NEW INDICES

OF

PERINATAL GROWTH

By

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PREFACE

To the best of the author's knowledge, this thesis contains no material previously published or written by another person, except where due reference is made in the text. It contains no material which has been submitted or accepted for the award of any other degree or diploma in any University.

(A. Hocking)

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SUMMARY

This thesis describes two new indices of perinatal morbidity relating to intrauterine growth retardation, namely neonatal water metabolism and protein metabolism.

Water metabolism was studied by determining body water turnover rates of 106 newborn infants, using the non-radioactive isotope of water, deuterium oxide (D_2O). The rate at which D_2O is excreted from the body can be expressed as a rate constant of water turnover. Water turnover rates of the infants were correlated with independent obstetric and paediatric assessments of the newborn infants. The neonates were classified by strict clinical criteria into three main groups, fully grown ("normal"), borderline (exhibiting some features of growth retardation) and clearly intrauterine growth retarded. There were highly significant differences between the water turnover rates of each of these groups, the medians of the rate constants ($x10^4$) being 73.3, 85.9 and 100.2 (h^{-1}) respectively. This test is also quantitative, in that infants who were more clinically growth retarded displayed higher water turnover rates.

Body water turnover rates are simple to perform by this method of isotope dilution and the collection of neonatal urine samples is convenient, using a paper towel placed inside the infant's nappy. However, 4-8 urine samples are necessary for this test to be performed.

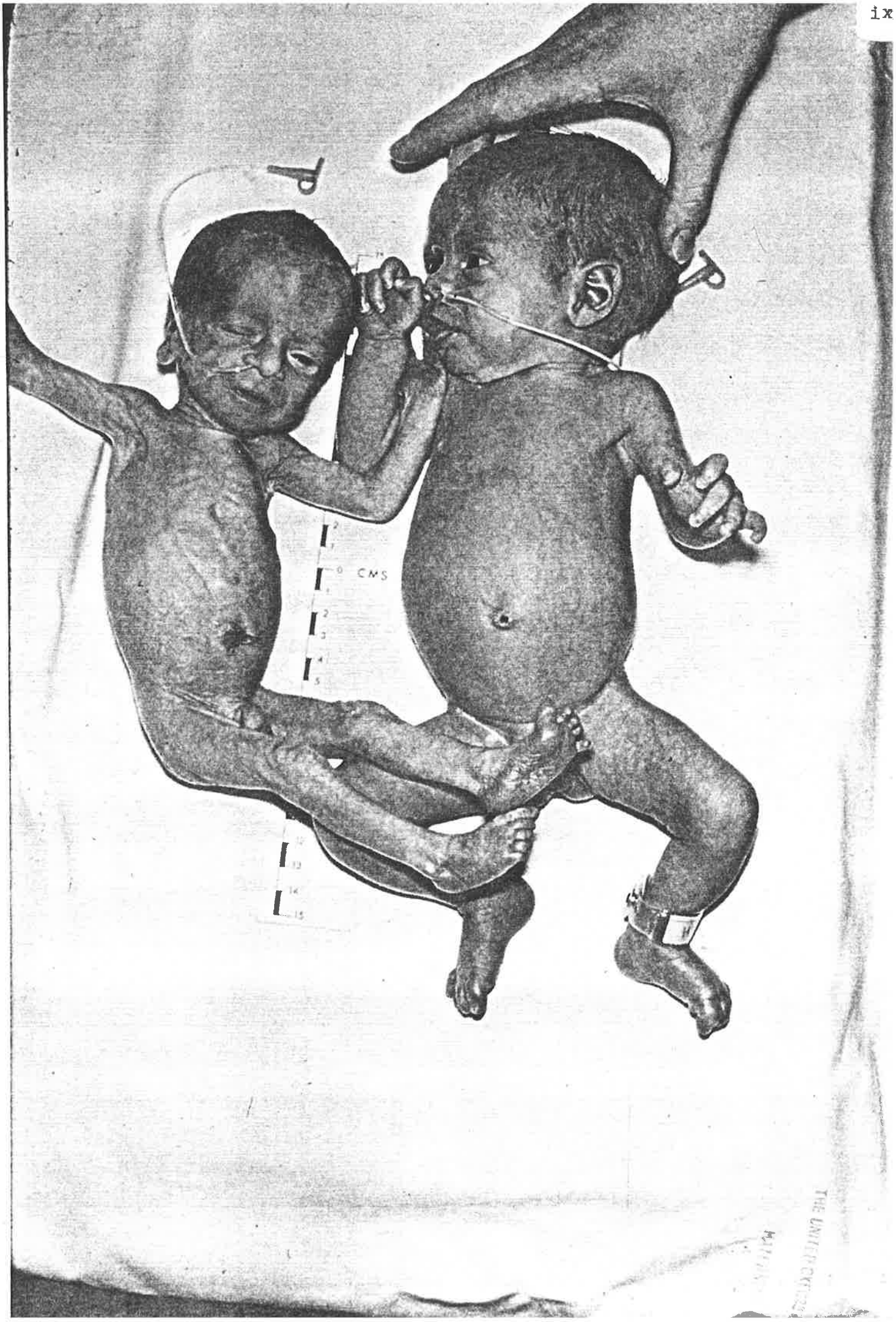
A test requiring only a single urine sample would be ideal and, for this reason, the measurement of 3-methylhistidine was considered. Urinary excretion of 3-methylhistidine has been shown to be an index of myofibrillar protein breakdown and 3-methylhistidine/creatinine ratios give a measure of protein turnover. Measurement of this amino acid has never before been undertaken in relation to intrauterine growth retardation in neonatal urine or amniotic fluid samples.

3-methylhistidine/creatinine ratios have been measured in amniotic fluid of foetuses ranging from 16-21 weeks and 27-42 weeks gestation. The ratio is high in amniotic fluid in early gestation (approximately 60 nmol/μmol),

decreasing at term to a level of approximately 10 nmol/μmol. The first neonatal urine ratio correlates well with the corresponding term amniotic fluid ratio. A rapid rise in the 3-methylhistidine/creatinine ratio occurs postnatally, reaching a plateau at around two days.

In a sample of eight normal and seven intrauterine growth retarded infants, the ratio in the first neonatal urine sample was consistently lower in the normal than in the growth retarded group. There is a possibility that the 3-methylhistidine/creatinine ratio may be used as an indicator of growth retardation.

The measurement of water turnover rates is a simple quantitative test for growth retardation and, although it is not certain whether 3-methylhistidine/creatinine ratios yield a quantitative result, it may potentially be used antenatally, as well as neonatally. Both tests are advantageous, in that they are independent of the gestational age of the infant, birth weight, behaviour and appearance.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ABBREVIATIONS

Abbreviations used in the text are as follows;

IUGR	=	intrauterine growth retardation
ln	=	natural logarithm
3MH	=	3-methylhistidine
Cr	=	creatinine
D_2O	=	deuterium oxide
ppm	=	parts per million

CHAPTER I

INTRODUCTION

1.1 GENERAL INTRODUCTION

1.1.1 Definition of Intrauterine Growth Retardation

The identification of abnormal intrauterine growth in the human infant at birth is imperative to assure appropriate management and to provide optimal conditions for postnatal physical growth and intellectual development. Infants who are growth retarded at birth present a major perinatal problem and this growth retardation is associated with a high mortality and morbidity. Infants who are small for gestational age face a perinatal mortality rate eight times higher than for those infants who are appropriate for their gestation, (Castelazo-Ayala, et al. 1976.) and the survivors display a high incidence of neurological and intellectual deficiencies (Fitzhardinge and Stevens, 1972).

In the literature there are many terms used for infants who are small for gestational age. Small-for-dates, dysmature, chronic foetal distress, intrauterine (or foetal) malnutrition, light-for-dates, placental insufficiency and intrauterine growth retardation are some of the synonyms in common usage. In this thesis the expression "intrauterine growth retardation" (IUGR) will be employed.

Definitions of IUGR are at the present imprecise and none are universally accepted. Currently, most aspects of growth are related to weights and many studies have based their definition of IUGR on an arbitrary weight limit. The weight limit is often taken as 2,500 grams. Infants less than 2,500 grams are called "low birth weight" infants, but this group is comprised of more than just growth retarded infants. It is comprised of approximately two thirds premature infants, one third growth retarded infants and a few abnormal infants (e.g. Down's Syndrome). This leads to considerable confusion between IUGR and prematurity since the gestational age of the infant is not always certain. If all preterm infants are omitted from the criteria of IUGR, infants who are both preterm and IUGR are completely ignored. This group could encompass a large number of neonates as it has been shown that many

factors associated with low birth weight are also observed with spontaneous preterm birth (Fredrick and Adelstein, 1978). Clearly, a weight limit assigned arbitrarily must lead to problems in interpretation. For example, it may be unclear where the weight limit lies in the statistical distribution of the population's birth weights (e.g. 50th percentile, 37th percentile, 10th percentile, etc.). Another disadvantage is the heterogeneity produced by not considering the gestational ages of the infants. The main advantage of this definition is that it is simple to use.

The introduction of foetal growth standards (for birth weight, length and head circumference) has greatly improved the understanding of IUGR. These growth standards are weight (length or head circumference) for gestation charts adjusted for maternal height, weight, sex of infant and birth order, etc. Initially, the distribution of birth weights at each gestational age was calculated by Lubchenko et al in 1963, and subsequently by many other investigators (Fig.1.1.1).

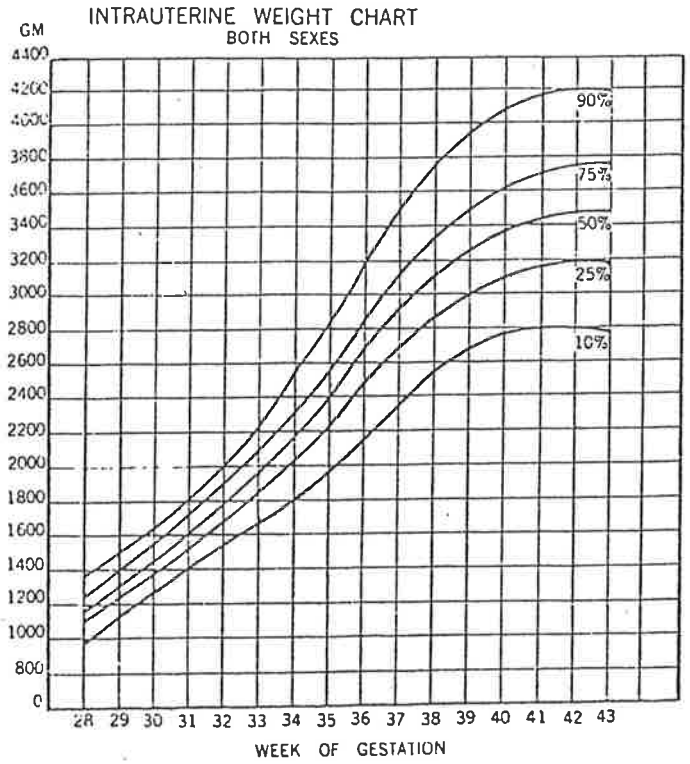


Fig. 1.1.1 Foetal body weight percentiles from 28-43 weeks gestation. (Reproduced from: Naeye and Dixon, 1978).

Although growth standards help in clarifying the definition of IUGR by improv-

ing the precision, many difficulties still exist. It is not possible to use foetal growth if the gestational age of the infant is uncertain. Also, weight-for-gestation charts must be produced for the particular community under study as varying genetic, socio-economic and environmental factors operate in the population (e.g. race, altitude, etc.). A major problem is that there is no consensus as to what constitutes a small-for-gestation infant. Investigators have used the 10th, 5th, 3rd and 2.3rd percentiles as well as two standard deviations below the average birth weight. Further, using fixed percentiles implies that IUGR occurs with the same frequency in every community. It is usually assumed that the population is normally distributed but this may not be true. If the distributions are assymetric, means and standard deviations become less meaningful. Therefore in preparing growth charts, consideration must be taken not only of birth weight and gestational age but also of community variations, parity, weight and height of the mother and sex of the infant. Although in theory the accuracy of growth standards is increased by including all these variables, the percentile charts become too complicated and the sample size for each gestational age is reduced, leading to inaccuracies in the percentiles themselves. (A more detailed discussion of foetal growth standards can be found in VAN Assche et al, 1981). A summary of the problems associated with defining IUGR is presented in table 1.1.1.

Table 1.1.1 Problems with defining IUGR

1.	The gestational age of the infant must be certain.
2.	The weight (length and head circumference) -for-gestation charts must be appropriate for the community under study.
3.	At present, there is no consensus as to what constitutes a small-for-gestation infant.
4.	IUGR does not necessarily occur at the same frequency in every community.
5.	The population under study may not be normally distributed.
6.	Growth charts must be adjusted for weight and height of the mother, parity and sex of the infant.

The incidence of IUGR varies widely depending on the diagnostic criteria used and the patient population under study, but the figure is generally between 2% and 3% (Carrera, 1976).

1.1.2 Clinical Identification of Growth Retarded Infants

The recognition of the clinical features of IUGR is not very difficult if the growth retarding effects are gross and if the correct gestational age of the infant is known. It is much more complicated to diagnose if the IUGR is mild and gestational age uncertain, which occurs in many instances. Preterm infants with mild IUGR are especially difficult to recognise. When the gestational age of the growth retarded infant is assessed neonatally (Dubowitz and Dubowitz, 1970), the external characteristics generally underscore the actual age and the neurological assessment slightly overscores it (Eggermont et al, 1981).

Generally, the clinical picture of IUGR is that of decreased body weight in relation to length, a relatively large head with wide skull sutures, muscle wasting, prominent ribs, an alert expression and dry, wrinkled skin with minimal subcutaneous tissue (Keirse, 1981). A detailed list of the clinical features of growth retarded term infants is presented in table 1.1.2. The extent to which these features are displayed varies greatly with the degree of IUGR.

1.1.3 Complications of IUGR

There are several complications resulting from IUGR in the neonatal period (table 1.1.3).

Hypoglycaemia is especially dangerous to the neonate. The incidence of hypoglycaemia in growth retarded infants varies considerably, but it is usually quoted as being between 29% and 67%. The symptoms encountered with hypoglycaemic neonates include high frequency tremor, lethargy, apnoea, cyanotic

Table 1.1.2 Clinical features of growth retarded term infants (modified from Babson et al, 1975).

	Growth retarded (38+ weeks)
Body size	Reduced body size for gestation, weight in general reduced more than length or head circumference, head circumference usually reduced least.
Weight loss	Minimal weight loss, if any, after birth, usually under 5%.
External appearance	
Skin	Minimal subcutaneous tissue, often loose and wrinkled; scaly and cracked in dysmaturity. Milia often present.
Colour	Whiter from thicker epidermis in spite of higher haematocrit.
Vernix	Minimal to absent.
Lanugo	None
Hair	Sparse, straight and silky.
Sole creases	Extend over whole sole.
Skull bones	Firm on palpation to edges - often suture separation without increase in spinal fluid pressure.
Ear cartilage	Erect, with sharp ridges.
Scrotum	Testes pendulous with well-developed rugae.
Labia	Labia minora tend to be covered.
Breast tissue	Nodule with or without gland swelling, depending on degree of foetal malnutrition.
Cord stump	Thin and often discoloured; dries early.
Behaviour	
Activity	Active - eyes open with anxious appearance.
Feeding	Takes nipple earerly, sucks fingers; gains rapidly.
Neurologic signs	
Tone	Increased tone - holds head well on traction; raises head from mattress.
Moro reflex	Brisk, complete, but often restricted.
Eye response	Fixes and follows with eye.
Transillumination	Under 2 cm of reflected light.
Electroencephalogram	Mature cerebral waves, short response to photic stimulation (157.4 $\pm$ 2 msec).
Physiologic signs	
Oxygen consumption	Increased per kilogram of body weight over preterm infant.
Temperature control	Better able to defend against heat loss (muscular activity, brown fat presence); however, heat conservation limited by reduced subcutaneous fat. Perspires and shivers.
Organ systems	Regular respiration unless airway obstruction. Concentrates urine well, minimal delay in bilirubin conjugation, decreased glycogen stores.
Ossification centres	
Knee	Presence of both proximal tibial and distal femoral epiphyses - delay influenced by degree of foetal malnutrition.

Table 1.1.3 Clinical conditions associated with intrauterine growth retardation. (After Lafeber et al, 1979).

Asymmetric organ growth	+ + +
Hypoglycaemia	+ +
Polycythaemia	+ +
Haemoglobin rise	+ +
Erythropoietin rise	+ +
Impaired gluconeogenesis	+
Depleted energy stores:	
glycogen	+
fat	+ +
Delay of skeletal growth	+ +
Higher incidence of asphyxia	+
Relatively few neurological disorders on examination	+ +

attacks, irritability, convulsions, reluctance to feed and a high pitched cry. If the hypoglycaemia is not treated, brain damage and mental retardation results. Deficiency in glycogen reserves, a high brain to liver ratio (resulting in glucose demands which are greater than production) and delayed onset of gluconeogenesis are three possible mechanisms which may produce hypoglycaemia in these infants (Chance, 1976).

Occasionally, hypocalcaemia develops in intrauterine growth retarded infants during the first few days of life. Quantitatively hypocalcaemia refers to serum calcium levels of less than 70 mg/l. Severely growth retarded infants can have quite drastic symptoms, such as convulsions.

Sometimes both hypernatraemia and hyponatraemia may develop. The mechanisms underlying these conditions are not yet fully understood. Hypernatraemia in growth retarded infants may cause convulsions and apnoea, while hyponatraemia below 120 mEq/l may produce cerebral oedema with convulsions.

Greater concentrations of erythropoietin have been found in the blood of growth retarded infants compared to appropriately grown neonates. Convulsions and neurological symptoms seen in some infants with polycythaemia have been

proposed by one group of experimentors (Wood, 1959) to be due to capillary sludging in cerebral vessels, whilst others (Humbert et al, 1969) attribute it to neonatal respiratory distress and priapism (Chance, 1976).

Neonatal asphyxia is frequently seen in growth retarded infants (Lovell, 1974; MacDonald et al, 1980). In growth retarded foetuses, the foetal: placental weight ratio is greater than in normally grown foetuses of the same gestation. This contributes to a high incidence of birth asphyxia and perinatal mortality. Asphyxia in growth retarded neonates has been quoted to be four times higher and pneumonia ten times greater compared to normal weight infants of more than 36 weeks gestation (Chance, 1976). This complication of IUGR is particularly prevalent because placental function, in many instances, is failing in growth retarded foetuses.

The most common causes of mortality in infants with IUGR are foetal malformations and chromosome alterations, neonatal asphyxia, aspiration pneumonitis, massive pulmonary haemorrhage and hypoglycaemia (Carrera, 1976).

1.1.4 Categories of Intrauterine Growth Retarded Infants

Intrauterine growth retarded infants can be classified into three clinicopathogenic types;

Type I - symmetrical, intrinsic IUGR

Type II - asymmetrical, extrinsic IUGR

Type III - symmetrical, extrinsic IUGR

In symmetrical, intrinsic IUGR (type I) the harmful factor begins to act from the start of pregnancy and the abnormalities are within the foetus itself. The weight, height and cephalic diameter of the foetus is uniformly affected. Causes of this type of IUGR are mainly genetic influences, infections, or toxic substances. The growth retardation is hypoplastic, with the major decrease being in foetal cell number due to impairment of cell division. Cell sizes are, in general, quite normal.

The causative factors in asymmetrical, extrinsic IUGR (type II) begin to

operate in the last trimester, and the growth retardation may be chronic, sub-acute or acute. In these cases, the growth retardation is hypotrophic. There is only a slight or moderate decrease in cell number compared to the decrease in cell size. Only the weight of the foetus is affected, with no effect on height and head diameter. The fundamental cause is placental insufficiency or dysfunction and foetal malnutrition. Some factors associated with type II IUGR are elderly primigravidas, uterine circulatory disturbances, prolonged gestation, multiple pregnancy, toxaemia, hypertension and diabetes. The most common form of IUGR is type II.

With type III IUGR the foetal insult is extrinsic, but it acts throughout gestation. The cause is a severe alteration of maternal nutrition because of the lack of an important factor such as folic acid or amino acids. Reduction in cell number is observed and the weight, length and head circumference of the fetus is reduced (Harding, 1976; Carrera, 1976; Chance, 1976).

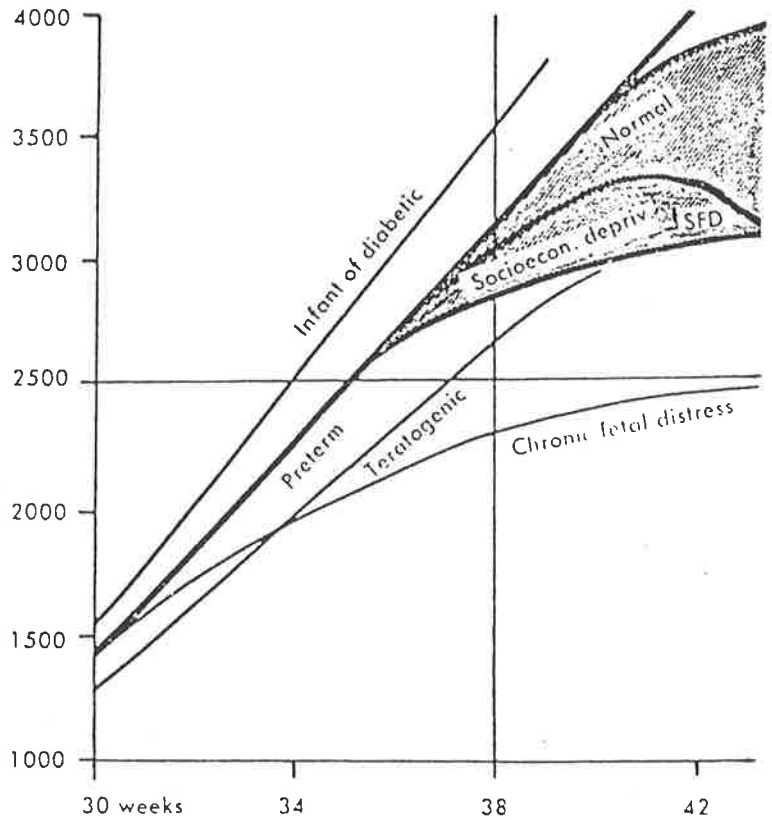


Figure 1.1.2 Birth weight curves of population (shaded range) and several abnormal groups (From Gruenwald, P. and Babson, S.G. In Davis, Gynecology and Obstetrics, Hagerstown, 1972, Harper and Low Inc.)

These three clinical classifications of growth retarded infants, are however, only theoretical. Mixtures of the features listed can occur.

1.1.5 Foetal Growth

Foetal growth represents the accretion of proteins and fat deposition and its regulation depends upon genetic determinants, the availability of growth substrates and growth promoting hormones and the vascular support of the mechanisms involved in these two factors. Theoretically, if there existed unlimited nutritional reserves the foetal growth curve would be linear. Normally, however, there is a plateauing of the growth curve near term, indicating that a rate limiting factor is acting (Fig. 1.1.2). It appears that there is unrestricted foetal growth until the total foetal weight approaches 3,000 grams (Harding, 1976). If abnormal influences are present the growth curve is displaced as indicated in figure 1.2. Protein deficiency in the foetus results in a symmetrically retarded foetal growth pattern in all viscera, including the liver and brain (Harding, 1976).

The rate of intrauterine growth in the human has been estimated by Hill (1978) to be 50% dependent on the maternal genotype and environment, 30% on other environmental factors and 15% on the foetal genotype, with an additional 2% due to the influence of the Y chromosome.

1.1.6 Causes of IUGR

At the present time it is difficult to identify direct or indirect causal influences on human foetal growth, firstly because the recognition of IUGR is a prerequisite for the recognition of its cause and secondly because of the confusion regarding the definition of IUGR. There exist numerous factors, however, which have been associated with growth retardation (Table 1.1.4). These factors can be grouped into four categories, namely:

1. foetal factors,

2. medical complications of pregnancy,

Table 1.1.4 Abnormal factors associated with intrauterine growth retardation. (From Miller and Merritt, 1979).

I	Foetal factors	Intrauterine infections Chromosomal aberrations Congenital malformations Inborn errors of metabolism Multiple births
II	Medical complications of pregnancy	Acute or chronic hypertension Preeclampsia Severe vaginal bleeding (third trimester) Severe chronic disease involving heart, liver, lungs, kidneys, gastrointestinal tract, thyroid or adrenal glands. Disseminated lupus erythematosus Sarcoidosis Severe chronic infection Anaemia (< 10 gm/dl) Leukaemia Malignant solid tumours Large ovarian cysts Multiple large fibroids of uterus Continuous medication with corticoids, immunosuppressive, teratogenic or growth-retarding drugs. Abnormalities of uterus, placenta or umbilical cord. Polyhydramnios Oligohydramnios
III	Selected maternal behavioural conditions associated with pregnancy	1. Abnormally low prepregnancy weight for height. 2. Low maternal weight gain in pregnancy. 3. Lack of any prenatal care. 4. Delivery before seventeenth birthday. 5. Delivery after thirty-fifth birthday. 6. Cigarette smoking during pregnancy. 7. Use of addicting drugs or large amounts of alcohol during pregnancy.
IV	Environmental factors	High altitude Exposure to toxic substances

3. selected maternal behavioural conditions associated with pregnancy and
4. environmental factors.

If growth of the foetus is impaired in the first half of pregnancy it is most likely to be due to embryonic injury, genetic defects, or the viral invasion of the foetus. Retardation of growth in the last half of pregnancy is usually associated with the reduction of the flow of nutrients from the mother to the foetus and/or the oxygen supply to the foetus being decreased.

The association of intrauterine infections with IUGR is well known. As many as 60% of infants with congenital rubella are below the 10th percentile in weight for gestation (Cooper et al, 1965). Cytomegalovirus infection can also produce marked IUGR. Infection with other viruses such as herpes simplex varicella zoster, vaccinia, hepatitis A and poliovirus have led to growth retardation but the information about these are scarce. A low incidence of IUGR has also been reported with congenital toxoplasmosis and syphilis. Little is known about the effect of bacterial infections on the foetus (Keirse, 1981).

High doses of ionizing radiation, cytotoxic drugs and administration of corticosteroids during pregnancy all may retard foetal growth. Although there has been controversy in the literature as to whether there are direct effects of maternal smoking, alcohol consumption and heroin addiction on foetal growth, there is no doubt that there exists an association of these with reduction in birth weight for gestation. Factors such as carbon monoxide effects, reduced utero-placental blood flow and reduced maternal food intake may contribute to the growth retardation observed (Keirse, 1981).

Chronic alcoholism may produce IUGR by a variety of effects. The direct effect of alcohol is one possibility and themetabolite of alcohol, acetaldehyde, may also play a role. Deficiencies in essential nutrients due to quantitative or qualitative malnutrition often occurs and may produce IUGR. Malabsorption secondary to alcoholism may also be important (Keirse, 1981). A daily intake of 28.5 mls of absolute alcohol or more during pregnancy presents a risk to the foetus, the risk rising with increasing alcohol intake. A multiplicity of malformations may occur (e.g. the characteristic facial features of the foetal alcohol syndrome, joint anomalies, ptosis) and the major anomalies are IUGR, cardiac defects, abnormal external genitalia in females and mental retardation (Newman and Correy, 1980).

There is growing evidence that IUGR is associated with congenital malformations. It has been observed not only with specific syndromes (Table 1.1.5), but also with non-specific types of major malformations involving musculo-skeletal, cardiac, renal, cerebral and other tissues (Miller and Merritt, 1979).

Table 1.1.5 Syndromes associated with intrauterine growth retardation (from Miller and Merritt, 1979).

Chromosomal abnormalities	Environmental
Aarskog	Aminopterin
Down	Fetal alcohol
Cri du chat	Fetal hydantoin
Noonan	Fetal trimethadione
Trisomy 18	Rubella
Trisomy 13	Thalidomide
Trisomy 8	Miscellaneous
Turner XO	Aerodysostosis
Williams	Bloom
XXXXY	Cerebro-costo-mandibular
4p	Coffin-Siris
13q	Cutis laxa - growth deficiency
18p	DeLange
18q	De Sanctis-Cacchione
21q	Dubowitz
Limb defect	Johanson-Blizzard
Fanconi pancytopenia	Leprechaunism
Femoral hypoplasia - unusual facies	Osteogenesis imperfecta
Limb reduction ichthyosis	Potter
Osteochondrodysplasias	Roberts
Achondrogenesis	Rothmund-Thomson
Achondroplasia	Russell-Silver
Camptomelic dwarfism	Rubinstein-Taybi
Diastrophic dwarfism	Seckel
Ellis-van Creveld	Smith-Lemli-Opitz
Grebe	Syndromes sometimes associated with fetal growth retardation
Hypochondroplasia	Cat-eye
Jansen metaphyseal chondrodysplasia	Cytomegalic virus
Kenny	Facio-auricular-vertebral
Lymphopenic agammaglobulinemia - short-limbed dwarfism	Hypophosphatasia
Metatropic dwarfism	Meckel-Gruber
Spondyloepiphyseal dysplasia congenita	Menkes
Thanatophoric	Prader-Willi
Storage syndromes	Riley-Day
Generalized gangliosidosis, Type I	Triploidy
Leroy I-Cell	

The association of poor foetal growth with hypertension disorders during pregnancy is well documented. These hypertension disorders include pre-eclampsia, essential and renal hypertension, and combinations of pre-eclampsia with one of the others (VAN Assche et al, 1981). Studies have shown that IUGR in severe pre-eclampsia often precedes the pre-eclampsia. The implication is therefore that placental insufficiency may predispose to pre-eclampsia, or that an unrecognized disease state resulting in foetal malnutrition is present for a longer period than the clinically apparent pre-eclampsia. IUGR is more frequent in early onset pre-eclampsia (18%) than in the late onset group (6%)

(Long et al, 1980).

Evidence is accumulating that foetuses of diabetic mothers not only have excessive deposition of fat, but also delayed initial growth. Growth retardation has been observed as early as the first trimester. Birth weight in these infants is not a good indicator of growth because it reflects the summation of retardation and macrosonic influences (VAN Assche et al, 1981).

The high incidence of IUGR in multiple pregnancies is well known. It has been noted that there is a progressive restriction of growth when the total foetal weight exceeds 3,000 grams. In twins, therefore, growth is retarded from 30 weeks gestation and from 26 weeks in quadruplets (Keirse, 1981).

Although factors such as low social class, poor socio-economic status, poor nutrition and high altitude exposure have all been associated with growth retarded infants, it is uncertain whether these are actually direct causes of growth retardation, or indirect ones. Similarly, anaemia has a negative effect on foetal growth if the condition is severe enough (Keirse, 1981).

As has been illustrated above, multiple foetal, maternal and environmental factors are associated with IUGR. However, in one study (Low and Galbraith, 1974) no cause could be demonstrated in half of their patients.

There is much discussion in the literature concerning the causes of IUGR and contradictory results are often observed. The aetiology of IUGR will continue to remain obscure until such time as the identification and definition of IUGR is resolved.

1.1.7 Neurological Abnormalities and the Long Term Prognosis for Intra-Uterine Growth Retarded Infants

Growth in humans is rapid from mid-pregnancy to about the second year of life. If malnutrition occurs during this critical period, it is harmful to brain development (Lovell, 1974). A significant failure of cell replication may occur in the brains of growth retarded infants if the foetus is restricted in acquiring adequate quantities of essential proteins. This leads to limited

amounts of available amino acids for structural and functional protein synthesis in the cells of the foetus (Zamenhof et al, 1968).

There is considerable debate in the literature over the degree of neurological impairment due to IUGR. Some investigators report that although there is a clear relationship between IUGR and suppressed brain development and learning in animal studies, in humans the impairment of intellectual development is only circumstantial (Babson et al, 1975). Others (Butler, 1973, Carrera, 1976, Chance, 1976) propose that it is unquestionable that mental/intellectual impairment occurs in growth retarded children.

Severe neurological alterations are infrequent in growth retarded infants. In a study of 11 year old children in Great Britain, only 5.8% of children who had been below the 10th percentile in weight for gestation at birth were severely physically or educationally handicapped (Butler, 1973). However, minor handicaps are much more frequent. These include poor reading ability, impaired co-ordination, mild speech defects, bed-wetting, visual problems, moderate deficiencies of IQ and dysfunctional E.E.G. disturbances (Butler, 1973, Carrera, 1976).

The results of follow-ups show that the intellectual development of growth retarded infants with congenital anomalies (e.g. hypoplastic) and of those with neurological problems, is not good (Chance, 1976). Hypotrophic growth retarded neonates, on the other hand, who survive the neonatal period without developing the complications of IUGR previously discussed, have a better prognosis both for physical growth and intellectual development.

A phenomenon called "catch-up growth" occurs in some growth retarded infants when the child reaches its appropriate weight, height and head circumference for its age. In severely hypoplastic children this catch-up growth appears unlikely because the underlying problem is a decrease in cell number rather than cell size.

The assessment of intellectual development of growth retarded infants on follow-up is hindered by wide variations on the child's opportunity for stimulation and socio-economic and environmental conditions (Chance, 1976).

These long term studies of intrauterine growth retarded infants have been potentially confounded by the difficulties in identifying, defining and quantitating IUGR in the study and control groups. Therefore, some of the variance in the results of different studies may be due to dilution of IUGR groups with "small" normals and normal groups with "large" growth retarded infants.

1.1.8 Purpose of Study

Consensus about the aetiology of IUGR and the future outcome of intrauterine growth retarded children cannot come about until the problems of identifying these neonates are unequivocally resolved. Not only infants who are below the 10th percentile (usually used weight for gestation limit for IUGR) in weight for gestation are necessarily growth retarded but also neonates in the normal weight range (10-90th percentile) may have suffered from growth retarding influences and have fallen from say the 90th to the 50th percentile. Conversely, there may be fully grown neonates under the 10th percentile in weight for gestation.

Clearly, a simple test is required for the identification of growth retardation in neonates which is quantitative and independent of gestational age, birth weight, race, behaviour and appearance at birth.

The aim of the studies reported in this thesis was to evaluate the potentials of two biochemical tests in the recognition of IUGR in newborn infants and to simplify the tests sufficiently to make them applicable to routine paediatric medicine. The measurements of both water turnover rates using deuterium oxide (D_2O) and protein turnover by 3-methylhistidine:creatinine ratios were explored.

1.1.9 Aims

Water metabolism and protein turnover form the basis of the two techniques which were investigated as identifiers of IUGR in neonates. The aims of the project were as follows;

1. to establish the reliability of body water turnover rates in assessing IUGR,

2. to determine the usefulness of urinary and amniotic fluid 3-methyl-histidine:creatinine ratios as an index of IUGR, both antenatally and postnatally,

3. to test the ideal conditions under which these tests should be performed and simplify them for routine use,

4. to correlate antenatal and neonatal events with body water turnover rates,

5. to evaluate water metabolism in patients with obstetric anomalies (e.g. multiple pregnancy, pre-eclampsia),

6. to observe the development of infants during the early stages of life (e.g. 0-3 years) and correlate water turnover rates with physical and intellectual development.

CHAPTER II

WATER TURNOVER RATES

2.1 INTRODUCTION

In this chapter, a detailed discussion of body water turnover rates in new-born infants will be undertaken and special reference will be made with respect to this parameter and to its relationship to intrauterine growth retardation. Firstly, it will be attempted to summarize existing information on water turnover, including the historical developments relating to its measurement. Next, the techniques employed in collecting and analysing the data will be described, followed by an extensive review of the results. Problems encountered and suggestions for improvement of the methodology will be covered in the discussion.

2.1.1 History

The idea that water turnover might be useful in the identification and quantification of intrauterine growth retardation in humans first arose from animal studies. MacFarlane et al (1966) conducted an experiment on merino sheep, in which they measured rates of water turnover in two groups of these animals. One group of sheep was grazed on dry pastures in November and the other was fed on lush green grass in January. They concluded from their results that the water turnover rates of sheep in November were greater than in January. It appeared that when the sheep were nutritionally deprived, they had less body fat. Since body fat is inversely proportional to total body water (Cheek, 1961), the poorly nourished sheep had a greater total body water and also a more rapid water turnover.

The determination of total body fat is very difficult, particularly in humans and total body water measurements also pose practical problems (e.g. the dose of tracer substance must be given very accurately). Further, the coefficient of variation total body water measurements is

approximately 10%; pathological changes of total body water can occur within this limit, (Schloerb et al, 1950). Therefore, since nutritionally deprived sheep exhibited faster water turnover rates than well fed sheep, the question arose as to whether infants malnourished in the intrauterine period also have faster water turnover soon after birth.

2.1.2 Measurement of Water Turnover

For the measurement of water turnover, a tracer substance must be introduced into the fluid compartments of the body. To qualify as an ideal agent for this purpose, the tracer should possess several important characteristics. It should rapidly equilibrate with all fluid compartments and not be selectively metabolised, stored or excreted. The tracer must be completely exchangeable with water and have no toxic or physiologic effects itself. The hydrogen isotopes of water, i.e. tritium oxide (T_2O) and deuterium oxide (D_2O), are "ideal" substances in studying water dynamics, since they are normal constituents of water and fit the above criteria. Preference for one or the other of these tracers depends on the experimental protocol.

In animals, T_2O (the radioactive isotope of water) was used extensively as the tracer substance in the measurement of water turnover rates. (Foy and Schnieden, 1960; MacFarlane et al, 1966; Richmond et al, 1961; Chapman and Black, 1967). Using the technique of isotope dilution in vivo (of a low dose of T_2O) provides precision, accuracy and ease of performance. This method is also relatively cheap. However, because T_2O is radioactive with a half-life of 12 years in man (Schloerb et al, 1950), it is potentially hazardous to humans.

2.1.3 Behaviour of D_2O in the Body

The non-radioactive isotope of water, D_2O, was discovered in 1932 and it proved to be a useful tracer substance in measuring water turnover in both animals and humans. D_2O forms an ideal solution with water (H_2O) (Longsworth et al, 1937) and when it constitutes less than 3% of the total body water, all the D_2O is present as DHO. (Stansell et al, 1968). During the equilibrium period, D_2O must mix with the blood, extracellular and intracellular water, exchange with free H groups and reach equilibrium stores such as the gastrointestinal contents and cerebrospinal fluid. Determinations of the urine/blood D_2O ratio in humans gives a result of 1.00 ± 0.04, indicating that the kidney does not differentiate between H_2O and D_2O (Hurst et al, 1952), thus, there is no selective excretion of D_2O via the kidneys.

Administered intravenously, D_2O equilibrates with body water within two hours. Absorption into body fluids after oral ingestion of D_2O is more gradual, reaching a peak in serum at 30 minutes and equilibrium in approximately 3 hours. (Schloerb et al, 1950). Fig 2.1.1 This indicates that water absorption from the gastrointestinal tract proceeds at a faster rate than the distribution of water throughout the body. After water equilibrium has been reached, blood and urine values of D_2O are identical. Therefore, for clinical purposes, oral administration of D_2O is adequate and less invasive than intravenous injection.

2.1.4 Toxicity of D_2O

D_2O toxicity has been carefully examined in many animals as well as in man. Various side effects have been noted, depending on the participating species and the concentrations used. An obvious question is how safe is the use of D_2O in human subjects and if it is safe, in what concentrations

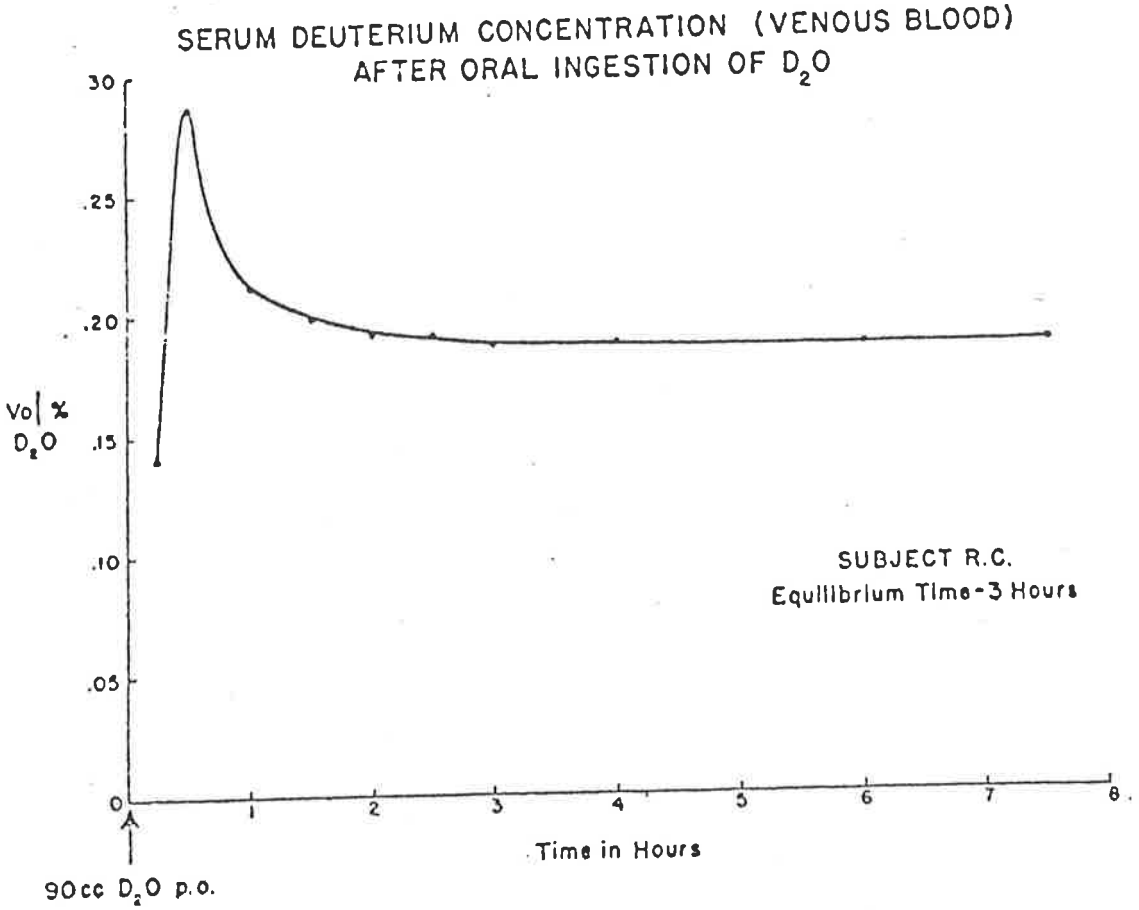


Figure 2.1.1 The curve represents the serum D_2O concentration after the oral administration of 90cc of D_2O (from Schloerb et al, 1950).

should it be administered?

Mammals can tolerate a maximum of approximately 35% deuteration (Katz et al, 1970), but higher concentrations are incompatible with life. The reason why mammals die from deuteration is still obscure, but is probably due to the combined effects of the impairment of vital functions and the mutual regulation of these functions in the body.

At lower concentrations, D_2O toxicity can produce several levels of effects; anaemia, disturbances of kidney function, muscle contraction and the central nervous system, dysfunction of biological timing processes, in particular cardiac and secretory rhythms, changes in morphology of tissues, cells and organelles, disturbances of enzymic reactions and alteration of the actions of antibodies and hormone responses. (Lacroix et al, 1975).

However, experiments conducted by Peng et al, 1972, showed that ingestion of doses of 5% D_2O for several months by rats resulted in no noticeable disturbances. They concluded that man could consume doses of D_2O of up to 1% for long periods of time. (Natural levels of D_2O in the water supply are about 0.015%). In another study (Katz et al, 1970), animals deuterated to about 25% D_2O in their body fluids lived a normal life span.

Many D_2O studies have since been performed on humans, including infants, children and adults. (Hanna, 1963; Cokington et al, 1963; Heald et al, 1963; and Coward et al, 1979). In humans, the half-life of D_2O is relatively short. In adults, the half-life is approximately 10 days, while in infants it varies between two and four days. At the low concentrations used, after a single D_2O dose of up to 2 mls per kilogram body weight (or up to a total of 0.30% of the total body fluids), no harmful effects have yet been demonstrated in man. (Coward et al, 1979).

2.1.5 Reliability of the Measurement of D_2O

The reliability of measuring body water turnover using D_2O dilution was considered in a recent experiment. Wang et al (1973), compared the use of D_2O and T_2O as a tracer substance in humans for the measurement of total body water. They concluded that the results obtained by the two methods were indistinguishable. Hence, the technique of D_2O dilution was employed in this present study to determine body water turnover rates in human infants.

2.1.6 Techniques for the Measurement of D_2O

There are five basic techniques available which can be used in the measurement of D_2O in aqueous media:

(1) mass spectrometry (Solomon et al, 1950),

(2) elevation of the freezing point (Reaser et al, 1958),

(3) falling drop (Schloerb et al, 1951),

(4) gas chromatography (Arnett and Duggleby, 1963) and

(5) infrared spectroscopy (Turner et al, 1964).

Infrared spectrophotometric analysis of D_2O in biological fluids is the most rapid and practical method to use in the standard laboratory. It requires relatively inexpensive equipment and is simple to perform. Analysis by mass spectrometry is not feasible for a biological laboratory because of the expensive equipment which is required and freezing point elevation necessitates lengthy sample preparations. The falling drop method entails rigid control of experimental conditions and is not practical when large numbers of samples are to be analysed. Gas chromatography has the advantage that very minute samples (i.e. 0.3 mls) can be used, but it shows inconsistencies in the results and there are practical difficulties in the technique at low D_2O concentrations.

Since, in dilute solutions, D_2O is essentially present only as DHO, it can be measured on the optical density vibrational band at 2,513 cm^{-1} in the infrared region of the spectrum. As many other substances also absorb near this region of the spectrum, analysis of biological fluids by infrared spectrometry first requires separation of interfering substances by distillation (Turner et al, 1960), sublimation (Byers, 1979) or treatment with charcoal at alkaline pH (Wang et al, 1973).

In the present study, the technique of sublimation was used to purify the neonatal urine samples, because the urine was collected in absorbent paper towels instead of the usual urine collection bags.

2.2 METHODS

2.2.1 Patients and Data Collection

Since this study required experimentation on human patients, consent was obtained from the Research and Ethics Committee of the Queen Victoria Hospital, Adelaide, for the clinical trial to be performed. The mother of each infant was interviewed and the aims and techniques used in the trial were explained to her. Only if informed consent was given by the parent(s) was the infant included in the study. Where appropriate, the mother was seen before the delivery of the child, in order to cause as little inconvenience as possible. In some cases the mothers were asked to help in the collection of the neonatal urine samples, and they seemed to be more motivated and co-operated better if they understood the purpose of the trial.

A total of 106 new-born infants, varying in gestational age between 32 and 43 weeks, participated in the study. No infants were included who were under intensive care treatment or who were receiving intravenous therapy, since their fluid balance regimens may have added in a significant variable. It was necessary to select critically the infants chosen for the trial, as these infants had to be classified accurately as growth retarded or non-growth retarded ("normal") by existing measures. This provided an independent assessment for later comparison with water turnover rates. For this reason, infants were excluded if their gestational age was uncertain or if there were large standard deviations of D_2O clearance.

Three methods were used to confirm the gestational age of the infants. The mothers were required to give an accurate menstrual history which correlated with each of the following tests:

(i) an ultrasound examination of the fetus before 20 weeks gestation;

(ii) uterine size at the first antenatal visit and

(iii) a Dubowitz test for maturity.

The Dubowitz test had to be accurate to within two weeks of calculated gestation.

The knowledge of the date of the mother's last menstrual period (L.M.P.) was essential, but if this was uncertain the infant was still included if the pregnancy was diagnosed early (5-6 weeks) and a pregnancy test performed. As many as one third of pregnant women have been found to have uncertain L.M.P.'s (Chapman et al, 1978). Note was also taken of the regularity of the menstrual cycle because all available pregnancy calendars assume a regular 28 day cycle, and no consideration is given to irregular and long cycles. If cessation of taking the "pill" was the cause of the L.M.P. (pill period), then calculating gestation from this date would be inaccurate, so in these cases the infants were also excluded.

Foetal ultrasound examination, employing the measurement of the biparietal diameter of the foetal head, has been proven to be a relatively reliable method of determining the gestation (Hasch et al, 1978). At the Queen Victoria Hospital the correlation of maturity with the ultrasonic measurement of the biparietal head diameter is ±4-5 days (10th and 90th percentile confidence limits) when performed before 20 weeks. However, the older the foetus, the less accurately can the gestational age be calculated. (There are also disease states, such as hydrocephaly, which produce distortions of the estimated gestation).

The paediatrician performed a Dubowitz test (Dubowitz and Dubowitz, 1970) on each infant to ascertain its maturity. This consists of a collection of physiological and neurological features of the neonate which are each assigned a score. The maturity of the infant is determined from the aggregate score.

Each of the above factors used by obstetricians to calculate the gestation of the foetus and neonate have limitations, but used in combination can produce a fairly accurate result.

2.2.2 Classification of Infants

Before any results of the body water turnover rates were known, the infants were classified into three main groups:

(i) normal infants, with no clinical signs of growth retardation;

(ii) borderline infants, exhibiting some features of growth retardation; and (iii) clearly intra-uterine growth retarded infants.

The classification was performed by two independent assessors (one obstetric and one paediatric). The obstetric assessment was based on 22 antenatal factors (appendix 1), serial ultrasound measurements of foetal growth and oestriol levels in maternal urine.

Neonatally, the infant was assessed by the paediatrician using several methods currently employed in the detection of growth retardation. These included the ponderal index (the weight of the infant divided by the cube of its length), triceps and subscapular skinfold thickness measurements, weight, head circumference and length for gestation percentile charts, and signs of hypoglycaemia and polycythaemia (appendix 2). All of these tests are of only limited value in assessing intrauterine growth retardation. The ponderal index, although independent of

gestational age, has limited accuracy because of the practical problems associated with measuring the crown-heel length of the infant. This percentage error is then increased threefold because the length must be cubed. The percentile charts are accurate, provided that the gestational age of the infant is correct. Measuring the skinfold thickness of the neonate should theoretically be a reliable test for growth retardation, since approximately 80% of body fat is subcutaneous, but this too is dependent on gestational age.

Only if both the obstetric and paediatric assessments were in agreement was an infant included in the trial.

2.2.3 Perinatal Risk Score

A perinatal risk score sheet (appendix 3) was prepared with the aim of determining the effectiveness of such a system in predicting the presence of I.U.G.R. retrospectively from the study. In all, 16 antenatal factors and 5 postnatal factors were considered, and each factor was allocated an arbitary risk score. The risk scoring system was modified from the system used by Edwards et al (1978) to incorporate only those factors which seemed relevant to I.U.G.R. and extra factors were included where appropriate. Correlations of antenatal, postantal and total risk scores were made with water turnover rates. (The total risk score was simply the sum of the antenatal and postnatal risk scores).

2.2.4 Skinfold Thickness Measurements

An indication of the proportion of body fat present in the infant can be obtained by measuring the skin thickness in the subcutaneous adipose layer. (Oakley et al, 1977).

The Harpenden caliper (acquired from John Bull, British Indicators Ltd., England) was used to make the measurements in the triceps and subscapular areas of each infant using the method described by Tanner and Whitehouse

(1975). The caliper applies a constant pressure of $10gm/mm^2$ over an area of ½ square inch in contact with the skin. Caliper readings were made after 60 seconds. To standardize the technique, only one paediatrician performed the skinfold thickness measurements. Unfortunately, the skinfold thickness percentile chart read only between 37 and 42 weeks gestation. Skinfold thicknesses of infants of less than 37 and greater than 42 weeks gestation could not be accurately calculated from these charts (Oakley et al, 1977).

2.2.5 Deuterium Oxide Administration

The infants were orally (bottle or gavage tube) administered approximately 2ml of 99.8% pure deuterium oxide (D_2O) per kilogram of body weight, within 48 hours of birth. This quantity of D_2O was sufficient to produce a urinary concentration which could be accurately determined by infrared spectrophotometry. In these low concentrations, D_2O has been shown to be completely safe to be ingested by man (see Introduction).

It was not imperative in this study for a precise amount of D_2O to be administered, because it was the rate of urinary excretion rather than the actual quantity which was under investigation.

2.2.6 Urine Collections

Infant urine samples were collected by a new technique described by MacLennan et al (1981). This involved placing an absorbent paper towel inside the nappy (Johnson's Day Lee All Purpose Towels; Johnson and Johnson Pty. Ltd., Sydney), and collecting the urine impregnated towels. This method alleviates the irritation of the perineal areas which is produced by the use of serial urine collection bags. Tests

have shown that results of D_2O concentrations were not significantly different for paper towels and urine collection bags. (MacLennan et al, 1981).

Collection of neonatal urine was carried out between 20 and 300 hours after the administration of D_2O. This corresponded to the linear excretion phase, during which time the D_2O concentration decayed exponentially. When the infant was rooming with the mother, the mother was asked to collect the urine-impregnated towels. When this was not so, the nursing staff in the Nursery organized the urine collection.

The urine-impregnated towels were stored in air-tight urine specimen containers at -4°C, until such time as they could be vacuum distilled.

2.2.7 Extraction and Purification of Neonatal Urine

The water and deuterium oxide was extracted from the urine-impregnated paper towels and purified by vacuum sublimation (Byers, 1979). Each towel was placed in a 50ml glass "Quick-fit" tube and frozen. The tubes were then fitted to an apparatus similar to that illustrated in fig. 2.2.1 and connected to 5ml glass D_2O-H_2O collecting tubes which were immersed in dewar flasks containing 20% dibutyl phthalate in 80% methanol and crushed, dry ice. A "Speedivac" High Vacuum Pump (model 2SC20A; Edwards High Vacuum Ltd., England) supplied the vacuum. Pressures of about 0.05mm Hg were used.

Eight urine samples could be extracted and purified at any one time using this apparatus. Approximately three hours was required for complete removal of the H_2O and D_2O from the urine samples. It was important to completely dry the towels, since D_2O sublimes more slowly than H_2O, and so understimates of the D_2O concentration in the urine

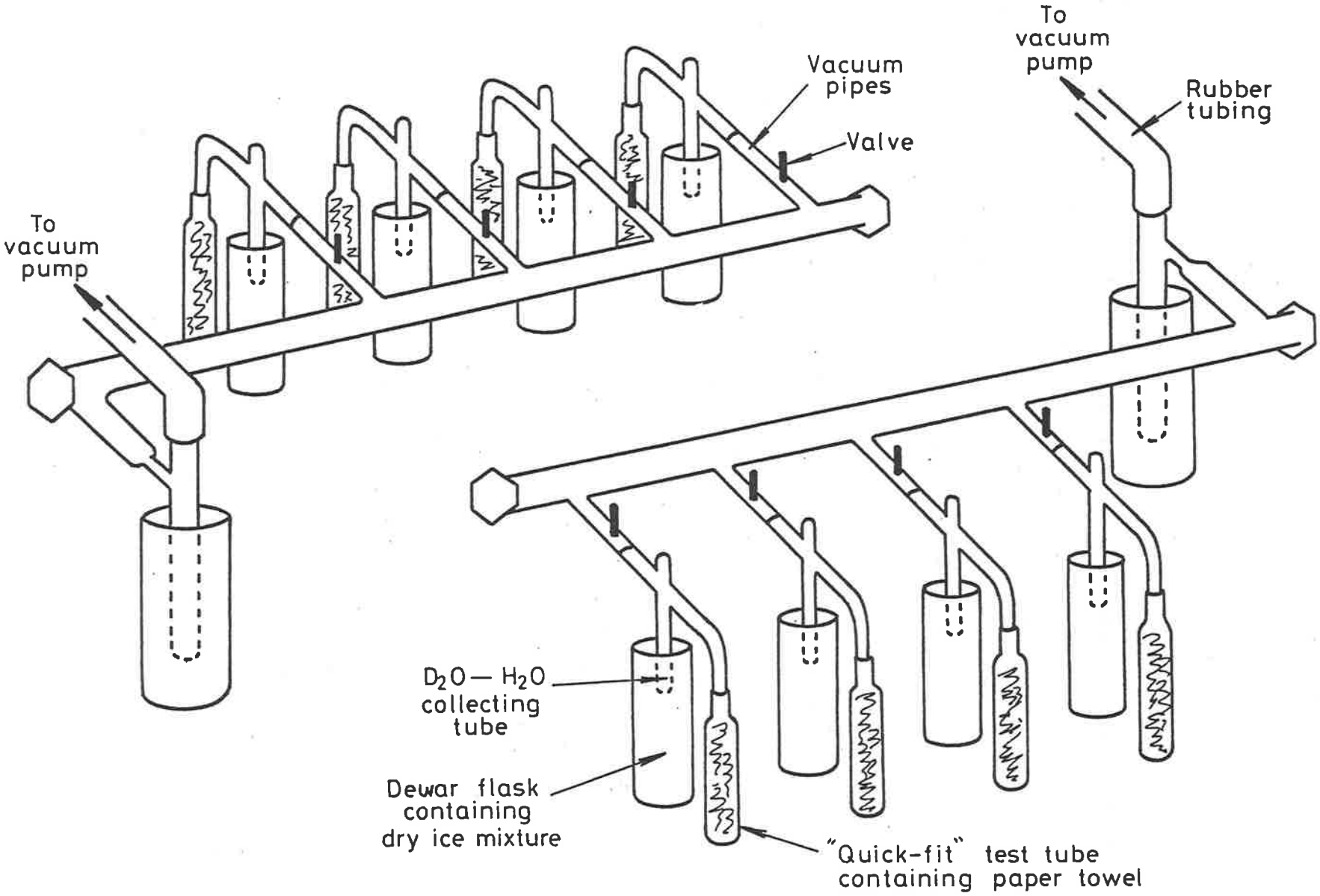


Figure 2.2.1 Illustration of vacuum sublimation equipment

sample could have resulted if the towels were not completely dried. The distillates were kept frozen in sealed 5ml vials until analysed.

2.2.8 Deuterium Oxide Assay Procedure

Infrared spectrophotometry was employed to determine the D_2O concentration in the urine distillates. This technique was chosen over the falling drop method, mass spectrometry, freezing point elevation and gas chromatography methods because it is relatively inexpensive, quick, easy and reliable (see Introduction). A Wilks Miran-1A Fixed Filter Infrared Analyser (Model no. 063-5651; Foxboro/Wilks Scientific Ltd., South Norwalk, Conneticut, U.S.A.) was used at a wavelength of 4.0 μm, extinction at 2510 cm^{-1}(3.9841 μm).

A water bath containing distilled water at 25°C was connected to the spectrophotometer and the water was circulated in an insulated jacket around the cell at a flow rate of 3 litres per minute to maintain a constant temperature in the cell. (The oxygen-deuterium resonance band exhibits a temperature dependence, and gross fluctuations in the readings occur without temperature control, (Byers, 1979)).

A zinc selenide precision sealed cell with a 0.2mm path-length was used. The cell and cell-holder were securely positioned in order to alleviate variations in abosrbance due to movement during filling and washing the cell. Naturally occurring levels of D_2O in water vary between 120 and 160 ppm. Therefore, the spectrophotometer was initially zeroed with distilled water.

The urine distillates were allowed to thaw and come to room temperature before the analysis was performed. Samples were slowly infused via a 1.0 ml plastic syringe, applying contant pressure. Care had to be taken to eliminate any air bubbles from the syringe and cell,

because even minute bubbles cause wide fluctuations in the readings. A possible method to alleviate this problem could be to place the urine distillate under a vacuum for a short period.

Flushing of the cell with the distillate three times (0.6 ml total) was required before an accurate reading could be taken. Cell and temperature equilibriation required between 10 and 20 seconds. The Miran analyser was set with an absorbance range of 0 to 0.1. The digital output (Wilks infrared analyser readout; Schneidr Electronics no. VT300) had a one second integration response time. The resolution of the technique was approximately 1 ppm. Urine distillate readings were taken in duplicate and at least 2 mls of distillate was required for an accurate determination. Between determinations, the cell was washed, using acetone and dried with air.

2.2.9 Determination of D_2O Concentration

The method used to calculate the concentration of D_2O in the neonatal urine distillate was the same as that used by MacLennan et al (1981). First, D_2O standards were prepared using 99.8% D_2O-distilled water (V/V). To determine the extinctions of various concentrations of D_2O, seven sets of seven standards were prepared, ranging in concentration from 385 to 2180 ppm. The extinctions of the triplicate samples of each standard was determined by infrared spectrophotometry, and estimates of analytical variance were made. Analysis of variance of the seven replicates showed that a straight line relationship between D_2O concentration and the photometer extinction was adequate to describe the data, and that the intercept of this line was not significantly different from zero.

(The variance at each of the seven points on the standard curve was calculated from the seven replicates, and the reciprocal variances were

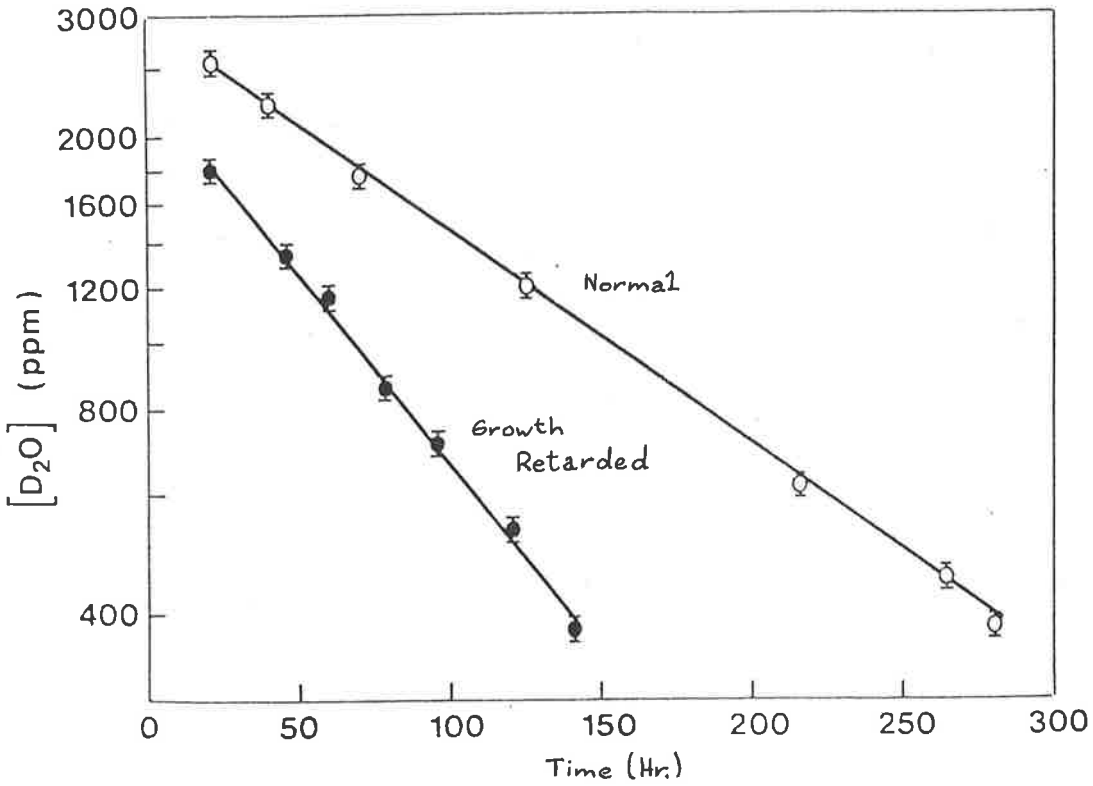


Figure 2.2.2 Clearance of oral D_2O from a normal and a growth retarded infant expressed as the decrease with time of the natural logarithmic concentration of D_2O in neonatal urine. The lines represent the calculated exponential decay process of the D_2O fitted to the data. (Reproduced from MacLennan et al, 1981).

used to weight a straight line fitted to the mean extinctions as a function of D_2O concentration (fig. 2.2.2). This linearity is in agreement with Wang et al (1973) who found that the standard curve was linear up to at least 25% D_2O.

For the estimation of the standard deviation in subsequent measurements of the extinction, a value of 4.4% was used. This value was arrived at by noting that the variance in extinction was proportional to the extinction with a constant coefficient of variance of 4.4%

Interpolation of values of D_2O concentration from the standard curves was acceptable because of the linearity of the extinction/D_2O concentration curve, and the intercept at zero.

Subsequently, a D_2O standard curve was prepared prior to the determination of each batch of urine distillates.

2.2.10 Calculation of Deuterium Oxide Clearance

After an oral administration of D_2O, the concentration of D_2O in the urine rises rapidly, and reaches a peak (equilibration phase) before 20 hours. The D_2O concentration then declines thereafter for up to 300 hours, following a simple exponential decay process during this time. This is to say that the concentration of D_2O at any time can be expressed by the relation

$$[D_2O]_t = Ae^{-kt} \qquad (1)$$

where

$|D_2O|_t$ = measured concentration of D_2O at time t after the administration of D_2O,

A = the hypothetical concentration of D_2O in the urine at zero time assuming instantaneous mixing of D_2O in all compartments of the body. (The fact that such instantaneous mixing does not occur is irrelevant),

and

k = the exponential coefficient of the single decay process.

The parameter k will be called the rate constant (MacLennan et al, 1981), and serves as an indicator of the rate of clearance of D_2O from the body. Since its value is typically about 70×10^{-4} to 120×10^{-4} , the numbers 10^4k will usually be presented for rate constants.

The formula (1) is only valid if certain assumptions hold. The first assumption is that the tracer (D_2O) and the non-tracer (H_2O) are uniformly mixed in the body and are affected similarly by chemical and physical processes. Secondly, it is assumed that all the tracer substance added at time t = 0 equilibrates rapidly and completely with the non-tracer substance. Thirdly, the intake and excretion are assumed to be continuous processes.

Although these assumptions are not absolutely correct when using D_2O as the trace substance, they are sufficiently accurate to provide a useful measure of water turnover.

Equation (1) can be transformed by taking logarithms, yielding

$$\ln [\underline{D_2O}]_t = \ln A - kt \qquad (2)$$

A graph of $\ln[\underline{D_2O}]$ concentration plotted against time t after D_2O administration will therefore produce a straight line between about 20 and 300 hours ("linear excretion phase") (fig. 2.2.3).

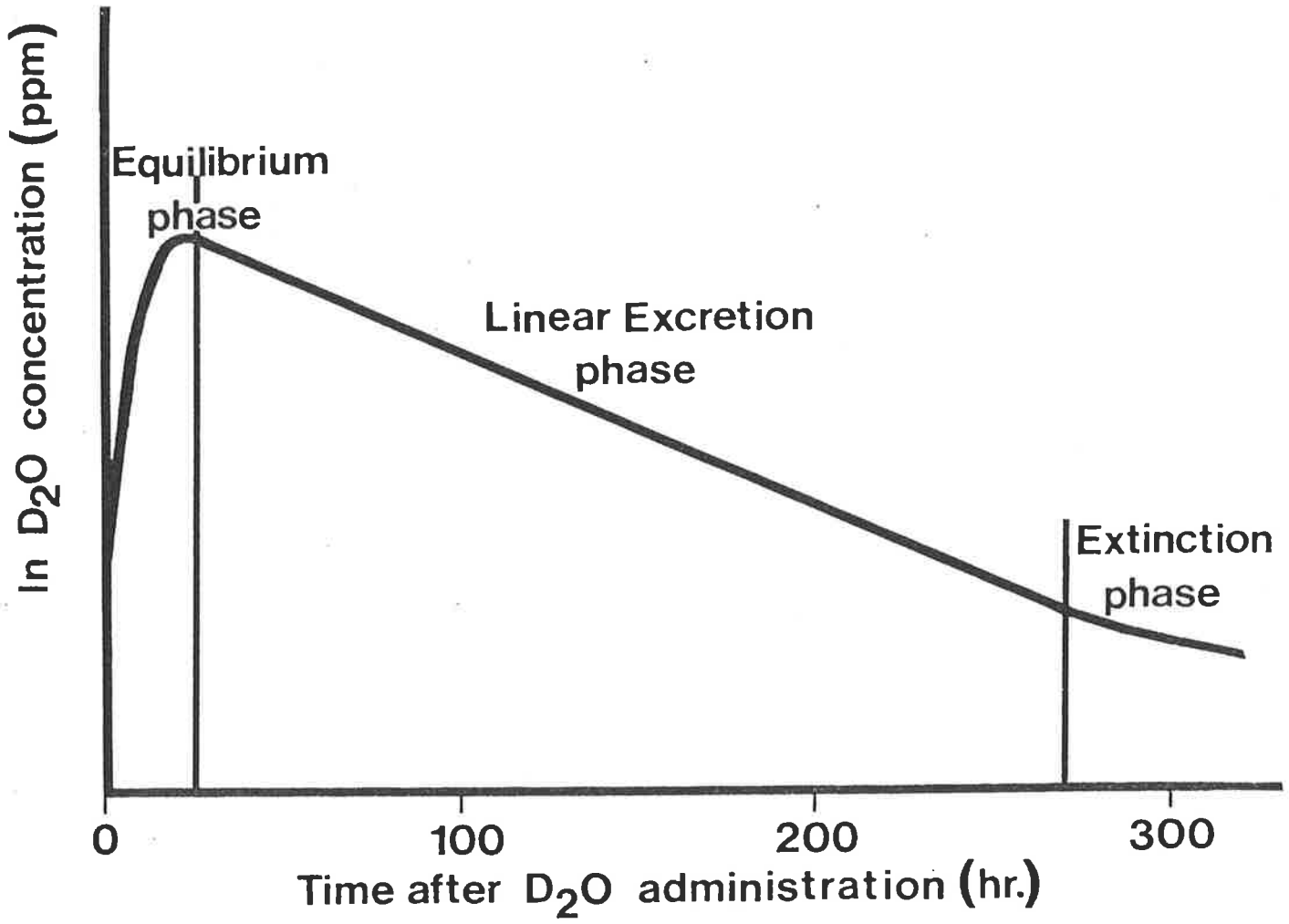


Fig. 2.2.3 The graph illustrates the time course of D_2O concentrations in neonatal urine after an oral administration of D_2O at zero time.

The half-life of D_2O in the body is related directly to k, and is given by

$t_{\frac{1}{2}}$ = ln 2/k hours.

As an example, for a typical normal baby with 10^4k = 70,

$t_{\frac{1}{2}}$ ≈ 100 hours ≈ 4 days.

2.2.11 Data Analysis

The Cyber computer at the University of Adelaide was used in the analysis of the data collected for the study. All the antenatal and postnatal details and test results were stored on a data file and later analysed using correlations.

To determine the rate constant of water turnover of each infant, the same method was used as in MacLennan et al (1981). A non-linear least squares technique was employed using program MODFIT in conjunction with a subprogram RESERVR; (McIntosh and McIntosh, 1980) in which each measurement of D_2O concentration was weighted by the inverse of its variance. The standard deviation of each estimation was also calculated.

The group of infants participating in the study did not represent a normally distributed population. Therefore non-parametric tests of significance were used to test differences between groups of infants rather than parametric test. (Colquhoun, 1971).

2.2.12 Follow-Up Study of Infants

An attempt was made to follow up the progress of each of the infants participating in the study. Where possible, the weights, head circumferences and lengths of the infants were recorded, graphed and percentiles calculated.

A developmental paediatrician with the Mothers and Babies Health Association, evaluated the mental progress of selected children using the Griffiths Mental Development Scales. The scales assess six factors of

development namely, locomotor, personal-social, hearing and speech, eye and hand co-ordination, performance and practical reasoning.

2.3 RESULTS

The water turnover rates of a total of 106 infants (45 females and 61 males) were determined in the study. All of these infants were Caucasian. Their gestational age ranged from 32 to 43 weeks and their birth weights from 1140 to 4440 grams. Maternal ages were between 14 and 40 years, with a mean of 24.7 years. The study cohort comprised 75 singleton infants, 11 sets of twins and 3 sets of triplets.

2.3.1 Optimal Sample Number

The optimal number of urine samples per infant necessary for the water turnover rates to be calculated had to be balanced between the need for statistical accuracy and the speed of performance of urine purification and spectrophotometric analysis.

In practice, the number of urine samples collected per infant ranged from 3 to 20. These samples were collected at various time intervals between 20 and 300 hours after D_2O administration. Using the Mann-Whitney Test it was resolved that the rate constant determined from 5 urine samples were not statistically different from those obtained from 8 or more samples. However, less than 5 urine samples produced significantly different rate constants ($p > 0.05$). Tests also showed that the urine samples should be collected at approximately evenly spaced intervals, well spread out between 20 and 300 hours rather than having them close together, in order to obtain the most accurate rate constant (Fig. 2.3.1).

As a rule, in the present study 8 urine samples were tested per infant because it was possible to purify 8 samples at one time with the distillation equipment.

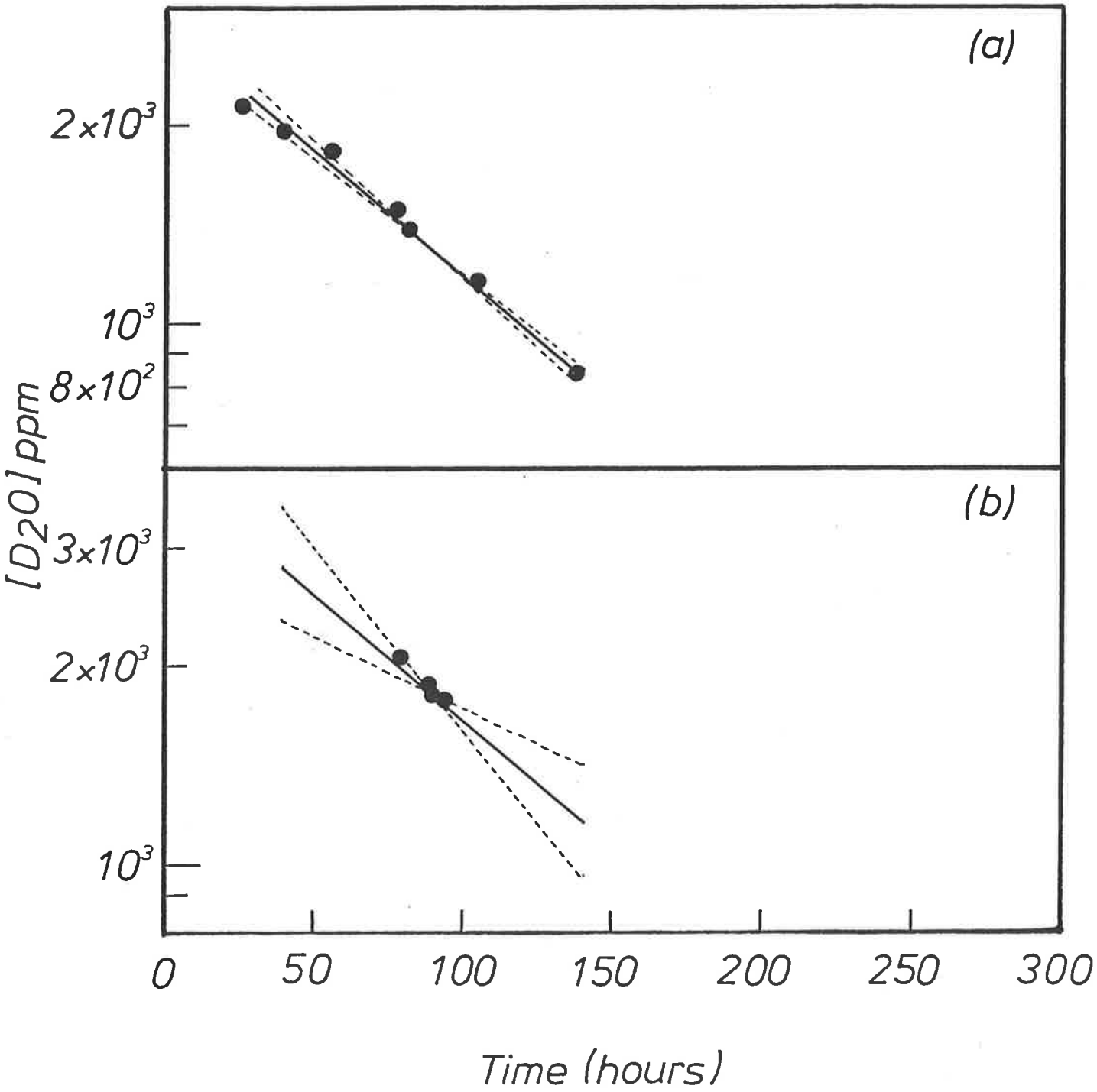


Figure 2.3.1 Illustration of ideal distribution of data samples to produce optimum rate constants.

The dots indicate the actual points, and the solid lines show the best fit straight line. The dotted lines have slopes equal to the mean slope (rate constant) $\pm$ one standard deviation, and hence given an indication of the reliability of the mean. A wide range of slopes indicates poor reliability.

Fig. (a) shows the situation when several well spaced urine samples were used. The mean rate constant is quite reliable. In contrast, fig. (b) shows the case where a few closely spaced points were used, and there is clearly a large error.

In most measurements made for this thesis, data samples similar in distribution to (a) were used.

2.3.2 Water Turnover Rates

Of the 106 infants who participated in the study 32 were classified as normally grown (normal), 25 as borderline and 46 as intrauterine growth retarded. Three infants were placed into different categories from these because one was the infant of a diabetic mother and two infants were postmature (i.e. more than 42 weeks gestation).

Figure 2.3.2 illustrates the linear phase of the D_2O excretion curve for a normal infant ($10^4K=72.0$), where K is the rate constant (10^4K was used in the study because of the convenience in reading the values) and for one who is growth retarded ($10^4K=101.0$). The slope of this phase of the D_2O excretion curve is greater for the growth retarded than the normally grown infant, indicating a faster water turnover rate.

The rate constants ($x10^4$) and standard deviations of the normal, borderline and intrauterine growth retarded categories are listed in appendix 4. The infant of the diabetic mother had a water turnover rate of 108.0 (S.D. = 8.9) and the two postmature infants had rates of 100.1 (S.D. = 7.0) and 122.0 (S.D. = 3.0).

Since it was not known initially that the study population was normally distributed non-parametric tests (i.e. medians, 97% confidence limits of the median) were used for the initial analysis of the results. Medians of the rate constant ($x10^4$) of the normal, borderline and growth retarded groups were significantly different ($p<0.005$, Mann-Whitney U Test (directional), Colquhoun, 1971) being 73.3, 85.9 and 100.2 respectively (table 2.3.1). The 97% non-

Table 2.3.1 Number of infants, medians of the rate constants and 97% confidence limits for normal, borderline and intrauterine growth retarded infants.

Clinical Group	Number	Median of the Rate Constant (h^{-1})	97% Non Parametric Confidence Limits of the Median
Normal Infants	32	73.3	66.9 - 78.0
Borderline Infants	25	85.9	81.9 - 91.3
Intrauterine Growth Retarded Infants	46	100.2	94.0 - 105.0

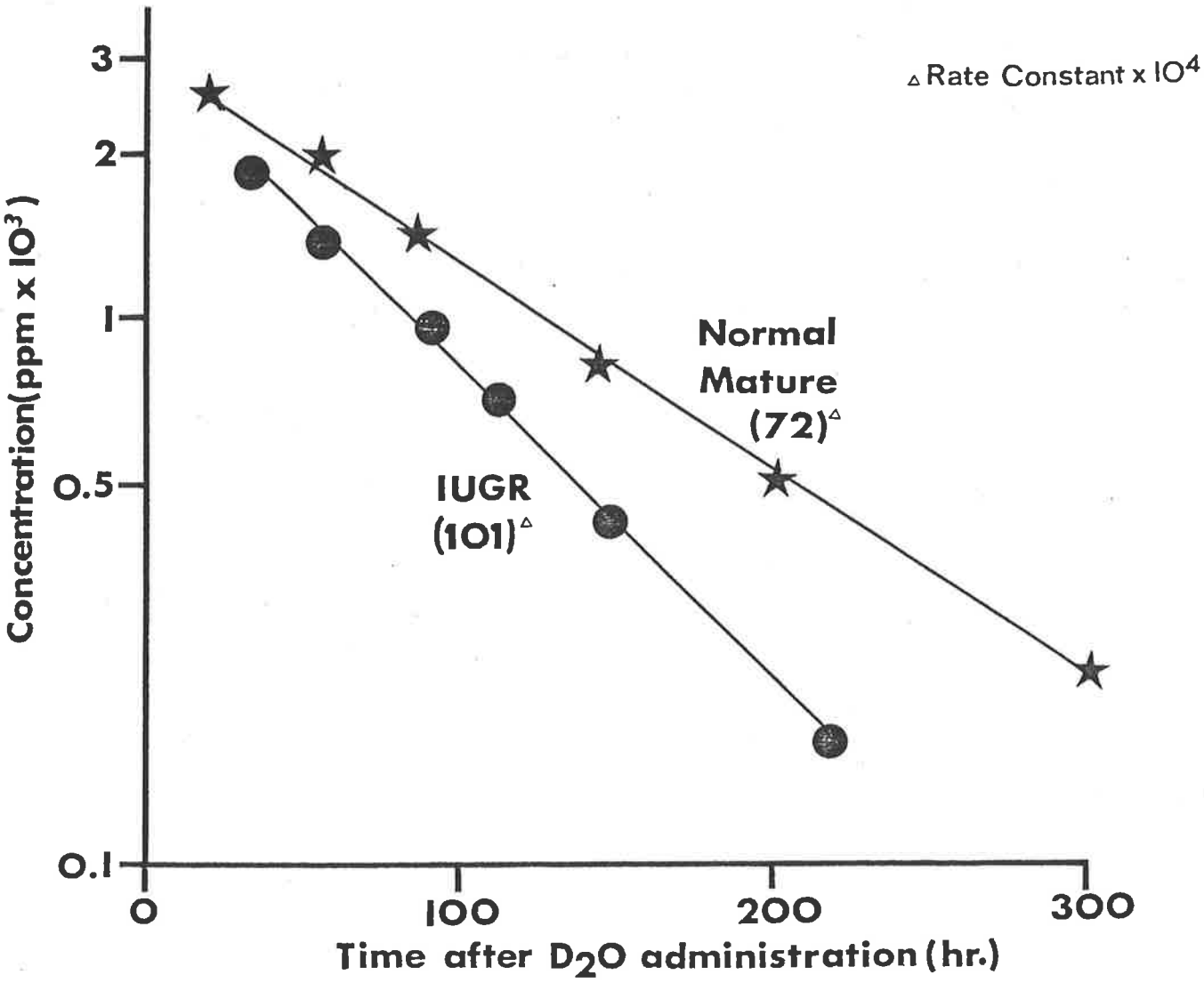


Figure 2.3.2 : Linear phase of the D_2O excretion curve for a growth retarded and a normal, mature infant.

parametric confidence limits of the median (Colquhoun, 1971) are not overlapping although some of the water turnover values themselves do overlap in the three clinical categories (Fig. 2.3.3). Clinically, the degree of growth retardation was generally directly proportional to the water turnover rates in the growth retarded group. That is, the more growth retarding features that were associated with the infant, the higher the rate constant.

The means of the rate constants ($x10^4$) of each clinical group were also determined, being 71.0 for the normal 87.0, for the borderline and 102.6 for the growth retarded infants. The means and medians of the water turnover rates were not statistically different and since parametric tests are more commonly used and better understood, the subsequent analyses were conducted using parametric tests.

Later the borderline class of infants were investigated more closely and it was found that within this group, the more retarded infants had higher rate constants. These borderline infants were then reclassified as either normal or growth retarded, with no allowance for a borderline group. The mean rate constant ($x10^4$) of the 12 borderline infants reclassified as being normal was 83.5 and for the 13 borderline infants reassessed as growth retarded it was 90.2 ($p<0.05$, Mann-Whitney U Test).

It was possible to set up arbitrary divisions using water turnover values such that infants with a rate constant ($x10^4$) less than 80.0 being regarded as normally grown, between 80.0 and 89.9 as borderline and greater than 89.9 as intrauterine growth retarded. If this were done then 31, 29 and 43 infants would belong in the normal, borderline and growth retarded groups respectively. When this predicted group membership is compared with the three clinically assessed groups, 73.8% of all the infants were correctly placed using water turnover rates (table 2.3.2).

2.3.3 Gestational Influence

When each of the three clinical groups were subdivided into premature

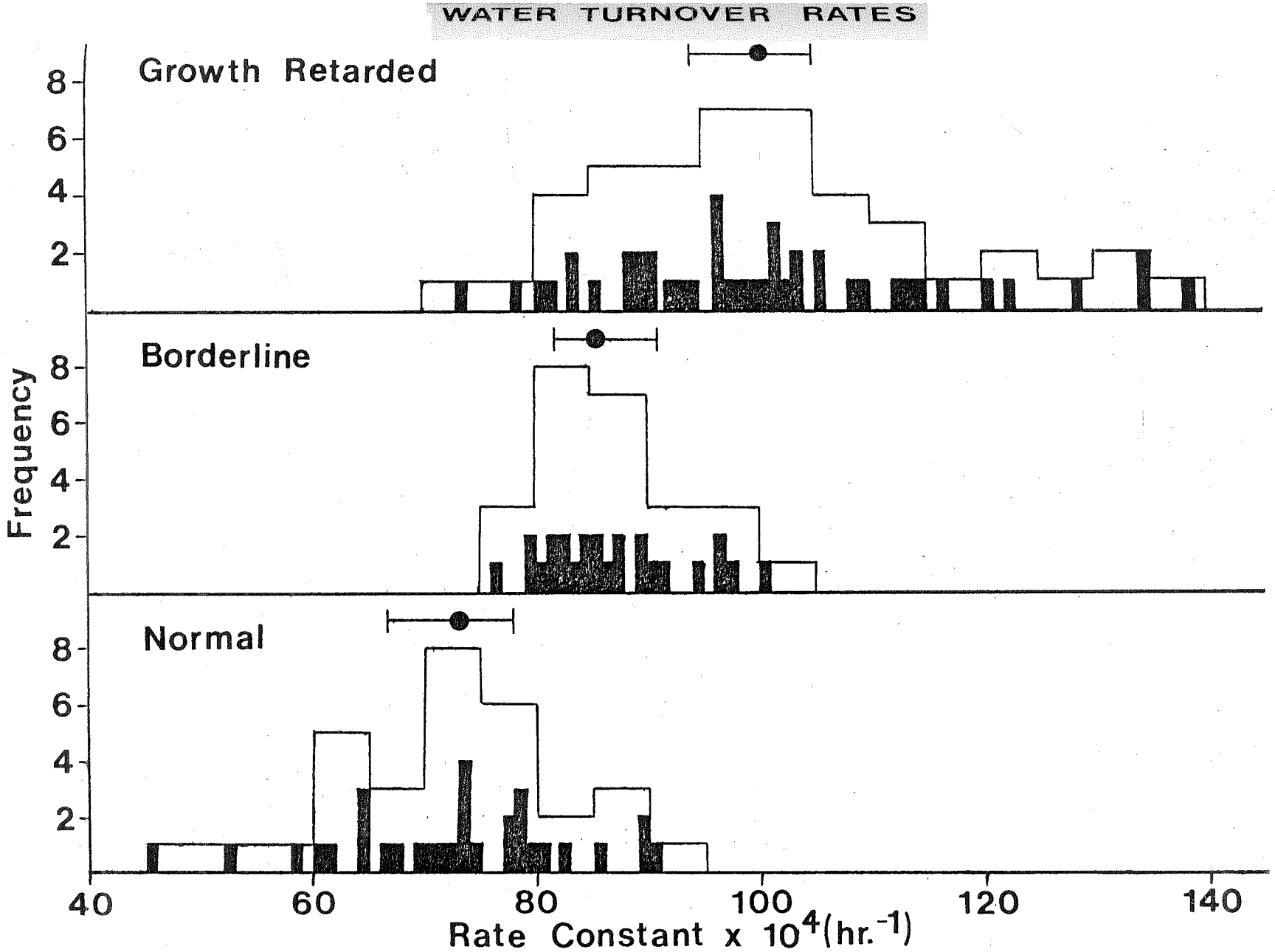


Figure 2.3.3 : Water turnover rates of 104 infants from the study are illustrated. The medians (dot) and 97% confidence limits are shown for each group. The rate constant ($x10^4$) of one normal infant was 26.5 (SD=9.3) and it was 167.0 (SD=24.0) for one growth retarded infant. These two values have been excluded from the graphical representation. The frequencies in steps of 5.0 rate constant ($x10^4$) are also illustrated.

Table 2.3.2 The table illustrates the number of infants for the predicted group membership and the percent of infants correctly classified by this method.

Clinical Group	Predicted Group Membership			
	Number of Infants	Normal	Borderline	Growth Retarded
Normal	32	26	5	1
Borderline	25	3	15	7
Growth Retarded	46	2	9	35
		31	29	43

Arbitrary divisions of rate constants ($x10^4$) for predicted group membership are:

<80.0 Normal

80.0 - 89.9 Borderline

>89.9 Growth Retarded

Percent of infants correctly classified = 73.8% (76/103).

(i.e. infants 32-36 weeks gestation) and mature infants (i.e. infants 37-42 weeks gestation), there was not statistically significant difference (Mann-Whitney U Test) in their water turnover rates (table 2.3.3). In each clinical

Table 2.3.3 Total number of mature and premature infants in the three clinical groups of normal, borderline and growth retarded and their respective mean rate constants ($x10^4$), mean weight percentiles and standard error of the means (S.E.M.).

Clinical Group	NORMAL		BORDERLINE		GROWTH RETARDED	
	Mature	Premature	Mature	Premature	Mature	Premature
Total Number	21	11	7	18	23	23
Mean of Rate Constant ($x10^4$)	70.1	72.5	83.8	88.2	99.8	105.3
S.E.M.	3.2	2.9	1.3	1.6	2.9	4.4
Mean of Weight Percentile	58.5	46.4	32.3	24.8	20.9	9.2
S.E.M.	5.8	4.4	9.6	2.5	6.3	2.8

group the mean of the rate constant ($x10^4$) was slightly higher for the premature infants than the mature ones, but the mean of the weight percentile was

also lower in each case for the premature infants. Figure 2.3.4 illustrates the mean of the water turnover rates for premature and mature infants in the normal, borderline and growth retarded groups as a function of the mean weight for gestation.

2.3.4 Clinical Correlations

The antenatal and postnatal factors listed in table 2.3.4 were correlated with the water turnover rates of the infants. None of the clinical parameters

Table 2.3.4 List of antenatal and postnatal factors investigated in the study.

Antenatal Factors	Postnatal Factors
Maternal Age	Weight Percentile
Maternal Smoking	Triceps Thickness Percentile
Maternal Alcohol	Subscapular Thickness Percentile
Mode of Delivery	Apgar at 1 Minute
Foetal Distress	Ponderal Index
Pre-Eclampsia	Fluid Intake/Kg. (Bottle Fed Infants)
Steroid Therapy	Cot Temperature
	Cot Humidity
	Birth Weight

individually correlated highly with the rate constant. The best correlation was with the total risk score (0.47) followed by the weight percentile (-0.41) and the ponderal index (-0.41) (table 2.3.5). The total risk score correlated

Table 2.3.5 Correlation coefficients of the rate constant (i.e. 10^4K) against various antenatal and postnatal factors.

	Correlation coefficient
Total risk score	.47
Weight percentile	- .41
Ponderal index	- .41
Maternal age	- .39
Subscapular thickness percentile	- .34
Birth weight	- .27
Triceps thickness percentile	- .23
Maternal alcohol	.13
Maternal smoking	.12
Pre-eclampsia	- .07

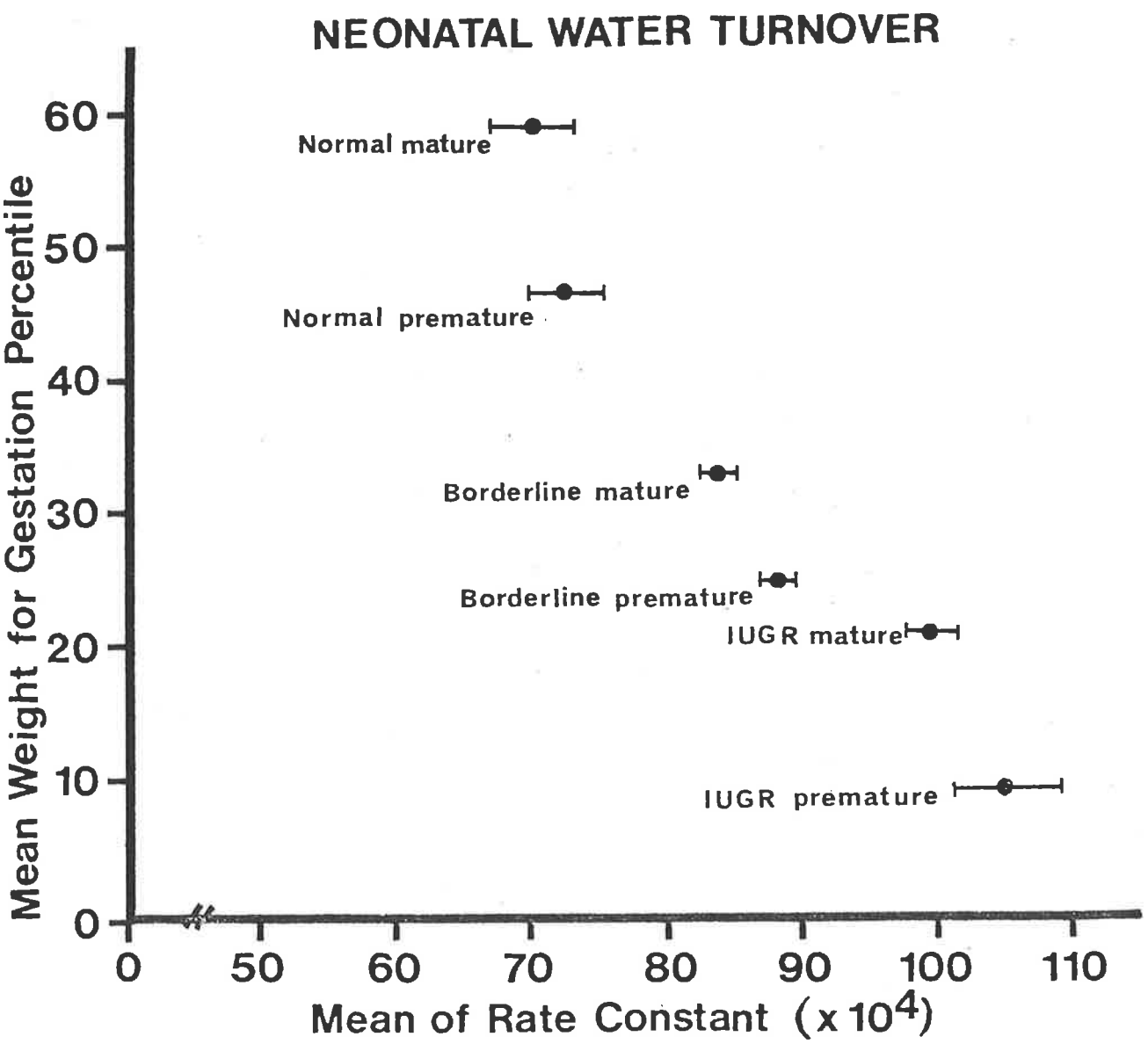


Figure 2.3.4 : Neonatal water turnover rates of mature and premature infants in relation to the mean weight for gestation percentiles. S.E.M. are also shown.

better than either the antenatal or postnatal risk scores. In the three clinical groups the total risk scores were significantly different ($p < .05$, Mann-Whitney U Test) (Fig. 2.3.5).

Information regarding the smoking habits of 93 mothers was obtained in the study. Of these 54 were non-smokers and 39 were smokers. 31 (79.5%) of infants of smoking mothers had rate constants ($x10^4$) greater than 80.0 and of these 16 (i.e. 41% of total infants of smoking mothers) had rate constants ($x10^4$) greater than 90.0. Only 8 of the smokers delivered infants with water turnover rates less than 80.0, and who were clinically classified as normally grown. 6 of the 7 infants whose mothers smoked more than 20 cigarettes per day displayed water turnover rates greater than 80.0.

In the normal group, infants who suffered foetal distress during labour had statistically higher water turnover rates ($p<0.05$, Mann-Whitney U Test).

Other clinical parameters that were measured (e.g. maternal pre-eclampsia, maternal alcohol consumption, neonatal disease, mode of delivery, apgar scores) did not correlate well with water turnover rates.

No correlations were found between the water turnover rates of the infants and antenatal steroid therapy (21 patients), humidity and temperature of the neonatal environment, or the fluid intake per kilogram body weight in bottle fed infants. There were few major differences in the neonatal environment however, as no infants were included in the study who were under 32 weeks gestation or who required intensive care treatment.

2.3.5 Antenatal Investigations

Information deduced from ultrasound scans performed during pregnancy was also recorded. Of the infants who had antenatal ultrasound scans, 25 infants showed consistently at several measurements biparietal diameters which were less than expected for their respective gestational ages. The biparietal diameters of these infants ranged from 2 to 9 weeks less than expected. The water turnover rate test showed that 24 out of 25 of these infants had high rate

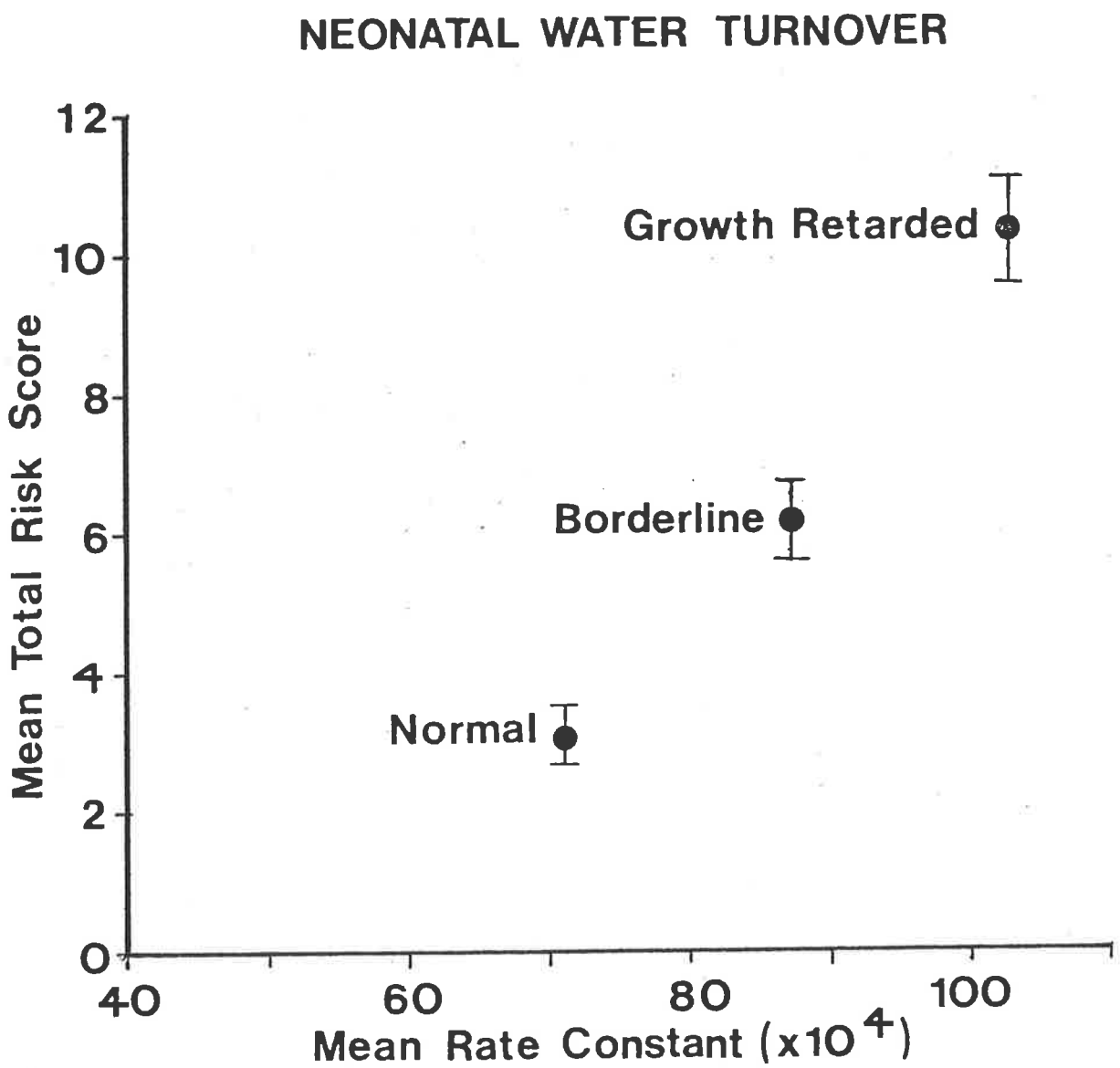


Figure 2.3.5 : Graphical representation of the relationship between neonatal body water turnover rates and the mean total risk score (and S.E.M.) of each of the 3 clinical groups.

constants. Only one infant of the 25 had a water turnover rate less than 80.0. The mean of the rate constant ($x10^4$) of this groups of infants was 101.9.

Six mothers of the study group had delivered an infant who was growth retarded in a previous pregnancy. 5 of the 6 infants born to these mothers in the current pregnancy were clinically growth retarded with high water turnover rates (mean rate constant ($x10^4$) = 108.1). The one infant who was clinically normally grown had a rate constant ($x10^4$) of 73.7.

2.3.6 Multiple Births

In the study group there were 11 sets of twins and 3 sets of triplets ranging between 32 and 39 weeks gestation. Six of these infants were clinically classified in the normal group, 10 in the borderline and 15 in the growth retarded group. The medians (means given in brackets) of the water turnover rate of the infants of multiple pregnancies for the normal, borderline and growth retarded groups were 75.3 (71.3), 84.2 (85.6) and 94.0 (94.9) respectively. No statistical difference existed between the medians and means of the multiple pregnancy births and those of singeltons.

2.3.7 The 10th Percentile Weight for Gestation

Thirteen infants who participated in the study were above the 10th percentile in weight for gestation but were clinically classified as growth retarded. All of these infants displayed high water turnover rates greater than 80.0, with a mean rate constant ($x10^4$) of 101.7. Their mean weight percentile was 43.1.

Two infants who were below the 10th percentile in weight for gestation (i.e. 5th and 7th percentiles) were not classified clinically as clearly growth retarded. They were both classified in the borderline group. If the borderline group was disallowed, then these 2 infants could have been regarded as normally grown. The water turnover rates of these 2 infants were 83.4 and 82.6.

2.3.8 Follow-up Studies

Follow-up studies of 25 infants were possible for periods ranging between five months and one year of age. Weight, length and head circumference measurements were performed at the time of the postnatal visit. Four of these infants were classified as clinically normally grown, 5 as borderline, 15 as growth retarded and 1 as postmature. The follow-up group included 2 sets of triplets and 3 sets of twins.

All 3 infants who were followed up and belonged to the group of infants who were above the 10th percentile in weight but were clinically assessed as growth retarded, caught up in physical growth (catch-up growth - see General Introduction) by 6 months of age. The infant who was below the 10th percentile in weight, but with a relatively low water turnover rate of 82.6 did not catch up in growth at one year of age and was termed a "small normal" child.

Catch-up growth was observed in 10 of the 15 infants who had been clinically classified as growth retarded at birth. The mean water turnover rate of the infants who caught up in growth was 98.2 and for the infants displaying no catch-up growth the mean water turnover rate was 115.7 (excluding one infant who developed a severe illness). One infant clinically classified as borderline, but with a rate constant ($x10^4$) of 80.1 did not catch up in growth at 6 months of age.

Griffith's Mental Development Scales was used to study 13 infants. The infants ranged between 6 and 19 months of age. No conclusions could be drawn about the mental development of growth retarded as compared with normally grown children using this test at such early ages. When considering the ages of these children there is a very large influence of environmental conditions, such as the child's opportunity for stimulation.

2.4 DISCUSSION

The results of this study show that body water turnover soon after birth is faster in intrauterine growth retarded infants than in normally grown infants. This confirms the suggestions made by MacLennan et al (1981). Such an increase in water metabolism is consistent with other reports (e.g. Cheek and Talbert, 1968) which have shown that growth retarded infants have excessive total body water per kilogram of body weight and that a rapid decrease in cell water takes place after birth (Cassady and Milstead, 1971). Similarly, Sinclair and Silverman (1966) claimed that neonates who suffered intrauterine growth retardation were hypermetabolic. They found that oxygen consumption per kilogram of body weight was higher for growth retarded infants than for normally grown infants of similar birth weight and that the degree of hypermetabolism correlated with the degree of under-growth.

Several explanations have been proposed to account for this increase in cellular metabolism after birth in intrauterine growth retarded infants. One possibility is that the increase in metabolism may reflect an increased cellular activity due to rapid cell growth and repair following intrauterine deprivation. This intrauterine deprivation takes the forms of malnutrition and/or hypoxia. Alternatively, because of inhibition of intrauterine cellular metabolism, there may be an increase in cell acidity and so a decrease in transport of intracellular solutes. This can result in an increased passive excretion of water along with the accumulated intracellular solutes after birth, since the neonatal kidney has not yet developed an effective mechanism to concentrate urine (Oh, 1981). A third possibility is that growth retarded infants may have a faster water turnover simply because they may have more cells per kilogram of body weight than normally grown infants. Lastly, the increased water turnover may be due to increased quantities of plasma, extracellular, intracellular and total body water. Such increases may be expected since growth retarded infants have diminished body fat.

The results of this study indicate that the optimal number of urine

collections necessary per infant is five. If less than 5 urine samples are collected the standard deviations of the calculated water turnover rates are too high. Collections of much larger number of urine samples, although improving the accuracy of the rate constant, are very time consuming both in the actual collection and in the purification and spectrophotometric analysis. (In this study usually 8 urine samples were analysed per infant because the distillation equipment could accommodate 8 samples to be vacuum distilled at one time, making it a convenient number to use). It is suggested that these 5 urine samples be collected at well spaced intervals between 20 and 300 hours after the administration of D_2O. Even with large numbers of urine collections it is not possible to further reduce the standard deviation of the individual water turnover rates. A possible explanation may be that the urine is stored for an unknown period of time in the bladder of the neonate before voiding. This may be up to 3 hours in duration.

It was essential that the infants in this study be first classified by conventional methods to test the value of neonatal water turnover rates as an index for intrauterine growth retardation. A combination of several conventional techniques were used, such as weight for gestation percentiles, skin-fold thickness measurements, ponderal indices and clinical signs of growth retardation. Great care was also taken to determine the correct gestational ages of the infants by recording accurate obstetric details, performing the Dubowitz test on each infant and carrying out antenatal ultrasound analysis. These methods, however, were still susceptible to subjective errors. Even with such scrutiny it was not possible to express quantitatively by these conventional means the degree of growth retardation suffered by the infant. It was only feasible to classify the infants into 3 groups; namely "normal", "borderline" and "intrauterine growth retarded". The possibility that some of the infants were clinically misclassified has to be considered. Hence the overlap in the individual rate constants in the 3 clinical groups could have been due to misclassification of some of these infants. Since clinically there were such large ranges in the degree of growth retardation in the infants in each class

it was not surprising that there also existed a wide spectrum in neonatal water turnover rates.

When the normal, borderline and growth retarded groups of infants were defined purely by the arbitrary water turnover values (i.e. <80.0 normal, 80.0-89.9 borderline, >89.9 growth retarded), then 73.8% of the infants were correctly assessed compared to the clinical classification. This is a relatively good correlation considering that some of the infants may have been clinically misclassified. Determination of the length of time after birth that water metabolism remains higher in growth retarded infants requires further research. It can be concluded from this study however, that the water turnover rate is higher for at least 12 days postnatally in growth retarded infants. During this period the logarithm of the excretion phase curve remained linear, indicating that excretion rates remained constant.

Gestational age did not appear to affect the water turnover rates of the infants in the study. Therefore, it is unlikely that the increased water metabolism observed in growth retarded infants was due to kidney immaturity. The neonatal environment (i.e. temperature and humidity) and the fluid intake of infants could not account for this increase in water metabolism either. Correlations of individual, antenatal clinical parameters with the rate constant were not significant. This result is not unrealistic since it is most probable that several factors operate simultaneously to produce growth retardation in foetuses. Further the degree to which each individual factor contributes to the growth retardation can vary widely.

From the study it appeared that smoking mothers had a higher percentage of growth retarded infants. This is despite the low correlation between water turnover rates and smoking mothers. The low correlation may have been because many other factors also operated (e.g. pre-eclampsia. alcohol consumption) as discussed above. Only a small number (8) of smoking mothers delivered normally grown infants. The occurrence of foetal distress during labour did slightly increase the water metabolism of the infants in the normally grown group. This is consistent with the theory that water metabolism is higher following

periods of hypoxia. The increase was not large enough however to account for the very high rate constant values observed with growth retarded infants.

The total risk score appeared to be a useful aid in assessing growth retardation in this study. Improvements to the risk score could be made by re-evaluating the arbitrarily chosen values in the light of the knowledge gained from the water turnover rates. Skinfold thickness percentiles did not correlate as well with the observed water turnover rates as may have been expected. This may at least partly have been due to the inadequacy of the available skinfold thickness percentile charts. The weight for gestation percentiles also did not correlate highly, supporting the theory that an infant can be in the "normal" weight range (i.e. between the 10th and 90th percentiles) and still be growth retarded (or conversely, that an infant below the 10th percentile can be normally grown).

Only 2 postmature infants and 1 infant of a diabetic mother were investigated in the study. High water turnover rates were observed with both of the postmature infants, but a larger study group is required before any conclusions can be made from this finding. The infant of the diabetic mother displayed a high rate constant supporting the finding that growth retardation may be present despite the excessive deposition of fat (see General Introduction). Again further studies are necessary.

The presence of 31 infants from multiple pregnancies did not significantly alter the mean water turnover rates in the normal, borderline or growth retarded groups. The water turnover rates of 22 of these 31 infants were greater than 80.0. The multiple births were not chosen at random for the study, so no conclusions can be drawn about the incidence of growth retardation as determined clinically or by water turnover rates.

Follow-up studies of only some of the infants in the study were possible because there were difficulties in locating them and, on occasions, some parents were unwilling to co-operate. The results obtained from the infants who were seen at several months of age were interesting although not conclusive because of the small numbers.

From the physical characteristics (i.e. weight, length and head circumference) the infant under the 10th percentile in weight for gestation but with a low rate constant did not catch up in growth by one year of age. This reinforces the assumption that this infant was indeed small, but not growth retarded at birth. The infant in the clinical borderline group with a low rate constant also did not catch up in growth, so possibly was misclassified and should have been placed in the clinically normal group.

It is interesting to note that of the infants who were growth retarded at birth, catch-up growth was observed with the ones whose water turnover rates were around 100, but not with infants with very high rate constants. Further investigations are required in this area.

No comments can be made about the mental development of the infants as they were too young when the investigations were performed. Repetition of Griffith's Mental Development test is recommended at 2 years of age.

The determination of water turnover rates in neonates has proven to be a relatively simple and inexpensive test to detect growth retardation. The techniques using paper towels placed in the nappy of the infant has greatly improved the collection of urine samples. The paper towels do not cause irritation on the skin around the infant's genitalia, as urine collection bags do and this fact also improves mothers' compliance in allowing the performance of the test.

Due to the improved method of analysis of the urine samples (see Methods), small quantities of D_2O need only be administered to the infants. The estimate of water turnover requires only a small quantity of urine (3-4 mls) due to the purification of urine by vacuum distillation (Byers, 1979). The one, single dose of D_2O can be given orally without the need for stomach tubes because the measurement of water turnover rates are not dependent on a known, total dose of D_2O.

Water turnover rate estimation has proven to be a useful, retrospective test to detect intrauterine growth retardation in neonates. The test is objective and not dependent on gestational age (tested in the range of 32-43 weeks gestation), birth weight, behaviour and appearance at birth. Besides

being qualitative, water turnover rates are also quantitative, giving an indication of the degree of growth retardation.

Potential applications of an objective, neonatal index of intrauterine growth retardation are numerous. It would solve the problem of the identification of growth retarded infants, and the definition of IUGR would no longer be dependent on arbitrary weight limits, or weight for gestation charts as are currently used. It would also eliminate the confusion of the identification of IUGR in premature infants as this test does not depend on gestational age.

As a research tool the test would assist in the retrospective analysis of the causes of IUGR. Follow-up studies of growth retarded infants would show the relationship between water metabolism (as measured by water turnover rates) and physical growth and mental development in later life. Obstetric management of these infants may be improved by assessing the effectiveness of currently used antenatal tests for IUGR by water turnover rate. Comparisons of the incidence of IUGR in different racial populations can also be studied with water turnover rates since it is independent of birth weight standards.

Table 2.4.1 Potential uses of water turnover rates.

1.	Identification of growth retarded infants.
2.	Retrospective analysis of the causes of IUGR.
3.	Correlations between water turnover rates and physical growth and mental development of infants in later life.
4.	Comparison of IUGR in different racial populations.
5.	Improve the treatment of growth retarded infants.

CHAPTER III

SKELETAL MUSCLE PROTEIN BREAKDOWN IN EARLY HUMAN DEVELOPMENT

INTRODUCTION

Determination of intrauterine growth retardation by the water turnover method is simple and easy to perform, but it does require the collection and analysis of 5 to 8 urine samples and D_2O has to be administered to the infants. The ideal test for the detection of intrauterine growth retardation would be one requiring a single urine sample. It should be non-invasive and should preferably be useful both antenatally and postnatally. The aim of this study was to determine whether an assessment of muscle protein breakdown could satisfy these criteria.

Muscle protein catabolism may be a good indicator of intrauterine growth retardation because several investigators have found a connection between the nutritional state of animals and humans and their protein metabolism. For example, during periods of inadequate protein intake, muscle protein is conserved through a decrease in protein catabolism. When there is a restriction of both energy and protein intake, the rate of muscle protein breakdown increases (Young and Munro, 1978). Children with conditions of protein-calorie malnutrition (i.e. marasmus, kwashiorkor and marasmic kwashiorkor) have been found to have lower rates of protein breakdown than well nourished children of the same age (Narasinga Rao and Nagabhashan, 1973). There is an increase in protein catabolism during acute starvation and a progressive decrease takes place during adaptation to chronic starvation (Seashore et al, 1980). During nutritional rehabilitation the rate of muscle protein turnover increases (Young and Munro, 1978).

3.1.1 Measurement of Muscle Protein Breakdown

To measure muscle protein breakdown, the excretion of the amino acid, 3-methylhistidine (3-MH) in the urine has been employed. Until recently, however, the measurement of 3-MH was fairly difficult and time consuming since an amino acid analyser had to be used. The automated technique of Murray et al (1980),

has greatly improved the measurement of 3-MH, so now studies of protein breakdown are much simplified. The analysis of muscle protein catabolism on large numbers of subjects are now possible, as in the present study.

Creatinine excretion in the urine is proportional to the total muscle mass in the body (Graystone, 1968). Therefore, 3-methylhistidine to creatinine (3-MH /Cr) ratios in the urine provide a measure of muscle protein breakdown per unit of lean body mass.

Since 3-methylhistidine and creatinine form the basis of this section of the thesis, an overview of their properties and details of previous studies will now be discussed.

3.1.2 3-Methylhistidine - Background

The amino acid 3-MH acts as an in vivo label for muscle protein breakdown rates. In humans, 3-MH was first recognised in 1954 (Tallan et al). In 1967, 3-MH was identified as a component of actin (Asatoor et al), and of myosin and actin by Johnson et al (1967). The rate of excretion of 3-MH in the urine has been proposed by Young et al, 1972, as an indicator of muscle protein breakdown. To validate the use of 3-MH as an index for muscle protein breakdown the following characteristics have to be met. It should not be reutilized for protein synthesis, it should exist in a non-protein bound form, have a rapid turnover time in the body and be quantitatively excreted in the urine in a readily identifiable form.

The majority of 3-MH formed in the body is present in skeletal muscle (Haverberg et al, 1975). In the adult male skeletal muscle constitutes approximately 45% of the total body protein, but it is only about 22% in the infant (Picou et al, 1976). Further, in infants, skeletal muscle is a smaller contributor to the total 3-MH pool than in the adult, being 17% and 70% respectively (Young et al, 1976). Small amounts of 3-MH are present in other tissues and organs because actin is generally present in eukaryotic cells (Young and Munro, 1978) and it has been estimated that the skin and intestines account for approx-

imately 10% of the total body 3-MH (Nishizawa et al, 1977). It has been found that 3-MH is absent from myosin of foetal skeletal muscle (Kuehl and Aldestein, 1970; Trayer et al, 1968).

When muscle protein is catabolized, 3-MH is liberated. It is not re-utilized for protein synthesis (Young et al, 1972). 3-MH is quantitatively excreted in the urine and in the human adult 95% or more is excreted as the parent compound. The remaining 5% or less of the total urinary 3-MH is excreted in the N-acetyl form (Long et al, 1975).

3.1.3 Creatinine in Urine and Amniotic Fluid

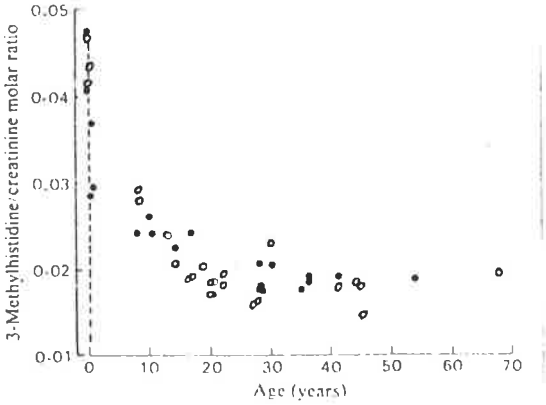
There is foetal urinary output as early as 14 weeks of gestation (Poulsen, H., 1955) and thus both the foetal urine and the amniotic fluid contain creatinine in increasing amounts late in pregnancy. Poulsen suggests that this reflects an increased foetal glomerular function. Droegemueller et al (1969), found that amniotic fluid creatinine concentrations did not correlate with foetal size, but Roopnarinesingh (1970) did find a correlation with birth weight and amniotic fluid creatinine in late gestation. De Voe and Schwarz, 1975, suggest that it is likely that both birth weight and foetal glomerular maturation play a role in the creatinine concentration in amniotic fluid, with the latter having the major influence.

3.1.4 3-Methylhistidine to Creatinine Ratios in Health and Disease

In man, there is a progressive decrease in the rate of 3-MH excretion per unit creatinine output with increasing age (Fig. 3.2.1).

Figure 3.2.1

Daily urinary 3-methylhistidine/creatinine ratios for male (●) and female (○) subjects ranging in age from 15 weeks pre term neonatal to 68 years. Zero age is birth after a full term gestation. (From Tomas et al, 1979).



In preterm neonates the 3-MH/Cr excretion rate is approximately 2.5 times greater than in the adult. The mean 3-MH/Cr molar excretion ratio for subjects older than 20 years was found by Tomas et al (1979) to be 0.0184 $\pm$ 0.0004.

Infants who are stressed (i.e. through illness) and/or had inadequate nutrient intake have been found to have higher 3-MH/Cr ratios (i.e. usually above the molar ratio of 0.023) than healthy, growing infants (Seashore, et al, 1980). A study by Pencharz et al (1981) on premature infants approximately one month old, showed that small-for-gestational age (intrauterine growth retarded) infants generally had higher rates of protein breakdown than appropriate-for-gestational age premature infants.

3.1.5 Aims

Prior to the studies undertaken for this thesis, no studies had been published concerning muscle protein breakdown during intrauterine life. Therefore no information was known about variations in foetal muscle metabolism and its relationship to abnormalities in the foetus or the mother. Only one study (Pencharz et al, 1981) has so far examined skeletal muscle protein breakdown in intrauterine growth retarded infants and compared them with values obtained from appropriate-for-gestational age infants. The first measurements in this study were taken, however, when the infants were at an average age of 24 days. Skeletal muscle protein breakdown rates in infants immediately after birth, or serial measurements over several days in intrauterine growth retarded infants have not been investigated. The first aim of this study, therefore, was to determine 3-MH/Cr ratios in neonates from the moment of birth until they were several days old (up to 10 days). In addition, serial investigations of 3-MH/Cr ratios were undertaken in a few normally grown and growth retarded infants to determine if any differences existed with regard to 3-MH/Cr ratios.

If the rate of 3-MH/Cr excretion in the urine does reflect the nutritional state of the neonate, then it seemed to the author that the same test could also be applied to amniotic fluid. This would then provide an antenatal as

well as a postnatal test for intrauterine growth retardation. Hence, the second aim of the project was to investigate 3-MH/Cr ratios in amniotic fluid and to determine whether these measurements correlated with growth retardation.

3.2 METHODS

3.2.1 Subjects

This project was undertaken with the approval of the Research and Ethics Committee, in The Queen Victoria Hosital, Adelaide, South Australia.

Muscle protein breakdown rates were evaluated in a total of 88 foetuses and infants. Informed consent was obtained from the parents of each of the infants before the sample collections were obtained.

In this section of the study, the infants were sub-divided into the clinical categories of "normal", "borderline" and "intrauterine growth retarded" by the same stringent criteria as those which were used in the neonatal water turnover study previously discussed.

3.2.2 Diet and 3-Methylhistidine

Studies on animals (Omstedt et al, 1978) and humans (Marliss et al, 1979), have shown that ingestion of meat protein proportionally increases the amount of 3-MH excreted in the urine. However, the 3-MH excretion is not affected if the sole protein source is milk. The infants in this study were only receiving milk; thus no dietary changes were necessary to obtain meaningful 3-MH values.

3.2.3 Sample Collections

The neonatal urine samples were collected using urine collection bags (Dansk Coloplast, Denmark). Amniotic fluid samples were obtained either

the delivery of the infant, or when amniocentesis was performed for the detection of foetal abnormalities. The urine samples were kept frozen at -20°C until the analyses were performed. The analysis of single urine voidings has been shown by Tomas et al (1979) to be a satisfactory method for the determination of muscle protein breakdown rates. This alleviates the use of the cumbersome 24 hour urine collections which are difficult to obtain in infants. This technique has been employed in this study.

3.2.4 Creatinine Determination

An automated method was used for the determination of creatinine in urine, serum and amniotic fluid, employing the Technicon Auto Analyser method N-11b (see appendix 5). This method uses the alkaline picrate reaction to determine the concentration of creatinine in the sample. The colour develops in a time delay coil and this is read in a Colorimeter at 505 mμ using a 15 mm tubular flow cell. Up to 60 creatinine determinations can be performed by this method per hour. A flow diagram of the method is given in figure 3.2.1.

3.2.5 3-Methylhistidine Determination

3-Methylhistidine concentrations in neonatal urine, amniotic fluid and serum were determined using the rapid, automated technique described by Murray et al (1981). This automated method uses a reaction of fluorescamine, with amines, and interference by histidine and histamine in the sample is removed by treatment of the sample with aldehydes prior to the addition of the fluorescamine (Fig. 3.2.2). Six to seven samples can be analysed per hour by this technique, rendering it extremely useful for the large numbers of analysers, required by this study.

Results from this automated method correlated well with the measurement of 3-methylhistidine by ion exchange chromatography with prior acid treatment of the sample (Burgoyne et al, 1982). Therefore N-acetyl-3-methylhistidine was

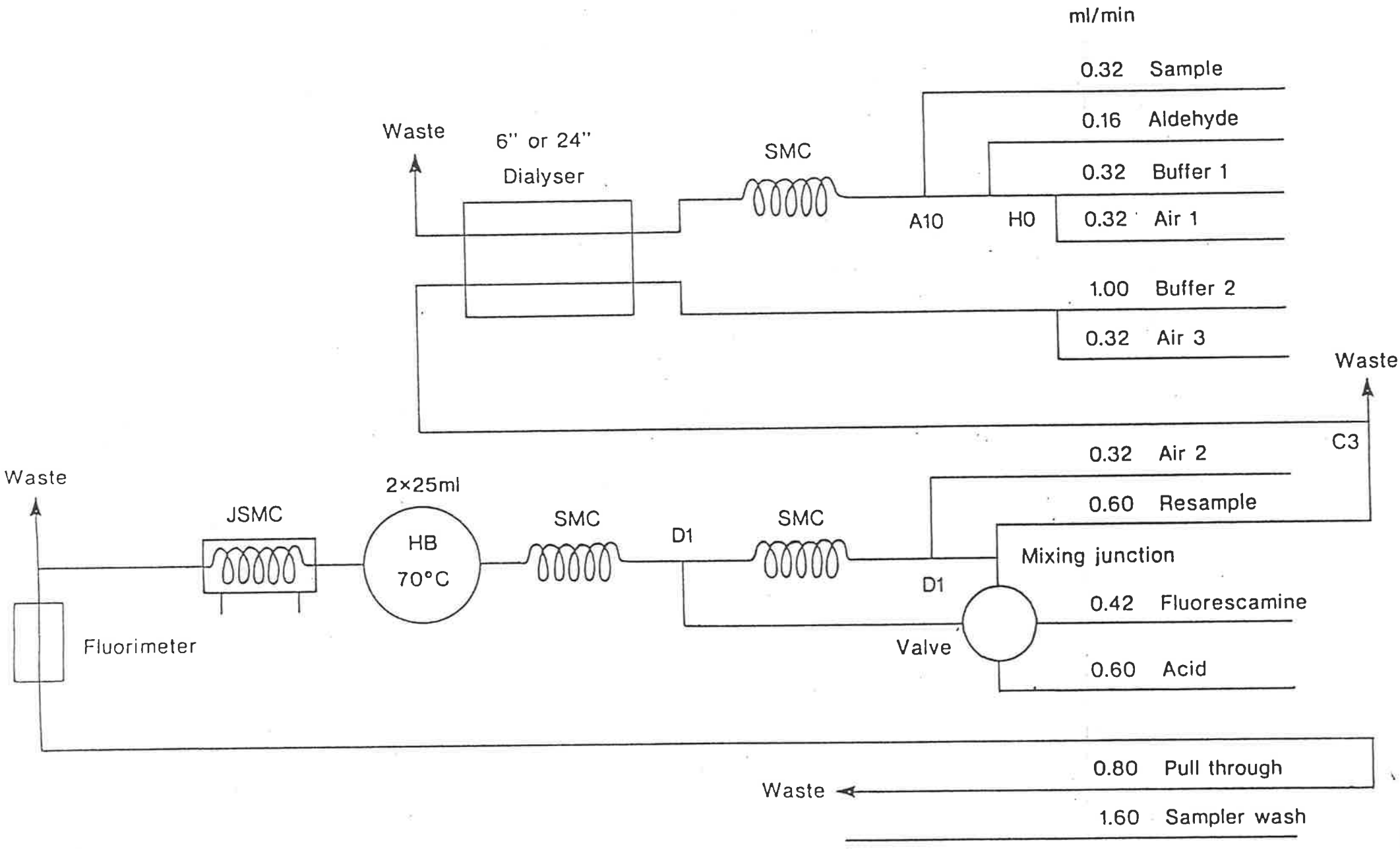


Figure 3.2.2 Flow diagram of 3-methylhistidine determination.

not present in significant quantities in the samples examined and so did not interfere with the results obtained by the automated technique.

3.2.6 Calculation of Muscle Protein Breakdown Rates

To calculate the rates of muscle protein breakdown in the infants in this study the following equation taken from Burgoyne et al (1982) has been used:

$$\% \text{ protein degradation per day} = 100 \times \frac{\mu\text{mol}}{\mu\text{mol}} \frac{[\text{3-MH}]/\text{ml}}{[\text{Cr}]/\text{ml}} \times \frac{1000}{2.42 \times 4 \times 113}$$

3.3 RESULTS

In the study of muscle protein breakdown in early human development, a total of 88 infants participated. 25 infants were clinically classified as normally grown, 22 as borderline and 31 as intrauterine growth retarded. One infant was postmature and two were infants of diabetic mothers. Four infants were found to have high amniotic fluid alpha feto protein levels, indicating the presence of a major neural tube abnormality in the foetus. These pregnancies were terminated. There was also a foetus with a 14/21 translocation, who was later aborted.

A total of 72 neonatal urine samples were collected from 40 infants and for 21 infants the first urine voided was collected. 44 amniotic fluid samples were examined and cord blood, maternal blood and maternal urine samples were collected where possible. Serial neonatal urine samples were analysed on 9 infants.

The infants in this study ranged in birth weight between 630 and 4,060 grams and the gestational age range was 31 to 40 weeks in infants from whom neonatal urine samples were collected. The gestational range of foetuses from whom amniotic fluid samples were taken was 12 to 43 weeks.

3.3.1 Neonatal Urine Samples

Table 3.3.1 summarizes the clinical classifications of the infants participating in this section of the study. Of the 40 infants in the study, 26

Table 3.3.1 Summary of the clinical classification of the infants in the study.

Classification	Number
Total Number of Infants	40
Normally Grown Infants	7
Borderline Infants	9
Growth Retarded Infants	21
Postmature Infants	1
Infants of Diabetic Mothers	2

were mature (37-42 weeks gestation) and 13 were premature (<37 weeks gestation). One infant was postmature.

On the initial analysis of the neonatal urine samples, there appeared to be a very wide range of 3-MH/Cr values (table 3.3.2) ranging from a molar ratio x 10^3 of 9.10 to 56.30. There was also no evidence of any variations of 3-MH/Cr ratios between normal, borderline or intrauterine growth retarded infants. On closer examination, however, it seemed to the author that the 3-MH/Cr values in neonatal urine were time dependent. Therefore the 3-MH/Cr values were plotted as a function of time of urine voidance after birth, and this is illustrated in figure 3.3.1. It is clear from this diagram that indeed the 3-MH/Cr ratios are very time dependent and the values vary considerably, especially in the first 50 hours after birth. This explains the wide range of 3-MH/Cr values recorded by Tomas et al (1978) in newborn infants, who placed all the data from neonates together irrespective of the time of voidance.

In the first 50 hours after birth, there is a rapid increase in the 3-MH/Cr ratio in normally grown infants. The values then plateau at approximately 50 hours, after which time there is a slow decrease. Burgoyne et al (1982) has shown with a larger number of urine samples (54) from 23 appropriate-for-gestational age (normally grown) infants, that there is a three-fold increase in the 3-MH/Cr x 10^3 molar ratio to about 38.0 within 40 hours after birth. In full term infants this represented a muscle protein breakdown rate of approximately 3.40% per day. The dashed line in figure 3.3.1 represents the time course of 3-MH/Cr ratios in the first 8 days of life as estimated from the normally grown, full-term infants in the study by Burgoyne et al (1982).

There appeared to be no statistical difference between the 3-MH/Cr values of normal, borderline or intrauterine growth retarded neonates in the first 50 hours after birth. Using least square regression analysis on the 3-MH/Cr values of growth retarded infants, however, a slope has been calculated for

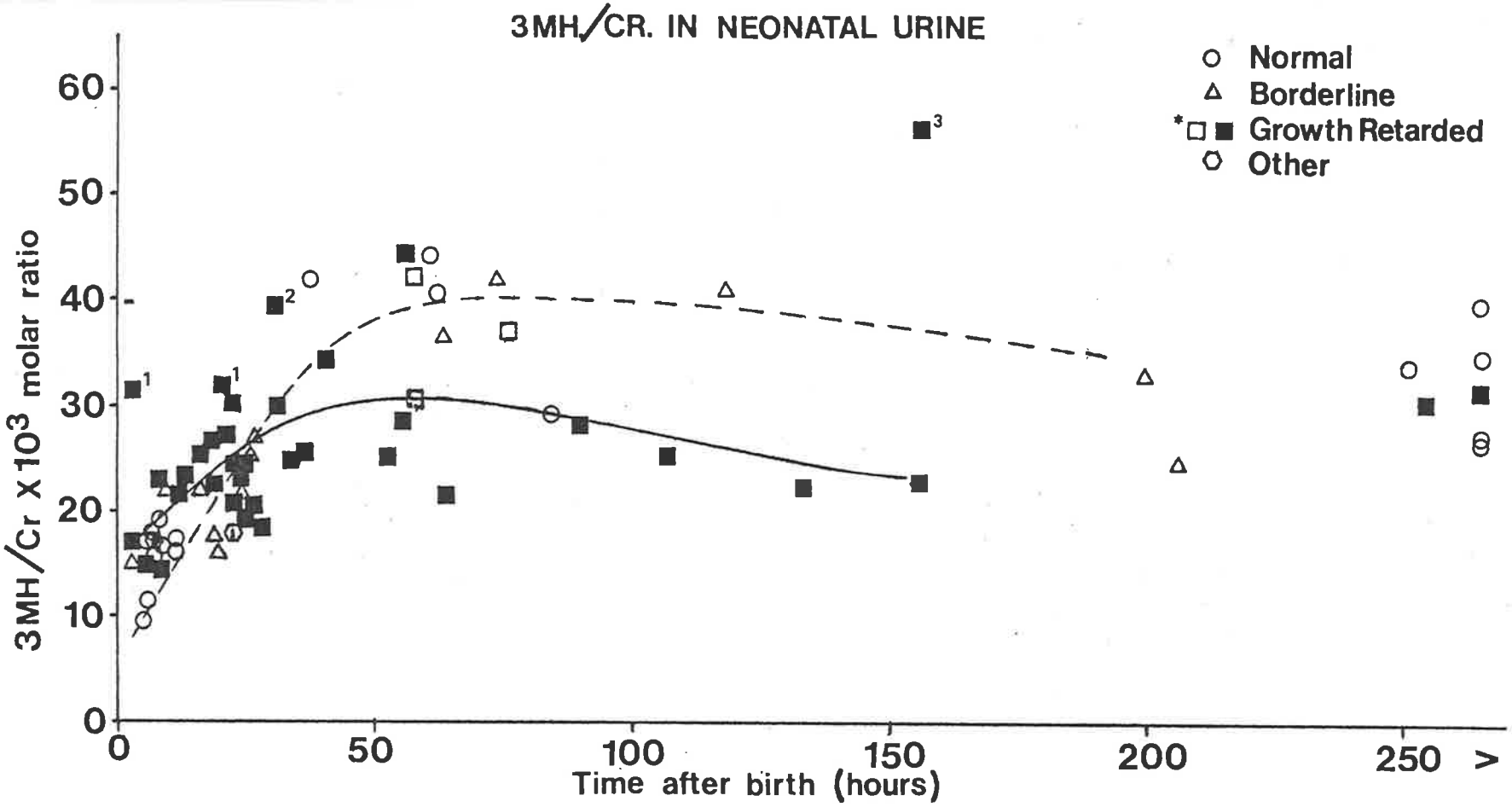


Figure 3.3.1 : 3MH/Cr x 10^3 molar ratios of neonatal urine samples plotted as a function of time after birth. The continuous and dashed lines represent the time course of 3MH/Cr x 10^3 in growth retarded infants and normal infants respectively.
The open squares represent infants clinically classified as growth retarded but assessed paediatrically as normal.
The same legend applies to figures 3.3.1 to 3.3.4.
"Other" categories;

1. Infants of diabetic mothers
2. Infants with neurotubular disease
3. Infants of mothers who were carriers of Duchenne Muscular Dystrophy
4. Infants with 14/21 translocations
5. Postmature infants

Table 3.3.2: 3-MH/Cr x 10^3 molar ratios of neonatal urine samples. 16 samples were obtained from 7 normally grown infants, 13 samples from 9 borderline infants and 39 samples from 21 growth retarded infants.

NORMALLY GROWN INFANTS	BORDERLINE INFANTS	GROWTH RETARDED INFANTS
3MH/Cr x 10^3 Molar Ratio	3MH/Cr x 10^3 Molar Ratio	3MH/Cr x 10^3 Molar Ratio
9.08	14.83	14.40
11.30	15.87	15.80
14.94	17.22	16.75
15.03	21.28	18.32
15.75	21.71	19.11
16.23	21.80	20.07
18.83	24.57	20.93
26.07	25.51	21.33
26.95	26.67	21.49
29.27	32.99	22.11
33.96	36.38	22.25
34.77	40.95	22.52
39.07	41.88	22.66
40.46		23.24
41.85		23.42
44.23		24.43
		24.94
		24.97
		25.00
		25.03
		25.36
		25.41
		26.70
		27.03
		28.21
		28.42
		29.70
		30.03
		30.06
		30.82
		31.17
		31.33
		31.90
		34.08
		37.00
		38.68
		41.95
		44.30
		56.31

the first 50 hour period and it is represented as an unbroken line in figure 3.3.1. From the 50 to 160 hour period after birth, the line for growth retarded infants has been drawn by inspection. The two growth retarded infants marked with number 1's in the diagram have much higher values than is expected for the time that these urine samples were collected. A possible reason for these high values may be that the infants were very premature, having gestational ages of 29 and 32 weeks. The 3-MH/Cr value marked with a number 2 was also higher than expected. This value was obtained from one of a series of urine samples taken from an infant and as it was much higher than all the other values from the same child, it seems probable that this value is incorrect.

For growth retarded infants, there was a slower increase in the urinary 3-MH/Cr values in the first 50 hours after birth than for normal infants (Fig. 3.3.1). The values then plateaued (50-100 hours) at a lower value of approximately 30.0 compared to the molar ratio of about 40.0 for normally grown infants in this study. Burgoyne et al (1982), found that the plateau for their normally grown infants was at a 3-MH/Cr x 10^3 molar ratio of 37.2. The mean 3-MH/Cr $\times 10^3$ molar ratio for growth retarded infants between 50 and 200 hours after birth was 29.6 (N=11). This mean was calculated by excluding the value marked with a number 3 because it was though to be erroneous. However, even if this value were included in the mean, it would not alter the mean significantly (i.e. mean = 31.9, N=12). For normally grown infants, in this period of time, Burgoyne et al (1982) had a mean of 36.0 (N=29). A mean of 29.6 for growth retarded infants represents a muscle protein breakdown rate of 2.71% per day, compared to 3.40% for normal infants. The degradation rate was significantly lower in growth retarded infants than normally grown infants.

Three growth retarded infants are represented in figure 3.3.1 by open squares. These infants were not severely growth retarded and were classified as normal by the paediatric assessment. Borderline infants in this study tended to follow the curve of the normally grown infants, rather than the growth retarded ones.

After the 3-MH/Cr values in normally grown infants reached the plateau between 50 and 100 hours, a slow decrease was observed with time (Fig. 3.3.1). This has been observed more conclusively in Burgoyne et al (1982). However, more values are necessary to substantiate this. The 3-MH/Cr values obtained after 250 hours and up to 14 days after birth had a mean of 32.2 (N=5) in normally grown infants. Two urine samples obtained from growth retarded infants in this period of time had 3-MH/Cr values which were not significantly different from those of normally grown infants. While it appears that the 3-MH/Cr ratio decreases with time after the plateau in normal infants, the values obtained from growth retarded infants firstly decreased (100-150 hours) and then increased after 250 hours.

The results of 3-MH/Cr ratios obtained from serial collections of urine samples up to 50 hours after birth are presented in table 3.3.3. 3-MH/Cr

Table 3.3.3 Serial measurements of 3-MH and Cr in neonatal urine of 2 normal, 2 borderline and 2 growth retarded infants.

SUBJECT	3MH/Cr x 10^3 MOLAR RATIO	[3MH] (nmol/ml)	[Cr] (μmol/ml)	TIME AFTER BIRTH (hours)	CLASSIFICATION
Infant 1	15.75	54.51	3.46	12	Normal
	41.85	77.85	1.86	38	
Infant 2	14.94	19.12	1.28	5	Normal
	16.23	23.86	1.47	9	
Infant 3	15.87	36.19	2.28	20	Borderline
	21.28	47.87	2.25	25	
Infant 4	25.51	179.85	7.05	16	Borderline
	26.67	29.34	1.10	18	
Infant 5	21.49	96.93	4.51	12	Growth Retarded
	23.24	66.94	2.88	13	
	27.03	16.76	0.62	21	
	20.93	14.86	0.71	23	
	20.07	22.28	1.11	27	
	38.68	29.01	0.75	31	
	24.94	24.94	1.00	34	
	25.41	18.80	0.74	36	
Infant 6	23.42	33.58	1.43	24	Growth Retarded
	24.97	35.23	1.41	25	
	25.36	35.38	1.40	26	
	26.70	50.79	1.90	27	

determinations of neonatal urine from one postmature infant and 2 infants of

diabetic mothers were all made within the first 24 hours after birth, and they did not vary significantly from the values from normal, borderline or growth retarded infants in this study.

3.3.2 Amniotic Fluid Samples

The measurement of 3-MH in amniotic fluid has never before been attempted prior to the present study and it seemed to the author that it may be possible to determine muscle protein breakdown rates antenatally in foetuses. With this aim in mind, amniotic fluid samples were collected (between October 1980 and December 1981) from 44 subjects of 12 to 43 weeks gestation and 3-MH and Cr concentrations calculated. Miodovik et al, 1982, have recently performed similar investigations and their findings will be discussed later in this presentation.

To ensure that the measured 3-MH in amniotic fluid was representative of the foetus and not the mother, diet histories were obtained from mothers in the study. Since dietary meat intake influences the maternal urinary 3-MH concentration, an estimate of the total ingested muscle protein per day was made (for 3 days prior to amniotic fluid collections) and these were compared with amniotic fluid 3-MH values. Also, maternal urine samples were collected and analysed for 3-MH concentrations at the same time as the amniotic fluid was collected. Neither the quantity of meat ingested by the mother nor the maternal 3-MH concentration correlated with the concentration of 3-MH in the amniotic fluid.

In table 3.3.4 a summary of the classification of foetuses from whom amniotic fluid samples were taken, are presented.

Table 3.3.4 Summary of foetuses from whom amniotic fluid samples were obtained.

Classification	Number
Total Number of Foetuses	42
Normally Grown Foetuses	18
Borderline Foetuses	6
Growth Retarded Foetuses	8
Postmature Foetuses	1
Foetuses of Diabetic Mothers	2
Foetuses of Carriers of Duchenne Muscular Dystrophy	2
Foetuses with Neurotubular Defect	4
Foetuses with a 14/12 Translocation	1

Figure 3.3.2 illustrates the 3-MH concentrations in amniotic fluid samples. The means and standard deviations of the 3-MH concentrations in amniotic fluid in early (12-22 weeks) and late (36-43 weeks) gestation are presented in table 3.3.5. Two foetuses had amniotic fluid sample collections in both early and

Table 3.3.5 Means and standard deviations of 3-MH concentrations in normal, borderline and growth retarded infants in early and late gestation amniotic fluid samples.

Gestational Age (weeks)	NORMAL		BORDERLINE		GROWTH RETARDED	
	12-22	36-33	12-22	36-43	12-22	36-43
Total Number	14	5	4	3	2	5
Mean (μM)	3.78	2.77	4.39	3.16	2.90	3.80
S.D.	0.78	1.17	0.90	2.00	0.48	1.66

late gestation. (These are joined by dashed lines in figure 3.3.2). One of these foetuses was normally grown and the other was clinically classified as borderline. These values are joined by the dashed lines in figures 3.3.2-4.

There was a statistically significant increase in the creatinine concentration in amniotic fluid from early to late gestation (Fig. 3.3.3 and table 3.3.6).

Table 3.3.6 Means and standard deviations of Cr concentrations in normal, borderline and growth retarded infants in early and late gestation amniotic fluid samples.

Gestational Age (weeks)	NORMAL		BORDERLINE		GROWTH RETARDED	
	12-22	36-33	12-22	36-43	12-22	36-43
Total Number	14	5	4	3	2	5
Mean (μM)	58.0	204.6	62.0	156.3	64.5	214.8
S.D.	6.8	47.9	5.7	23.5	12.0	58.3

No significant difference was observed between Cr concentration values from the 3 clinical classifications of infants (i.e. normal, borderline and growth retarded). A comparison of late gestation Cr concentrations with birth weights of the infants showed that there was no correlation and this is in agreement with

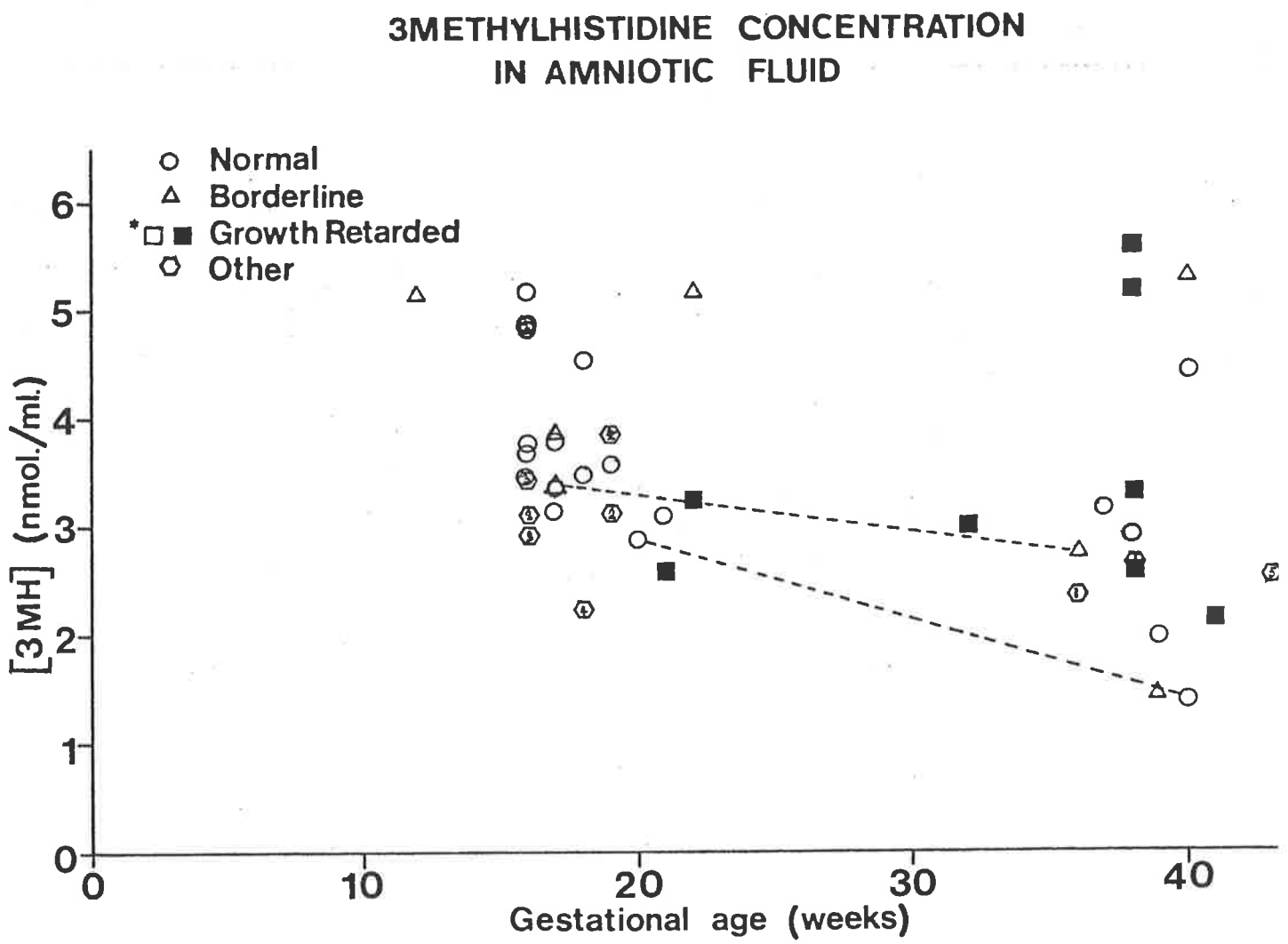


Figure 3.3.2 : 3Methylhistidine concentration in amniotic fluid taken from foetuses from 12 to 43 weeks gestation. The dashed lines join amniotic fluid values taken from the same foetus.

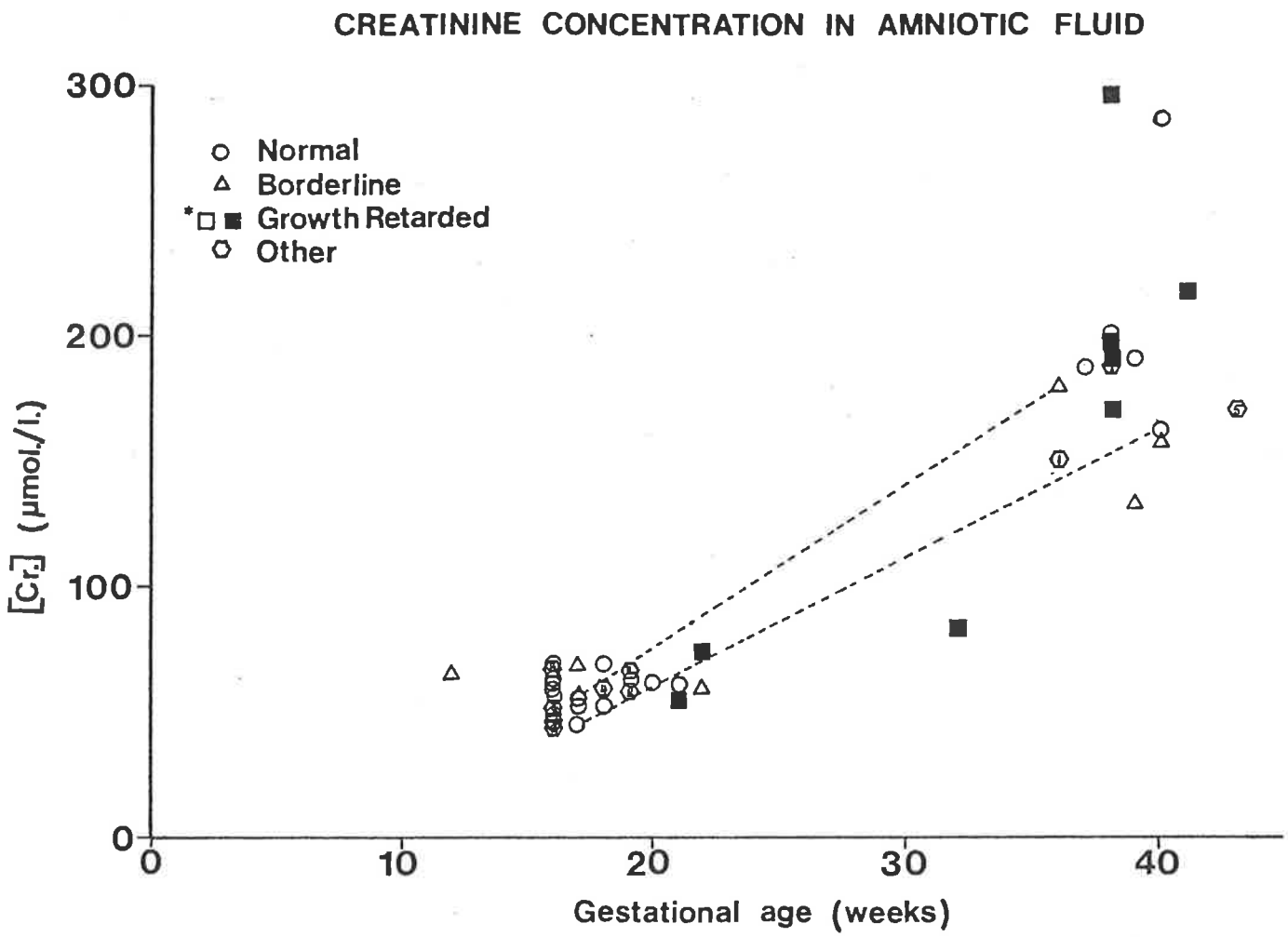


Figure 3.3.3 : Creatinine concentration in amniotic fluid taken from foetuses from 12 to 43 weeks gestation. The dashed lines join amniotic fluid values taken from the same foetus.

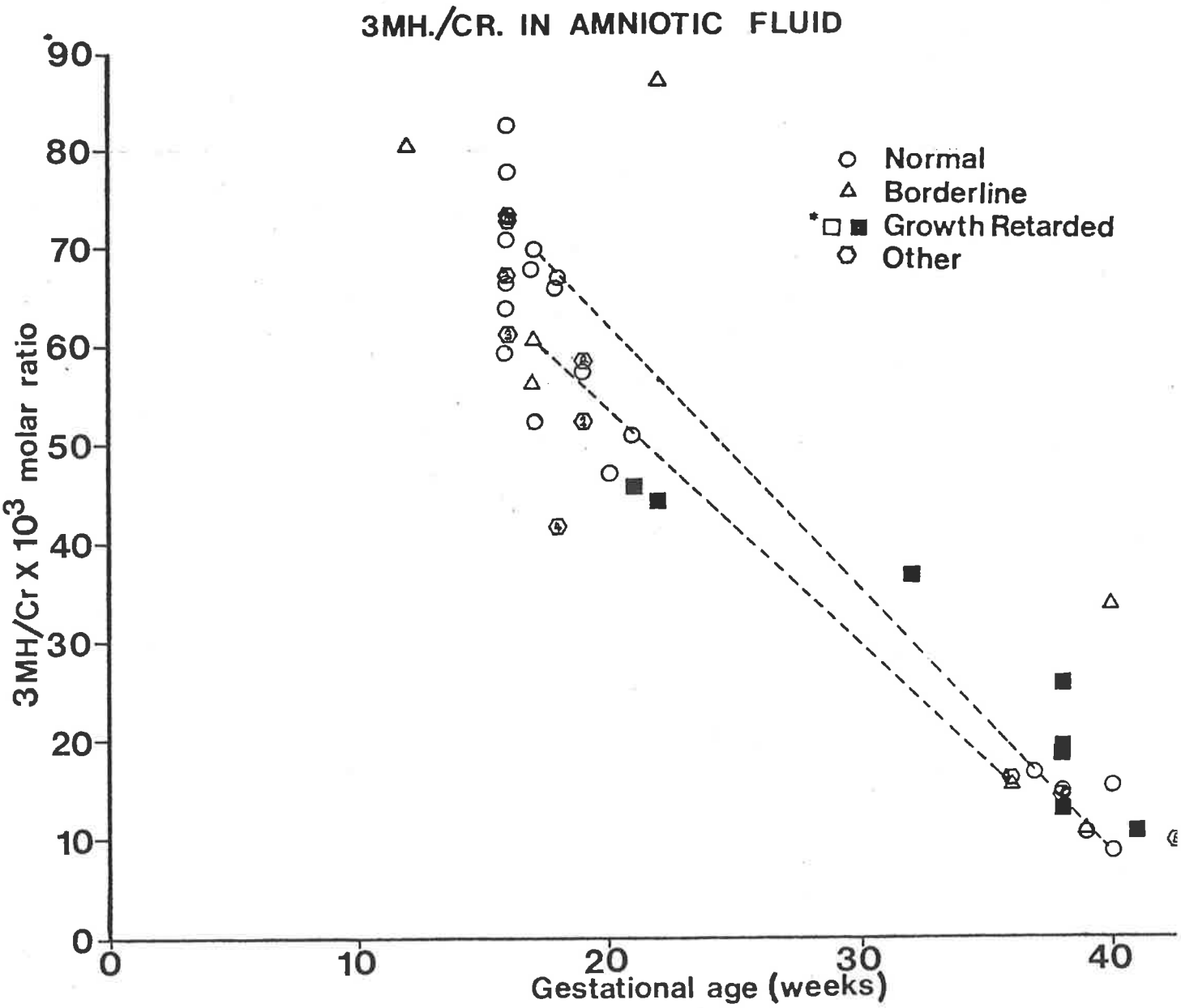


Figure 3.3.4 : 3MH/Cr ratios in amniotic fluid taken from foetuses from 12 to 43 weeks gestation. The dashed lines join amniotic fluid values taken from the same foetus.

Droegegemueller et al (1969).

Figure 3.3.4 illustrates the amniotic fluid 3-MH/Cr molar ratios of normally grown, borderline, growth retarded and other foetuses as a function of gestational age. There was a marked decrease in the 3-MH/Cr molar ratio from early to late gestation which was due to the substantial increase in the Cr concentration and only a slight decrease in the 3-MH concentration (table 3.3.7).

Table 3.3.7 Means and standard deviations of 3-MH/Cr x 10^3 molar ratios in normal, borderline and growth retarded infants, in early and late gestation amniotic fluid samples.

Gestational Age (weeks)	NORMAL		BORDERLINE		GROWTH RETARDED	
	12-22	36-43	12-22	36-43	12-22	36-43
Total Number	14	5	4	3	2	5
Mean x 10^3 Molar Ratio	64.12	13.21	71.07	19.96	45.05	17.58
S.D.	9.88	3.45	15.14	12.08	0.94	5.93

Four of the 6 growth retarded foetuses in the period between 32 and 43 weeks gestation had higher 3-MH/Cr ratios than the normally grown foetuses, although this result was not statistically significant (Wilcox test, $p<0.10$) with the small number of samples tested. There was only one amniotic fluid sample collected between 23 and 35 weeks gestation inclusive. This resulted because amniocenteses are not usually performed in this period for the detection of foetal abnormalities and relatively few infants are delivered before 35 weeks gestation.

Amniotic fluid samples collected at delivery as well as the corresponding first voided neonatal urine samples were obtained from 11 infants (table 3.3.8). In these infants, the 3-MH/Cr molar ratios, from amniotic fluid samples, correlated very closely with the values obtained from the first neonatal urine samples, provided that they were voided within 12 hours after birth ($r=0.86$). The mean 3-MH/Cr x 10^3 molar ratio in late amniotic fluid was 16.5, while the mean in the first voided urine samples was 16.0. In the first urine samples voided more than 12 hours after birth, the 3-MH/Cr ratios were significantly

Table 3.3.8 Late gestation amniotic fluid and the corresponding first voided neonatal urine 3-MH/Cr x 10^3 molar ratios in 11 infants.

Late Gestation Amniotic Fluid	FIRST VOIDED NEONATAL URINE	
3MH/Cr x 10^3 Molar Ratio	3MH/Cr x 10^3 Molar Ratio	Time of Collection (Hours)
15.40	14.83	3
19.24	16.75	4
10.37	9.08	5
14.50	14.94	5
18.70	15.80	6
15.60	17.48	7
15.52	15.03	7
25.99	22.66	8
13.58	16.98	12
45.71	31.90	21
10.66	30.06	23

higher than in the amniotic fluid. Although the 3-MH/Cr molar ratios were almost identical in first urine samples and amniotic fluid, as described above, the actual 3-MH and Cr concentrations themselves in the first voided urine samples were much higher than in amniotic fluid. This is because the large volume of amniotic fluid in the amniotic fluid sac dilutes the concentrations of these two substances, but does not change their ratio.

Maternal serum and amniotic fluid was obtained from 7 subjects, and in all cases the amniotic fluid 3-MH concentration was higher than in the maternal serum. From one, normally grown infant it was possible to obtain a cord serum sample, first neonatal urine and amniotic fluid as well as a maternal urine and serum sample. Results from these samples are presented in table 3.3.9.

Table 3.3.9 3-MH/Cr ratios, 3-MH and Cr concentrations from samples collected from a mother and her normally grown infant.

SAMPLE	3MH/Cr x 10^3 Molar Ratio	3MH (n mol/ml)	[Cr] (μmol/ml)
Amniotic Fluid	15.52	4.44	0.286
Maternal Urine	15.87	163.3	10.29
First Neonatal Urine	15.03	209.5	13.94
Cord Serum	63.21	5.31	0.084
Maternal Serum	41.55	3.49	0.084

The 3-MH/Cr ratios in the amniotic fluid of foetuses with neural tube defects, the postmature foetus, foetuses of carriers of Duchenne muscular dystrophy and foetuses of diabetic mothers were not significantly different from the 3-MH/Cr values of normally grown foetuses in this study. The two foetuses of carriers of Duchenne muscular dystrophy were, however, both normal females. The foetus with the 14/21 translocation did have a much lower 3-MH/Cr ratio in the amniotic fluid than any other foetuses in early gestation.

3.4 DISCUSSION

Investigations have been performed in the present study on muscle protein breakdown rates (using 3-MH/Cr ratios in urine as a marker), in the first few weeks following birth, as well as in amniotic fluid from 12 to 43 weeks gestation.

Both from serial determinations of the 3-MH/Cr ratio and from individual urine samples, it is evident that there is a marked increase in the urinary 3-MH/Cr molar ratio in the first 50 hours after delivery of the infant. In normally grown infants, the rise is steep and it plateaus at a 3-MH/Cr x 10^3 molar ratio of about 40.0. The increase observed with growth retarded infants is lower than for normally grown infants, with the plateau occurring at a lower level, the mean 3-MH/Cr x 10^3 molar ratio being 29.6. The scatter of the 3-MH/Cr values for the two groups is however very wide in the first 50 hours after birth. Therefore, this time span is not useful for the determination of IUGR.

Two suggestions have been proposed by Burgoyne et al, 1982, for the observed rapid increase in the 3-MH/Cr ratio in the first 50 hours following birth. Firstly, it may reflect a combination of birth-associated trauma and poor nutrition in the postnatal period. Alternatively, they suggest that it may not be due to a rapid rise in muscle protein breakdown in the newborn infant from a value of 1.45%/day to 3.40%/day, but rather reflects a more effective passage of 3-MH than Cr from the foetus to the mother prior to birth. Following birth, when the mother is no longer removing the 3-MH and Cr from the foetal circulation, the neonatal urine gradually attains the true levels, these occurring after 50 hours following birth.

The Cr concentration in the neonatal urine in the first 50 hours was relatively steady, or sometimes decreased dlightly, but the 3-MH concentration increased. In serial collections of neonatal urine, the first urine voided usually had higher concentrations of Cr and sometimes 3-MH than subsequent

samples and the level of Cr and 3-MH was time dependent. The longer it took for the first urine to be voided, the higher the concentration of 3-MH and Cr in the urine sample. From two normally grown infants, serial urine samples collected before 50 hours after birth showed a marked increase in the 3-MH/Cr ratio and there was also a rapid increase in the actual 3-MH concentration. In contrast, two growth retarded infants showed a slow increase in the 3-MH/Cr ratio in the first 50 hours and the 3-MH concentration decreased initially in one case and only marginally increased in the other. The slower increase in the 3-MH/Cr concentration and plateauing at a lower level in growth retarded infants compared to normally grown infants, may be due to the lower amount of muscle protein being present in growth retarded infants.

The plateauing of the 3-MH/Cr molar ratio in growth retarded infants between 50 and 200 hours after birth occurred at a lower level in growth retarded infants than in normal infants, with a muscle protein breakdown rate of 2.71% per day in growth retarded infants, compared with 3.40% per day estimated by Burgoyne et al (1982) in normally grown infants. More neonatal urine samples need to be analysed in this period of time to conclusively prove that growth retarded infants consistently have lower rates of muscle protein breakdown between 50 and 200 hours following birth than normal infants. Results indicate that it may be possible to detect IUGR from neonatal urine collected from infants between 50 and 200 hours after birth. The lower rate of breakdown of muscle protein in growth retarded infants in this study is in agreement with results in children suffering from conditions of protein-calorie malnutrition (see Introduction). However this result is contrary to that found by Seashore et al (1980) on stressed infants, and also the results of Pencharz et al (1981) on normal and growth retarded infants. Notwithstanding this, these two studies were conducted on older infants who were probably in a state of nutritional rehabilitation, in which case it would be expected that their muscle protein breakdown rates would be higher than those of normal infants.

The growth retarded infants in this study still reflected the state of nutritional deprivation which they suffered in the intrauterine environment.

In the present study, the infants who were clinically classified as borderline seemed to follow the same curve of 3-MH/Cr excretion as those of the normally grown infants. It appears that the 3-MH/Cr ratio in neonatal urine may only be a qualitative test for IUGR rather than a quantitative one. The 3-MH/Cr ratio distinguishes severely growth retarded infants from normally grown infants.

The total number of neonatal urine samples analysed for 3-MH/Cr ratios in this study were greater in the period before 50 hours following birth, than between 50 and 200 hours. This occurred because it was thought at the time the urine collections were made, that the region before 50 hours may be a more useful time to detect IUGR in infants if it were present. However, it now seems that in the 50-200 hour period, it may be easier and more meaningful to identify IUGR. Analysing the first urine samples voided by the infants to detect growth retardation did not prove very useful as a wide scatter of values resulted. Both gestational age and time after birth has to be considered, which complicates the analysis of the resultant 3-MH/Cr ratios.

In amniotic fluid, it was interesting to observe a decrease in the 3-MH/Cr ratio with increasing gestational age. This decrease was so significant because there was a marked increase in the Cr concentration with only a slight decrease in the 3-MH concentration. No differences between 3-MH/Cr values were observed in normally grown, borderline or growth retarded infants in early gestation (12-22 weeks) but this was expected because growth retardation only becomes pronounced in late gestation. In amniotic fluid samples obtained at the time of delivery (i.e. late gestation samples), the growth retarded infants appeared to have higher 3-MH/Cr ratios than normally grown infants, although the results were not statistically significant because of the small numbers of samples analysed.

A study was performed recently by Miodovnik et al, 1982, measuring 3-MH/Cr ratios in amniotic fluid collected within 6 days of delivery, from 15 foet-

uses under the 10th percentile in weight (i.e. growth retarded) and 27 normal birth weight foetuses. They found that the growth retarded foetuses had significantly higher 3-MH/Cr ratios than the normal infants with the mean molar 3-MH/Cr x 10^3 ratio being 15.9 and 6.2 respectively.

In the present study, the normally grown foetuses also had lower 3-MH/Cr values than the growth retarded foetuses. However, the normal foetuses had higher 3-MH/Cr values than those found by Miodovnik et al (mean = 13.2). The growth retarded infants had similar 3-MH/Cr values (mean = 17.6). The higher 3-MH/Cr values found in this study compared to the study by Miodovnik et al may have been due to the simple colorimetric method used by Miodovnik et al to measure creatinine which gives erroneously high values. Also, their study only used the 10th percentile in weight to classify infants as intrauterine growth retarded. The techniques used in this study to identify growth retarded infants were more stringent.

In growth retarded foetuses the 3-MH/Cr ratios in late gestation amniotic fluid may be greater than for normal foetuses, since these foetuses are probably restricted in both protein and energy intake and muscle protein is degraded in the body to provide the necessary energy requirements (Young and Munro, 1978).

CHAPTER IV

CONCLUSION

CONCLUSION

In this thesis an attempt has been made to identify infants affected by IUGR using two methods which are easier and more accurate than currently used techniques. Weight-for-gestational age charts do not consider community variations, sex and parity, weight and height of the mother, have all been proven to affect the birth weight of the infant. In theory, if all these factors are considered, the accuracy of the growth standards are increased, but then errors in the percentiles themselves are produced because of the decrease of sample size for each gestational age. Clinical, paediatric examination of the infant soon after birth can detect IUGR if it is severe enough, as illustrated in the infant on the left of the photography at the front of this thesis (page ix). However, when the growth retarding effects are not quite so severe, or when the infant is premature as well (e.g. infant on the rightof the photograph), then the diagnosis of IUGR is not quite as simple.

At the present, the effects of IUGR in later life are controversial mainly because of the confusion over the actual definition of IUGR and the problems of its identification (see General Introduction). The degree of growth retardation is also very difficult to assess by current methods. Although Butler (1973) has found that severe neurological defects are rare in growth retarded infants, minor handicaps, for example learning difficulties, are quite frequent. By identifying and quantifying IUGR, a better understanding of future effects may be possible and then an attempt can be made to correct them soon after birth rather than in later life when the sequelae of IUGR are well established.

The two biochemical markers used to identify IUGR in this thesis were the determination of water turnover rates and muscle protein breakdown employing 3-MH/Cr ratios. The rate constant gives a measure of the rate of water turnover in the infant. This method is a useful retrospective test for the identification of growth retardation in neonates. It is valuable in that the test

is objective, independent of gestational age, behaviour and appearance at birth. The results suggest that the test is not only qualitative but also quantitative.

Several urine samples are however required to determine the rate constant and spectrophotometric analysis takes some time to perform; thus, a faster method of identifying IUGR was sought. The measurement of muscle protein breakdown by the determination of 3-MH/Cr ratios seemed a feasible alternative. Using this technique it is possible that only a single uring sample need be collected between 50 and 200 hours after birth to detect IUGR. If this proves to be a reliable test, then growth retardation could be easily and quickly identified. It appears, however, that the 3-MH/Cr ratio is only a qualitative index of IUGR and does not indicate the degree of growth retardation in the infant. Hence, it may be valuable to initially detect IUGR by determining muscle protein breakdown rates and then if the infant had suffered growth retardation it can be quantified using water turnover rates. One additional advantage of using 3-MH/Cr ratios is that it may be possible to identify growth retardation antenatally. A recently published study by Miodovnik et al (1982), has shown that amniotic fluid collected within 6 days of delivery from 15 foetuses under the 10th percentile in weight had significantly higher 3-MH/Cr ratios than foetuses greater than the 10th percentile. These findings are in agreement with the results of this study. However a greater number of amniotic fluid samples need to be analysed, before it can be confidently used as an index for growth retardation.

Further research in this area is vital. Suggestions for possible future investigations are as follows:

1. Neonatal urine samples should be collected in large numbers between 50 and 200 hours after birth to determine whether 3-MH/Cr ratios determined in this period of time reliably identifies IUGR.

2. Water turnover rates as well as muscle protein breakdown rates could be determined on each infant so that the rate constant and 3-MH/Cr ratio can

be compared.

3. Large numbers of amniotic fluid samples could be collected before delivery and analysed to determine whether antenatal 3-MH/Cr ratios are reliable indicators of growth retardation.

4. It is important to know the reason for the initial, rapid increase in the 3-MH/Cr ratio in the first 50 hours following birth. Cord blood (both arterial and venous), maternal serum, amniotic fluid and first urine samples could be analysed to provide more information about possible 3-MH and Cr transport across the placenta.

5. Serial neonatal urine samples could be collected from 50 hours after birth till several months of age to determine whether there is a gradual decline in the 3-MH/Cr ratio in normal infants and perhaps a gradual increase in the values of growth retarded infants.

6. Using water turnover rates, and possibly 3-MH/Cr ratios, the incidence of IUGR in the population could be determined.

7. The incidence of growth retardation in mutliple pregnancies could be found using water turnover rates.

8. The degree of IUGR as determined by water turnover rates could be correlated with handicaps found in later life in growth retarded infants. It may then be possible to provide early intervention to minimize or avoid these handicaps.

9. It has been found (Bank et al, 1971; McKeran et al, 1977) that sufferers of Duchenne muscular dystrophy have higher than normal rates of muscle

protein breakdown, but currently there is no antenatal test to diagnose this disease. There is a possibility that Duchenne muscular dystrophy may be identified using 3-MH/Cr ratios from amniotic fluid obtained in early pregnancy. In this study, both of the foetuses of carriers of Duchenne muscular dystrophy were females and so were not affected by the disease, hence higher than normal 3-MH/Cr ratios were not observed. However, high 3-MH/Cr ratios may occur in affected males.

APPENDIX 1 Fetal growth survery: Antenatal details

1. Name _____ Mother's Initials _____

2. Order at birth: 1 / 2 / 3 / 4 (1 = singleton 2 = 1st Twin)

3. Obstetric case no. _____

4. Accession no. _____

5. Infant's sex: M / F

6. Address: (a) Personal _____

_____

(b) Stable for follow-up, e.g. parent's address:

_____

_____

7. Date of birth _____

8.

8. Date and time of D_2O administration _____

9. Infant's weight at that time _____ gr.

10. Dose _____ gr.

11. Vomiting after administration of D_2O: Yes / No When _____

12. Bromide space Infant Yes / No

Data pairs _____

_____

Osmolality results _____

_____

Fetal growth survey: Antenatal details

1. Name _____ Initials _____

14. Gestation _____ certain/uncertain

15. Mother's age _____

16. Mother's parity _____

17. Mother's height _____

18. Race: Caucasian / Indigenous / Asian / Other _____

19. Social factor index: 1 2 3 4 5

20. Pre eclampsia: none / mild / moderate / severe

21. Diabetes: none / gestational / pre gestational

22. Previous S.F.D.: Yes / No Number _____

23. Antenatal suspicion of S.F.D.: Clinical _____

Hormone tests _____

Scans _____

C.T.G. _____

24. Drugs: None / Dexamethazone / Others _____

25. Smoking: 0 1-10 11-20 20+ per day

26. Alcohol: Nil / moderate / heavy

27. Other antenatal diseases: APH / Prem Rupt. Membs. / ess. hypertension/

other _____

28. Mode of delivery: Normal / forceps / elective C.S. / C.S. after labour

29. Fetal distress in labour: No / possibly / definitely

Cause _____

30. Mother on I.V. drip: Yes / No Amount _____

Solution _____

APPENDIX 2 Fetal growth survey: neonatal details

Name _____ No. _____

31. Birth weight _____ Percentile _____

32. Apgars _____ Percentile _____

33. Dubowitz _____ Percentile _____

34. Length _____ Percentile _____

35. Head circumference _____ Percentile _____

36. Triceps thickness _____ Percentile _____

37. Subcutaneous fat thickness _____ Percentile _____

38. Gross appearance: 1. Normal 2. Normal Premature
3. Small Normal 4. Growth Retarded
5. Prem. Growth Retarded 6. Large Growth Ret.
7. Postmature

39. Behaviour: Mature / immature

40. Hypoglycaemia: Detrostix <45 / <25

41. Polycythaemia: PCU <60 / >65 / >70

42. Fluid input: Oral - bottle amount _____ type _____
- breast amount _____
I.V. amount _____ type _____

43. Drugs _____

44. Temperature: <36° / normal / >37°

45. Phototherapy: Yes / No duration _____

46. Radiant heat warmer: Yes / No duration _____

47. Heat shield: Yes / No duration _____

48. Nursed in incubator: Yes / No duration _____

49. Vomiting or diarrhoea: _____

50. Infection _____

51. Congenital malformations: None / _____

APPENDIX 3

PERINATAL RISK SCORE FOR GROWTH RETARDATION

ANTENATAL:

Risk Score	Risk Factors
2	gestation: >42 weeks
1	age <17
1	social factor index 4 or 5
1	pre eclampsia: mild
2	moderate
3	severe
2	diabetes: gestational
3	pre-gestational (insulin dependent)
3	previous small for dates
2	antenatal tests: clinically S.F.D.
2	↓ oestriols
2	↓ B.P.D.
2	C.T.G. significant dips
1	maternal smoking: 1-10/day
2	11-20/day
3	20+/day
1	maternal alcohol: moderate
2	heavy
1	threatened miscarriage
2	placenta praevia in late pregnancy
3	antepartum haemorrhage
3	premature rupture of the membranes (>4 days)
3	intrauterine infection
3	essential hypertension
1	fetal distress in labour: possibly
2	definitely
3	multiple births: twins
4	triplets

POSTNATAL:

Risk Score	Risk Factors
1	Apgars: 1 minute <7
2	1 minute <4
2	5 minutes <7
4	5 minutes <4
3	Ponderal Index: <2.32
4	<2.26
2	fat thickness: <20 percentile
3	<10 percentile
2	hypoglycaemia (<25)
2	polycythaemia >70

APPENDIX 4 Rate constants ($x10^4$) and standard deviations of normal, borderline and growth retarded infants.

NORMAL		BORDERLINE		GROWTH RETARDED	
10^4K	S.D.	10^4K	S.D.	10^4K	S.D.
26.5	9.3	76.9	4.8	73.3	3.7
45.8	6.6	79.6	8.2	78.0	12.0
52.9	7.4	79.7	3.5	80.1	2.5
58.9	9.3	80.1	3.8	81.7	18.8
60.0	9.0	81.3	7.6	83.2	2.0
61.7	8.2	81.9	3.7	83.4	9.5
64.3	4.1	82.5	1.9	85.7	4.6
64.4	8.4	82.6	2.3	88.8	12.4
64.5	4.6	83.4	3.3	88.9	15.7
66.9	6.0	84.0	3.0	89.2	5.6
67.0	4.9	84.4	2.2	89.4	4.6
69.2	8.2	85.7	4.7	90.2	2.5
70.3	2.4	85.9	2.0	90.6	7.0
71.8	3.2	86.2	2.8	92.7	7.2
72.0	1.7	87.0	10.3	93.7	5.8
73.2	3.2	87.7	10.8	94.0	8.3
73.4	1.9	89.2	2.5	96.1	3.3
73.4	13.6	89.9	8.6	96.3	3.4
73.7	7.2	90.4	6.8	96.5	3.4
74.9	1.7	91.3	3.2	96.6	9.7
77.1	10.3	94.5	1.6	97.8	2.4
77.2	4.4	96.2	13.2	98.1	1.8
78.0	7.6	96.8	3.8	99.7	2.9
78.4	13.0	97.2	8.3	100.6	2.0
78.6	5.1	100.5	14.9	101.0	4.0
79.1	2.3			101.2	5.6
80.2	8.0			101.6	9.5
82.2	2.2			102.0	3.0
85.5	2.9			103.8	6.6
89.0	3.6			103.8	2.1
89.9	7.8			105.0	14.0
90.7	9.2			105.2	12.0
				108.8	2.0
				109.4	11.0
				110.6	8.9
				112.0	7.0
				113.8	3.2
				114.6	15.8
				116.2	12.2
				120.0	10.0
				122.0	20.0
				128.0	4.0
				134.0	11.0
				134.6	5.3
				138.5	9.2
				167.0	24.0

APPENDIX 5

Flow diagram of the automated method

for the determination of creatinine.

(Auto Analyser methodology N-11b).

CREATININE

GENERAL DESCRIPTION

The method employed is a modification of the procedure of Folin and Wu taken from the text "Practical Physiological Chemistry" by Hawk, Oser, and Summerson, The Blakiston Co., 12th Ed., p.506. The automation of the creatinine technique was accomplished by the following:

1. D. L. Stevens and L. T. Skeggs
2. A. L. Chasson, H. J. Grady and M. A. Stanley

The sample stream, segmented with air, is diluted with 0.9% sodium chloride. This combined stream enters the sample side of the Dialyzer. The recipient stream consists of water segmented with air. After emerging from the Dialyzer it is joined with one formed by a combination of saturated picric acid and 0.5 normal sodium hydroxide. The streams are mixed, sent through a time delay coil and then into the Colorimeter. The developed color is read at 505 mμ using a 15 mm. tubular flowcell.

REAGENTS

SALINE - Concentrate Solution: Technicon Formula T21-0029 (formerly 29-58)
Liquid Solution: T01-0029
Powder: T11-0029

Chemical Composition

1 - Sodium Chloride		9.0	gm.
2 - Distilled Water	q.s.	1000	ml.

Preparation

1.- Place the sodium chloride in a one liter volumetric flask.

2 - Add approximately 500 ml. of distilled water and shake the flask until the sodium chloride is completely dissolved.

3 - Dilute to volume.

4 - Add 0.5 ml. Brij-35, mix.

SODIUM HYDROXIDE, 0.5N - Technicon Formula T01-0044 (formerly 44-59)

Chemical Composition

1 - Sodium Hydroxide		20.0	gm.
2 - Distilled Water	q.s.	1000	ml.

Preparation

1 - To 20.0 grams of sodium hydroxide in a one liter volumetric flask add distilled water to the mark.

2 - Shake until dissolved.

SATURATED PICRIC ACID - Technicon Formula T01-0043 (formerly 43-59)

Chemical Composition

1 - Picric Acid		13 gm.
2 - Distilled Water	q.s.	1000 ml.

Preparation

1 - To 13 grams of reagent grade picric acid in a one liter volumetric flask add distilled water to the mark.

2 - Allow the excess picric acid to remain in contact with the water and shake occasionally.

3 - Filter and store in a polyethylene bottle.

OPERATING PROCEDURE NOTES

1 - The creatinine method can be run at 60 determinations per hour.

2 - The samples should consist of clear serum or urine. Lower values were experienced with whole blood and plasma.

3 - Standards covering a range of 1 to 10 mg. creatinine/100 ml. are adequate. Serum containing values of creatinine higher than this range should be diluted.

4 - The 40 ft. time delay coil used for color development should be immersed in a container of water at room temperature to protect against rapid fluctuations in ambient temperature.

5 - When running the creatinine determination a check should be made of the noise level. This can be done by continually aspirating a 5 mg./100 ml. creatinine standard. The noise level should be no greater than $\pm$ 0.5 transmission line. If the noise level is greater, a check of the manifold and Dialyzer should be made to insure that a good bubble pattern is being obtained. Noise is generally related to a poor bubble pattern which gives poor proportioning of reagents.

6 - For optimal bubble pattern and low noise use 0.5 ml. of Brij-35 per liter of saline and distilled water recipient.

7 - The noise with serum may sometimes be due to the formation of a precipitate. If this occurs it is advisable to try a different lot of picric acid. It may also be helpful to clean the picric-sodium hydroxide lines and coils as well as the flow cell with 10% acetic acid.

CREATININE PROCEDURE

STANDARDS

STOCK CREATININE STANDARD (1 mg.creatinine/ml.) Technicon Formula T03-0045

Chemical Composition

1 - Creatinine		1.000	gm.
2 - Hydrochloric Acid, 0.1 N	q.s.	1000	ml.

Preparation

1 - Weigh out creatinine on analytical balance and transfer to one liter volumetric flask.

2 - Dissolve and dilute to volume with 0.1 N HCl.

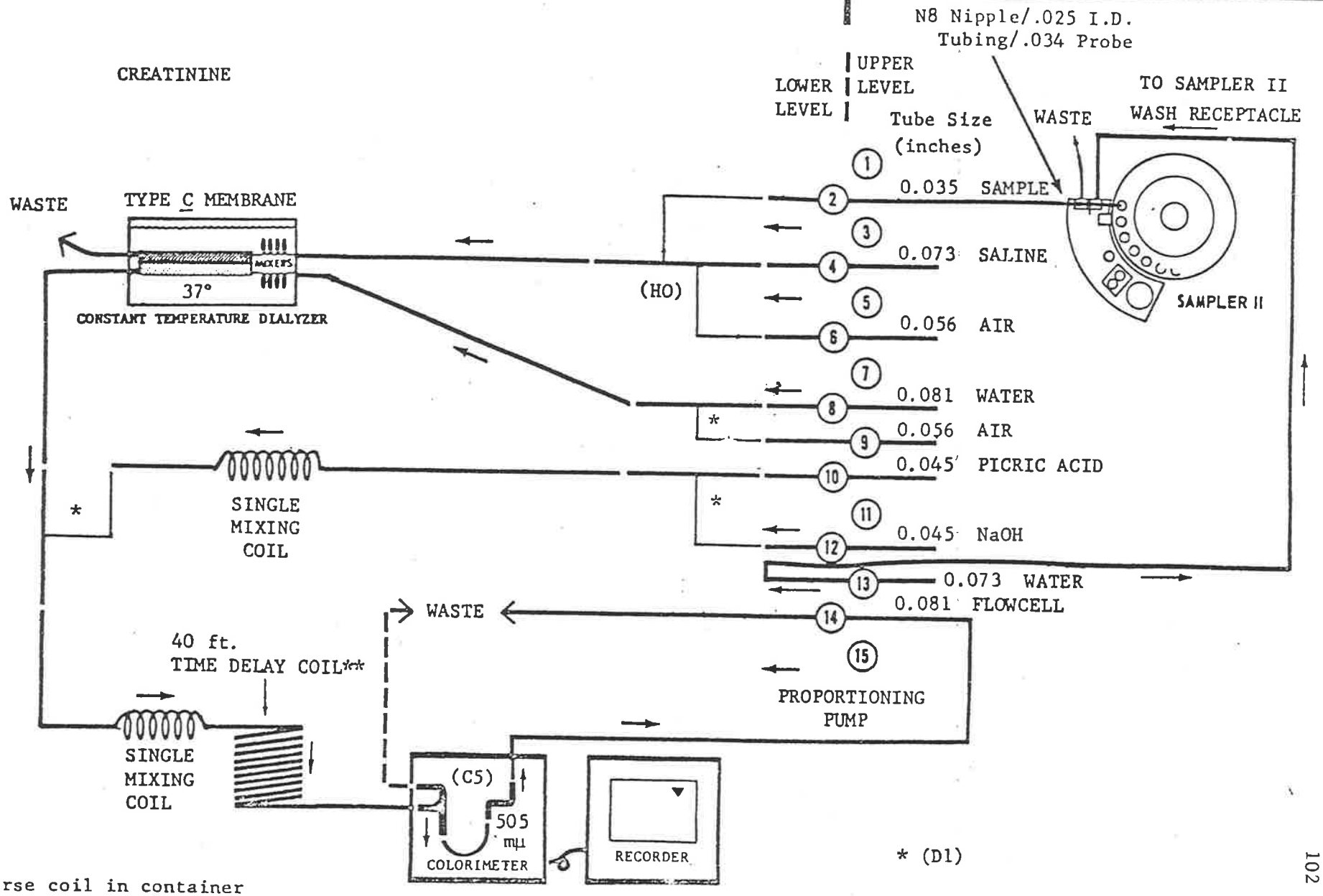
WORKING CREATININE STANDARDS

Dilute stock creatinine standard with 0.02 N HCl.

ml. stock	Dilute to	mg. Creatinine/100 ml.
1	100 ml.	1
3	100 ml.	3
5	100 ml.	5
7	100 ml.	7
10	100 ml.	10

# # #

N-11b
flow diagram



APPENDIX 6

Neonatal Water Metabolism: An Objective Postnatal Index of Intrauterine Fetal Growth

by

A.H. MacLennan, A. Hocking, R.F. Seamark,
B. Godfrey and R. Haslam.

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Neonatal water metabolism: an objective postnatal index of intrauterine fetal growth

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Summary

The water metabolism of 103 newborn babies was determined over the first 10 postnatal days, by measuring water turnover rates by means of an isotope dilution technique. This technique involves the oral administration of the non-radioactive isotope of water, ^2H_2O, and the measurement of its urinary excretion by infrared spectrophotometry. The slope of the excretion curve after equilibration with the infant's body water was mathematically expressed as the rate constant. Using multiple obstetric and paediatric criteria, the babies were clinically classified into one of three categories, fully grown ('normal'), borderline or clearly growth retarded. The median values of the rate constants $\times 10^4$ (h^{-1}) for the three groups were 73.3, 85.9 and 100.2 and were highly significantly different from each other ($P < 0.0005$) with no overlap of the 97% non-parametric confidence limits of each group. Neonatal water turnover increased with the clinical degree of intrauterine fetal growth retardation and within the limits of this study, this finding was unaffected by gestational age, birth weight or the neonatal environment. The results suggest that neonatal water metabolism is an objective postnatal index of fetal growth retardation.

neonatal water metabolism; water turnover rate; intrauterine fetal growth retardation; perinatal morbidity

Introduction

The accurate identification of growth retardation in the newborn and the quantitative estimation of the degree of impaired growth present have thwarted perinatolo-

0378-3782/83/0000–0000/$03.00

gists since Gruenwald [6] showed that many low birth weight infants were small for their gestational age rather than premature. Even babies that are considered average in size for their gestational age may be growth retarded and may not have reached their full growth potential during intrauterine life. Studies using serial ultrasonic fetal measurements [5] have shown that some fetuses have growth arrest near the end of pregnancy but conventional neonatal criteria for the assessment of intrauterine growth, e.g. percentile charts, may fail to identify these babies as growth retarded unless they are under the tenth percentile of size for their gestation. Similarly, babies classified as being small for their gestational age, i.e. under the tenth percentile of weight for gestation, may, in fact, have reached their full growth potential and not be growth retarded. The estimation of relative fetal growth at birth based on any size-for-gestation percentile growth chart is vulnerable to miscalculations of the gestational age and also to the epidemiological comparability of the baby being assessed to the group of infants from which the standard chart was drawn. Ideally, neonatal indices of intrauterine growth retardation should be independent of birth weight, gestational age, or subjective assessments of the infant's appearance or behaviour at birth. An objective neonatal index of intrauterine growth is necessary to better identify and quantify the effects of intrauterine fetal growth retardation, thus facilitating studies of its aetiology and sequelae.

In seeking such an objective index of intrauterine growth retardation, we firstly considered the measurement of total body fat, which may be reduced in relation to the degree of fetal growth retardation. Total body water is inversely proportional to total body fat but the measurement of total body water is technically difficult in the neonate. However, water turnover rates are more simply measured. This latter parameter was investigated because there is evidence of both disturbance of water dynamics in the growth retarded infant [3,4] and a faster water turnover rate in animals moving from states of undernutrition to improved nutrition [7,8]. In a pilot trial of 46 infants, water turnover rates were significantly increased in infants clinically classified as growth retarded compared to infants who were apparently fully grown [9]. This study seeks to examine in detail whether neonatal body water turnover can be used as a quantitative postnatal index of intrauterine growth retardation.

Patients and Methods

103 infants whose parents had given informed consent were included in the study, the protocol of which had been approved by the Research and Ethics Committee of the Queen Victoria Hospital, Adelaide. The gestational ages of all infants were confirmed by all three of the following: (1) an accurate menstrual history correlating with early uterine size; (2) ultrasonic examination before the 20th week of gestation; and (3) a Dubowitz assessment of maturity at birth, confirming within two weeks, the calculated gestation. The following antenatal data were collected: mother's age, height, race, parity, past obstetric, menstrual, medical and drug histories, clinical and laboratory antenatal estimates of fetal growth, antenatal and intrapartum complica-

tions, mode of delivery and maternal intravenous fluids during labour. Paediatric assessment included birth weight, head circumference, length, Apgar scores, Dubowitz maturity assessment, subscapular and triceps fat thickness, gross appearance, general health and behaviour, blood sugar, haematocrit, fluid intake (when not breast fed), humidity and temperature of environment, including the influence of phototherapy, heat shields, radiant warmers and incubators. The gestational age of the infants studied varied from 32 to 42 weeks and the birth weights, from 1140 to 4440 g. The weight for gestation charts [1] used in this study have been found to be appropriate for the Adelaide population and they allow for adjustments of the percentile with respect to the maternal height and weight and the infant's sex and birth rank.

On the basis of the above data and without knowledge of the test data, the obstetrician (A.H.M.) and the paediatrician (R.H.) had to clinically and independently classify the pregnancies and infants into three groups: (1) normally grown infants with no antenatal or neonatal signs of growth retardation; (2) an intermediate group of infants with borderline evidence of growth retardation and (3) clearly growth retarded infants. Within these three groups, infants were also classified as premature (< 37 weeks) or mature (37–42 weeks). Classification of the infants on the basis of the clinical data was facilitated by using a scoring system where the data recorded was arbitrarily scored with stronger weightings being given to objective measurements of fetal growth, e.g. the adjusted weight percentile at birth, the fat thickness percentiles. Infants were only included in the study where there was no conflict in the clinical classifications made by two clinicians and the gestational age had been strictly confirmed as above. Infants requiring intravenous therapy or antibiotics were excluded from the study.

The rate of body water turnover was determined by the oral administration, body water dilution and urinary excretion of the non-radioactive isotope of water, deuterium oxide (^2H_2O). The methodology and justification of the mathematical model used has already been published [9]. In this study, ^2H_2O (2 ml/kg) was given between 2 and 48 h after birth. 4–8 aliquots of urine were simply obtained from each baby from small paper towels placed under the baby's napkin. Purified samples were obtained from the urine impregnated towels by vacuum distillation. The concentration of ^2H_2O in the distillate was determined with the use of a MIRAN 1A infrared spectrophotometer. After an initial equilibration phase with the infant's body water, urine concentrations of ^2H_2O plotted on a semi log curve were found to be linear (i.e., in keeping with a single exponential decay process) and the values declined during the next 300 h (Fig. 1).

The rate of loss of ^2H_2O from the body, i.e. the slope of the plotted linear excretion phase, was expressed as the rate constant of water turnover. In this paper, the rate constant (k) per h (h^{-1}), e.g. 0.00692, has been multiplied by 10^4 (giving 69.2) to simplify the presentation. A standard deviation (S.D.) can be calculated for each rate constant and is computed from the analytical variance in each set of points on the excretion curve.

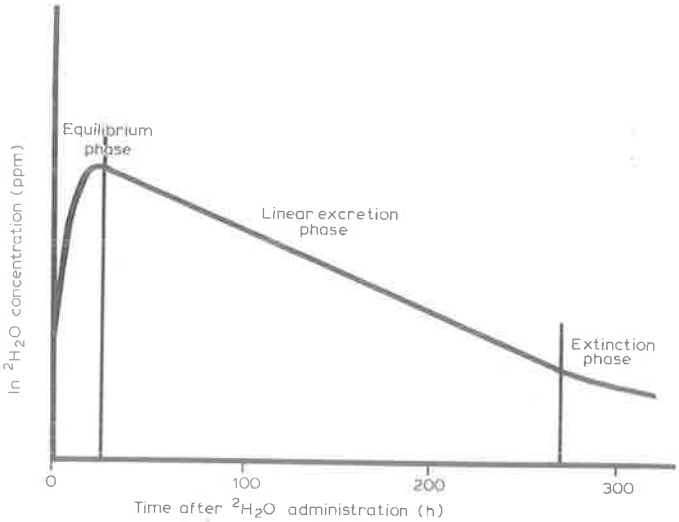

Fig. 1. Graphic representation of the log concentration of ^2H_2O in the infant's urine plotted against time after oral administration at time 0.

Results

Examples of the time courses of concentration of ^2H_2O in the urine of a baby clinically classified as normally grown ($k = 72.0$), and a baby classified as growth retarded ($k = 101.0$), are shown in Fig. 2. The rate of ^2H_2O excretion, i.e. the rate of body water turnover, is faster in the growth retarded baby.

Using the strict clinical criteria and data outlined in the methodology, 32 infants were classified as normally grown, 25 as borderline and 46 as growth retarded. The median of the rate constant of each of these three groups of infants compared to each other was highly significantly different ($P < 0.0005$, Mann-Whitney U-test (1-tailed); Table I). The 97% non-parametric confidence limits of the three groups do not overlap, although the values at the ends of the groups do overlap (Fig. 3) *. The median rather than the mean of each group and non-parametric tests has been used in the analysis of the results, as the patients were not necessarily derived from a normally distributed population. The means of the rate constant for each group were 71.0, 87.0, 102.6 for normal, borderline and intrauterine growth retarded groups respectively. Thus, with respect to the three clinical groups, neonatal water turnover increased with the clinical signs of growth retardation. Within the intrauterine

* The 97% non-parametric confidence limits on the median may be considered as an equivalent to the mean with 95% confidence limits (mean $\pm$ 2 S.E.M.), which are applicable to normal distributions. The 97% confidence limits delineate the range of values between which one can be 97% sure that the true group median lies. Just as in the parametric case, many measured values lie outside the mean $\pm$ S.E.M., in the non-parametric case many values do not fall within the 97% confidence limits on the median.

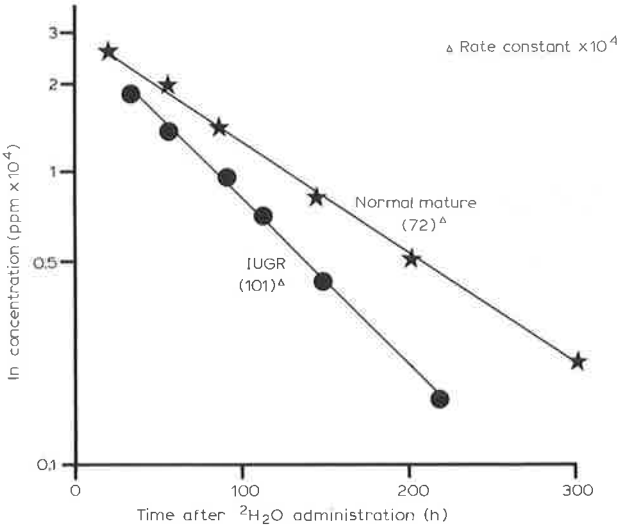

Fig. 2. Examples of the linear excretion phase of ^2H_2O in the urine of a baby clinically classified as normally grown ($k = 72.0$) and one classified as growth retarded ($k = 101.0$).

growth retarded group, in general, the clinically most growth retarded babies (by weight for gestation percentile) had the highest rate constants. This tendency was also seen in the borderline group, where the clinician classifying the borderline infant was asked to indicate whether the baby should be classified as normal or growth retarded if the borderline classification could not be given. The mean rate constant of the (12) borderline infants reclassified as normal by clinical assessment was 83.5 and for the 13 borderline infants reclassified as growth retarded, the mean of the rate constant was 90.2 ($P < 0.05$) (Mann–Whitney U-test).

Clinical correlations

No single clinical parameter highly correlated with the infant's water turnover

TABLE I

Neonatal body water turnover

Clinical group	Number	Median of rate constant	97% non-parametric confidence limits of median
Normal infants	32	73.3	66.9– 78.0
Borderline infants	25	85.9	82.6– 89.9
Intrauterine growth retarded infants	46	100.2	94.0–105.0

Neonatal body water turnover rates expressed as the rate constant ($\times 10^4$) in each of the three clinically classified groups of infants.

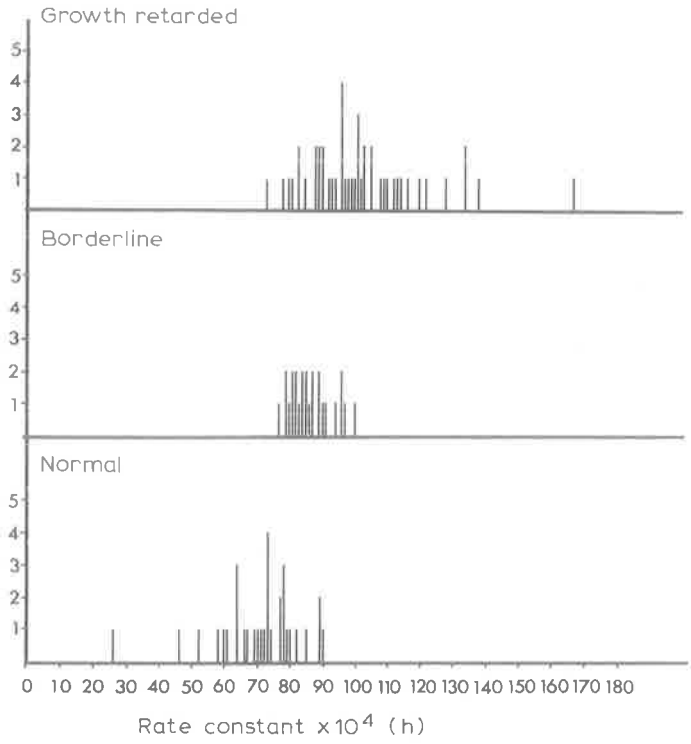

Fig. 3. The rate constants of all babies in the three clinical groups.

rate. The clinical parameters with the highest coefficient of correlation (non-parametric test) were the weight for gestation percentile (−0.41), the ponderal index (−0.41), maternal age (−0.39), subscapular fat thickness (−0.34) and the birth weight (−0.27). All other clinical parameters measured, e.g. Apgar scores, smoking, alcohol consumption, had coefficients of correlations below ± 0.20. There was also no correlation of the water turnover rate with the mode of delivery, antenatal steroid therapy (21 patients), maternal intravenous therapy during labour, the fluid intake per kg in bottle fed babies or the humidity and temperature of the neonatal environment. However, there were few major differences in the neonatal environment of the infants in this study, as infants under 32 weeks and infants requiring intensive care were not included.

Influence of gestation

There was no statistically significant difference in the water turnover rates between premature infants (32–36 weeks gestation) and mature infants (37–42 weeks gestation), when each of the three clinical groups was subdivided by gestation (Table II). The mean of the rate constant (± S.E.M.) of these subgroups is plotted against the mean weight percentile of each group in Fig. 4. In each clinical group,

TABLE II

The mean rate constants and the mean weight percentiles of the three clinical groups subdivided by gestation

Clinical group	Normal		Borderline		Intrauterine growth retarded	
	Mature	Premature	Mature	Premature	Mature	Premature
Total number	21	11	7	18	23	23
Mean of rate constant ($\times 10^4$)	70.1	72.5	83.8	88.2	99.8	105.3
Mean of weight percentile	58.5	46.4	32.3	24.8	20.9	9.2

the premature infants' mean rate constant is marginally higher than that of the mature infants but the mean weight percentile of the premature infants is lower in each case.

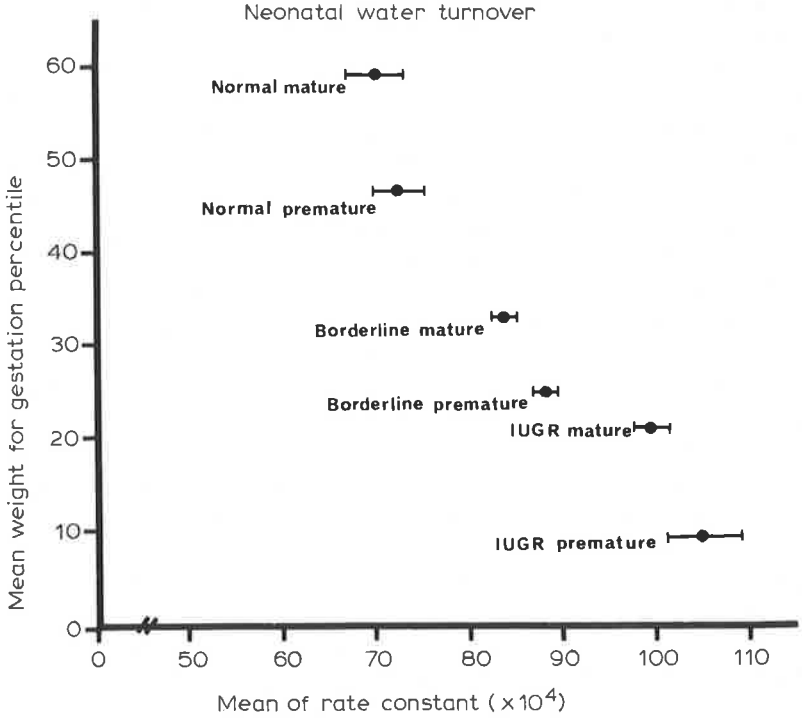

Fig. 4. The mean of the rate constant (±S.E.M.) plotted against the mean of the weight for gestation percentile for mature and premature infants in each of the three clinical groups.

Multiple pregnancy

31 infants were born of a multiple gestation (3 sets of triplets and 11 sets of twins). Of these infants, 6 were classified as normally grown (median of the rate constant 75.3), 10 were in the borderline group (median rate constant 84.2) and 15 were classified as clearly growth retarded (median rate constant 94.0). The medians of these groups are not significantly different from those of the singleton infants.

The 10th percentile of weight for gestation

13 babies were clinically classified as being clearly growth retarded but were above the 10th percentile of weight for gestation. All had rate constants above 80.0 and their mean was 101.7. Two babies below the 10th percentile (on the 5th and 7th percentiles) were not clinically classified as clearly growth retarded. Both were classified in the borderline group, or as normal, when the borderline classification was disallowed. These babies had rate constants of 83.4 and 82.6, respectively.

Discussion

This larger study confirms our preliminary communication [9] that neonatal water turnover is faster in infants where intrauterine growth retardation has occurred. Within the limits of this study, differences in gestational age or the neonatal environment did not account for this increase in water metabolism. Such an increase in the water metabolism of the growth retarded neonate is in keeping with reports that growth retarded infants have a higher than normal total body water content [4] and that during the first 24 h after birth, the growth retarded infant loses water at a faster rate than normally grown infants [3]. The growth retarded infant has also been shown to have a higher oxygen consumption than its normally grown counterpart [11] and this finding together with an increased water turnover rate, suggests the growth retarded infant is hypermetabolic after birth.

There are several possible explanations for an increase in water metabolism in the growth retarded neonate. Firstly, it may reflect an increased cellular metabolism associated with cell growth and repair, following a prolonged period of intrauterine malnutrition and/or hypoxia. Secondly, the neonatal kidney has yet to develop an effective capacity to concentrate its urine [10] and there may be increased passive excretion of water along with solutes built up during episodes of intrauterine hypoxia. Thirdly, the high levels of body water in the growth retarded infant may also influence the rate of its turnover. The length of time that an increased water turnover rate persists requires further investigation with further measurement of ^2H_2O excretion rates at later dates. However, in this study, infants were followed up to 14 days after birth and as the excretion phase of the semi log curve remained linear, it appears that excretion rates may remain constant during this time.

To assess the potential value of neonatal water turnover as an index of intrauterine growth retardation, it was necessary to define intrauterine growth retardation by conventional clinical criteria, some of which are subjective in nature and many of which are open to clinical error. To offset this problem, as much as

possible, errors of gestational age were eliminated and objective neonatal measures of fetal growth were included to facilitate the final overall clinical classification of the baby, e.g. the subcutaneous fat thickness, the ponderal index and adjusted size for gestation percentiles. Only three broad clinical classifications were allowed, as it was felt that even when used together, conventional clinical criteria, such as weight for gestation percentiles, did not identify retardation of growth during intrauterine life from, for example, the 90th to the 50th percentile. In view of the inherent weaknesses of traditional indices of growth retardation, it is perhaps not surprising, therefore, that no single conventional clinical parameter correlated closely with the rate of water turnover whilst there was significant correlation of neonatal water turnover with the three broad clinical classifications based on the combined clinical data. It is possible that by including only babies in the study whose growth status could be defined by conventional clinical criteria, that babies with less recognizable growth retardation have been misclassified or excluded from the study. Babies with congenital anomalies and infants of diabetic mothers were not studied and further studies are required in these groups.

The concept that some babies under the tenth percentile of weight for gestation are not growth retarded and that some above the 10th percentile are growth retarded, is supported in this study as 13 babies over the 10th percentile on close clinical scrutiny did show definite signs of growth retardation and all these infants had rate constants well above the normal range, whilst two babies under the 10th percentile had minimal clinical signs of intrauterine growth retardation and had rate constants close to the normal range.

The inclusion of 31 infants born of a multiple pregnancy did not significantly affect the mean values of the rate constants in the three clinical groups. However, only 6 of the 31 infants were classified as showing no signs of intrauterine growth retardation. These multiple pregnancies were not randomly chosen and the incidence of growth retardation in multiple pregnancy, as judged clinically or by high water turnover rates (e.g. over an arbitrary level of 80), requires further analysis in a consecutive or random series.

The estimation of neonatal water turnover is a relatively simple and inexpensive test, which does not involve sampling of blood and the paper towel method of collecting aliquots of urine means that little discomfort is experienced by the infant being studied. ^2H_2O can be given orally with a feed and without a stomach tube, as the measurement of water turnover is not dependent on a known loading dose of ^2H_2O and smaller quantities per kg of ^2H_2O than used in this study can now be given, due to improved sensitivity of the measurement of small quantities of ^2H_2O in the distilled urine [2].

This study suggests that neonatal water turnover is an objective, retrospective index of intrauterine growth retardation and that within the gestational limits of the study, the results were independent of gestational age, birth weight and the neonatal environmental factors studied. There are several potential applications of an objective neonatal index of intrauterine growth retardation. For example, by defining accurately the presence and the approximate degree of intrauterine growth retardation present at birth, the test provides a research tool to facilitate retrospective

analysis of the aetiology of fetal growth retardation. Accurate definition of growth retardation would allow better assessment of its sequelae and allow more specific identification of children in need of follow up. At present, selected babies from this study are being followed in a long term paediatric developmental program and the relationship of neonatal water turnover rates to growth and psychological development in later years will be the subject of another paper. As an index of perinatal morbidity, the test may provide a more sensitive parameter of obstetric outcome than perinatal mortality and thus be of value in auditing obstetric management, policies and the cost effectiveness of currently used antenatal tests of intrauterine growth. Lastly, although only infants from a Caucasian population were studied in this paper, the test may allow a better comparison of the incidence of fetal growth retardation in different racial groups, as birth weight may reflect genetic differences rather than nutritional differences.

Perinatal mortality has been the yardstick of obstetric services for many years but as mortality rates diminish to less than 10 per 1000 births, the objective assessment of the quality of the remaining live-born infants becomes paramount. However, perinatal morbidity with regard to retarded fetal growth, has been difficult to identify and quantitate. The study suggests that the water turnover rate of the newborn infant may be a useful objective retrospective index of intrauterine fetal growth.

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APPENDIX 7

Measurements of Muscle Protein Breakdown in Newborn Human Infants

by

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In the list of references, the following abbreviations will be used:

ACTA. PHYSIOL. SCAND.	ACTA Physiologica Scandinavia
ANAL. BIOCHEM.	Analytical Biochemistry
ANAL. CHEM.	Analytical Chemistry
ANN. N.Y. ACAD. SCI.	Annals New York Academy of Science
ARCH. DIS. CHILDH.	Archives of Disease in Childhood
ARCH. NEUROL.	Archives of Neurology
ARCH. PATHOL.	Archives of Pathology
AUST. J. AGRIC. RES.	Australian Journal of Agricultural Research
BIOCHEM. BIOPHYS. RES. COMMUN.	Biochemical and Biophysical Research Communications
BIOCHEM. J.	Journal of Biochemistry
BIOCHIM. BIOPHYS. ACTA.	Biochimica et Biophysica ACTA
BR. J. NUTR.	British Journal of Nutrition
BRI. J. OBSTET. GYNAECOL.	British Journal of Obstetrics and Gynaecology
BRIT. MED. J.	British Medical Journal
CLIN. CHEM.	Clinical Chemistry
CLINICS IN PERINATOL.	Clinics in Perinatology
CLIN. SCI.	Clinical Science
J. AM. CHEM. SOC.	Journal of the American Chemical Society
J. APP. PHYSIOL.	Journal of Applied Physiology
J. BIOL. CHEM.	Journal of Biological Chemistry
J. CELLULAR & COMP. PHYSIOL.	Journal of Cellular and Physiology
J. CLIN. INVESTIGATION	Journal of Clinical Investigation

J. LAB. CLIN. MED.	Journal of Laboratory and Clinical Medicine
J. NEUROL. NEUROSURG. PSYCHIATR.	Journal of Neurological and Neurosurgical Psychiatry
J. NUTR.	Journal of Nutrition
J. OBSTET. GYNECOL. BRIT. COMMONW.	Journal of Obstetrics and Gynaecology in the British Commonwealth
J. PEDIATR.	Journal of Pediatrics
J. PEDIATR. SURG.	Journal of Pediatric Surgery
J. PHYSIOL.	Journal of Physiology
LANCET	The Lancet
LIFE SCI.	Life Science
MED. J. AUST.	The Medical Journal of Australia
MICRO. CHEM. J.	Microchemical Journal
OBSTET. & GYNAECOL.	Obstetrics and Gynaecology
OBSTET. GYNAECOL. SURV.	Obstetrical and Gynaecological Survey
OBSTET. GYNECOL.	Obstetrics and Gynecology
PEDIATR.	Pediatrics
PEDIAT. RES.	Pediatric Research
PERINAT. MED.	Perinatal Medicine
POULTRY SCI.	Poultry Science
SCI.	Science

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