the incidence of congenital abnormality. Glucose is the major foetal substrate (Gillmer et al 1977) and it has been proposed that congenital abnormalities may be the result of a minor disturbance of maternal plasma glucose levels (Gillmer et al 1975) which might have more serious
implications for the foetus than can be assessed by the measurement of maternal glucose alone. The interference of glycolysis in the rat foetus in early pregnancy has been studied and found to cause teratogenesis (the production of deformity in the developing foetus) and intra-uterine growth retardation (Villee 1984). It has been postulated that a similar interference with glycolysis may occur in humans, leading to congenital abnormalities. An unsatisfactory diet has been cited as one of the putative causes of this particular derangement of glucose metabolism (Freinkel et al 1984).

The development of the foetus is associated metabolically with the placenta, an organ which arises from foetal tissue. Since the mother and foetus are anatomically separate, the two are linked by the placenta which provides the mechanism for the exchange of nutrients (Beaconsfield et al 1980). At implantation, the trophoblast penetrates the maternal endometrium, destroying the epithelium, connective tissue and the endothelium of the maternal blood vessels so that maternal blood directly bathes the placental chorion, allowing exchange to take place between maternal and foetal circulations - the placenta is therefore said to be haemochorionic (Passmore & Robson 1976). The placenta has a high rate of metabolism (Meschia et al 1980), synthesising protein at a rate of upto 7.5 g per day at term. It transfers substrates from the mother to the foetus by a variety of processes:- passive diffusion for gases, free fatty acids, steroids, electrolytes and fat soluble vitamins; facilitated diffusion for sugars; active transport for amino acids, cations and water soluble vitamins; and pinocytosis for proteins (Hill & Longo 1980). In addition to being an organ for the exchange of substrates, the placenta is able to actively concentrate amino acids, which are then passed to the foetus by 3 transport mechanisms (Enders et al 1976). The placenta
is mature and fully developed by the end of the first trimester of pregnancy (Munro 1980) and is sensitive to malnourishment - under these circumstances, substrate transfer to the foetus is reduced (Rosso 1980). This is thought to be caused by an under-production of hPL, which occurs during malnutrition (Munro 1980).

One of the most studied metabolic derangements occurring in humans is diabetes mellitus. Diabetes is brought about by a relative or absolute insulin deficiency which leads primarily to glucose intolerance and also to other metabolic derangements associated with defective or absent insulin action (Montague 1983). The progress of diabetes is affected by pregnancy because of the increased requirement for insulin in the antenatal period, owing to the insulin resistance described above. When diabetes is badly controlled in pregnancy, the foetus is larger than normal because of maternal hyperglycaemia (Pedersen 1954) – excess glucose is passed across the placenta and, in the latter months of pregnancy, is converted by the foetus to fat under the influence of foetal insulin. This does not occur in the first 3 months of pregnancy because the islets of Langerhans have not yet differentiated and foetal insulin is therefore absent. Insulin secreting B cells are present after the third month of embryonic development (Passmore & Robson 1976). The hyperinsulinaemia elicited in the foetus by maternal hyperglycaemia also has a growth hormone-like effect, since insulin promotes protein synthesis (Newsholme & Start 1973). Babies of diabetic mothers are therefore large and fat, i.e., macrosomic (Oakley et al 1978). The size of the baby can be controlled to a certain extent by the strict control of maternal blood sugar levels throughout pregnancy. However, one notable feature of diabetic pregnancies is that there is a higher level of perinatal mortality and congenital abnormality in the offspring.
of these mothers. Major improvements in the incidence of neonatal mortality and morbidity have been made - it is now known that the incidence of perinatal mortality is lower when the blood sugar is well controlled (Karlsson & Knulmer 1982) - but the rates are still higher than in the non-diabetic population.

In addition to the problems faced by existing diabetics who become pregnant, it has also been observed that a woman may become diabetic during pregnancy but return to normoglycaemia post-partum - this condition is called gestational diabetes mellitus (GDM). Pregnancy can be regarded as the only physiological event which is of a diabetogenic nature (Metzger et al 1979). Whatever the causes and origins of diabetes, the complications of pregnancy seen in this condition can be minimised by metabolic control. Congenital abnormalities are lesions which are linked with "incomplete metabolic compensation" (Freinkel 1981). All metabolic fuels are ultimately regulated by insulin and therefore the fuels destined for placental transfer may be affected by any agent which modifies insulin production or action. There is also recent evidence to suggest that the prenatal environment, where the mother is diabetic, can lead to metabolic problems in the offspring, such as childhood obesity and possibly diabetes, whose causes originate in foetal over-nutrition (Pettitt et al 1983).

It is clearly imperative that the metabolism of the pregnant diabetic is as strictly controlled as possible. If control is achieved, the likelihood of an abnormal foetal outcome is reduced. However, the rate of abnormality and fatality in these babies is still higher than in babies of normal mothers. Any metabolic derangement in pregnancy, however slight, must therefore be looked upon as a potential danger to
the viability of the foetus.

**Congenital abnormality and perinatal mortality**

Perinatal mortality is quantified by calculating the perinatal mortality rate (PMR), which is the number of babies who are still born together with those who die in the first week of life, expressed in deaths per thousand live and still births. A congenital abnormality is an abnormality which, if untreated, will have an adverse effect on a person's normal role in society - this may be a physical or a mental abnormality, or both. In a study of handicap conducted in France, 35 per cent of handicaps arose from congenital abnormality and a further 34 per cent from prematurity, low birthweight and social problems (Robertson 1981). Where congenital abnormalities are sufficiently serious or multiple, the foetus is often so compromised that its abnormalities are incompatible with survival. The cause of congenital abnormality is multifactorial, and the mechanism by which it occurs is unknown. In recent years, with the advent of improved obstetric care, although neonatal mortality from causes other than congenital malformations has been reduced (Kitzmiller et al 1978), congenital malformations have had an increasing impact on the PMR (Kalter & Warkany 1983). In addition, despite improvements in the PMR, the gap between the PMR in black and white patients has widened because the birth weight distributions are different - the prevention of low birth weight being one of the most effective ways of improving pregnancy outcome (Williams & Chen 1982).

Numerous factors have been found to contribute to the incidence of perinatal mortality. For example, zinc deficiency in pregnancy is potentially damaging to the foetus. A lack of zinc affects the metabolism
of arachidonic acid, thereby increasing the concentration of the vasoconstrictor prostaglandins - this leads to decreased uterine blood flow (Cunnane et al 1982). Pregnancy is a vulnerable period for zinc deficiency because it is a time of active growth. In addition, low socio-economic status can lead to zinc deficiency because of the increased use of meat substitute items based on vegetable protein. This is also true of the vegetarian diet in any social class, the best sources of zinc being meat, fish and eggs (Williams 1977). A combination of a vegetarian or low-quality diet with pregnancy would therefore tend to lead to zinc deficiency and increased perinatal mortality by the mechanism described. Poor diet has also been implicated in the cause of spina bifida (a congenital neural tube defect) and it is thought that supplementation of vitamins (particularly folic acid) pre-conceptually and in the first weeks of pregnancy could reduce the incidence of this defect (Laurence 1982).

It has already been stated above that low birth weight predisposes to a higher PMR. Retardation of growth (known as foetal growth retardation; FGR, or intra-uterine growth retardation; IUGR) correlates with perinatal mortality, and also with the output of oestriol in the urine (Dobson et al 1981). The amount of oestriol excreted is a measure of foeto-placental unit function and can be increased by improving maternal nutrition (Benny et al 1978). If the maternal diet is deficient in essential nutrients, IUGR may be established at an early stage in pregnancy - this can be detected by measuring the plasma levels of the various placental proteins (Salem et al 1981).

In summary, there are numerous factors causing perinatal mortality and congenital abnormality, many of which have not yet been elucidated. One
of the major dangers to the foetus is that of maternal malnutrition, which leads both to growth retardation and perinatal death. It is also possible that in some instances, malnutrition leads to the birth of babies with congenital abnormalities. Dietary habits vary with culture - socio-economic status and religious restrictions are major influences on what is eaten.

**Birth weight**

Deviation from normal growth leads to an increase in perinatal mortality and morbidity. It is thought that in some infants, a common factor may lead to specific organ damage as well as general growth failure, thus providing a mechanistic link between IUGR and congenital abnormality. The placenta is a major determinant of foetal size, but a growth retarded foetus will tend to outgrow its placenta in an attempt to achieve a "normal" size (Jones & Battaglia 1977). Birth weight is therefore a good measure of the quality of the outcome of pregnancy, since the natural tendency is to increase the birth weight as much as possible, even within the constraints of a small placenta which produces comparatively small quantities of hormones for the maintenance of the pregnancy.

IUGR is usually identified by taking the tenth centile of birth weight allowing for gestational age. Those babies weighing less than the tenth centile (in this country, 2500 g) are classed as growth retarded. However, this practice has a clear disadvantage, since by the nature of its calculation, all groups in the population studied will have the same rate of IUGR. This reduces the definition of IUGR to one of sub-average, rather than sub-optimal, growth (Keirse 1981). There are also indications
that the relationship between birth weight and perinatal mortality is
not as simple as it first appears, because there is a higher percentage
of female babies weighing less than 2500 g at birth, and yet the PMR is
lower in the female population (Wilcox & Russell 1983). Nevertheless,
in Britain in 1968, 7 per cent of all infants weighed less than 2500 g
at birth; these babies accounted for 65 per cent of the PMR (Brimblecombe &
Ashford 1968). It is interesting to note that in 1982 these figures
were effectively unchanged, 6 per cent of infants being growth retarded
and accounting for 60 per cent of mortalities (Dawson et al 1982).
Continued improvements in obstetric care over 14 years have failed to
significantly reduce the death rate in small babies and it must therefore
be assumed that death in these babies is largely unavoidable. It is
clear that there is an important relationship between birth weight and
mortality, but that the assessment of growth retardation must be made
with caution and must not be merely a statistical exercise.

Environmental factors have an effect on birth weight, along with the
mother's age and parity (Yudkin & Harlap 1983) and therefore differences
in the socio-economic status would be expected to lead to differences
in birth weight. The same is true of ethnic differences, especially
where the groups concerned inhabit the poorer areas of the town or city.
The study of birth weight is a useful adjunct to the study of perinatal
mortality because, whereas there are only a few deaths in a given
population, the birth weights of all babies can be studied to gain a
clearer picture of the risk to the foetus.

Special problems in pregnancy faced by ethnic groups

An ethnic group is "a recognisable socio-cultural unit based on some
form of national or tribal distinction which lives among other peoples
rather than in its own country" (Barron 1983). Whenever large-scale immigration has occurred, the ethnic minorities have tended to settle in particular areas of the country. Leicester has a large concentration of Asians, which fall into 2 main groups:- Indians from the Gujurat area, who are of the Hindu or Moslem religions, and East Africans from Kenya, Uganda, Malawi, Tanzania and Zambia, who are mostly Hindus (Henley 1979). In addition there are a few Sikh Indians who originate from the Punjab region. The PMR is higher in the ethnic groups living in this country; for example, in London, the PMR among West Indians in the early 1970s was significantly greater than in the indigenous population (Robinson et al 1982) and in the city of Leicester, the Asian PMR in 1979 was almost twice the non-Asian rate (MacVicar & Clarke 1980). Epidemiological studies have shown that Asians in Leicester are more prone to endocrine, nutritional and metabolic disease, in addition to problems in pregnancy (Donaldson & Taylor 1983). Clearly there are genetic factors which influence the general health of a particular group of people. Genetic factors also determine the eventual size of adults in a population, but it is doubtful whether the birth weight is solely determined by inheritable factors (Barron 1983); the environment must also exert some influence. Therefore, because they are a genetically distinct population and because they tend to live in different areas of the city from the indigenous white population, the Asians in Leicester face special problems in their pregnancies, which may affect birth weight and the mortality rate. Some differences between Asian and Caucasian pregnancies have already been found, for example, that the Asians have a lower plasma volume during pregnancy (Hutchins 1980) but because the plasma volume was related to the body weight, this observation could not be implicated as one of the causes of the higher PMR in the Asians. Some comparative assessment of the growth of foetuses from different ethnic groups has been made by
other workers. Maternal diet is known to exert some influence on foetal size and it has been suggested that low birth weight in ethnic groups may be due in part to the retention of eating habits which are inappropriate to the country of adoption (Ong et al. 1983). In addition, women originating in India and Africa may have been malnourished for long periods of time prior to pregnancy whilst living in their own country, due to the low availability of food – in these women the majority of protein consumed would be derived from vegetable rather than animal sources (Pushpamma et al. 1982). Asians living in this country are often unwilling to eat English food because it may not have been prepared in the correct way, i.e., according to religious customs. For this reason, some Asians say that they are vegetarians when in hospital, while at home they are happy to consume at least some meat (Leicester Royal Infirmary Information Sheets for Social Workers). In pregnancy, certain foods are avoided by Asians, and non-vegetarians may refrain from eating meat during this time. Furthermore, Asians often fast in the last weeks of pregnancy in an effort to keep the birth weight down, because they believe that this will lead to an easier birth (Henley 1979).

Finally, it is more difficult to effect regular antenatal care in the immigrant population because many Asians tend to wait until the pregnancy is well established before reporting it to their doctor, often until after the twentieth week (MacVicar & Clarke 1980). In Leicester, the Hindu vegetarians have an excess of mortality over other groups, and the later start of their antenatal care has been implicated as one of the possible causes, since the mothers at risk are not being seen early enough to institute positive health behaviour, such as eating a balanced diet (Clarke & Clayton 1983).
Nutritional problems in Asians

Asians living in this country are at a disadvantage from a nutritional viewpoint. The climate is much colder than in their own country and the amount of sunlight considerably less. The amount of vitamin D which can be synthesised by exposure to sunlight is therefore less than in India and, as a result, British Asians are at risk of vitamin D deficiency, especially during pregnancy, which can lead to rickets and have adverse effects on foetal dentition (Brooke et al 1981). The Asian religions (Hinduism, Islam and Sikhism) impose considerable restrictions on the diet, and Asian mothers living in England may find that familiar and trusted foods are too expensive to buy in adequate quantities; additionally, there may be a language barrier—if these women were told what English foods contained or how they were cooked (especially vegetables) they might be more able to incorporate them into their diet. A further problem faced by Asian women is that the husband often has first priority at meal times, the mother eating the left-overs (Butler 1971). The pregnant woman is subjected to considerable pressure from members of the family and is told what she should eat (Eaton 1982). For this reason, nutritional advice given during pregnancy should be available to the whole family and not just to the mother herself.

In the Islamic religion followed by Moslems, one lunar month of the year forms a period of fasting called Ramadan. During this time, no food is consumed during the hours of daylight, and the main nutrient intake of the day consists of a large meal taken after sunset. Metabolic studies of Moslems during Ramadan have shown that there is a significant degree of weight loss in this period, accompanied by a rise in serum
cholesterol levels (Fedail et al 1982). Clearly there are distinct metabolic changes associated with fasting, which may have serious implications during pregnancy. In other Asian religions, there is a fast day once a week where only liquids are taken. These too may adversely affect a growing foetus because of the effects of accelerated starvation.

In Birmingham, nutritional studies of Asians showed evidence of inadequate nutrition in poorly grown babies. However, dietary supplementation given during pregnancy did not improve foetal growth (Viegas 1982). In mothers of babies weighing less than the tenth centile, the intake of almost all nutrients was lower, particularly fat and vitamin B6 (Doyle et al 1982). In Asians, the lacto-vegetarian diet which is generally followed can lead to vitamin D deficiency, along with a lack of folic acid and vitamin B12 (Robertson et al 1982). It has been suggested that the gut bacteria of South Indians can produce vitamin B12, thereby alleviating the dietary requirement for this vitamin (Baker & Mathan 1981). However, the babies of vegans (strict vegetarians who do not consume any animal product whatsoever) who are exclusively breast-fed may suffer from severe vitamin B12 deficiency and develop megaloblastic anaemia (Higginbottom et al 1978). Folic acid (vitamin B9) supplementation in pregnancy is commonly practised, and it has been found that the duration of pregnancy can be increased by up to a week in supplemented patients, allowing the foetus to be more adequately prepared for post-natal life (Blot et al 1981). Another aspect of the vegetarian diet is that a greater amount of bran is consumed - this shortens the transit time of food in the gastro-intestinal tract and results in an increased loss of nitrogen and energy by excretion. Increased amounts of phytate in the diet
(found in vegetable products) decrease the amount of calcium absorbed from the gut (Ong et al 1983).

Vitamin B6 deficiency is also common in pregnancy. Lack of this vitamin can affect carbohydrate metabolism, possibly leading to impaired secretion of insulin (Toyota et al 1981). The mechanism by which a vitamin B6 deficiency disturbs carbohydrate metabolism has not been fully elucidated. An interruption of the tryptophan degradative pathway leading to a build-up of xanthurenic acid, which then inactivates insulin, has been proposed (Spellacy 1975), but this has not been proved because exogenous xanthurenic acid does not lead to impaired glucose tolerance. Nevertheless, impaired glucose tolerance in patients taking oral contraceptives can be mitigated by taking vitamin B6 (Safaya & Bamji 1981). Irrespective of the mechanism, vitamin B6 deficiency in pregnancy can lead to impaired glucose tolerance, and therefore put the foetus at risk in the same way as in the diabetic patient. In low income American women, the Apgar score of their babies at birth was lower when the mothers were vitamin B6 deficient (Schuster et al 1981).

The vegetarian diet has been studied in Gujuratis living in Harrow, and it has been found that the foods eaten are low in zinc and copper (Abraham 1982).

It is evident that Asian immigrants have a greater tendency towards malnutrition that the Caucasian population, and that this can have serious implications during pregnancy. The metabolism of these women may be disturbed in such a way as to be detrimental to the foetus.
Metabolic studies of pregnant women in Leicester

In 1980, 12.5 per cent of births in the city of Leicester were to Asian mothers (MacVicar & Clarke 1980). In the same year, the PMR in Leicestershire as a whole fell in line with the national average of 12.3 deaths per thousand live and still births, while the city rate remained at 20.5 deaths per thousand live and still births - this was attributed to the high Asian population in the city. A pilot study carried out in Leicester showed that the incidence of perinatal mortality and growth retardation was higher in Hindu vegetarians than in Caucasian non-vegetarians. It was found that at 36 weeks' gestation, the Hindu vegetarians had mean diurnal blood glucose levels which were similar to those of chemical diabetics, though the babies of these mothers were not macrosomic; in fact they were lighter than the Caucasian non-vegetarian babies. It was concluded that malnutrition was probably a larger problem than diabetes in these women, and that if malnourished, the foeto-placental unit would be unable to synthesise normal amounts of hormones such as hPL. This was confirmed by experiments which showed that Hindu vegetarians had lower levels of hPL than Caucasian non-vegetarians (Benny et al 1980).

These studies were a useful starting point for an investigation of perinatal mortality and congenital abnormality in Leicester's Asian population. In the present study, the scope of the investigation has been widened to include all Asians. Non-vegetarian Asians have formed a third group of patients, along with Asian vegetarians and Caucasian non-vegetarians. In this way it was hoped that any differences in metabolism between vegetarians and non-vegetarians, or between Asians and Caucasians could be elucidated. Whereas the pilot study was conducted at 36 weeks' gestation, this study has been expanded to include measurements of blood metabolites in each trimester of pregnancy. The
problem of excess perinatal mortality in the Asians has endocrine, metabolic and nutritional components which can be studied using biochemical techniques. Blood metabolites have been measured during pregnancy in order to study nutrient homeostasis. The metabolic status of pregnant Asians and Caucasians in Leicester has been investigated at regular intervals. The purpose of this study was to investigate possible causes of the increased PMR in the Asian population, one of the main objectives being to ascertain whether the Asians were gestational diabetics, or whether the higher mean diurnal blood glucose levels previously measured were of little significance. The birth weights of Asian and Caucasian babies have been compared to ascertain whether there are differences in the outcome of pregnancy which would account for the raised incidence of congenital abnormality and perinatal mortality in the Asians.

The study described in this thesis has been carried out on patients attending the Antenatal and Gynaecology Clinics at Leicester Royal Infirmary, between October 1981 and June 1984. The metabolic status of the three study groups of women has been studied using both static and dynamic biochemical tests. All of the measurements have been made from maternal blood samples in order to make the tests as non-invasive as possible. Several aspects of carbohydrate and lipid metabolism have been studied, along with an estimation of plasma free amino acid levels. These measurements have given some idea of the quality of the nourishment of the foetus, since the majority of material passing to the foetus does so via the umbilical cord (Battaglia & Meschia 1978) and thus via the maternal blood.
CHAPTER TWO

PATIENTS AND METHODS
INTRODUCTION

This chapter describes in detail the methods used to obtain the data presented in this thesis. There are three sections; the first describes the clinical protocol, the second deals with the statistical methods employed in the analysis of the data, and the third details the experimental methods used to analyse blood metabolite levels.
REAGENTS

Glucose GOD-Perid test combination, Uranyl acetate deproteinising solution, Precilip E, Precilip EL, Cholesterol C-system, HDL-cholesterol precipitant, $\beta$-Nicotinamide-adenine dinucleotide, disodium salt, grade 2, 98% (NADH), Adenosine-5'-triphosphate, crystallised disodium salt, special quality (ATP), Triacylglycerol acylhydrolase EC 3.1.1.3 (Lipase), Carboxylic-ester hydrolase EC 3.1.1.1 (Esterase), Phosphoenolpyruvate, crystallised monosodium salt (PEP), ATP:pyruvate 2-0-phosphotransferase/L-lactate:NAD oxidoreductase EC 2.7.1.40/1.1.1.27 (PK/LDH mixture), and ATP:glycerol 3-phosphotransferase EC 2.7.1.30 (Glycerokinase) were obtained from The Boehringer Corporation (London) Ltd., Lewes, E. Sussex.

Insulin binding reagent and Human insulin standard were purchased from Wellcome Reagents Ltd., Beckenham, Kent.

Radio-iodinated ($^{125}$I) insulin was obtained from Amersham International Ltd., Amersham, Buckinghamshire.

Bovine albumin powder (Fraction V from bovine plasma) was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

All inorganic salts and reagents for buffers were of Analar grade and were obtained from Fisons Scientific Apparatus, Loughborough, Leicestershire.

Sodium dodecyl sulphate (SDS) was purchased from BDH Chemicals Ltd., Poole, Dorset.
One hundred and thirty nine patients were selected at random from the Antenatal Clinic at Leicester Royal Infirmary Maternity Hospital. Patients were rejected only if they were diabetic, or over 35 years of age, or greater than 120 per cent of their ideal body weight, since these factors are known to affect the response to an oral glucose tolerance test (GTT) (Beard & Hoet 1982).

Each patient was seen during each trimester of pregnancy at approximately 14, 28 and 36 weeks' gestation. Most women present themselves at the clinic at 10 to 12 weeks, and 14 weeks is therefore the earliest time at which a suitable appointment for a GTT can be made. Most women return to the clinic at 28 and 36 weeks, and these dates were therefore chosen to coincide with ordinary clinic appointments. In addition, 36 weeks was the latest date at which most women could be guaranteed not to have delivered. The exact gestational age was calculated from the date of the last normal menstrual period (LNMP) or from ultrasound scan dates. It can be seen from Table 2.1 that the mean gestational ages of the various groups of patients at each visit did not vary significantly. The patients gave their consent to their participation in the study, and the protocol was deemed acceptable by the Local Ethical Committee.

For the purpose of this study, the patients were divided into 3 groups; Caucasians (who were all non-vegetarians), Asian non-vegetarians and Asian vegetarians. A patient was classed as a vegetarian only if, on taking a dietary history, she said that she never ate meat. The Asian population in Leicester is divided as follows:- 60 per cent are Hindus,
Table 2.1

Table showing median gestational age of the 3 groups of women, when GTTs were performed.

The gestational age was calculated from the first day of the last normal menstrual period.

CNV = Caucasian non-vegetarian
ANV = Asian non-vegetarian
AV = Asian vegetarian
<table>
<thead>
<tr>
<th>Trimester</th>
<th>Ethnic Group</th>
<th>Median Gestation (weeks)</th>
<th>Interquartile Range (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CNV</td>
<td>15</td>
<td>12 - 16</td>
</tr>
<tr>
<td></td>
<td>ANV</td>
<td>13</td>
<td>11 - 15</td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>15</td>
<td>12 - 16</td>
</tr>
<tr>
<td>2</td>
<td>CNV</td>
<td>28</td>
<td>27 - 30</td>
</tr>
<tr>
<td></td>
<td>ANV</td>
<td>28</td>
<td>28 - 30</td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>28</td>
<td>28 - 29</td>
</tr>
<tr>
<td>3</td>
<td>CNV</td>
<td>36</td>
<td>36 - 36</td>
</tr>
<tr>
<td></td>
<td>ANV</td>
<td>36</td>
<td>36 - 36</td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>36</td>
<td>36 - 36</td>
</tr>
</tbody>
</table>
25 per cent are Moslems and 15 per cent are Sikhs (Clarke & Clayton 1983). In our randomly selected population, the ratios of Hindus, Moslems and Sikhs were 64 per cent, 17 per cent and 19 per cent respectively, suggesting that the study population had approximately the same ethnic composition as the Asian population in Leicester as a whole.

**Oral glucose tolerance tests** Each patient was asked to fast from midnight prior to her visit to the clinic, ie, for a minimum of 8 hours. On arrival (8 am - 9 am), a 10 ml fasting blood sample was taken. An oral glucose tolerance test was then performed as follows:- 75 g of glucose dissolved in 250 ml warm water, flavoured with pure lemon juice, was administered orally to the patient. Five ml blood samples were obtained at 30, 60 and 120 minutes after the glucose drink. It was intended that samples should be obtained at 15 minute intervals for 2 hours but this was not possible because many of the Asian women had veins which were too fine to extract this number of samples from. Attempts were made to use a catheter to enable the extraction of multiple samples from a vein in the back of the hand, but this proved unsatisfactory. The blood samples were collected into heparinised tubes and were kept on ice for a maximum of 30 minutes prior to further processing. Duplicate 50 μl aliquots of fasting whole blood were added to 1 ml of isotonic saline (0.9%) for the haemoglobin A\textsubscript{1c} assay. (Later in the study, a 5 ml sample of fasting whole blood was obtained in an EDTA coated bottle and sent to the Haematology Department for the Haemoglobin A\textsubscript{1c} assay to be performed there.) All of the blood samples were then centrifuged in an MSE bench centrifuge for 10 minutes at maximum speed. The plasma supernatant was aspirated and kept on ice; the remaining red blood cells were discarded. Duplicate 50 μl aliquots of each sample were added to 0.5 ml of uranyl acetate deproteinising
solution (0.16%) in preparation for estimation of their glucose content. An aliquot of fasting plasma (0.6 ml) was added to 0.15 ml of 5-sulphosalicylic acid solution (15 g/100 ml) in preparation for amino acid analysis. These specially prepared samples were processed according to the procedures described later in this chapter.

Preservation of samples The remainder of each plasma sample was frozen at -20 °C in 0.4 ml aliquots for subsequent analysis. None of these samples were kept for more than 6 months before assay. The losses of most of the metabolites studied over this period have been investigated and shown to be negligible (Demacker & Jansen 1983; Siddiqui & Craig 1975).

Clinical data After delivery, patients' case notes were studied, and any relevant information about the pregnancy and its outcome, such as the weight of the mother through pregnancy and the weight of the baby, were noted.

Pre-pregnancy studies In addition to the study on pregnant women described above, a small study of 38 patients who were not pregnant was carried out in order to investigate metabolism prior to pregnancy. It was not possible to follow up the pregnant patients post-partum in the time allowed for this study because breast feeding would affect their metabolism for several months after the birth. The non-pregnant women were selected randomly from the Gynaecology Out-patients Department at Leicester Royal Infirmary. None of the patients were taking oral contraceptives. In this part of the study, a single 10 ml fasting blood sample was taken from each of the patients and the plasma separated as described previously. Samples were frozen at -20 °C in 0.4 ml aliquots, and subsequently assayed for glucose, insulin,
triglyceride, total cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol.

**Study design** The main reservation in embarking upon this type of study, as in many other studies employing human subjects, is that it was not possible to control or assess all of the experimental conditions. In particular, the quality and quantity of food consumed during pregnancy could not be controlled. The Caucasian group were regarded as "controls" but were strictly a randomly selected group with which to compare the Asians' results. The random selection of patients is said to be the logical basis of tests for statistical significance and can "replace the mythical doctrine that a study group and its controls should be 'alike in all respects' except the factor or factors under investigation" (Mainland 1984). The pre-pregnancy patients were regarded as a "baseline" for pregnancy to enable comparison of pregnant metabolite levels with non-pregnant ones. However, these patients were not the same women as those in the antenatal study and therefore longitudinal comparison, ie, that between pregnancy and the non-pregnant state should be made with reservations. In addition, most of the patients did not attend for GTTs at every trimester. There was some drop-out and therefore in the latter half of the study, patients were asked to attend only at the first and third trimesters. In order to obtain larger groups for the statistical analyses, the second and third trimester groups were supplemented with patients who came for a single GTT at 28 or 36 weeks. So again, longitudinal comparison between trimesters was not strictly possible, but it was possible to make cross-sectional comparisons between the ethnic groups at a particular trimester. In addition, because some of the patients were studied right through pregnancy, it was permissible to make "semi-longitudinal" comparisons, provided that the
problems outlined were carefully considered. The numbers of patients in each group are shown in Table 2.2

**STATISTICAL ANALYSIS**

"It is now widely accepted that any set of biological results which can be described on a numerical scale running from zero upwards will show a skewed distribution . . . the results are weighted by the occurrence of a few relatively high values." (Chard 1976). In most biological assays, therefore, the distribution of results is not Gaussian (normal). Student's t test is often used to assess differences between groups of measurements, but this test assumes a normal distribution in its calculations. A set of results can be assessed for normality by plotting the frequency distribution (Figure 2.1). An approximate guide to normality is that the mean, mode and median values are equal, whereas for a moderately skewed distribution, the following equation applies:

\[
\text{Mean} - \text{Mode} \approx 3(\text{Mean} - \text{Median}) \quad (\text{Petrie 1982})
\]

Accurate assessment of the shape of a frequency distribution requires a large number of results – at least 100. As none of the groups in this study had more than 60 patients, it was impossible to assume a normal distribution, and therefore distribution-free (non-parametric) statistics were used for the analysis of the data.

All of the results obtained in the antenatal study were stored in a data file, ROUDT1, on the Leicester University Cyber 73 computer. This file was too large to fit on the departmental disc and was therefore stored archived on magnetic tape, and accessed on to the scratch (temporary) disc only when needed. Some of the calculations were performed on a micro-computer in the Community Health Department,
Table 2.2

Number of patients participating in the study

CNV = Caucasian non-vegetarian
ANV = Asian non-vegetarian
AV = Asian vegetarian
<table>
<thead>
<tr>
<th></th>
<th>CNV</th>
<th>ANV</th>
<th>AV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number at 1st trimester</td>
<td>30</td>
<td>43</td>
<td>37</td>
</tr>
<tr>
<td>Number at 2nd trimester</td>
<td>17</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Number at 3rd trimester</td>
<td>20</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>Number at 1st and 3rd trimesters</td>
<td>16</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Number at all 3 trimesters</td>
<td>3</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Number in pre-pregnancy study</td>
<td>19</td>
<td>11</td>
<td>8</td>
</tr>
</tbody>
</table>

TOTAL NUMBER OF PATIENTS STUDIED = 177
Figure 2.1

Two types of frequency distribution

This figure shows the variation in mean, mode and median, which occurs when the frequency distribution is skewed away from the normal distribution.

Definitions:

Mean - the sum of all the observations divided by the number of observations.

Median - the middle observation of a set of observations arranged in order of magnitude.

Mode - the most commonly occurring value in a set of values.
a) Normal, or Gaussian, distribution
eg, heights in 8 year olds

Variable (height in metres)

b) Positively skewed distribution
eg, plasma insulin concentrations

Variable (insulin concentration in ng/ml)
and for this, the data file was sent to the micro-computer using the Midnet system.

The statistical manipulations which were performed on the data were used only to confirm observations which were obvious from the raw data. Powerful statistical packages such as the ones used in this study have the danger of "over-analysis" of data simply because the computations can be performed quickly and in numerous combinations. In order to avoid this, only one test was used for all of the analyses; if a significant variation between groups of results was suspected, the data was analysed using the Mann-Whitney U test (Siegel 1956), which is the non-parametric equivalent of Student's t test. This test made comparisons between the median values of 2 groups to see if they were equivalent or significantly different from each other. The test was performed using the Statistical Package for the Social Sciences (SPSS) on the Cyber 73 computer (Nie & Hull 1975; Hull et al 1981). A result was deemed to be significant if the probability that the 2 groups were statistically equivalent was less that 5% ($p < 0.05$). If $p < 0.05$, a result is said to be probably significant; if $p < 0.01$ it is significant; if $p < 0.001$ it is highly significant.

Because non-parametric statistics were used, medians and interquartile ranges were used to describe the data instead of the more common means and standard errors. Version 8.3 of SPSS was used, although the interquartile ranges could not be obtained from this program. Therefore a version of the Minitab program (Ryan 1981) available in the Community Health Department was used to obtain median values with interquartile (IQ) ranges. The results were tabulated to show medians and IQ ranges for each group. Significant differences between groups, where
present, were indicated by an asterisk.

**EXPERIMENTAL**

Quality control of assays

All of the assays used in this study were performed in batches of 20 samples, and several steps were taken to minimise possible errors caused by inter-assay variation.

**Standard curves** A standard solution of the substance under assay was provided with some commercial assay kits, while others contained a commercially prepared plasma sample containing a known concentration of the substance to be estimated. These have been named Primary and Clinical Primary standards respectively (Henry et al 1974). In an assay where an optical density (OD) reading was obtained, the OD of the standard solution was used to calculate the concentration of the substance under investigation in the samples. This method was acceptable for simple assays, for example glucose, but where the assay involved several coupled steps, for example triglyceride, it was preferable to construct a standard curve using a range of dilutions of the standard (Edwards 1983). This is especially true where the response to the assay is known or suspected not to be linear over the range of concentration studied. In all of the assays described in this thesis, except glucose, a standard curve was constructed. The range exceeded, at both high and low extremes, the expected distribution of the sample results.

**Plasma pool** In this method of quality control, variation between
successive assays was assessed by running one aliquot of a pooled plasma sample with each batch of experimental samples. This was known as a Reference Sample (Henry et al 1974), and was particularly useful in radio-immunoassay procedures, where 2 plasma pools, one containing high and one low concentrations of the substance to be estimated, were prepared and used. If the results did not lie on the operative part of the standard curve, the appropriate dilution of sample was made and the sample re-assayed (Siddiqui & Craig 1975). The 2 plasma pools used in this study were prepared from plasma remaining after aliquots had been reserved for the various assays. In each assay where they were used, the mean values obtained over a number of assays were calculated along with the standard deviations. An individual assay was deemed to be suspect if the values of the pooled plasma samples were outside the range of 2 standard deviations from the mean. In this case the assay was repeated for that batch of samples.

Repitition of assays Most assays have an unavoidable margin of error, and in this study, samples were assayed for a second time of their duplicates differed by more than 10 per cent, i.e., if the error on each of the duplicates was more than 5 per cent. This repetition minimised avoidable errors, such as careless pipetting.

Glucose assay

Principle The glucose concentration of plasma samples was estimated using reagents available commercially (Boehringer GOD-Perid Colorimetric Method). In this assay, glucose was oxidised to gluconate by glucose oxidase. This reaction yielded a stoichiometric amount of hydrogen
peroxide, which was then reacted with a chromogen using the enzyme peroxidase, to yield a coloured complex. The concentration of this complex was directly proportional to the initial glucose concentration - see Figure 2.2 (Werner et al 1970). Paracetomol and other drugs are known to interfere with some glucose analyses (Roddis 1981), although the glucose oxidase method seems to be unaffected (Farah et al 1982). Consequently, all patients were asked if they were currently receiving medication likely to affect the assay (National Diabetes Data Group 1979). None were being prescribed any of these.

Procedure For the assay, 100 $\mu$l of deproteinised plasma (see section on oral GTTs) was added to 2.5 ml of 0.1 M phosphate buffer (pH 7.0) containing glucose oxidase (10 U/ml), peroxidase (0.8 U/ml) and the chromogen di-ammonium 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonate) (1 mg/ml). The samples were incubated at 25 °C for 30 minutes, following which their optical density was measured within 20 minutes at 610 nm, using a Unicam SP600 spectrophotometer. Measurements were made with reference to a blank made by carrying 100 $\mu$l of distilled water through the assay procedure. Each sample was assayed in duplicate. A standard solution was supplied with the kit. The concentration of glucose in the samples was calculated with reference to the standard:-

$$\text{Concentration} = \frac{5.55 \times \text{OD sample}}{\text{OD standard}} \quad \text{[mM]}$$
Glucose assay

This diagram shows the test principle of the colorimetric reaction in the Boehringer glucose oxidase method for the estimation of glucose in plasma. The amount of coloured complex formed was estimated by measuring the optical density at 610 nm.
Glucose + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconate} + H_2O_2

H_2O_2 + ABTS \xrightarrow{\text{Peroxidase}} \text{Coloured complex} + H_2O

ABTS = \text{di-ammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonate)}
**Insulin assay**

**Principle** Plasma insulin concentration was estimated by the double antibody procedure of Hales & Randle (1963). The reagents were obtained commercially and were prepared as follows. Porcine insulin, which is immunologically equivalent to human insulin, was injected into a guinea pig to produce anti-insulin serum. Guinea pig gamma globulin, which was made from an ammonium sulphate precipitation of anti-insulin serum, was then injected into a rabbit to yield rabbit anti-guinea pig globulin. When mixed, the anti-insulin serum and the anti-guinea pig globulin combined to form an immune precipitate, the insulin binding reagent. Insulin present in plasma, added to this reagent, bound to the precipitate. In the radio-immunoassay, there was competition for the fixed number of binding sites on the immune precipitate between unlabelled "cold" insulin present in the sample, and $^{125}$I labelled "hot" insulin which was added in a constant amount to each sample. The separation of bound insulin from free insulin was effected by centrifugation. The bound insulin precipitate was counted for radio-activity, allowing an estimate to be made of the amount of unlabelled insulin present in the original sample, by reference to a standard curve ranging from 0 to 8 ng/ml of human insulin, which was constructed each time the assay was performed. Haemolysis of the samples, ie, the presence of haemoglobin in the plasma caused by erythrocyte lysis during venepuncture, is known to affect the assay (Dwenger & Trautschold 1982) and therefore haemolysed samples were not assayed for insulin.

**Procedure** Aliquots of plasma to be assayed were thawed, mixed, and then centrifuged for one minute in a Beckman microfuge to precipitate the fibrin clot which had formed on thawing. The assay was carried
out in triplicate in 3 ml plastic tubes. 100 \( \mu \)l of sample was added to each tube. 100 \( \mu \)l of insulin binding reagent was then added - this was supplied freeze-dried and was re-constituted with 8 ml of distilled de-ionised water. The tubes were mixed and incubated at 4 °C for 4 to 6 hours. One ml of \( ^{125} \text{I} \) insulin (\( \simeq 1 \mu \text{Ci/ml} \)) was made up to 8 ml with diluent buffer. The diluent buffer used was 0.04 M sodium phosphate (pH 7.4) containing 9 g/l sodium chloride and 5 g/l bovine albumin.

100 \( \mu \)l of this solution was added to each assay tube, mixed, and incubated at 4 °C over night. One additional tube, containing 100 \( \mu \)l of \( ^{125} \text{I} \) insulin alone was prepared to determine the total amount of radio-activity added. The next day, 1 ml of diluent buffer was added to each tube. The tubes were mixed and then centrifuged at 3000 rpm for 30 minutes at 0 °C. The supernatant from each tube was immediately decanted, wiping the edge of the tube with tissue to remove the last drops. The precipitate was counted using a Beckman \( \& 4000 \) counter.

A standard curve of counts per minute against insulin concentration for each of the standard solutions was constructed, using a human insulin standard supplied freeze-dried at 2.6 mU per vial. The standard was diluted to 1 mU/ml by adding 2.6 ml of diluent buffer, and kept at -20 °C in 0.4 ml aliquots. For the preparation of the standards, one aliquot was thawed and diluted to 200 \( \mu \)U/ml by the addition of 1.6 ml of diluent buffer. Serial dilutions were made to give standard concentrations of 200, 100, 50, 25, 12.5 and 6.3 \( \mu \text{U/ml} \). A zero consisting of diluent buffer only was also run through the assay. As 25 \( \mu \text{U} \) is approximately equal to 1 ng insulin, these standards were expressed as 8, 4, 2, 1, 0.5 and 0.25 ng/ml. As a measure of the efficiency of the binding reagent, the ratio of the number of counts in the zero tube (0 ng/ml) to that in the total tube was calculated - this should have been approximately 30 per cent. Values of insulin concentration were
determined from the standard curve (Figure 2.3) and the means of each triplicate calculated.

Samples of pooled plasma, containing low and high concentrations of insulin, labelled X and Y respectively, were run through each assay to monitor inter-assay variation. The insulin concentration of X was \(2.50 \pm 0.43\) ng/ml, and that of Y was \(6.68 \pm 1.34\) ng/ml (mean \(\pm\) standard deviation.)

Haemoglobin A\(_{1c}\) assay

Glycosylated haemoglobin is formed non-enzymatically in 2 stages, the first being a rapid and reversible step, where a Schiff base with glucose is formed on the N terminal of the \(\beta\) chains of haemoglobin. This is then slowly and irreversibly converted to haemoglobin A\(_{1c}\) (HbA\(_{1c}\)) (Figure 2.4). The Schiff base is called "labile" HbA\(_{1c}\) and the amount present in the blood varies according to diurnal fluctuations in blood glucose concentration. The final product of glycosylation is referred to as "stable" HbA\(_{1c}\) and reflects the average blood glucose concentration over the previous 2 to 3 months (Goldstein et al 1980). The labile HbA\(_{1c}\) can be extracted from erythrocytes by incubating 0.05 ml of whole blood in 1 ml of isotonic saline (0.9% w/v) for 6 hours at 37 °C prior to analysis (Bolli et al 1981).

Assisted electrophoresis separation of HbA\(_{1c}\) The principle of this assay has been described by Ambler et al (1983). Samples of blood were collected in EDTA coated bottles and sent to the Haematology Department, where a haemolysate was prepared. The buffer used contained dextran sulphate, which attached to the \(\beta\) chain of haemoglobin at the
Insulin was estimated in plasma samples by radio-immunoassay. Displacement of $^{125}\text{I}$ insulin from insulin binding reagent was assessed using increasing concentrations of unlabelled human insulin. The number of counts remaining in the precipitate after centrifugation was plotted as a function of insulin concentration. The points represent the means of triplicate observations.
Steps in the formation of haemoglobin $A_{1c}$

Haemoglobin is glycosylated non-enzymatically by the addition of glucose to the N terminal of the $\beta$ chains of the molecule. Glycosylation takes place in 2 stages; the first is reversible, the second irreversible, as shown.
\[ \beta - \text{NH}_2 + \text{HC} = 0 \]

\[ \text{HCOH} \]
\[ \text{HOCH} \]
\[ \text{HCOH} \]
\[ \text{HCOH} \]
\[ \text{CH}_2 \text{OH} \]

\[ \text{HbA}_{II} + \text{Glucose} \]

\[ \text{(amino terminal of the } \beta \text{ chain)} \]

\[ \text{RAPID, REVERSIBLE} \]

\[ \text{HC} = N \beta \]
\[ \text{HCOH} \]
\[ \text{HOCH} \]
\[ \text{HCOH} \]
\[ \text{HCOH} \]
\[ \text{CH}_2 \text{OH} \]

\[ \text{Labile HbA}_{1c} \]
\[ \text{(Schiff base)} \]

\[ \text{SLOW, IRREVERSIBLE - AMADORI REARRANGEMENT} \]

\[ \text{H}_2 \text{C-AH}_2-\beta \]
\[ \text{C}=0 \]
\[ \text{HOCH} \]
\[ \text{HCOH} \]
\[ \text{HCOH} \]
\[ \text{CH}_2 \text{OH} \]

\[ \text{Stable HbA}_{1c} \]
\[ \text{(Ketamine)} \]
glycosylation site, i.e., it had an affinity for unglycosylated haemoglobin (HbA\textsubscript{d}). The dextran sulphate effectively added charge to the haemoglobin. The haemolysate was separated by mobile affinity electrophoresis on a cellulose nitrate strip, where the HbA\textsubscript{d} moved more rapidly than the HbA\textsubscript{lc} due to its extra charge. The strips were scanned using a densitometer at 520 nm, and the peaks were integrated to determine the ratio of HbA\textsubscript{lc} to total haemoglobin.

**Total cholesterol assay**

**Principle** The concentration of total cholesterol in fasting plasma was estimated using reagents available commercially (Boehringer Cholesterol C-system). In the assay, cholesterol esters in the sample were first converted to cholesterol and fatty acid by cholesterol esterase (\geq 0.4 U/ml). The cholesterol was then oxidised by cholesterol oxidase (\geq 0.25 U/ml) to \Delta^4\text{-}cholestenone and hydrogen peroxide. The hydrogen peroxide was reacted with 4-aminophenazone (1 mM) and phenol (6 mM) in the presence of peroxidase (\geq 0.2 U/ml), to give 4-(p-benzoquinone-mono-imino)-phenazone, which can be detected spectrophotometrically at 500 nm, and the concentration of which is stoichiometrically related to the original concentration of cholesterol in the sample (Figure 2.5). All of these reagents were present in a stock reagent solution provided. In addition, the stock solution contained tris buffer (100 mM, pH 7.0), magnesium aspartate (50 mM), 3,4 dichlorophenol (4 mM) and hydroxypolyethoxy - n-alkanes (0.3%). (Siedel et al 1981; Stahler et al 1977; Trinder 1981).

**Procedure** One aliquot of frozen fasting plasma from each patient was thawed and centrifuged for one minute in a Beckman microfuge to remove
Total cholesterol assay

This diagram shows the test principle of the enzymatic assay for total cholesterol by the Boehringer C-system method. The amount of $4-(p$-benzoquinone-mono-imino)$\cdot$phenazone formed was estimated by measuring optical density at 500 nm.
Cholesterol ester + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{RCOOH}

\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \Delta^4\text{-cholestenone} + \text{H}_2\text{O}_2

2\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} 4\text{-}(\text{p-benzoquinone-mono-imino})\text{-phenazole} + 4\text{H}_2\text{O}
the fibrin clot which formed on thawing. A stock solution of reagent was made up by adding 100 ml of distilled water to the dried reagents supplied. The solution was left to stand at room temperature for at least 10 minutes before use. 10 µl aliquots of each sample were pipetted in duplicate into plastic 1 ml cuvettes. One ml of stock reagent solution was added to each cuvette and mixed. The samples were incubated for 10 minutes at room temperature. The optical density was read at 500 nm within 60 minutes, against a blank of stock solution. A standard curve was prepared using a range of dilutions of the Precilip EL standard obtained from Boehringer, and the sample values calculated from this graph (Figure 2.6). A pooled plasma sample (X) was run through each assay to detect inter-assay variation. The total cholesterol concentration of X was 5.17 ± 0.7 mM (mean ± standard deviation).

High density lipoprotein (HDL) cholesterol assay

Principle Chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated when phosphotungstic acid and magnesium ions were added to the plasma sample. Centrifugation of the sample left HDL in the supernatant (Burstein et al 1970; Lopes-Virella et al 1977). The cholesterol concentration of the HDL present in the supernatant was then determined using the cholesterol assay described above. The precipitating solution was available commercially (Boehringer Test Combination, HDL cholesterol).

Procedure The precipitant was prepared by mixing phosphotungstic acid (4.8 g/l) and magnesium chloride (3 M) together in a 5:1 ratio as required. One aliquot of fasting plasma from each patient was
Total cholesterol in fasting plasma was estimated enzymatically. The assay yielded a coloured compound whose concentration was stoichiometrically related to the concentration of cholesterol in the sample. The optical density of this compound was plotted as a function of increasing concentration of a standard plasma sample containing a known amount of cholesterol.

Each point represents the mean of duplicate observations.
Optical density (500 nm) vs. Cholesterol concentration (mM)
thawed as described previously. 0.1 ml of each sample was added to 10 μl of precipitant in a 1.5 ml microfuge tube. The samples were mixed and left to stand for 10 minutes at room temperature. The tubes were then centrifuged in a Beckman microfuge for 2 minutes. Duplicate 20 μl aliquots of each sample supernatant were assayed for cholesterol using the total cholesterol method. The total cholesterol standard curve was used to calculate the results (Figure 2.6). A pooled plasma sample (X) was used to detect inter-assay variation. The concentration of HDL cholesterol in X was 0.89 ± 0.15 mM (mean ± standard deviation).

**LDL cholesterol estimation**

**Principle** The usual method of estimating LDL cholesterol concentration involves separating the LDL from the other lipoproteins in the sample using an ultracentrifuge, followed by analysis of the LDL fraction for cholesterol. However, a method for estimating the LDL cholesterol content of serum or plasma is available, which does not require this technique. LDL cholesterol concentration can be calculated using values for total cholesterol, triglyceride and HDL cholesterol. The results gained by calculation have a correlation coefficient of 0.94 - 0.99 with the preparative ultracentrifuge method (Friedewald et al 1972).

**Procedure** The calculation used made 2 assumptions:-

1. That the ratio of the mass of triglyceride to that of cholesterol is relatively constant at 5:1 - this is true in all patients except those with the rare type III hyperlipoproteinaemia.
2. That in the absence of chylomicrons (ie, in fasting plasma), most of the triglyceride is contained in the VLDL.
The equation used to calculate the concentration of LDL cholesterol was:

\[
\text{LDL cholesterol} = \left( \frac{\text{total cholesterol}}{\text{mg/100 ml}} \right) - \left( \frac{\text{triglyceride}}{5} \right) - \text{HDL cholesterol}
\]

In order to convert this expression to millimolar quantities, as in this study, the equation was modified to:

\[
\text{LDL cholesterol} = \left( \frac{\text{total cholesterol}}{\text{mM}} \right) - \left( \frac{\text{triglyceride}}{2.2} \right) - \text{HDL cholesterol}
\]

**Triglyceride (triacylglycerol) assay**

**Principle** Triglyceride in fasting plasma was assayed enzymatically (Boehringer 1978) according to the reactions shown in Figure 2.7. The triglyceride in a sample was initially hydrolysed by a lipase/esterase mixture to glycerol and fatty acids. The glycerol was then assayed using a coupled enzyme system. The conversion of NADH to NAD was followed at 340 nm.

**Development of the assay** The assay was first performed using a "kit" and following the method of Wahlefeld (1974). However, a standard sample, Precilip EL, having a triglyceride concentration of 3.46 mM, showed a concentration of 2.36 mM by this method. This suggested that the reaction was not going to completion, even though the protocol stated that 10 to 15 minutes' incubation at room temperature was adequate. The time course for the assay was determined by connecting a chart recorder to the spectrophotometer. This showed that the reaction took 90 minutes to reach completion at room temperature. The assay consisted of 2 stages:-- the hydrolysis of triglyceride to glycerol,
Triglyceride assay

This diagram shows the test principle of the ultraviolet enzymatic method for the estimation of triglyceride concentration in plasma. The conversion of NADH to NAD was measured by observing the fall in optical density at 340 nm.
Triglyceride $\xrightarrow{\text{lipase/esterase}}$ Glycerol + Fatty acid

Glycerol + ATP $\xrightarrow{\text{glycerokinase}}$ Glycerol-3-phosphate + ADP

ADP + PEP $\xrightarrow{\text{pyruvate kinase}}$ Pyruvate + ATP

Pyruvate + NADH + H$^+$ $\xrightarrow{\text{lactate dehydrogenase}}$ L-lactate + NAD$^+$
and the coupling of glycerol to the NADH/NAD conversion. When the assay was performed using a 3.46 mM solution of glycerol, the end-point was reached more rapidly, suggesting that the rate-limiting step was the hydrolysis of triglyceride. It was necessary, therefore, to increase the activity of the lipase/esterase mixture, either by adding more enzyme or by increasing the temperature of the assay. The latter had the most desirable effect, the end-point being reached after 15 minutes at 37 °C. Although the kit protocol stated that the concentration of triglyceride in the samples could be calculated from the optical density measurements of the samples themselves, i.e., concentration of triglyceride = 8.13 x change in OD \text{[mM]}, it was decided that a standard curve should be constructed using Precilip standard solutions, and the results calculated from the graph.

**Procedure** An assay buffer was made up (20 mM phosphate, 4 mM magnesium sulphate and 0.35 mM SDS, pH 7.0). Aliquots of dry NADH, ATP and PEP were kept in brown bottles at 4 °C in silica gel-dried containers until required (7.2 mg NADH, 13.4 mg ATP and 3.8 mg PEP). Precilip E and EL standards were prepared by reconstituting one bottle of lyophilised plasma with 3 ml of distilled water. The solution was left at room temperature for 30 minutes and then stored at -20 °C in 0.15 ml aliquots. One aliquot was thawed for each assay. One aliquot of fasting plasma from each patient was thawed as described previously. A stock reagent solution was made up immediately prior to the assay by mixing together, at room temperature, 50 ml of assay buffer, the contents of one vial of NADH/ATP/PEP dissolved in 1 ml of distilled water, 1 ml of LDH/PK mixture (supplied as a mixture of 4 mg/ml containing ≥300 U/ml LDH and ≥50 U/ml PK), 0.08 ml of lipase (50,000 U/ml) and 0.03 ml esterase (100 U/mg and 10 mg/ml, i.e., 1000 U/ml). This stock solution contained all of the
reagents needed for the assay except glycerokinase.

Twenty \( \mu l \) of each plasma sample was placed, in duplicate, in 1 ml plastic cuvettes. One ml of stock reagent solution was added to each cuvette and the contents mixed by inversion. The cuvettes were incubated in a 37\(^\circ\)C water bath for 15 minutes. The optical density of the samples was measured at 340 nm in a Cecil CE272 Linear Readout UV Spectrophotometer, using the tungsten lamp (OD 1). The spectrophotometer was zeroed against air. Five \( \mu l \) of glycerokinase (1 mg/ml, 85 U/mg) was then added to each cuvette and mixed by inversion. The cuvettes were incubated for 15 minutes at 37\(^\circ\)C, and the optical densities read (OD 2). The change in optical density due to hydrolysis of triglyceride (\( \Delta OD \)) was calculated as \( OD_2 - OD_1 \). A reagent blank containing 20 \( \mu l \) of distilled water instead of sample was carried through the assay. The change in optical density occurring in this reagent blank was in the range 0.005 to 0.010, and was subtracted from the \( \Delta OD \) for each sample. A standard curve was prepared using a range of dilutions of the Precilip E and EL standards (Figure 2.8) and the results calculated from this graph. Pooled plasma samples (X and Y) were run through each assay to detect inter-assay variation. The triglyceride concentration of X was 2.27 \( \pm \) 0.16 mM and that of Y was 2.13 \( \pm \) 0.13 mM (mean \( \pm \) standard deviation).
Figure 2.8

Standard graph for triglyceride

Triglyceride concentration in fasting plasma was estimated enzymatically. The assay was coupled to a reaction involving the conversion of NADH to NAD, and the fall in optical density at 340 nm corresponded to the amount of triglyceride originally present. The change in optical density was plotted as a function of increasing concentration of the 2 standard plasma samples, Precilip E and EL, which contained a known amount of triglyceride.

Each point represents the mean of duplicate observations.

○ = Precilip E
● = Precilip EL
Triglyceride concentration (mM)

Change in optical density (340 nm)
CHAPTER THREE

CARBOHYDRATE METABOLISM
INTRODUCTION

Previous studies of carbohydrate metabolism during pregnancy in the Asian population of Leicester showed that the Asian vegetarian women had higher mean diurnal blood glucose levels than Caucasian non-vegetarian women (Benny et al 1980). These results indicated that the control of carbohydrate metabolism might be impaired in the Asians and it was suggested that these changes might contribute to the increased perinatal mortality rate seen in the Asians. It was therefore decided to investigate in more detail, carbohydrate metabolism during pregnancy in Asian and Caucasian women living in Leicester.

Plasma glucose and insulin concentrations were measured after an overnight fast of at least 8 hours in order to assess basal levels of glucose, and to measure the amount of insulin secreted to maintain these levels. In addition, an oral GTT was performed to obtain a more dynamic assessment of carbohydrate metabolism, since this test measures the ability of the body to return the blood glucose concentration to the fasting value following an oral glucose dose of 1 to 2 g per kilogram of body weight. HbA1c was also measured in fasting whole blood, since this may give a retrospective assessment of the integrated blood glucose concentration over the 2 to 3 months prior to the test (Cerami 1978).

METHODS

Glucose tolerance tests were carried out on Asian non-vegetarian, Asian vegetarian and Caucasian non-vegetarian women at approximately 14, 28 and 36 weeks' gestation. Blood samples were obtained at fasting, and
30, 60 and 120 minutes after the administration of a 75 g oral glucose load. The samples were centrifuged to obtain plasma, which was assayed for glucose and insulin, using methods described in Chapter 2. In addition, aliquots of whole blood were sent to the Haematology Department, where their HbA\textsubscript{1c} content was estimated.

**RESULTS**

The results were analysed using non-parametric statistics and tabulated to show median values and interquartile ranges.

**Glucose**

Table 3.1 shows the fasting plasma glucose concentration in each trimester of pregnancy for the 3 groups of women studied. There was no significant difference between the groups at any time during pregnancy, although the Asian vegetarians appeared to have lower fasting levels of glucose in early to mid pregnancy. There was a slight downward trend in fasting glucose as pregnancy advanced in Asian non-vegetarians and Caucasian non-vegetarians. This, together with the slight rise in fasting glucose concentration seen in the Asian vegetarians in the third trimester, resulted in all groups having virtually the same fasting glucose concentration in the third trimester. Fasting glucose concentrations remained between 4.25 and 4.50 mM throughout pregnancy in all groups studied.

In Table 3.2, the plasma glucose concentrations during an oral GTT are shown for each group of women in each trimester of pregnancy. The maximum level, to which the glucose concentration rose, increased from
Table 3.1

Median fasting plasma glucose concentrations in pregnancy

Fasting plasma glucose concentration was measured in the following groups of women: Asian non-vegetarians (ANV), Asian vegetarians (AV) and Caucasian non-vegetarians (CNV). Median values are shown for each group, with the interquartile ranges in brackets.
<table>
<thead>
<tr>
<th>Group</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>ANV</td>
<td>4.56 (4.25-4.94)</td>
</tr>
<tr>
<td>AV</td>
<td>4.43 (4.14-4.74)</td>
</tr>
<tr>
<td>CNV</td>
<td>4.51 (4.08-4.66)</td>
</tr>
</tbody>
</table>
Table 3.2

Profiles of median plasma glucose concentrations during an oral glucose tolerance test.

Plasma glucose concentrations were measured during an oral glucose tolerance test, at each trimester of pregnancy, in the following groups of women: Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values for each group are shown, with interquartile ranges in brackets.
<table>
<thead>
<tr>
<th>Trimester of pregnancy and group</th>
<th>Time of blood sample (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1st trimester (14 weeks)</td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>4.56 (4.25-4.94)</td>
</tr>
<tr>
<td>AV</td>
<td>4.43 (4.14-4.74)</td>
</tr>
<tr>
<td>CNV</td>
<td>4.51 (4.08-4.66)</td>
</tr>
<tr>
<td>2nd trimester (28 weeks)</td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>4.39 (4.18-4.97)</td>
</tr>
<tr>
<td>AV</td>
<td>4.21 (3.82-4.86)</td>
</tr>
<tr>
<td>CNV</td>
<td>4.35 (4.03-4.74)</td>
</tr>
<tr>
<td>3rd trimester (36 weeks)</td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>4.27 (4.01-4.58)</td>
</tr>
<tr>
<td>AV</td>
<td>4.32 (4.02-4.66)</td>
</tr>
<tr>
<td>CNV</td>
<td>4.32 (4.00-4.60)</td>
</tr>
</tbody>
</table>
6 - 7 mM, to 7 - 8 mM in all groups as pregnancy proceeded. In addition, the 60 minute glucose concentration became progressively higher, so that in the third trimester, it was almost equal to the 30 minute concentration. This is in sharp contrast to the first trimester, where the glucose concentration had fallen to basal levels by 60 minutes. Therefore the shape of the glucose tolerance curve was observed to change as pregnancy advanced. At no time during the GTT did the Asians (vegetarian and non-vegetarian) differ significantly from the Caucasians, or from each other, with respect to their plasma glucose concentrations.

Insulin

Table 3.3 shows the fasting plasma insulin concentration in each trimester of pregnancy for the 3 groups of women studied. There was no significant variation in plasma insulin concentration during pregnancy in the Caucasian non-vegetarians. However, the Asians (both vegetarian and non-vegetarian) showed a significant rise in fasting insulin concentration during pregnancy - the fasting plasma insulin concentration doubled between 14 and 36 weeks' gestation in these 2 groups. The Asians also had lower fasting insulin concentrations than the Caucasians in the first trimester (although this difference was not statistically significant), and their fasting insulin concentration in the third trimester was significantly higher than that of the Caucasian non-vegetarians.

During the GTT, the insulin concentrations (Table 3.4) showed similar trends to those of the glucose concentrations, ie, as pregnancy advanced, the maximum insulin levels became progressively higher,
Table 3.3

Median fasting plasma insulin concentrations in pregnancy

Fasting plasma insulin concentrations were measured in the following groups of women: Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values are shown for each group, with interquartile ranges in brackets.

* $p < 0.05$ for both the difference between Asian non-vegetarians and Caucasian non-vegetarians, and the difference between Asian vegetarians and Caucasian non-vegetarians.

Biological activity of insulin: 1 ng/ml $\approx 25 \mu U/ml$
<table>
<thead>
<tr>
<th>Group</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>ANV</td>
<td>0.35 (0.22-0.60)</td>
</tr>
<tr>
<td>AV</td>
<td>0.34 (0.26-0.50)</td>
</tr>
<tr>
<td>CNV</td>
<td>0.45 (0.29-0.60)</td>
</tr>
</tbody>
</table>
Profiles of median plasma insulin concentrations during an oral glucose tolerance test

Plasma insulin concentrations were measured during an oral glucose tolerance test, in each trimester of pregnancy, in the following groups of women: Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values are shown for each group, with interquartile ranges in brackets.

* p < 0.01 for the difference between ANV and CNV
  p < 0.05 for the difference between AV and CNV

** p < 0.01 for the difference between ANV and CNV
  p < 0.05 for the difference between AV and CNV

*** p < 0.05 for both the difference between ANV and CNV, and the difference between AV and CNV

Biological activity of insulin: 1 ng/ml ≈ 25 µU/ml
<table>
<thead>
<tr>
<th>Trimester of pregnancy and group</th>
<th>Time of blood sample (minutes)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st trimester</strong> (14 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>0.35 (0.22-0.60)</td>
<td>3.60 (1.95-4.43)</td>
<td>* 2.82 (2.01-3.68)</td>
<td>**2.18 (1.46-2.85)</td>
<td></td>
</tr>
<tr>
<td>AV</td>
<td>0.54 (0.26-0.50)</td>
<td>3.27 (2.10-4.60)</td>
<td>2.79 (1.86-3.72)</td>
<td>2.05 (1.20-3.17)</td>
<td></td>
</tr>
<tr>
<td>CNV</td>
<td>0.45 (0.29-0.60)</td>
<td>2.30 (1.76-4.40)</td>
<td>1.65 (1.17-2.95)</td>
<td>1.35 (0.88-2.10)</td>
<td></td>
</tr>
<tr>
<td><strong>2nd trimester</strong> (28 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>0.70 (0.33-0.79)</td>
<td>4.65 (2.31-6.64)</td>
<td>4.37 (2.81-7.34)</td>
<td>3.90 (1.83-4.85)</td>
<td></td>
</tr>
<tr>
<td>AV</td>
<td>0.58 (0.45-1.33)</td>
<td>4.87 (3.03-7.19)</td>
<td>4.75 (1.40-6.47)</td>
<td>2.73 (1.59-7.05)</td>
<td></td>
</tr>
<tr>
<td>CNV</td>
<td>0.60 (0.43-0.70)</td>
<td>4.45 (2.40-5.53)</td>
<td>3.07 (2.27-5.59)</td>
<td>2.02 (1.37-3.93)</td>
<td></td>
</tr>
<tr>
<td><strong>3rd trimester</strong> (36 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>***0.79 (0.51-1.02)</td>
<td>5.47 (3.08-8.00)</td>
<td>5.35 (3.03-8.00)</td>
<td>4.10 (2.35-6.60)</td>
<td></td>
</tr>
<tr>
<td>AV</td>
<td>0.73 (0.49-1.07)</td>
<td>4.79 (3.40-7.70)</td>
<td>4.63 (3.38-7.26)</td>
<td>3.97 (3.07-5.97)</td>
<td></td>
</tr>
<tr>
<td>CNV</td>
<td>0.47 (0.37-0.70)</td>
<td>4.78 (3.00-5.20)</td>
<td>4.82 (3.03-6.88)</td>
<td>3.88 (2.33-4.88)</td>
<td></td>
</tr>
</tbody>
</table>
increasing by up to 3 ng/ml between the first and third trimesters. There was also a similar shift in the shape of the curve, with the 60 minute level approaching the 30 minute level in late pregnancy. At no time during pregnancy did the insulin concentration return to its basal value (< 1 ng/ml) by 120 minutes.

The Asians (both vegetarian and non-vegetarian) had higher plasma insulin concentrations than the Caucasians during the GTT throughout pregnancy, but this observation reached statistical significance only in the first trimester, 60 and 120 minutes after the glucose challenge.

Haemoglobin A\textsubscript{1c}

Table 3.5 shows the HbA\textsubscript{1c} results obtained by electrophoretic separation of the haemoglobin fractions. The results show that all of the groups of women had normal levels of HbA\textsubscript{1c} throughout pregnancy and that there was no significant difference between the groups at any time during pregnancy. Furthermore, the haemoglobin A\textsubscript{1c} concentration did not change significantly in any of the groups as pregnancy advanced.

DISCUSSION

Effects of pregnancy on blood glucose concentration

Glucose tolerance tests It is known that the response to an oral glucose challenge changes during pregnancy (Phelps et al 1981), and it has been suggested that one cause of this change is the pressure exerted by the foetus on the abdomen, which delays the absorption of
Table 3.5

Median fasting whole blood $\text{HbA}_{1c}$ concentrations in pregnancy

The concentration of $\text{HbA}_{1c}$ in whole blood was determined in the first and third trimesters of pregnancy in the following groups of women:— Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV).

$\text{HbA}_{1c}$ content was expressed as a percentage of total haemoglobin. The number of determinations for each group was shown in brackets.

Normal range of $\text{HbA}_{1c} = 5 - 9\%$
<table>
<thead>
<tr>
<th></th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANV</td>
</tr>
<tr>
<td>% HbA1c at 1st trimester (14 weeks)</td>
<td>6.5 (7)</td>
</tr>
<tr>
<td>% HbA1c at 3rd trimester (36 weeks)</td>
<td>6.1 (13)</td>
</tr>
</tbody>
</table>
foodstuffs. However, this would only account for the change in the shape of the glucose tolerance curve observed as pregnancy advanced (Table 3.2) and would not explain why the maximum blood glucose concentration rose with increasing gestational age. Clearly there is an element of metabolic adaptation occurring during pregnancy. It is possible to use an intravenous GTT to by-pass the gastro-intestinal tract (and thus the possible effects of the foetus on glucose absorption) (Spellacy 1975) but the oral glucose challenge is a more clinically relevant method for studying carbohydrate metabolism, and since it has been shown that the oral and intravenous GTTs show the same distribution of results in pregnancy (Hadden 1975), it is doubtful whether differential absorption is affecting the GTT results. The oral GTT was therefore used in this study. Elaborate methods are available for the numerical analysis of GTT results (Billewicz et al 1973), but for this study it was felt that if there were differences between the groups, they should be obvious without the need to resort to lengthy mathematical manipulations. The British Diabetic Association (BDA) criteria were chosen for the investigation of individual GTT results, in order to ascertain whether any of the patients became diabetic or showed impaired glucose tolerance during pregnancy. The BDA support the criteria suggested by the European Association for the Study of Diabetes (EASD), based on a 75 g glucose dose (Keen et al 1979). Other studies have used 100 g, but it has been found that there is no significant difference in the results obtained with these 2 doses (National Diabetes Data Group 1979). The BDA criteria can be summarised as follows:

1 Impaired Glucose Tolerance - if the fasting plasma glucose concentration is less than 8 mM, and, 2 hours after the glucose dose, the plasma glucose concentration is between 8 and 11 mM.
2 Diabetes - if the fasting plasma glucose concentration is greater than 8 mM, and, 2 hours after the glucose dose, the plasma glucose concentration is greater than 11 mM. In both cases, one of the glucose measurements made between 0 and 2 hours should exceed the 2 hour value (Keen et al 1979). The identification and careful management of patients whose blood glucose levels exceed the "normal" limits of the mean + 2 standard deviations during a GTT, helps to lower the perinatal mortality rate, but insulin treatment is only required when the fasting glucose level is raised (Carpenter & Coustan 1982; Coustan 1983).

Glucose tolerance in pregnancy Using the BDA criteria, none of the patients were found to be diabetic. Only one patient, a Caucasian non-vegetarian, showed impaired glucose tolerance in the second trimester, having a plasma glucose concentration of 10.29 mM, 2 hours after the glucose dose, with one intervening value of 13.70 mM (the patient did not attend for the third trimester GTT).

None of the groups studied, when their median values were considered, showed impairment of glucose tolerance, as assessed by the BDA criteria. However, the plasma glucose concentrations during a GTT increased in pregnancy in all of the groups. It was also observed that the plasma glucose concentration did not return to basal levels by 120 minutes in the second and third trimesters. This is illustrated in Figure 3.1, which shows the changing plasma glucose response to a GTT during pregnancy in the Caucasian non-vegetarians. Both of these observations indicate that the patients' glucose tolerance became progressively lower during pregnancy. This appears to be a normal physiological response, making the extra glucose in the blood available to the
Figure 3.1

Median plasma glucose concentrations during an oral glucose tolerance test in each trimester of pregnancy, as observed in Caucasian non-vegetarian women.

Plasma glucose concentrations were measured during a GTT in Caucasian non-vegetarians at the following times in pregnancy:

- **First trimester (14 weeks)**
- **Second trimester (28 weeks)**
- **Third trimester (36 weeks)**

Median values are plotted.
growing foetus. However, careful metabolic control is still essential if metabolite levels are to be maintained within physiological limits.

**Insulin secretion during a GTT in pregnancy**

It is known that the insulin response to glucose is increased in late pregnancy (Freinkel 1980). However, since the glucose level also rises, the sensitivity to insulin must be reduced. Placental hormones can augment the islet insulin secretory response, affect insulin receptors on target tissues, and alter the sensitivity to insulin (Kalkhoff & Kim 1979). These hormones are therefore implicated in the cause of these changes in insulin secretion and action. The result of the increased islet response to glucose is that the extra insulin, though having less effect on blood glucose levels and thereby allowing the blood glucose concentration to remain higher, maintains control over the metabolism and prevents degeneration to the diabetic state. The Asian women studied had higher fasting plasma insulin concentrations in the second and third trimesters, when compared with the Caucasians, whose fasting plasma insulin concentrations did not change significantly during pregnancy. These results suggest that the Asian women required higher levels of insulin to maintain their fasting blood glucose concentrations, and as pregnancy proceeded, they became progressively more resistant to the biological effectiveness of basal concentrations of insulin.

Plasma insulin concentrations during a GTT were higher in all groups in late pregnancy, when compared with early pregnancy, and did not return to basal levels by 120 minutes. This is illustrated in Figure 3.2, which shows the changing insulin response to a GTT during pregnancy in the Caucasian non-vegetarians. This hyperinsulinaemia, occurring in
Figure 3.2

Median plasma insulin concentrations during an oral glucose tolerance test in each trimester of pregnancy, as observed in Caucasian non-vegetarian women.

Plasma insulin concentrations were measured during a GTT in Caucasian non-vegetarians at the following times in pregnancy:—

- **First trimester (14 weeks)**: ●—●
- **Second trimester (28 weeks)**: ■—■
- **Third trimester (36 weeks)**: ▲—▲

Median values are plotted.
Median plasma insulin concentration (ng/ml)

Time after glucose load (min)
parallel with increased glucose levels, indicated that the patients were becoming more resistant to the action of their insulin, as pregnancy advanced. In addition to the higher fasting plasma insulin concentrations which were observed in the Asians in mid to late pregnancy, this group of women also had significantly higher plasma insulin concentrations than the Caucasians during a GTT in the first trimester of pregnancy.

**Insulin dose-response patterns**  These observations may be explained in part by a consideration of the dose-response curve for insulin action, whose shape has been determined (Sandra & Fyler 1982; Sugden & Smith 1982), and is represented diagramatically in Figure 3.3. The y axis represents the extent of the removal of glucose from the plasma in response to the insulin concentration shown on the x axis. The diagram shows the normal sigmoid shape of the dose-response curve. If the response in the Asian population was displaced to the right of that of the Caucasian population, then at low insulin concentrations (ie, at fasting), the Asians would require more insulin (\( \times \)) than the Caucasians (a) to maintain the same level of removal of glucose from plasma (A), and thus the same plasma glucose level. This would explain why the Asians had elevated fasting insulin concentrations in the second and third trimesters. However, it would not explain why they do not have elevated fasting insulin concentrations in the first trimester, unless the relationship between the Asian and Caucasian dose-response curves were to change during pregnancy. The diagram can also be used to explain why the Asians had higher plasma insulin concentrations than the Caucasians, during a GTT in the first trimester, where the rise in plasma glucose concentration above basal levels was moderate, leading to a rate of removal equivalent to AA. This situation changed in mid to late pregnancy, where the plasma glucose concentration during a GTT rose.
Figure 3.3

Dose-response curve for insulin

This figure shows a hypothetical dose-response curve for the action of insulin with respect to the removal of glucose from plasma in Caucasian and Asian women.

- - - hypothetical response in Caucasians
- - - hypothetical response in Asians
Plasma insulin concentration (arbitrary units)
significantly higher, demanding a higher rate of removal from the plasma (B), in order to maintain homeostasis. Because of the shape of the curves, both Asians and Caucasians would require the same amount of insulin (b) to maintain control at this level. In order to define these observations in more detail, it would be necessary to study target cell insulin receptors to investigate their role in the change in insulin action which takes place in pregnancy.

**Dietary effects on glucose tolerance** Some of the vegetables of the normal Asian diet have been found to affect carbohydrate metabolism. The most notable of these are Karela (Leatherdale et al 1981) and those which contain the non-absorbable carbohydrate, Guar (Simpson et al 1982). The viscous fibre contained in these foodstuffs delays gastric emptying, and thereby attenuates carbohydrate absorption (Gabbe et al 1982). However, these foods have no effect on insulin secretion (Ray et al 1983) and therefore their presence in the diet would not be expected to have any effect on GTTs performed on fasted individuals. It has been suggested that the results of a GTT are dependent on the diet consumed in the few days prior to the test (Schwartz & Brenner 1982), because a diet which was low in carbohydrate would lead to the down-regulation of the insulin receptors, and therefore when an oral glucose challenge was administered, the insulin response would be blunted and the glucose tolerance thus appear to be impaired.

The plasma glucose concentrations in the Asians did not differ from those of the Caucasians, and therefore their diet clearly had no effect on glucose levels. In addition, there was no significant difference, either in fasting plasma glucose and insulin concentrations, or in the response to a GTT, between Asian vegetarians and non-vegetarians.
It is unlikely, therefore, that the differences in insulin response to a GTT which were observed between Asians and Caucasians could be attributed to differences in meat-eating habits.

**Haemoglobin A$_1c$ in pregnancy**

The results from this study show that there was no significant difference between the 3 groups of women, and no significant variation in the HbA$_1c$ concentration throughout pregnancy. Since HbA$_1c$ is known to correlate with blood glucose concentration (Compagnucci et al 1981), this observation was not unexpected - the Asians would be expected to have the same blood levels of HbA$_1c$ as the Caucasians because their glucose tolerance curves are the same. However, as glucose tolerance declined in all groups through pregnancy, the HbA$_1c$ concentration should increase because of the raised mean diurnal blood glucose levels; in this study HbA$_1c$ remained in the range 5.2 to 6.8 per cent throughout pregnancy. It has been observed by other workers that HbA$_1c$ does not vary in pregnancy (Leslie et al 1978), and it is suggested that this is because a major glucose intolerance is needed in order to affect significantly the concentration of HbA$_1c$ in the blood (Davies & Welborn 1980).

**SUMMARY**

Uncontrolled maternal hyperglycaemia in pregnancy is associated with increased risk of congenital abnormality, and foetal and neonatal morbidity (Beard & Hoet 1982). Whilst fasting hyperglycaemia was not observed in the Asians in this study, it has been shown that they have increased insulin resistance. Since maternal plasma glucose concentrations do not necessarily reflect the foetal glucose environment (Gillmer et al 1975), this increased insulin resistance in the Asians may have
serious implications for the foetus. In pregnancy, most of the maternal metabolites are controlled by maternal insulin, which means that metabolites destined for transplacental transfer would be affected by anything which modified maternal insulin production or action (Freinkel 1981). Thus, the Asians' comparative insulin resistance in the first trimester may mean that their control of glucose homeostasis is less stable at this crucial time of organogenesis. Furthermore, the continuing struggle for blood glucose control, even when fasting, in the Asians in late pregnancy, may also put the foetus at greater risk of perinatal death than the foetus of the Caucasian mother. It may be, therefore, that the increased insulin resistance we have observed in the Asian women is a factor which contributes to the higher incidence of congenital abnormality and perinatal mortality seen in this group of women.
CHAPTER FOUR

LIPID METABOLISM
INTRODUCTION

In Chapter Three, it was shown that Asian women require significantly higher fasting plasma insulin concentrations than Caucasian women in late pregnancy to maintain fasting blood glucose concentrations. These results indicated a greater struggle for glucose homeostasis in the Asian women and may be a factor in the raised perinatal mortality rate in the Asian group of women. Since insulin affects the blood levels of the various lipids (Phelps et al 1981), and there are marked alterations in lipid metabolism during pregnancy, the levels of plasma lipids in pregnant Asians and Caucasians were measured in order to determine whether the changes in carbohydrate observed in the Asian population were paralleled by changes in lipid metabolism.

Plasma lipid content was analysed using the protocol of the Association of Clinical Pathologists (Mount et al 1982), ie, plasma samples obtained after an overnight fast of at least 8 hours were assayed for total cholesterol, HDL cholesterol and triglyceride. In addition, these values were used to estimate the plasma concentration of LDL cholesterol.

METHODS

Fasting blood samples were obtained from Asian non-vegetarians, Asian vegetarians and Caucasian non-vegetarians at approximately 14, 28 and 36 weeks' gestation. The samples were centrifuged to obtain plasma, which was stored frozen at -20 °C until required. Aliquots of plasma were thawed at a later date and assayed for triglyceride, total
cholesterol and HDL cholesterol, using methods described in Chapter Two. Plasma LDL cholesterol concentration was calculated using the mathematical relationship between the various plasma lipid concentrations described in Chapter Two.

RESULTS

The results were analysed using non-parametric statistics and tabulated to show the median values and interquartile ranges for each group of patients studied.

**Triglyceride** Table 4.1 shows the fasting plasma concentration of triglyceride in each trimester of pregnancy for the 3 groups of women studied. There was no significant difference between the groups at any time during pregnancy. Plasma triglyceride concentration doubled in all groups during pregnancy, the most dramatic increase being between the second and third trimesters. The Asians (both vegetarian and non-vegetarian) showed a relatively constant rise in triglyceride throughout pregnancy, whereas the Caucasians tended to have comparatively low levels of triglyceride in the second trimester, the rise in triglyceride concentration in this group of women occurring almost entirely in the third trimester.

**Total cholesterol** In Table 4.2, the fasting plasma concentration of total cholesterol is shown for each group in the 3 trimesters of pregnancy. There was a 25 per cent increase in total cholesterol concentration in the Asian non-vegetarians and the Caucasian non-vegetarians (16 per cent in the Asian vegetarians), this increase occurring solely between the first and second trimesters. There was
Table 4.1

Median fasting plasma triglyceride concentrations in pregnancy

Fasting plasma triglyceride concentration was measured in the following groups of women:- Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values are shown for each group, with the interquartile ranges in brackets.

Triglycerides: 1 mM = 88 mg/100 ml
<table>
<thead>
<tr>
<th>Group</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>ANV</td>
<td>1.23 (0.89–1.68)</td>
</tr>
<tr>
<td>AV</td>
<td>1.44 (1.13–2.11)</td>
</tr>
<tr>
<td>CNV</td>
<td>1.38 (1.16–1.58)</td>
</tr>
</tbody>
</table>
Table 4.2

Median fasting plasma total cholesterol concentrations during pregnancy.

Fasting plasma total cholesterol concentration was measured in the following groups of women: Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values are shown, with interquartile ranges in brackets.

Cholesterol: 1 mM = 39 mg/100 ml
<table>
<thead>
<tr>
<th>Group</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>ANV</td>
<td>4.59 (4.18-5.33)</td>
</tr>
<tr>
<td>AV</td>
<td>4.85 (4.13-5.50)</td>
</tr>
<tr>
<td>CNV</td>
<td>4.72 (4.20-5.75)</td>
</tr>
</tbody>
</table>
no significant change in the level of total cholesterol between the second and third trimesters - in fact the results indicate a tendency for the plasma level of total cholesterol to fall towards the end of pregnancy. There was no statistically significant difference between the plasma concentrations of total cholesterol in any of the groups at any time during pregnancy. The Asian vegetarians appeared to have noticeably lower levels in mid to late pregnancy (in contrast with the first trimester, where the Asian vegetarians had the highest plasma concentration of total cholesterol), but this difference, although striking, was not significant owing to the large interquartile range for the distribution of the results.

**HDL cholesterol** Table 4.3 shows the fasting plasma concentration of HDL cholesterol in each trimester of pregnancy for the 3 groups of women studied. There was no significant difference between the groups at any stage of pregnancy, although the Caucasian non-vegetarians had marginally higher plasma levels than the Asians in the first and third trimesters. The plasma concentration of HDL cholesterol did not vary significantly during pregnancy in any of the groups. The maximum fluctuation observed was 0.15 mM in the Caucasian non-vegetarians.

**LDL cholesterol** The calculated fasting plasma concentrations of LDL cholesterol are shown for each group of women studied in the 3 trimesters of pregnancy (Table 4.4). In each of the 3 groups of women, the plasma concentration of LDL cholesterol rose to a maximum value in the second trimester and fell in the third trimester. There was an overall increase in LDL cholesterol during pregnancy (although this was not statistically significant in any of the groups), which was most marked in the Caucasian non-vegetarians. There was no
Table 4.3

Median fasting plasma HDL cholesterol concentrations in pregnancy

Fasting plasma HDL cholesterol concentration was measured in the following groups of women: - Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values are shown, with interquartile ranges in brackets.

Cholesterol: $1\ \text{mM} = 39\ \text{mg/100 ml}$
<table>
<thead>
<tr>
<th>Group</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>ANV</td>
<td>0.89 (0.67-1.14)</td>
</tr>
<tr>
<td>AV</td>
<td>0.89 (0.59-1.13)</td>
</tr>
<tr>
<td>CNV</td>
<td>1.05 (0.81-1.29)</td>
</tr>
</tbody>
</table>
Median fasting plasma LDL cholesterol concentrations in pregnancy

Fasting plasma LDL cholesterol concentration was measured in the following groups of women: - Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV). Median values are shown, with interquartile ranges in brackets.

Cholesterol: 1 mM = 39 mg/100 ml
<table>
<thead>
<tr>
<th>Group</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>ANV</td>
<td>3.50 (2.64-3.80)</td>
</tr>
<tr>
<td>AV</td>
<td>3.31 (2.48-3.96)</td>
</tr>
<tr>
<td>CNV</td>
<td>3.02 (2.51-3.98)</td>
</tr>
</tbody>
</table>
statistically significant difference between the groups at any time
during pregnancy, although, as with total cholesterol, the Asian
vegetarians had noticeably lower concentrations in mid to late pregnancy
(again in contrast with the first trimester, where the Asian vegetarians,
together with the Asian non-vegetarians, had higher plasma LDL cholesterol
levels than the Caucasian non-vegetarians).

**DISCUSSION**

The results presented in this chapter show that the plasma concentrations
of triglyceride and cholesterol rose as pregnancy advanced. This is
illustrated in Figures 4.1 and 4.2, which show the increases in plasma
triglyceride and cholesterol levels respectively, in Caucasian non-
vegetarian women during pregnancy. These observations confirm those of
other workers (Aurell & Cramer 1966; Darmady & Postle 1982). The most
dramatic rise in plasma lipid concentration was observed in the
triglyceride, whose plasma concentration increased 2-fold during
pregnancy (increases of up to 3-fold have been reported by Ordovas
et al 1984). There was also a significant rise in plasma cholesterol
levels of up to 25 per cent. In the non-pregnant subject, a rise in
plasma lipid levels of this magnitude would be extremely worrying, and
it is clear that the pregnant patient is a special case. In order to
discuss the significance of these pregnancy-induced changes in lipid
metabolism, it is first necessary to consider the distribution of lipids
amongst the various carrier lipoproteins.
Median fasting plasma triglyceride concentrations in each trimester of pregnancy, as observed in Caucasian non-vegetarian women.

Fasting plasma triglyceride concentrations were measured in Caucasian non-vegetarians at the following times in pregnancy:

- First trimester (14 weeks)
- Second trimester (28 weeks)
- Third trimester (36 weeks)

Median values are shown.
Weeks of pregnancy

Median fasting plasma triglyceride concentration (mM)
Figure 4.2

Median fasting plasma cholesterol concentrations in each trimester of pregnancy as observed in Caucasian non-vegetarian women.

Fasting plasma concentrations of total cholesterol (●—●), LDL cholesterol (■—■) and HDL cholesterol (▲—▲) were measured in Caucasian non-vegetarians at the following times in pregnancy:

First trimester (14 weeks)
Second trimester (28 weeks)
Third trimester (36 weeks)

Median values are shown.
Median fasting plasma cholesterol concentration (mM) over weeks of pregnancy.
The composition and function of lipoproteins

Lipids are insoluble in aqueous solutions and are therefore carried in blood plasma associated with proteins as components of lipoprotein particles. There are 4 types of plasma lipoprotein, which are named according to their relative densities when separated by ultracentrifugation. They are: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Because pure lipid is less dense than water, the proportion of lipid to protein determines the density of the lipoprotein - the more lipid present, the less dense the lipoprotein (Harper et al 1977).

Chylomicrons These are triglyceride-rich particles formed in gut epithelial cells from dietary lipid. In this study, chylomicrons were not present in the plasma samples, since the patients were fasted. Their role in lipid metabolism will therefore be disregarded for the purpose of this discussion.

VLDL The main function of VLDL is the transport of endogenous triglyceride, synthesised in the liver, to its target sites for utilisation or storage (mainly the latter). Triglyceride reaching adipose tissue is released from VLDL as fatty acids by the action of tissue lipoprotein lipase.

LDL The LDL normally carry up to 70 per cent of plasma cholesterol, most of which is esterified. They are responsible for the transport of cholesterol synthesised in the liver to peripheral tissues for utilisation, and are produced from VLDL following lipoprotein lipase activity. The utilisation of LDL cholesterol is dependent on the
possession by peripheral tissues of LDL receptors, which bind circulating LDL as the first step in a process which eventually leads to the entry of the cholesterol into the cell. High circulating levels of LDL may be associated with the development of atherosclerosis (Montgomery et al 1980).

**HDL** HDLs are formed in the liver, and are mainly concerned with the transport of cholesterol from peripheral tissues to the liver for degradation. The cholesterol carried in HDL is esterified, and this traps the cholesterol within the HDL, preventing it from diffusing into the tissues. High circulating levels of HDL are thought to protect against atherosclerosis.

**Triglyceride in pregnancy**

The observed rise in triglyceride levels in pregnancy is thought to be caused by an increased rate of synthesis throughout pregnancy, accompanied by a decrease in utilisation towards term (Knopp et al 1975). The extra triglyceride made available by these processes is not primarily destined for the foetus (Lorenzo et al 1983), but for use by the mother as an energy supply during and following pregnancy. As a result, less glucose is required by the mother, and glucose is thereby released for placental transfer to the foetus (Warth et al 1975). In addition to its use for an energy source by the mother, the extra triglyceride is used by the placenta in steroid synthesis (Warth et al 1975) and by the breast tissue as a substrate for milk synthesis (Darmady & Postle 1982). This last factor may explain the large increase in circulating triglyceride between the second and third trimesters (triglyceride was the only lipid investigated where such an increase was observed), since at this time, the breast tissue
is being prepared for the onset of lactation which is triggered by the abrupt decrease in progesterone output post partum (Banks et al 1976).

**Cholesterol in pregnancy**

The results presented in this chapter show that the rise in plasma total cholesterol concentration occurring during pregnancy is due in part to an increase in the amount of cholesterol carried by LDL. This cholesterol is available for uptake by the peripheral tissues. The placenta is a substantial target tissue for LDL cholesterol because it is dependent on maternal plasma cholesterol as a substrate for progesterone synthesis, as the placenta is not able to synthesise progesterone from acetate in sufficient quantities (Ryan et al 1966).

The amount of circulating HDL cholesterol did not vary significantly in pregnancy. Since HDL is thought to carry cholesterol from peripheral tissues to the liver for eventual excretion, the amount of cholesterol lost from the maternal circulation in this way did not appear to change during pregnancy.

The fraction of total cholesterol not accounted for by LDL and HDL cholesterol must, in fasting samples, be carried by VLDL. In the calculation used to determine the concentration of cholesterol carried by the LDL, the concentration of VLDL cholesterol was assumed to be directly proportional to the plasma triglyceride concentration, and thus to parallel the rise in triglyceride levels observed in pregnancy. This assumption, while being accepted in routine lipid research, may not necessarily apply in pregnancy, and
it may therefore be preferable to measure VLDL and LDL cholesterol by
direct means.

Hormonal effects on HDL/LDL ratios The overall increase in LDL
cholesterol concentrations observed in pregnancy, in conjunction with
the maintenance of a constant level of HDL cholesterol, means that the
HDL/LDL ratio decreased as pregnancy advanced. This phenomenon has
also been observed in women using oral contraceptives (Wynn &
Niththyananthan 1982), and is thought to be caused by the progestin
component of the pill (Wahl et al 1983). Clearly this change in the
cholesterol content of the lipoproteins may well be related to the
increase in circulating progesterone levels known to occur in pregnancy
(Freinkel 1980).

The relationship between triglyceride and cholesterol in pregnancy

The hypertriglyceridaemia of mid to late pregnancy observed in this
and other studies is thought to be different from that occurring in
hyperlipidaemic, non-pregnant patients. In pregnancy, the HDL
cholesterol concentration has been shown to remain constant,
irrespective of the plasma concentration of triglyceride. Conversely,
in other types of hypertriglyceridaemia, the concentration of HDL
cholesterol falls (Warth et al 1975). The lipoproteins have a limited
capacity for the carriage of lipid (Fielding et al 1983) and therefore
in cases of hypertriglyceridaemia in the non-pregnant subject, there
is an excess of circulating triglyceride over and above that which can
be carried by the VLDL. The extra triglyceride is then carried by
the HDL at the expense of cholesterol (Warth et al 1975), and thus
the concentration of HDL cholesterol falls. In the pregnant state,
the observed rise in plasma cholesterol concentration may lead to an
increase in the amount of available lipoprotein, since cholesterol from the diet is known to be used for the structural composition of lipoproteins (Frederickson et al 1967). If this is so, then the amount of HDL would increase, enabling a constant concentration of HDL cholesterol to be maintained, while at the same time allowing enrichment of the HDL by triglyceride. In order to confirm this hypothesis, it would be necessary to measure the amount of triglyceride carried by each of the lipoproteins. This could be achieved by separating the plasma lipoproteins either by ultracentrifugation or by electrophoresis.

The effect of diet on lipid metabolism

The hypercholesterolaemia observed in pregnancy is not thought to be caused by over-eating (Aurell & Cramer 1966), since the dietary intake of cholesterol has no effect on plasma cholesterol levels (Goldstein et al 1983), and the rise in circulating cholesterol during pregnancy cannot be prevented by a hypocholesterolaemic diet (Green 1966). Studies carried out on Moslems who fasted in their festival of Ramadan showed that plasma cholesterol levels were increased during the one month fasting period, where one large meal per day was consumed instead of several small meals. The composition of the food consumed before and during the fast was similar and the weight loss small. It was therefore concluded that the increase in plasma cholesterol concentration was caused by the change in food intake patterns, from several spaced meals in a day, to one very large meal in the evening (Fedail et al 1982). It is therefore possible that changes in feeding habits during pregnancy (rather than changes in nutritional intake) may give rise to the observed hyperlipidaemia. There was no
significant difference in the plasma lipid concentrations between
Asian vegetarians and non-vegetarians, indicating that meat-eating
habits did not affect the lipid metabolism of the women participating
in this study.

**SUMMARY**

There is a dramatic rise in plasma lipid levels during pregnancy. The
increase in circulating triglyceride probably occurs in preparation
for lactation. The rise in total plasma cholesterol concentration
is due largely to an increase in the amount of cholesterol carried by
LDL. This cholesterol is available for uptake by the peripheral tissues,
notably the placenta, which uses cholesterol to synthesise progesterone.
There was no significant difference in lipid metabolism during pregnancy
between different ethnic groups, or between patients taking different
diets. The observed differences in carbohydrate metabolism between
Asians and Caucasians were not mirrored by alterations in lipid
metabolism, and it is unlikely therefore, that lipid metabolism has
any association with the higher perinatal mortality rate observed in
patients of Asian origin.
CHAPTER FIVE

AMINO ACID ANALYSIS
INTRODUCTION

Dietary protein deficiency is a serious nutritional problem occurring frequently in the third world, where, because of the vegetarian habit, the diet is often low in protein. It may also be a problem in pregnancy, where the added demands on metabolism made by the growing foetus may exhaust maternal nutrient supplies. The most important function of dietary protein is to supply the amino acids required for the biosynthesis of protein and other nitrogen containing compounds, such as haem.

Amino acids are classified as either essential or non-essential. The former are available to the body only from dietary sources, whereas the carbon skeletons of the latter can be synthesised in vivo from glucose. In adults, the essential amino acids are: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Tyrosine is essential when phenylalanine is absent from the diet. In addition, arginine and histidine may become essential during periods of active growth, eg, childhood (Montgomery et al 1980). A diet which is deficient in the essential amino acids may, during pregnancy, impair the growth of the foetus and therefore lower its chances of survival. Since Asians normally have a different diet from Caucasians, and might therefore have a different dietary amino acid profile, the plasma concentrations of a number of amino acids were measured in the Asian women to determine whether they were deficient in any of the essential amino acids.

PRINCIPLE

Amino acids can be separated in an automatic analyser, using ion exchange columns. The most commonly used ion exchange resins are derived from
styrene co-polymerised with the cross-linking agent divinyl benzene, giving a 3-dimensional network. Sulphonation of this material gives a cation exchanger. Most commercially available resins have on average, slightly less than one sulphonic acid group per benzene ring (Leggett-Bailey 1967).

Samples to be analysed are applied to the ion-exchange column in a buffer of low pH (usually pH 2.0). At this pH, all of the amino acids have a net positive charge and bind to the negatively charged beads of the cation exchange resin. The column is eluted with a series of 4 buffers of increasing pH. As the pH increases, the carboxyl groups of the amino acids ionise, and eventually the amino groups lose their positive charge. The amino acids become negatively charged and are eluted from the column. Amino acids having acidic R-groups elute first, followed by neutral and then basic acids. The order of elution is also affected by size, and therefore bulky amino acids such as tyrosine and tryptophan will be retained on the column for longer than expected because their bulky R-groups will cause them to become trapped between the beads of the resin. The elution of all the amino acids is a lengthy process which can be cut down by having 2 columns, one to separate the acidic and neutral amino acids, the other to separate the basic amino acids (Spackman 1967). The analyser used in this study had only one column but, because pressure was applied to the column, the run time was reduced to approximately 4 hours for each sample.

For the quantitative analysis of the amino acids in the eluate, the effluent stream from the column is mixed with ninhydrin reagent and passed through a Teflon coil immersed in boiling water. This process develops a blue colour, Ruheman's purple (Figure 5.1). This product
Figure 5.1

Formation of Ruheman's purple from ninhydrin and amino acids

When a mixture of amino acids and ninhydrin are heated to boiling, there is a series of chemical reactions leading to the formation of Ruheman's purple, a pigment which has a maximum absorbance at 570 nm and can be used to estimate the concentration of amino acids present in a plasma sample.
Ninhydrin (triketohydrindene hydrate) + $\alpha$-amino acid $\rightarrow$ Hydrindantin

Hydrindantin + $\mathrm{NH}_3$ $\rightarrow$ Ninhydrin

Ninhydrin $\rightarrow$ Ruhemann's Purple
has an absorption maximum at 570 nm. Proline, hydroxyproline and
cysteine, however, follow different reactions with ninhydrin to give
yellow products absorbing at 440 nm. Under controlled conditions,
the colour follows Beer's law. The coloured effluent stream is passed
through a photometer where the extinction at both 570 and 440 nm is
measured and plotted on a chart recorder. The trace obtained consists
of a series of peaks. The position of each peak with respect to the
others identifies the amino acid, and the area under the peak is a measure
of the amount present in the sample. The shape of the peak is determined
by the size of the column, and the resin. In general, smaller diameter
columns and finer resin particles will give tall, narrow peaks, from
which the amount of amino acid present can be more accurately estimated
than from short, broad peaks (Hamilton 1967).

Each batch of ninhydrin reagent is stable for up to 2 weeks. During
that time, several calibration runs are made using a mixture of known
amounts of various amino acids. In addition, for each batch of
ninhydrin, it is necessary to run a baseline with no sample input,
because each buffer change in the run can cause a "blip" on the
otherwise flat trace, which in a sample run could be confused with a
trace from an amino acid running close to that point. In order to
measure the reproducibility of the determinations, an internal
standard of a non-physiological amino acid such as norleucine may
be incorporated into the samples before application to the column.
This addition was not made in this study, although norleucine was
added to each batch of calibration standards. As a calibration
standard was run every day, the daily batches of 6 runs could be
compared with each other, even though the reproducibility of the
individual runs was not assessed.
All of the reagents used in the analysis, along with the equipment making up the analyser itself, are prone to contamination with ammonia (Hamilton 1967). Ammonia is extremely ninhydrin reactive, and will therefore interfere with the estimation of amino acids. In particular, if the ninhydrin reagent itself is exposed to ammonia, the baseline will drift because of the change in the reagent due to darkening. This problem was avoided by storing the ninhydrin reagent under nitrogen. Ammonia was kept from the ion exchange column by first passing the buffer input through a smaller column containing a strongly cationic ammonia-trapping resin, which filters out ammonia and particulate matter.

METHODS

Materials

Thiodiglycol (30% solution) and Ninhydrin were obtained from Pierce and Warriner (UK) Ltd, Chester, Cheshire.

All inorganic salts and reagents for buffers were of Sepramar grade, or the highest grade commercially available, and were purchased from BDH Chemicals Ltd, Poole, Dorset.

Microbead Cation (LA 6/7.5μ) ion exchange resin, and column filters, were obtained from Locarte, London.

Apparatus

The apparatus used in this study was a Locarte amino acid analyser. The column dimensions were 30 x 0.6 cm. The resin used was Locarte Microbead Cation (LA 6/7.5μ). The analyser is represented in the block diagram in Figure 5.2. The buffer to be pumped onto the column was selected by a timed control junction. The buffer passed
Figure 5.2

Block diagram of the Locarte amino acid analyser

The various components of the automatic amino acid analyser are inter-related as shown. The function of the individual components is discussed in the text.
through the pump and a valve onto the loading column, where a sample had been applied. From there, the sample was eluted onto the top of the column. The column was surrounded by a jacket through which warm water was circulated, to maintain a constant temperature during the analysis. The column eluate was mixed with ninhydrin (which was pumped through the ninhydrin pump at half the flow rate of that of the buffer pump) at A, and the mixture was then heated as described above. The eluate was then passed through the colorimeters and into a waste vessel. Between the colorimeters and the waste vessel, a flow meter was attached to check that the flow of reagents was sufficiently rapid.

Reagents

Buffers All of the buffers used were citrate based. Brij 35, a polyoxyethylene lauryl alcohol detergent, was added to the buffers to give a constant drop size. Thiodiglycol was added to buffers 1 to 3 as an antioxidant, to protect the sulphur-containing cysteine and methionine; it was not needed in buffer 4 as these amino acids elute earlier in the run. Pentachlorophenol was added to the buffers to act as a preservative, along with the fungicide n-octanoic acid (caprylic acid). The pH of the buffers was set to within 0.01 pH units of the pH stated, and was checked regularly.

Buffer 1 - 6.24 g citric acid monohydrate, 17.64 g trisodium citrate dihydrate, 22.2 ml thiodiglycol (30%), 1.5 ml BRIJ 35 (30%), 3 drops pentachlorophenol, 9 ml concentrated hydrochloric acid and 0.1 ml octanoic acid were made up to 1 litre with distilled water and set to pH 3.25.
Buffer 2 - 7.00 g citric acid monohydrate, 19.60 g trisodium citrate dihydrate, 22.2 ml thiodiglycol (30%), 1.7 ml BRIJ 35 (30%), 3 drops pentachlorophenol, 7.8 ml concentrated hydrochloric acid, and 0.1 ml n-octanoic acid were made up to 1 litre with distilled water and set to pH 3.25.

Buffer 3 - 7.00 g citric acid monohydrate, 19.60 g trisodium citrate dihydrate, 22.2 ml thiodiglycol (30%), 1.7 ml BRIJ 35 (30%), 3 drops pentachlorophenol, 5.6 ml concentrated hydrochloric acid and 0.1 ml n-octanoic acid were made up to 1 litre with distilled water and set to pH 4.15.

Buffer 4 - 34.3 g sodium citrate, 2.7 g sodium hydroxide, 2.06 g boric acid, 1.5 ml BRIJ 35 (30%), 6 drops pentachlorophenol and 0.1 ml n-octanoic acid were made up to 1 litre with distilled water and set to pH 10.0.

Ninhydrin reagent (2.5 litres) The ninhydrin reservoir was cleaned with distilled water. 625 ml of 4 M sodium acetate buffer pH 5.5, and 1.87 l methyl cellosolve (2-methoxyethanol), and a stirring bar, were added to the reservoir and the lid clamped. The mixture was stirred for 20 minutes while bubbling nitrogen through it using the nitrogen line and adaptor from the analyser. This nitrogen was passed out through citrate buffer pH 2.2 to destroy excess methyl cellosolve (which is carcinogenic). The nitrogen supply was then turned off, the reservoir lid removed and 50 g ninhydrin added to the solution. The lid was clamped again and nitrogen bubbled through for 30 minutes with stirring. The nitrogen supply was again turned off and 1.035 g stannous chloride dihydrate added to the reservoir through a funnel
attached to the nitrogen outlet. At this stage the solution was oxygen sensitive. Nitrogen was bubbled through the solution for a further 90 minutes, after which the reservoir was connected to the analyser and kept under nitrogen pressure.

The 4 M sodium acetate buffer pH 5.5 was obtained commercially, but can be made up as follows: 2.72 kg sodium acetate trihydrate was added to 2.5 l distilled water and heated. 500 ml glacial acetic acid was added to the solution, which was made up to 5 litres with distilled water and the pH set to 5.5.

Regenerating reagent After each analysis, the column was regenerated using 0.2 M sodium hydroxide solution.

Loading reagent In order to keep the loaded samples acidic, the loading columns were washed with 0.02 M hydrochloric acid before the sample was added.

Methanolic loading buffer The samples on the loading columns were buffered with buffer 2 containing 33% v/v methanol, pH3.0. The methanol was added to aid the separation of threonine and serine. The buffer was shaken before use.

Standard mixture A standard mixture of amino acids was prepared at a concentration of 0.1 mM for each amino acid. The mixture contained lysine, histidine, ammonia, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, ½ cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine and tryptophan. The internal standard of norleucine was added to this mixture at a concentration
of 0.1 mM. The mixture was stored at -20 °C in 1 ml aliquots.

**Plasma samples** Plasma samples from the first and third trimesters were analysed. Since the samples were to be analysed for free amino acids, it was necessary to precipitate the protein in the plasma. This was achieved by adding 0.15 ml of a 15 g/100 ml solution of 5-sulphosalicylic acid to 0.6 ml plasma. The sample was centrifuged in a Beckman microfuge for one minute, and the protein-free plasma aspirated from the precipitate using a pasteur pipette. Samples were stored at -20 °C until required. Twenty per cent of the final volume of the sample was precipitating agent – this was allowed for in the calculations.

**Procedure**

**Preparation of the column** The column was packed using a suspension of the ion exchange resin in 0.2 M sodium hydroxide. The water pump was switched on to keep the column at the operating temperature (50 °C). A filter was placed at the bottom of the column to support the resin, and a second filter at the top of the column to protect the resin from dirt. It was essential that there was as little dead space as possible at the top of the column between the filter and the tube through which the sample and buffers flowed onto the column. The larger the dead space, the greater the possibility of mixing of the sample and buffers before they reached the resin, and therefore the more diffuse the peaks on the chart recorder trace. The column was equilibrated using buffer 1, and the pumps adjusted so that the buffer pump was pumping twice as fast as the ninhydrin pump.
Sample loading  Each sample (up to a maximum of 12) was loaded onto a numbered glass loading column containing a filter, to await selection for analysis as follows: the top of the column was removed and any liquid in the column washed through with nitrogen until the meniscus lay on the filter. A little loading reagent (hydrochloric acid) was added and washed through with nitrogen. The maximum volume of sample that could be loaded was 0.5 ml - this is because the tubes leading out of the loading columns (into which the samples were washed) lead to waste, except in the column connected to the analyser. Samples therefore wait in these tubes, just below the column selected, and it is essential not to wash them too far along the tubes. The standard mixture was loaded in aliquots of 0.1 ml, and the samples were loaded in 0.2 ml aliquots (this difference was accounted for in the calculations). The column was again washed with a little hydrochloric acid. Finally, the loading column was filled up with methanolic loading buffer and the top replaced.

Each loading column was connected to the ion exchange column via a tube connected to a set of valves. The valves enabled any of the loading columns to be selected for analysis at any time. Columns were automatically selected in ascending order unless manually over-ridden.

Run procedure  The buffer and water pumps were switched on at the beginning of a batch of runs and the system equilibrated in buffer 1 for 60 minutes. The samples were loaded and the column before the loading column required, selected. The auto-load was activated and the buffer selector set on automatic. The ninhydrin pump was switched on. The run timer was set so that the next loading column (the one
required) would be switched into the system as soon as the equilibration
time was completed. Once the auto-load effected this switch, the
sequence of events in the run was as follows:— elution with buffer 1
for 15 minutes, buffer 2 for 15 minutes, buffer 3 for 30 minutes,
buffer 4 for 60 minutes, regeneration with sodium hydroxide for 20 minutes
and equilibration with buffer 1 for 60 minutes, next sample. The
temperature of the circulating water surrounding the ion exchange column
was automatically raised from 50 °C to 60 °C at 65 minutes in order to
speed the separation of the basic amino acids. The total run time was
200 minutes. During the equilibration phase, the ninhydrin pump was
automatically switched off so that the lines would be washed free of
ninhydrin after each run, since this reagent can solidify and clog the
lines, causing a build-up of pressure. An auto-shutdown device was
used to stop the analyser after a batch of runs, but this was
occasionally unreliable and therefore not used if the machine was
to be unattended for more than the overnight period.

**Calculations** The trace produced by a standard mixture of amino acids
is shown in Figure 5.3. Arginine did not appear on the trace because
buffer 4 was not at the correct pH. Numerous alterations were made to
this buffer, but no improvement was possible with respect to arginine.
Two traces were plotted by the chart recorder — a red trace corresponding
to measurements made at 570 nm, and a blue trace corresponding to
measurements made at 440 nm. The trace was plotted at a speed of
5 mm/min and the blue trace was plotted slightly later than the red.
(Only that part of the trace at 440 nm showing the proline peak is
illustrated in Figure 5.3)

The trace consisted of a number of peaks which had to be integrated
Figure 3.3

Analysis of a standard mixture of amino acids

A standard mixture containing $100 \mu M$ (0.1 mM) of the amino acids listed in the text was separated using an automatic amino acid analyser, and produced the chart recorder trace shown.

--- 570 nm trace

--- shows part of the 440 nm trace identifying proline.
manually. For the most accurate results, the peaks must be well resolved from each other, and should be as tall and narrow as possible. The integration used approximated the area of the peak to that of a triangle, such that Area = width at $\frac{1}{2}$ height $\times$ height. The height of the peak was measured from the baseline of that peak (the baselines of the peaks should lie on a straight line corresponding to the baseline for that particular batch of ninhydrin). If 2 peaks occurred as an unresolved duplet, it was theoretically possible to integrate by eye if the duplet was symmetrical. If 2 amino acids co-eluted, giving rise to an irregularly shaped peak with only one maximum, it was not possible to separate the 2 traces. The analysis was accurate to within 10 per cent if all of the peaks were well resolved.

The area of the norleucine peak was compared from day to day, and the measured areas of all of the peaks adjusted by the ratios of the norleucine peak of the day and the norleucine peak of the first day of the batch of ninhydrin, ie, if day 1 norleucine peak area = 100 units, and day 2 norleucine peak = 90 units, then all of the day 2 peaks would be smaller than they should be, and would be multiplied by $\frac{100}{90}$. This calculation was made to compensate for the decline in ninhydrin reactivity over the lifetime of the batch of reagent.

The peaks in the sample traces were identified by their position compared with the standard trace. The amount of amino acid present in each peak was calculated by comparing the area with that of the 0.1 mM trace of the standard solution. The amount of sample applied and the dilution of the original sample by the addition of the deproteinising agent were also accounted for.
Concentration of amino acid in the sample (μM) equals:

\[
\frac{(\text{area of sample peak}) \times (\text{concentration}) \times (\text{volume of standard used})}{(\text{area of standard peak}) \times (\text{of standard}) \times (\text{volume of sample used})} \times \left(1 + \frac{\text{volume of deproteinising agent}}{\text{total volume of sample + deproteinising agent}}\right)
\]

For example, if a valine peak in the standard trace had an area of 600 for 0.1 ml applied, and the same peak in a sample had an area of 565 for 0.05 ml applied, the concentration of valine under the conditions described would be:

\[
\frac{565 \times 100 \times 0.1}{600 \times 0.05} \times \left(1 + \frac{0.15}{0.6 + 0.15}\right) = 226 \text{ [μM]}
\]

RESULTS

The results were assessed using non-parametric statistics and expressed in tabular form to show median values, with interquartile ranges (Table 5.1). The number of results obtained was as follows:– Asian non-vegetarians, n = 16; Asian vegetarians, n = 14; Caucasian non-vegetarians, n = 11.

Technical difficulties Two problems were encountered with the analysis. The first was that arginine did not elute at the end of the run. The pH of buffer 4 was changed several times, but with no improvement. The second occurred at the beginning of the run, where it was found that aspartic acid, threonine and serine co-eluted in one large peak. The 3 acids were not resolved when the pHs of buffers 1 and 2 were changed. It was suggested that the pH of the methanolic loading buffer was too
Table 5.1

Plasma free amino acid concentrations after an overnight fast during pregnancy [\( \mu M \)].

The results are expressed as medians, with interquartile ranges in brackets.

ANV = Asian non-vegetarian
AV = Asian vegetarian
CNV = Caucasian non-vegetarian

<table>
<thead>
<tr>
<th></th>
<th>First trimester concentration</th>
<th>Third trimester concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>175 (117-204)</td>
<td>186 (140-241)</td>
</tr>
<tr>
<td>AV</td>
<td>190 (168-230)</td>
<td>191 (119-235)</td>
</tr>
<tr>
<td>CNV</td>
<td>136 (104-204)</td>
<td>198 (178-254)</td>
</tr>
<tr>
<td>Proline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>119 (94-157)</td>
<td>157 (141-189)</td>
</tr>
<tr>
<td>AV</td>
<td>97 (94-143)</td>
<td>140 (113-215)</td>
</tr>
<tr>
<td>CNV</td>
<td>108 (86-126)</td>
<td>168 (107-211)</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>172 (141-203)</td>
<td>177 (126-224)</td>
</tr>
<tr>
<td>AV</td>
<td>153 (132-202)</td>
<td>185 (148-200)</td>
</tr>
<tr>
<td>CNV</td>
<td>140 (128-192)</td>
<td>149 (135-222)</td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>253 (187-349)</td>
<td>339 (285-369)</td>
</tr>
<tr>
<td>AV</td>
<td>243 (228-274)</td>
<td>319 (274-375)</td>
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<tr>
<td>CNV</td>
<td>276 (257-330)</td>
<td>322 (290-381)</td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>90 (66-121)</td>
<td>107 (85-132)</td>
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<tr>
<td>AV</td>
<td>94 (85-100)</td>
<td>98 (77-110)</td>
</tr>
<tr>
<td>CNV</td>
<td>85 (71-152)</td>
<td>116 (63-151)</td>
</tr>
<tr>
<td>Valine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>188 (145-204)</td>
<td>167 (147-194)</td>
</tr>
<tr>
<td>AV</td>
<td>160 (126-183)</td>
<td>155 (137-173)</td>
</tr>
<tr>
<td>CNV</td>
<td>175 (149-185)</td>
<td>181 (121-183)</td>
</tr>
<tr>
<td></td>
<td>First trimester concentration</td>
<td>Third trimester concentration</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td><strong>Methionine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>29 (24-34)</td>
<td>29 (27-32)</td>
</tr>
<tr>
<td>AV</td>
<td>26 (23-35)</td>
<td>26 (24-32)</td>
</tr>
<tr>
<td>CNV</td>
<td>26 (25-51)</td>
<td>27 (20-52)</td>
</tr>
<tr>
<td><strong>Isoleucine</strong></td>
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<td></td>
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<tr>
<td>ANV</td>
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<td>50 (43-55)</td>
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<td>AV</td>
<td>48 (42-56)</td>
<td>48 (43-54)</td>
</tr>
<tr>
<td>CNV</td>
<td>43 (40-52)</td>
<td>47 (37-59)</td>
</tr>
<tr>
<td><strong>Leucine</strong></td>
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<td></td>
</tr>
<tr>
<td>ANV</td>
<td>89 (76-103)</td>
<td>87 (79-86)</td>
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<td>AV</td>
<td>84 (73-91)</td>
<td>84 (75-91)</td>
</tr>
<tr>
<td>CNV</td>
<td>92 (77-102)</td>
<td>81 (65-105)</td>
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<tr>
<td><strong>Tyrosine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>47 (41-53)</td>
<td>45 (32-52)</td>
</tr>
<tr>
<td>AV</td>
<td>42 (37-54)</td>
<td>45 (37-50)</td>
</tr>
<tr>
<td>CNV</td>
<td>44 (40-49)</td>
<td>46 (41-49)</td>
</tr>
<tr>
<td><strong>Phenylalanine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>68 (60-82)</td>
<td>65 (52-87)</td>
</tr>
<tr>
<td>AV</td>
<td>75 (57-87)</td>
<td>70 (61-72)</td>
</tr>
<tr>
<td>CNV</td>
<td>79 (62-90)</td>
<td>75 (52-87)</td>
</tr>
<tr>
<td><strong>Histidine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>79 (74-97)</td>
<td>97 (85-118)</td>
</tr>
<tr>
<td>AV</td>
<td>86 (76-105)</td>
<td>86 (76-97)</td>
</tr>
<tr>
<td>CNV</td>
<td>89 (82-98)</td>
<td>118 (91-121)</td>
</tr>
<tr>
<td><strong>Tryptophan</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>49 (26-56)</td>
<td>67 (56-72)</td>
</tr>
<tr>
<td>AV</td>
<td>80 (43-110)</td>
<td>56 (48-68)</td>
</tr>
<tr>
<td>CNV</td>
<td>50 (46-70)</td>
<td>52 (43-55)</td>
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<tr>
<td><strong>Lysine</strong></td>
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<td>ANV</td>
<td>132 (93-161)</td>
<td>126 (90-156)</td>
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<td>AV</td>
<td>116 (99-143)</td>
<td>105 (81-128)</td>
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<tr>
<td>CNV</td>
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<td><strong>Total amino acids</strong></td>
<td>1544</td>
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<tr>
<td>ANV</td>
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<td>1608</td>
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<tr>
<td>AV</td>
<td>1465</td>
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<td>688</td>
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<tr>
<td>amino acids</td>
<td>675</td>
<td>650</td>
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<tr>
<td>CNV</td>
<td>676</td>
<td>696</td>
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<td><strong>Total branched</strong></td>
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<tr>
<td>chain amino acids</td>
<td>292</td>
<td>287</td>
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<tr>
<td>ANV</td>
<td>310</td>
<td>309</td>
</tr>
<tr>
<td>AV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNV</td>
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</tbody>
</table>
high, and that a pH of 2.0 would have been more appropriate. However, bringing the pH down to 2.6 had no appreciable effect on the results and therefore values for these 3 amino acids could not be calculated.

**Observations from the results** In all groups, as pregnancy proceeded, the plasma levels of most of the amino acids stayed the same. There was a small rise in glutamic acid, proline, glycine, alanine, cysteine and histidine between the first and third trimesters, but this reached statistical significance only for proline and alanine. There were no significant differences between the groups for any of the amino acids, except for histidine, where in the third trimester, the Asian vegetarians had a significantly lower plasma concentration than the Asian non-vegetarians ($p<0.08$) and the Caucasian non-vegetarians ($p<0.03$).

The total concentration of detected plasma amino acids was calculated using the median values for each amino acid, along with the total concentration of detected essential amino acids and the total concentration of the branched chain amino acids (BCAA), ie, leucine, isoleucine and valine. Interquartile ranges were not computed for these totals and therefore no assumption about statistical significance could be made. However, the general trend was that all groups had similar concentrations of total and essential amino acids in the first trimester, but in the third trimester, the Asian vegetarians appeared to have lower concentrations of total and essential amino acids - the total amino acid concentration for this group was $90 \mu M (\approx 5\%)$ lower than that of the Asian and Caucasian non-vegetarians, while the concentration of essential amino acids was $60 \mu M (\approx 9\%)$ lower. The Asian vegetarians had lower levels of BCAA through pregnancy than the other 2 groups.
DISCUSSION

The technical difficulties encountered with the amino acid analyser prevented the analysis of all of the samples from patients studied, and for this reason, the statistical errors on the results are relatively high. Very few of the individual amino acids measured showed any significant change during pregnancy, or any significant differences between the groups studied. Nevertheless, some interesting observations have been made, which merit further discussion.

There appears to be no metabolic reason for the significant rise in plasma proline levels during pregnancy, which was observed in all groups. However, the significant rise in plasma alanine levels may be of interest. It is thought that in periods of brief starvation, the rise in ketone bodies which occurs results in the stimulation of alanine output from muscle (Fery & Balasse 1982). The purpose of the resultant increased rate of supply of alanine to the liver would be to restrict hepatic ketogenesis by means of a regulatory feedback system (Nosadini et al 1982). This would ensure an adequate balance between gluconeogenesis (GNG) and ketogenesis, the mechanisms which supply substrates to the body during food deprivation. In pregnancy, especially the second half, a phenomenon called accelerated starvation occurs during fasting, where some of the metabolic symptoms of starvation are present even after a short overnight fast (Metzger et al 1982). This results in an increase in ketone output, which if uncontrolled, is thought to impair the intellectual development of the offspring by inhibiting pyrimidine biosynthesis in the foetal brain (Metzger et al 1982; Bhasi et al 1981). The increase in circulating alanine levels observed in late pregnancy may therefore be a result of
a "safety mechanism" whereby some of the maternal energy requirement during fasting would be satisfied by GNG, thus reducing the demand for the production of ketone bodies. This adaptation occurred to the same extent in all of the groups studied.

The branched chain amino acids are known to act as regulators or precursors for various metabolic reactions (Adibi 1980), as well as being substrates for protein synthesis. One such role for the BCAA is that of a major source of alanine nitrogen - over 28 per cent of leucine nitrogen has been shown to go to alanine *in vitro* in dog studies (Haymond & Miles 1982). The results presented in this chapter showed that the Asian vegetarians had a lower level of BCAA in both the first and third trimesters. Therefore, the Asian vegetarians were not as well equipped to produce alanine (a substrate for GNG) as the Asian or Caucasian non-vegetarians. If the hypothesis of alanine regulation of ketone bodies described above is correct, then the Asian vegetarian may be at a metabolic disadvantage during pregnancy and this may have adverse effects on their foetuses.

The Asian vegetarians had lower levels of total and total essential amino acids in the third trimester of pregnancy. Since essential amino acids are, by definition, those which the body is unable to synthesise de novo and which are therefore required in the diet, it is clear that the Asian vegetarians' potential for protein synthesis may be reduced in such a way that the body is unable to make up for the shortfall by other metabolic pathways. As with the BCAA, this may have serious implications for the well-being of the foetuses of these women.
When considering the amino acids individually, only in the case of histidine was there any difference between the groups, and again the Asian vegetarians had significantly lower plasma histidine concentrations in the third trimester than the other groups. Histidine is normally a non-essential amino acid, but in periods of active growth (and therefore presumably in pregnancy), it becomes essential in the diet. Thus, the Asian vegetarians were deficient in another aspect of their amino acid metabolism in pregnancy.

**SUMMARY**

In general, the plasma concentration of individual amino acids did not change significantly through pregnancy. However, it is clear from these studies that there was a difference in the plasma amino acid levels in women who did not eat meat. The studies of other blood metabolites presented in this thesis did not show differences of this nature. It is possible that deficiencies in amino acids may be linked with the observed increased perinatal mortality rate in Asian vegetarians. However, because the majority of these differences were observed in the third trimester only, it would be difficult to implicate impaired amino acid metabolism in the increased incidence of congenital abnormalities reported in these women, since organogenesis occurs in the first trimester. Nevertheless, a deficient diet in the third trimester may retard the growth of the foetus, leading to the birth of a smaller than average child, which may be at a greater risk of neonatal death. Finally, since the Asian non-vegetarians had similar amino acid levels to the Caucasian non-vegetarians, the different amino acid levels in the Asian vegetarians can be attributed to dietary rather than ethnic differences.
CHAPTER SIX

OUTCOME OF PREGNANCY
INTRODUCTION

In 1980 the Asian population in Leicestershire had a perinatal mortality rate of 19.7 deaths per 1000 live and still births, compared with 11.3 in the non-Asian population (Dhariwal 1982). Because Asian infants are, on average, 300 g lighter at birth than Caucasian infants (Grundy et al 1978) and low birthweight infants are known to make a disproportionately large contribution to the perinatal mortality rate (Barron 1983), the outcome of pregnancy in the women who had taken part in the previously described metabolic studies was investigated.

Low birthweight can arise either because the baby is born before the "term" date of 37 completed weeks (ie, it is premature), or because the development of the baby has been retarded in some way and it is small for its gestational age (SGA) - this condition is called intra-uterine growth retardation (IUGR). The relative contribution of these 2 factors to the incidence of low birthweight babies is variable, depending on the socio-economic status of the population under study (Villar & Belizan 1982). It has been suggested that the differences in birthweight between Asians and Caucasians in Leicestershire might be related to their different diets. It is not certain whether the differences between Asian vegetarian and non-vegetarian diets are significant in this respect. However, there are marked differences between the Asian and Caucasian diets, regardless of whether or not meat is consumed, which might account for the differences in pregnancy outcome. The aim of this investigation was to ascertain whether the reported differences in pregnancy outcome between Asians and Caucasians were present in the study population, and if so, whether they could be correlated with various measurable characteristics of the mother.
(weight, parity, length of gestation) or the baby (sex, birthweight, placental size), in addition to the metabolic status of the mother (as determined by the blood metabolite levels), which has already been discussed in Chapters 3 to 5.

METHODS

The information presented in this chapter was obtained from files compiled by medical and nursing staff at Leicester Royal Infirmary Maternity Hospital. Previous obstetric histories were taken, and maternal weights measured at the Antenatal Clinic. The outcomes of pregnancy were recorded by the Labour Ward staff at the time of delivery. All of this information was available from files stored in the Antenatal Clinic approximately one month after delivery.

RESULTS

Age and Parity The age in years of each patient was recorded at her booking visit to the Antenatal Clinic. The mean age (plus or minus the standard error of the mean) was calculated for each group studied (Table 6.1). The Asian non-vegetarians were significantly younger by 2 to 3 years than the Asian vegetarians \( (p<0.001) \) and the Caucasian non-vegetarians \( (p<0.01) \) in the pregnancy studied. Not all of the patients were primigravida and therefore the percentage of patients in their first, second, third and subsequent pregnancy was calculated for each group (Table 6.1). Although there were slight differences in the distribution of parity between the groups, most of the women were in their first or second pregnancy, ie, 75 per cent of Asian non-vegetarians, 69 per cent of Asian vegetarians and 69 per cent
Table 6.1

Age and Parity of patients in the study

The mean maternal age in whole years was taken from the case notes, along with information about any previous pregnancies. For each group, the percentage of women in their first, second, third and subsequent pregnancy was calculated, in addition to the percentage of women having one or more previous foetal or neonatal deaths.

ANV = Asian non-vegetarian (n = 50)
AV = Asian vegetarian (n = 37)
CNV = Caucasian non-vegetarian (n = 36)

* $p < 0.001$ for the difference between ANV and AV
$p < 0.01$ for the difference between ANV and CNV
<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANV</td>
<td>AV</td>
<td>CNV</td>
</tr>
<tr>
<td>Mean maternal age</td>
<td>*23.8 ± 0.5</td>
<td>26.7 ± 0.7</td>
<td>26.1 ± 0.8</td>
</tr>
<tr>
<td>at 14 weeks' gestation (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of group for which this was the</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First pregnancy</td>
<td>42</td>
<td>33</td>
<td>47</td>
</tr>
<tr>
<td>Second pregnancy</td>
<td>33</td>
<td>36</td>
<td>22</td>
</tr>
<tr>
<td>Third pregnancy</td>
<td>15</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Subsequent pregnancy</td>
<td>10</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Percentage of patients with previous abortion (spontaneous) or perinatal death</td>
<td>10</td>
<td>36</td>
<td>19</td>
</tr>
</tbody>
</table>
of Caucasian non-vegetarians. The maternal age of this group was lower, and this may partially explain why there was a slightly higher percentage of first and second pregnancies in the Asian non-vegetarian group, despite the fact that the groups were randomly selected.

**Previous obstetric history** Table 6.1 shows the percentage of patients for each group having a previous foetal or neonatal death. The Asian vegetarians had double the percentage of previous deaths than the Caucasians and 3 times the percentage of the Asian non-vegetarians. Despite the fact that fewer of the Asian vegetarians were primigravid (ie, there were more previous pregnancies in this group), these figures represent a significantly higher rate of foetal and neonatal mortality in this group of women.

**Maternal weight** Figure 6.1 shows the weight of the mother at each trimester of pregnancy. Throughout pregnancy, the Asian non-vegetarians were significantly lighter than the Caucasian non-vegetarians ($p<0.001$). The Asian vegetarians were also lighter than the Caucasian non-vegetarians, although this was statistically significant only in the first and third trimesters ($p<0.05$). The Asian vegetarians were heavier than the Asian non-vegetarians, though this difference was not significant. The weight gained by the mother through pregnancy was statistically similar in all of the groups studied (12 to 13 kg), as was the weight gain when expressed as a percentage of the first trimester weight (21 to 25 per cent) (Table 6.2).

**Foetal and placental weights** The babies of the Asian non-vegetarians were significantly lighter than those of the Caucasian non-vegetarians ($p<0.001$), as were the babies of the Asian vegetarians ($p<0.05$).
Maternal weight gain during pregnancy

The weight of the patients taking part in the study was measured in each trimester of pregnancy. The median weight was plotted for the following groups: Caucasian non-vegetarians (CNV) (○—○), Asian non-vegetarians (ANV) (□—□) and Asian vegetarians (AV) (■—■). The interquartile ranges are shown.

* $p < 0.001$ for the difference between ANV and CNV
  
  $p < 0.05$ for the difference between AV and CNV

** $p < 0.001$ for the difference between ANV and CNV

*** $p < 0.001$ for the difference between ANV and CNV

  
  $p < 0.05$ for the difference between AV and CNV
A graph showing maternal weight (kg) plotted against weeks of pregnancy. The x-axis represents weeks of pregnancy with values at 14, 28, and 36. The y-axis represents maternal weight with values ranging from 45 to 85 kg. Three lines are plotted, each representing different groups or conditions, indicated by different symbols and error bars. The graph includes symbols for statistical significance: * for p < 0.05, ** for p < 0.01, and *** for p < 0.001.
However, the birthweight expressed as a percentage of the mother's first trimester weight was the same in all groups (5.9 to 6.0 per cent) (Table 6.2). The placentas of the Asian non-vegetarians were significantly lighter than those of the Caucasian non-vegetarians (p<0.05) but the Asian vegetarians had placentas of similar weight to the Caucasian non-vegetarians. The placental weight expressed as a percentage of the mother's first trimester weight was the same in all groups (1.1 per cent) (Table 6.2).

Centile birth weight  Centile birth weights are calculated using recorded weights of babies from the whole population in a particular country. The tenth centile weight is that weight above which 90 per cent of all babies are born. The ninetieth centile weight is that weight below which 90 per cent of all babies are born. In this country, the tenth centile weight is 2.5 kg and the ninetieth centile weight is 4.0 kg, for new-born babies. It can be seen from Table 6.2 that, while the Caucasian non-vegetarians had approximately equal numbers of babies at the low and high ends of the birth weight distribution, as expected, the Asians produced only 2 babies heavier than the ninetieth centile, giving birth to 9 babies weighing less than the tenth centile, 3 of which were born pre-term.

Abnormal outcomes  The incidence of abnormal outcome seen in the pregnancies studied is shown in Table 6.3. The number of congenital abnormalities was higher in the Asians than in the Caucasians. The Asians also had 4 incidences of foetal and neonatal death, 3 of which occurred in Asian vegetarians, whereas all of the Caucasian babies survived. The percentage of Asian non-vegetarian babies who died or had congenital abnormalities was 4 times as high as in the Caucasian
Maternal weight was measured in each trimester of pregnancy, along with foetal and placental weights at delivery. Weights are expressed as median values (kg) with interquartile ranges in brackets, for each of the following groups: Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV).

The foetal birth weight and placental weight were also expressed as a percentage of maternal weight at 14 weeks' gestation.

The weight gained by the mother between 14 weeks and term was calculated and expressed as a percentage of the maternal weight at 14 weeks' gestation.

* A baby is pre-term if it has not completed 37 weeks in utero.
<table>
<thead>
<tr>
<th>Measurement</th>
<th>ANV</th>
<th>AV</th>
<th>CNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal weight at 14 weeks</td>
<td>50.8 (45.0 - 54.9)</td>
<td>53.1 (44.8 - 61.6)</td>
<td>60.4 (49.7 - 67.3)</td>
</tr>
<tr>
<td>Maternal weight at 28 weeks</td>
<td>57.7 (51.2 - 63.9)</td>
<td>62.6 (54.3 - 69.3)</td>
<td>67.1 (58.1 - 77.9)</td>
</tr>
<tr>
<td>Maternal weight at 36 weeks</td>
<td>62.4 (54.7 - 67.5)</td>
<td>64.2 (56.2 - 71.6)</td>
<td>71.6 (60.9 - 81.0)</td>
</tr>
<tr>
<td>Birth weight</td>
<td>2.95 (2.60 - 3.32)</td>
<td>3.08 (2.77 - 3.51)</td>
<td>3.47 (3.04 - 3.89)</td>
</tr>
<tr>
<td>Birth weight as percentage of 14 week weight</td>
<td>6.0 (5.1 - 6.5)</td>
<td>5.9 (4.9 - 6.6)</td>
<td>5.9 (5.4 - 6.6)</td>
</tr>
<tr>
<td>Placental weight</td>
<td>0.53 (0.48 - 0.63)</td>
<td>0.56 (0.50 - 0.60)</td>
<td>0.62 (0.52 - 0.68)</td>
</tr>
<tr>
<td>Placental weight as percentage of 14 week weight</td>
<td>1.1 (0.9 - 1.3)</td>
<td>1.1 (0.9 - 1.3)</td>
<td>1.1 (0.8 - 1.3)</td>
</tr>
<tr>
<td>Weight gain</td>
<td>12.0 (8.0 - 15.0)</td>
<td>13.0 (9.5 - 15.0)</td>
<td>13.0 (10.3 - 15.0)</td>
</tr>
<tr>
<td>Weight gain as percentage of 14 week weight</td>
<td>22.5 (17.7 - 28.7)</td>
<td>24.6 (17.4 - 31.7)</td>
<td>21.7 (18.4 - 26.8)</td>
</tr>
<tr>
<td>Number of babies &gt; 90th centile birth weight</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Number of babies &lt; 10th centile birth weight</td>
<td>6 (2 pre-term)*</td>
<td>3 (1 pre-term)*</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 6.3

Abnormal outcomes of pregnancy

The outcomes of the pregnancies studied were recorded by Labour Ward staff in the case notes. Congenital abnormalities and deaths are recorded in this table for the following groups of women:-- Asian non-vegetarian (ANV), Asian vegetarian (AV) and Caucasian non-vegetarian (CNV).

ANV - number in group = 50
AV  - number in group = 37
CNV - number in group = 36
<table>
<thead>
<tr>
<th></th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANV</td>
</tr>
<tr>
<td>Number of congenital</td>
<td>5</td>
</tr>
<tr>
<td>abnormalities</td>
<td></td>
</tr>
<tr>
<td>Number of perinatal</td>
<td>1</td>
</tr>
<tr>
<td>deaths</td>
<td></td>
</tr>
<tr>
<td>Number of spontaneous</td>
<td>0</td>
</tr>
<tr>
<td>abortions</td>
<td></td>
</tr>
<tr>
<td>Total abnormalities and</td>
<td>6</td>
</tr>
<tr>
<td>deaths</td>
<td></td>
</tr>
<tr>
<td>Percentage of babies with</td>
<td>12</td>
</tr>
<tr>
<td>abnormality or death</td>
<td></td>
</tr>
</tbody>
</table>
non-vegetarians; the percentage of Asian vegetarian babies who died or had congenital abnormalities was almost 5 times as high as in the Caucasian non-vegetarians.

**Foetal sex** The sex of the babies born to women participating in the study was noted, in order to determine the ratio of males to females in each group. The percentages of male babies produced by each group of women were as follows: Asian non-vegetarians, 52%; Asian vegetarians, 43%; Caucasian non-vegetarians, 44%.

**DISCUSSION**

**Obstetric history**

The average age of the groups of women taking part in the study ranged from 23 to 27 years. Although the Asian non-vegetarians were younger on average than the other women, the difference was small and unlikely to account for any differences in the quality of outcome between the groups. The risk of foetal death is greatest in the first pregnancy, falling to a minimum for the second and third babies, and then increasing progressively with subsequent pregnancies. The parity of the groups studied was approximately the same and therefore any increase in the mortality rate in the Asians could not be attributed to their having had significantly more or less previous pregnancies.

The previous obstetric histories of the women studied showed that the Asian vegetarians had the greatest number of previous deaths or abnormalities (this held true when corrected for group size). This observation lends support to the conclusion of Benny et al (1980),
that the Asian vegetarians were more at risk of perinatal mortality than the Caucasian non-vegetarians. However, Benny et al did not study Asian non-vegetarians, and in the present study it was the Asian non-vegetarians who had the lowest number of previous deaths and abnormalities. There is, therefore, a clear distinction between the Asian vegetarians and non-vegetarians. Excess mortality in term babies of normal weight has been attributed to avoidable factors, such as poor patient compliance, sub-optimal antenatal care, care at delivery etc (Robinson et al 1982), and therefore if the Asian vegetarians and non-vegetarians produced babies weighing the same on average, it could be assumed that some other factor was causing the difference in the rate of mortality and morbidity. In order to investigate this further, it was necessary to compare the mortality rate in the babies from this study with the birth weight of the babies.

Pregnancy associated weight gain

The maternal weight gain through pregnancy was approximately linear in all of the groups studied (Figure 6.2). This phenomenon has been reported in part by other workers, who observed that the increase in weight is linear between the second and third trimesters (Gueri et al 1982). In the present study, the Asian non-vegetarian mothers were significantly lighter than the Caucasians throughout pregnancy, with the Asian vegetarians falling between the 2 weights. However, the maternal weight gain was the same in all of the groups studied, being on average between 21.7 and 24.6 per cent of the weight at 14 weeks (as compared with 20 per cent in Gueri's Dominican population). None of the groups showed a disproportionately low weight gain, and therefore the raised perinatal mortality rate cannot be attributed to poor maternal weight gain. Because all of the groups had the same
Figure 6.2

Increments in maternal weight during pregnancy

The weights of the patients taking part in the study were measured at each antenatal visit by the nursing staff. Mean weights were calculated for each group of women, in each of the 5-week periods shown (eg, 10 - 15 weeks, >15 - 20 weeks, etc) and plotted as a histogram.

- = mean weight of the ANV
- - = mean weight of the AV
- - - = mean weight of the CNV
percentage weight gain, the amount of weight gained in pregnancy appears to be regulated by the woman's size prior to pregnancy.

**Foetal and placental weights**

Despite the fact that all of the mothers gained between 12 and 13 kg on average during pregnancy, the weights of the babies varied significantly, the babies of the Asian non-vegetarians being the lightest, followed by the Asian vegetarians, with the Caucasian babies being significantly heavier than those in the other groups. This difference could not be attributed to differences in the sex ratio - male babies are heavier than females (Haines et al 1982) - since the percentage of male babies was relatively constant in all groups, ranging from 43 to 52 per cent. The birth weight differences observed follow the same trend as the maternal weights and it is therefore not surprising to find that the weight of the baby expressed as a percentage of the mother's weight at 14 weeks' gestation was an average of 5.9 to 6.0 per cent in all of the groups. This suggests that all of the groups were producing normally sized babies and that there is a constant relationship between the birth weight and the mother's pre-pregnant weight. This has already been suggested by Jones and Battaglia (1977) but has not previously been studied in different ethnic groups. If it is assumed that a healthy baby needs to attain a certain percentage of the mother's weight, then the Asian babies in this study were just as healthy as the Caucasian babies, and were lighter merely because their mothers were smaller.

In the same way as the birth weights, the placental weights were a constant percentage (1.1 per cent) of the mother's weight at 14 weeks.
Placental size is a major determinant of foetal size (Jones & Battaglia 1977). None of the groups had an abnormally low average placental weight, and therefore none of the groups were put at risk by having smaller placentas. The increased mortality rate in the Asians cannot therefore be attributed to a possible hormonal deficiency in pregnancy resulting from a small placenta.

Birth weight analysis in the whole population

The birth weight distribution for this country has been determined (see results section). The general practice is to use centile cut-off points for all babies, ie, for both Asians and Caucasians, although it has recently been suggested that "accurate assessment of foetal growth at birth . . . requires growth standards reflecting the pattern of growth and development in the population at risk" (Forbes & Smalls 1983) - in other words, that we should calculate a separate set of centiles for the Asian population. Using the United Kingdom standard for both Asians and Caucasians, it was found, in this study, that the Caucasians had an equal percentage of babies at the extreme low and high ends of the distribution, whereas the Asians had a larger percentage of babies weighing less than the tenth centile, and few babies weighing more than the ninetieth centile. Clearly, the weights of the Asian babies lie towards the lower end of the birth weight distribution. Three of the Asian babies weighing less than 2500 g were pre-term, ie, born before the completion of the 37th week in utero. These babies are classed as premature, whereas the other babies in this category were small for gestational age, due to intra-uterine growth retardation (Villar & Belizan 1982).
Quality of outcome of pregnancy

Because the sample used in this study was relatively small, it was not expected that the number of perinatal mortalities would correspond with the expected value given for the whole population. However, we anticipated that there would be one or possibly 2 mortalities in our study population, given a perinatal mortality rate of about 15 per 1000 live and still births. In fact, there were 2 perinatal deaths, both of which were Asian babies, in addition to 2 spontaneous abortions in the first trimester, both of which were in the Asian vegetarian group. The number of congenital abnormalities was 4 times higher in the Asians than in the Caucasians. Both of the perinatal mortalities occurred because the babies had multiple congenital abnormalities which were incompatible with life (anencephaly and microcephaly with hydrocoele respectively). The congenital abnormalities in the surviving infants were not severe in the main, but, having been confirmed by a paediatrician, were evidence that the outcome of pregnancy in that particular patient was not as good as it might be.

Possible reasons for a poor outcome of pregnancy

In 1982, Williams & Chen reported that low birth weight results from adverse maternal circumstances, and that it is associated with either prematurity or low weight for gestational age. They observed that in California, two thirds of the observed decrease in the PMR between 1960 and 1974 was due to an increase in birth weight. This would suggest that in our study population, the PMR could be reduced by achieving higher birth weights in the Asian population. The Asians in Leicester are known to have nutritional problems (Donaldson &
Taylor 1983) and it is thought that intra-uterine growth retardation is a symptom of starvation and low social class, brought about by long-term sub-optimal nutrition (Howie 1982). Congenital abnormalities are caused by a number of factors acting in concert, ie, genetic, and environmental factors, the latter of these being made up of maternal, chemical and nutritional elements (Kalter & Warkany 1983). Clearly, mortality and morbidity could be related to birth weight in some way. It has not been found possible to raise birth weight by the use of nutritional supplements in pregnancy, which suggests that if the nutritional factor is a major contributor to the problems in Asian pregnancies, it is a long-term one, being the result of years of malnutrition prior to pregnancy (Wharton et al 1982).

Though we have observed a higher PMR and congenital abnormality rate in our Asian patients, we have not been able to conclusively show why this is so. Although the Asian babies were lighter than their Caucasian counterparts, all of the babies' weights were related to those of their mothers by a simple mathematical relationship, and therefore the only conclusion we can make with any confidence about the Asian babies is that they are small because their mothers are small. In addition, there were no obvious differences in outcome between the Asian non-vegetarians and vegetarians, and it would therefore be difficult to envisage dietary differences during pregnancy as a major cause of increased perinatal mortality and congenital abnormality, ie, the vegetarians and non-vegetarians had the same outcome with different diets, and therefore the ethnic difference (ie, genetic) between Asians and Caucasians is more likely to be the cause of the different mortality rates.
SUMMARY

Differences in the quality of outcome of pregnancy between Asians and Caucasians have been observed, but there were no differences between Asian non-vegetarians and Asian vegetarians. The Asians had smaller babies and higher numbers of spontaneous abortions, congenital abnormalities and perinatal deaths than the Caucasians. Previous to this study, it was thought that the raised perinatal mortality rate in the Asian population could be accounted for by the smaller size of their babies. However, it has now been shown that the Asian baby is small merely because the mother is small, and that birth weight is a constant proportion of the mother's weight in the first trimester (which is approximately equal to her pre-pregnant weight), namely 6 per cent. Similarly, the placental weight was about one per cent of the mother's weight in all of the groups, and therefore the difference in outcome between Asians and Caucasians cannot be attributed to placental insufficiency. The Asian babies fall towards the lower end of the birth weight distribution for this country, but instead of attributing their perinatal disadvantage to this factor, it may be more informative to measure the birth weight distribution for the Asians alone, because many of their "low birth weight" babies could be normal when the mother's size is taken into account.

With respect to nutrition, there is no way of retrospectively assessing the long-term nutrition of these patients, and therefore we can only postulate that the Asians may have been under-nourished for long periods of time prior to pregnancy, and that this may have a more detrimental effect on their foetuses than a relatively short period of malnutrition which may or may not have occurred during pregnancy.
CHAPTER SEVEN

METABOLIC STUDIES ON NON-PREGNANT WOMEN
INTRODUCTION

Having investigated fasting blood metabolite levels in pregnant Asian and Caucasian women, it was decided that, instead of using the first trimester results as a baseline with which to compare the other data, the study would be more complete if the results obtained in pregnancy were compared with data from women who were not pregnant. Ideally, the patients who were followed through pregnancy should have been studied post-partum, so that each patient formed her own non-pregnant control. However, the duration of lactation is variable and it is known that metabolism does not return to a normal non-pregnant pattern until at least 6 months after delivery, depending on whether the mother is breast feeding (Darmady & Postle 1982). Because of the limited amount of time available for this study, it was decided that a separate group of patients should be investigated before pregnancy. These patients were selected at random from the Gynaecology out-patients department (GOPD) and were known not to be using oral contraceptives, which affect lipid metabolism (Wynn et al 1979) and possibly mimic some of the effects on metabolite levels observed in pregnancy.

The aim of this investigation was to ascertain whether the levels of blood metabolites observed in the first trimester of pregnancy were the same as those prior to pregnancy, and also whether the relationships between the results obtained in the 3 groups of non-pregnant women studied were the same as those in early pregnancy.
METHODS

Thirty eight non-pregnant women (11 Asian non-vegetarians, 8 Asian vegetarians and 19 Caucasian non-vegetarians) were selected at random from the GOPD at Leicester Royal Infirmary. None of these patients was taking oral contraceptives.

A blood sample was obtained from each patient following an over-night fast of at least 8 hours. Samples were centrifuged to obtain plasma, which was assayed for glucose, insulin, triglyceride, total cholesterol, HDL cholesterol and LDL cholesterol, using methods described in Chapter 2.

RESULTS

Median values and interquartile ranges were calculated for the results in each of the 3 groups studied. These were compared with the results obtained in the study of pregnant women in the first trimester (Tables 7.1 and 7.2). The degree of significance of the difference between the results in the non-pregnant women and those of the pregnant women was calculated, along with the degree of significance of the differences between blood metabolite levels in non-pregnant Asians and Caucasians.

Glucose and insulin Table 7.1 shows that the fasting plasma concentrations of glucose and insulin did not vary significantly between non-pregnant patients and those in early pregnancy. There was no significant difference between the fasting plasma glucose and insulin levels in Asians and Caucasians in the non-pregnant state.
Table 7.1

Median fasting plasma glucose and insulin concentrations in the non-pregnant state.

Fasting plasma glucose and insulin concentrations were measured in groups of non-pregnant Asian non-vegetarians (ANV), Asian vegetarians (AV) and Caucasian non-vegetarians (CNV). Median values (with interquartile ranges) were calculated for each group of women and tabulated alongside the values obtained from women in the first trimester of pregnancy (Chapter 3). The significance of the differences between the results obtained from non-pregnant and pregnant women was shown.

NS = not significant
<table>
<thead>
<tr>
<th>Group</th>
<th>Non-pregnant value (mM)</th>
<th>1st trimester value (mM)</th>
<th>Significance of difference between non-pregnant and pregnant results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting glucose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>4.39 (4.26–4.78)</td>
<td>4.56 (4.25–4.94)</td>
<td>NS</td>
</tr>
<tr>
<td>AV</td>
<td>4.56 (3.79–4.68)</td>
<td>4.43 (4.14–4.74)</td>
<td>NS</td>
</tr>
<tr>
<td>CNV</td>
<td>4.70 (4.31–4.85)</td>
<td>4.51 (4.08–4.66)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Fasting insulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>0.43 (0.32–0.58)</td>
<td>0.35 (0.22–0.60)</td>
<td>NS</td>
</tr>
<tr>
<td>AV</td>
<td>0.56 (0.36–0.75)</td>
<td>0.34 (0.26–0.50)</td>
<td>NS</td>
</tr>
<tr>
<td>CNV</td>
<td>0.41 (0.32–0.67)</td>
<td>0.45 (0.29–0.60)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 7.2

Median fasting plasma lipid concentrations in the non-pregnant state

Fasting plasma triglyceride, total cholesterol, HDL cholesterol and LDL cholesterol concentrations were measured in groups of non-pregnant Asian non-vegetarians (ANV), Asian vegetarians (AV) and Caucasian non-vegetarians (CNV). Median values (with interquartile ranges) were calculated for each group of women and tabulated alongside the values obtained from women in the first trimester of pregnancy (Chapter 4). The significance of the differences between the results obtained from non-pregnant and pregnant women was shown.

NS = not significant
<table>
<thead>
<tr>
<th>Group</th>
<th>Non-pregnant value (mM)</th>
<th>1st trimester value (mM)</th>
<th>Significance of difference between non-pregnant and pregnant results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting triglyceride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>0.89 (0.79-1.08)</td>
<td>1.23 (0.89-1.68)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>AV</td>
<td>0.71 (0.63-0.80)</td>
<td>1.44 (1.13-2.11)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CNV</td>
<td>0.98 (0.77-1.24)</td>
<td>1.38 (1.16-1.58)</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>Fasting total cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>4.74 (4.35-4.88)</td>
<td>4.59 (4.18-5.33)</td>
<td>NS</td>
</tr>
<tr>
<td>AV</td>
<td>3.85 (3.63-4.59)</td>
<td>4.85 (4.13-5.50)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CNV</td>
<td>5.11 (4.39-5.63)</td>
<td>4.72 (4.20-5.75)</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>1.26 (1.03-1.39)</td>
<td>0.89 (0.67-1.14)</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>AV</td>
<td>1.07 (0.95-1.42)</td>
<td>0.89 (0.59-1.13)</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>CNV</td>
<td>1.22 (0.97-1.52)</td>
<td>1.05 (0.81-1.29)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Fasting LDL cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td>2.92 (2.76-3.40)</td>
<td>3.30 (2.64-3.80)</td>
<td>NS</td>
</tr>
<tr>
<td>AV</td>
<td>2.53 (2.12-2.84)</td>
<td>3.31 (2.48-3.96)</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>CNV</td>
<td>2.97 (2.53-4.16)</td>
<td>3.02 (2.51-3.98)</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Triglyceride** Table 7.2 shows that fasting plasma triglyceride concentration was lower in women who were not pregnant than in the first trimester of pregnancy ($p < 0.01$ for ANV, $p < 0.001$ for AV, $p < 0.005$ for CNV). In the women who were not pregnant, there was a significant difference in fasting plasma triglyceride concentrations between Asian non-vegetarians and vegetarians ($p < 0.05$) and between Asian vegetarians and Caucasian non-vegetarians ($p < 0.02$), the Asian vegetarians having the lowest plasma triglyceride concentration at this time.

**Total cholesterol** There was no significant difference between plasma total cholesterol concentrations in non-pregnant and pregnant Asian and Caucasian non-vegetarians, although the fasting plasma cholesterol levels were slightly higher in the non-pregnant women in these groups (Table 7.2). However, in the Asian vegetarians, the converse was true, the pregnant women having significantly higher levels of total cholesterol ($p < 0.001$). In the women who were not pregnant, the Asian vegetarians had a significantly lower plasma cholesterol concentration than the Asian non-vegetarians ($p < 0.01$) and the Caucasian non-vegetarians ($p < 0.001$).

**HDL cholesterol** Table 7.2 shows that the plasma concentration of HDL cholesterol was significantly lower in the first trimester of pregnancy than in the non-pregnant state in all groups. There was no significant difference between the HDL cholesterol levels in any of the non-pregnant patients.

**LDL cholesterol** The fasting plasma concentration of LDL cholesterol was lower in the non-pregnant women than in those in the first trimester
of pregnancy, although this difference was statistically significant only in the Asian vegetarians \(p<0.005\). The LDL cholesterol concentrations in the 3 groups of non-pregnant women were similar, except that the Asian vegetarians had significantly lower levels than the Caucasian non-vegetarians \(p<0.02\).

**DISCUSSION**

In discussing the relationships between the blood metabolite levels of non-pregnant and pregnant women which have been investigated in this chapter, it is essential to bear in mind that the non-pregnant and pregnant patients were completely separate groups of women and as such, longitudinal comparisons between these 2 states must be made with reservation. Additionally, the women attending the GOPD were doing so mainly because of problems with fertility, which may have had an effect on their metabolism. Conclusions drawn from this chapter must therefore be tentative.

**Carbohydrate metabolism**

There were no differences in carbohydrate metabolism (as assessed by measurements of plasma glucose and insulin concentrations) between the pregnant and non-pregnant patients in any of the groups studied. In addition, there were no differences between Asians and Caucasians in the non-pregnant state. This reflected the results obtained in the first trimester of pregnancy, described in Chapter 3, where the Asians and Caucasians had similar fasting levels of glucose and insulin. Fasting levels of carbohydrates in non-pregnant Asian women, and in those in the early stages of pregnancy, appear to be normal, and
therefore the increased incidence of congenital abnormality in the Asian population is not likely to be a result of deranged carbohydrate metabolism at conception and during organogenesis. However, it would be useful in the future to carry out GTTs on non-pregnant Asian women to find out whether they show the same pattern as the pregnant women, ie, raised insulin levels, 60 and 120 minutes after the oral glucose dose (Chapter 3). If this occurred in the non-pregnant women, it would suggest that the Asians had less control over their blood glucose levels than the Caucasians, even when not pregnant, and that this feature of Asian metabolism is normal and not likely to endanger the growing foetus. Conversely, if the Asians were found to have the same GTT response as the Caucasians prior to pregnancy, this would suggest that the Asians in the first trimester of pregnancy had difficulty in maintaining control over their carbohydrate metabolism because of the extra demands of the foetus, whose health might be compromised as a result.

Lipid metabolism

Although the amount of weight gained in the first trimester of pregnancy is very small, there was a significant increase in the fasting plasma concentration of triglyceride in the first trimester when compared with non-pregnant patients, in all groups studied. Plasma triglyceride is destined either for storage in adipose tissue, or for use as an energy source. The rise in plasma triglyceride levels in the first weeks of pregnancy therefore indicates early adaptation of lipid metabolism to the demands made by the foetus - increased energy supplies for growth, and maternal storage of potential energy in preparation for the dramatic growth of the foetus in late pregnancy. The Asian vegetarians had
slightly lower plasma concentrations of triglyceride than the Asian and Caucasian non-vegetarians prior to pregnancy, but this does not necessarily mean that they are at a disadvantage, because they had the same plasma triglyceride levels as the other groups in the first trimester of pregnancy (Chapter 4).

The plasma concentration of total cholesterol did not vary between non-pregnant and pregnant women in the Asian and Caucasian non-vegetarian groups, but the Asian vegetarians showed a significant increase in plasma total cholesterol with the onset of pregnancy, which was caused by their having significantly lower levels in the non-pregnant state than the other groups. The pattern is therefore the same as that observed in the triglyceride, where the Asian vegetarians seem to be deficient in these 2 metabolites prior to pregnancy, but catch up to the levels observed in the other groups in the early stages of pregnancy.

There was no significant difference between the groups of non-pregnant women in either the HDL cholesterol or the LDL cholesterol concentrations in plasma. In all groups, the concentration of cholesterol carried by HDL decreased with the onset of pregnancy, whereas the concentration of cholesterol carried by LDL increased (although statistically this was not as significant as the change in the concentration of HDL cholesterol). Clearly, the HDL/LDL ratio decreased as a result of the onset of pregnancy, beginning a trend which has been shown to continue through pregnancy (Chapter 4). The amount of cholesterol destined for excretion (ie, that carried by HDL) decreased, making more cholesterol available to the peripheral tissues (carried by LDL). This cholesterol is available to the placenta for use in hormone biosynthesis to help
maintain the pregnancy, as discussed in Chapter 4.

SUMMARY

Bearing in mind the reservations already discussed with respect to the comparison of results from unrelated groups of patients, it appears that Asians and Caucasians have similar carbohydrate metabolism in the non-pregnant state, and that this relationship does not change with the onset of pregnancy, fasting glucose and insulin concentrations remaining the same in the first trimester. However, studies of lipid metabolism show that plasma triglyceride and total cholesterol levels were increased in early pregnancy in all women. It was also found that, because they had lower levels of these metabolites prior to pregnancy, Asian vegetarian women had a larger increase in their plasma concentration of triglyceride and total cholesterol, bringing their levels up to those of the other women, in early pregnancy. The proportion of plasma cholesterol available to peripheral tissues increased with the onset of pregnancy in all of the groups studied. Thus, the metabolic changes observed throughout pregnancy begin to manifest themselves in the first trimester, since even at this early stage, the levels of some blood metabolites are different from those of women who are not pregnant.
CHAPTER EIGHT

GENERAL DISCUSSION
SUMMARY OF AIMS OF THE STUDY

The aim of the present study was to assess the extent to which metabolic adaptation to pregnancy occurred in Asian and Caucasian women in Leicester. Because the risk of an infant dying in the immediate neonatal period was significantly raised in the Asian population, it was suggested that there might be differences in the ways Asians and Caucasians responded metabolically to pregnancy. Such differences might be reflected by the levels of metabolites in the blood, and as such would be amenable to biochemical study. A pilot study carried out by Benny (Benny et al. 1980) showed that Asian vegetarians had higher mean diurnal blood glucose concentrations than Caucasian non-vegetarians, but the babies of Asian mothers were smaller than those of Caucasian mothers. However, the Asian vegetarians had blood glucose levels akin to those of chemical diabetics in the third trimester of pregnancy, but their babies did not have the macrosomic features typical of the offspring of established diabetics. It was hoped that the more detailed studies described in this thesis would help to explain these results. In addition, it was possible that there might be a metabolic explanation for the increased rate of congenital abnormality and perinatal mortality in the Asian population.

METABOLIC STUDIES

From the studies of blood glucose and insulin levels described in Chapter 3, it is clear that although Asians and Caucasians had the same blood glucose levels during a GTT, (Table 3.2) the Asians secreted more insulin in order to achieve the same level of response (Table 3.4). This was particularly notable in the first and second
trimesters. In the third trimester, the Asians showed the same degree of insulin secretion in response to the GTT as the Caucasians, but their fasting insulin concentration was raised (Table 3.3). As discussed in Chapter 3, it is not clear how these 2 observations are linked, but they indicate that Asians had increased resistance to the effects of their insulin in pregnancy, which became more pronounced in the third trimester because it occurred at basal glucose levels. This did not lead to increased impairment of glucose tolerance in the Asians, and this is reflected in the HbA$_{1c}$ levels, which were similar in all groups studied. In gestational diabetes, the insulin binding capacity of the placenta is reduced owing to a decrease in the number of receptors (Harrison et al 1977). This occurs because the raised insulin concentration seen in gestational diabetes leads to a modulation of the concentration of its receptors in the target cells, ie, down-regulation (Duran-Garcia et al 1979). It would therefore be useful to investigate whether there were differences between the insulin binding capacities of the placentas of Asians and Caucasians, as this might indicate why the raised insulin levels were needed to maintain normal glucose tolerance in the Asians. A detailed analysis of the pregnancy hormones (progesterone, oestrogen and hPL), along with glucagon, might also yield useful information on the extent of contra-insulin activity in the Asians and Caucasians.

The measurements of the various plasma lipid levels described in Chapter 4 showed that there were dramatic changes in the lipid composition of plasma, which in a non-pregnant subject, would indicate gross metabolic derangement. These changes led to an increase in the amount of circulating triglyceride (Table 4.1), which was available for use by the mother as an energy source. In addition, more cholesterol,
carried as LDL, was made available to the peripheral tissues as a precursor for steroid biosynthesis. There was no difference between the lipid levels of the Asians and Caucasians. The distribution of cholesterol among the lipoproteins was measured indirectly, but the distribution of triglyceride was not assessed. It would therefore be interesting to separate plasma lipoproteins by electrophoresis or ultracentrifugation, and directly measure the amounts of triglyceride and cholesterol associated with each type of lipoprotein. The adaptation of lipid metabolism to pregnancy occurred in all of the groups, to the same extent.

The amino acid studies, presented in Chapter 5, gave rise to the only observed differences between Asian vegetarians and non-vegetarians. Asian vegetarians had lower plasma levels of BCAA throughout pregnancy. Since BCAA are a potential source of alanine, these women had fewer reserves of substrate for gluconeogenesis than non-vegetarians, and therefore the effects of accelerated starvation would be expected to be more rapidly felt in the vegetarian group. Asian vegetarians also had lower levels of essential amino acids in late pregnancy, particularly histidine. The Asian vegetarians were therefore at a metabolic disadvantage with respect to their amino acid levels, particularly in late pregnancy.

The secondary study, which was undertaken to compare non-pregnant patients with those in the first trimester (Chapter 7), revealed that carbohydrate metabolism was the same in both groups of women, and therefore that there was no significant change in glucose or insulin levels in early pregnancy, ie, during organogenesis. There were, however, slight changes in the levels of the various lipids. Triglyceride
levels increased with the onset of pregnancy, as did the amount of cholesterol carried by LDL. Clearly, the changes in lipid levels which were observed in pregnancy were initiated in the few weeks following conception. There were no major differences between non-pregnant Asians and Caucasians, except that the non-pregnant Asian vegetarians had lower levels of triglyceride and cholesterol. However, as there were no differences in the lipid levels between the groups in the first trimester, the Asian vegetarians clearly adapted rapidly to the demands of pregnancy.

OUTCOME OF PREGNANCY

The data which was collected regarding the outcome of pregnancy (Chapter 6) showed that Asian babies were significantly lighter than Caucasian babies (Table 6.2). This difference was not attributable to variation in the male:female ratio, nor to significant differences in age and parity between the groups of mothers. The most striking observation was that the weight of the baby at birth was 6 per cent of the mother's first trimester weight, in all 3 groups. It was therefore concluded that the Asian babies were smaller because they were in proportion to their mothers' size. The excess perinatal mortality reported in the Asians may be related to infant size - because the frequency distribution of birth weight lies more towards the lower end in the Asians, a higher percentage of these babies will be growth retarded and have less chance of survival. In response to the results presented in this thesis, it has been suggested that those babies weighing less than 6 per cent of their mothers' weight at birth would be most at risk. The potential risk could therefore be assessed by ultrasound in utero, where the expected birth weight
could be measured and related to the mother's weight before pregnancy. This, of course, would be dependent on an accurate date for the last normal menstrual period.

The increased incidence of abnormalities in Asian babies (Table 6.3) is caused by a disturbance in organogenesis, which occurs in the first 7 to 8 weeks of foetal life (Freinkel 1980). This study did not show any major disturbance of metabolism in the first trimester, nor any significant change between the non-pregnant state and early pregnancy which could account for the adverse effects on organogenesis in the Asians. However, the Asians did secrete greater quantities of insulin in order to maintain a normal GTT response, and this may point to a struggle for glucose homeostasis in these women, which could have had more serious implications for the foetus than for the mother.

The conflicting findings of small babies and raised mean diurnal blood glucose levels in the Asians are less of a problem in the light of the present study, because it has been found that the Asians had normal blood glucose levels after an over-night fast, and during a GTT. However, if the average concentration of plasma glucose was raised, especially in early pregnancy, it is possible that these patients would become like chemical diabetics, and that this might lead to growth delay in the first 7 weeks. This hypothesis was put forward by Pedersen (1983), but the mechanism by which this "early growth delay" might occur has not been postulated - Pedersen suggests delayed ovulation or delayed fertilisation as alternatives. If this hypothesis were correct, and if the degree of diabetes was mild or well controlled, macrosomia would not occur, and the baby suffering from "early growth delay" would not catch up in size with his normal
counterpart. In the present study, it is probably preferable to say that the Asian babies were smaller, despite indications that carbohydrate tolerance may be impaired, not because of them, and that malnutrition in the Asian population as a whole over a long period of time is responsible for their size.

GENERAL COMMENTS

This study of pregnancy in Leicester's Asian population is unique because the Asian women taking part were immigrants, most of whom had been resident in the United Kingdom for less than 5 years. These women therefore tended to retain their own dietary habits and religious customs. Consequently, any further study of this nature would need to be initiated within the next few years, because the second generation of Asians who were born in Leicester, and who are now reaching adolescence, have begun to adopt Western dress and behaviour. If the incidence of smaller babies is attributable to long-term under-nutrition in the Asians, rather than malnutrition during pregnancy, the Asians who were born in Britain are less likely to suffer problems in pregnancy than their mothers, and we should see a gradual decline in perinatal mortality in this group to the level of the indigenous population.

SUMMARY

The study described in this thesis has shown that there were no major derangements of the metabolic adaptation to pregnancy in Leicester's Asians. The raised perinatal mortality rate is therefore unlikely to be a result of differences in blood metabolite levels. The birth weight was lower in the Asians, although it was a constant proportion of the
mother's weight. Low average birth weight for the group may lead to increased mortality simply because of the higher likelihood of babies being growth retarded. The smaller size of the Asian population as a whole is probably a result of many generations of malnutrition. The study has not explained the higher incidence of congenital abnormality in the Asians, although the mechanism which leads to general growth failure (IUGR) could also lead to specific growth failure affecting certain tissues during organogenesis. In addition, it is presumed that any foetus which does not achieve 6 per cent of its mother's weight is at a higher risk of perinatal mortality and/or morbidity, regardless of race. Finally, no evidence was found to support the hypothesis that Asian vegetarians are more susceptible to perinatal death than Asian non-vegetarians.
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This study was carried out to determine whether the raised perinatal mortality rate observed in Leicester's Asian population was related to abnormalities in the metabolic response to pregnancy. This was assessed by measuring blood metabolite levels in groups of Asian non-vegetarian, Asian vegetarian and Caucasian non-vegetarian women during pregnancy.

Maximal plasma glucose and insulin concentrations during a glucose tolerance test increased as pregnancy advanced. The glucose levels were similar in all groups, but the Asians had higher insulin concentrations following an oral glucose dose in the first trimester. Asians therefore had a greater degree of insulin resistance than Caucasians.

Plasma triglyceride and low density lipoprotein cholesterol levels rose during pregnancy; there was no significant difference between the groups of women.

Asian vegetarians had lower levels of branched chain amino acids throughout pregnancy, and lower levels of essential amino acids, particularly histidine, in the third trimester.

In all groups, the percentage maternal weight gain was the same, and the birth weight and placental weight were a constant percentage of the maternal weight at 14 weeks. Asian women gave birth to smaller babies and had a greater number of spontaneous abortions, congenital malformations and perinatal mortalities.

Similar metabolic studies on non-pregnant women showed little difference from the first trimester of pregnancy, although there was some evidence of early adaptation to pregnancy in lipid metabolism.

The results of this study show that there are small but significant differences in the metabolic adaptation to pregnancy in the groups of women studied, and these may contribute to the higher perinatal mortality rate observed in Leicester's Asians. However, it is clear that Asians give birth to small babies because they themselves are small, and this fact alone may put these babies at greater risk of mortality and morbidity.