TO ROB

for understanding me
if not the thesis.

(ii)
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SECTION I

INTRODUCTION

CHAPTER 1
TERATOLOGY - DEFINITION AND HISTORY

An interest in human malformations dates back to ancient times and the moral attitudes towards the afflicted individuals have varied through the ages reflecting the cultural tendencies and knowledge of the people at the time. Early Greek and Roman cultures are known to have worshipped monsters and considered the birth of a malformed child as an omen. Belief in these and many other superstitions associated with birth defects has been upheld throughout the centuries, and even in today's enlightened times, inherent prejudices still exist.

An early scientific explanation concerning causation was proposed by William Harvey in 1651 who suggested that the premature halting of a process in embryological development could result in a malformed child. Although now accepted as a fundamental cause of birth defects, at that time, Harvey's theory received little credit.

In the 19th century, a French zoologist, Saint Hilaire, proposed that the environment could be responsible for some human abnormalities (Saint Hilaire, 1832). He supported his theory by creating defects in developing chicks by either starving the embryo of oxygen or by pricking it through the shell. He thus produced chicks with spina bifida and anencephaly and their
grotesque appearance gave rise to the first use of the term teratology, from the Greek word teras, meaning monster.
Thus teratology was originally defined as the study of monstrosities. Unfortunately, the work of Harvey and other early teratologists attained little public acceptance as their use of lower life forms was considered irrelevant to the human situation. Also, the general belief that the human uterus was virtually impervious to external influences, did little to strengthen their case.
It was not until the beginning of the 20th century, with the rediscovery of Mendel's work of 1866 and the founding of the science of genetics, that teratology as a scientific study was really started. With the new understanding of genetics, hereditary factors became the chief suspect in the aetiology of all malformations. In light of this, other work, including that of Stockard (1921) and Bagg (1922) implicating environmental factors, was largely disregarded. Even the studies of Goldstein and Murphy (1929), demonstrating the adverse effects of ionising radiations on pregnant women was little acknowledged. The theory of genetic control was too strongly accepted—especially as most experimental studies, including Hale's 1933 work on vitamin A deficient sows and Warkany's 1940s experiments on maternal nutrient deficiencies in rats, reported results obtained from non-human models.
In 1941, Gregg's discovery of the first human teratogen, and his description of the embryopathic effect of rubella, created widespread interest in the scientific world, but once again there
was very little concern within the general public. In fact, the
ture implications of teratology were not realised until the
catastrophe with thalidomide in 1961 (Lenz, 1961; McBride, 1961). The fact that an agent, which appeared to be acceptably safe for
use in postnatal patients was responsible for severe
malformations manifested in more than 8000 children, at last
heightened general awareness to the risk of environmental changes
and drug use during pregnancy. Only then did the question of
environmental influences receive attention. Scientists moved
quickly into the area and since then many naturally occurring and
artificial teratogens have been recognised. From this milestone,
the science of teratology has moved with great impetus. From its
origins as a topic of little interest it has become a science
affecting many other scientific disciplines and both industrial
and governmental bodies. Nowadays the government will not permit
the release of a new drug or household chemical without stringent
testing by regulating agencies. The science has progressed from
observational studies to mechanistic investigations and has
enlarged our understanding of normal embryology and genetics.
Even now, questions are being raised about environmental agents
and surroundings which were previously presumed harmless. Along
with drugs and chemicals other less obvious agents such as
alcohol have been identified as human teratogens. There has also
been a great increase in the number of animal studies to reveal
the effects of dietary excesses and deficiencies and
environmental stresses during pregnancy. Also, the previously
disregarded findings of earlier workers has at last been
recognised as relevant and important.

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CHAPTER 2

EXPERIMENTAL EMBRYOLOGY

Within the last 20 years, experimental embryology has been advanced by the introduction of more refined methods for \textit{in vitro} culture of post-implantation embryos (New et al, 1976a).

Although \textit{in vitro} culture of mammalian embryos dates back to the late 19th century (Schenk, 1880; Heape, 1890), most of the early work was restricted to pre-implantation eggs and blastocysts. This was mainly due to their greater availability and also the lack of success in culturing post-implantation embryos. Earliest experimentation was carried out largely on chicks and amphibians whose maternal independence made them readily accessible and therefore easier to culture. For these groups, knowledge of development was enhanced and proficiency in culture techniques achieved, well in advance of their mammalian counterparts.

However, by the 1930's several groups did have some success in culturing post-implantation mammalian embryos. Nicholas and Rudnick (1934 & 1938) reached the 16 somite stage in rat embryos and similar results were obtained in the guinea pig (Jolly & Lieure, 1938). Development to 6 somites was also attained in rabbit embryos (Waddington & Waterman, 1933). In these early experiments, although \textit{in vitro} culture could not be successfully maintained much beyond 36 hours, embryologists gained valuable information about heart development (Goss, 1935; Dwinnel, 1939) and embryonic induction (Toro, 1938).

Progress in methodology ensued and \textit{in vitro} culture, for several species, can now be maintained for periods of up to 4 days. This
has enabled much more detailed study of embryonic growth and in particular organogenesis which is the time of onset of some of the most serious malformations.

To the experimental embryologist or teratologist, the advantages of better *in vitro* culture systems are many. The ability to study both normal development and the effects of external variables in a controlled and isolated environment, overcomes many of the problems associated with inaccessibility and interference from the maternal system. The embryologist is able to observe both closely and continuously the processes involved in embryonic growth and the teratologist can monitor any adverse effects during the very critical and susceptible period of organogenesis.

The *in vitro* model system for the culture of post-implantation rat embryos (New et al, 1976a; New, 1978) has many advantages over the equivalent *in vivo* system. Isolation of the embryo both physically and physiologically from the maternal system removes variables introduced by maternal metabolism. This allows for assessment of the effect of an environmental agent directly on the embryo and facilitates distinction between this and an effect mediated by maternal metabolism or by possible maternal dysfunction. Although many agents cross the placenta and thereby act on the embryo directly, in the *in vivo* situation clear distinction of any such effect would not be possible.

However, with any *in vitro* system, the question always arises as to how comparable the system is to the *in vivo* situation. With New's culture system, it has been established that rat embryos cultured by this method, from the primitive streak or early head-
fold stage to the limb bud stage (9.5-11.5 days), are essentially normal and almost indistinguishable from 11.5 day old *in vivo* embryos (New et al, 1976b; Cockroft, 1976; New, 1978). Having ascertained that the *in vitro* culture system compares favourably with *in vivo*, the problem of extrapolating results from animal to man then arises. Obviously man is unacceptable for teratological study and therefore experimentation with animals is the best possible alternative. The choice of test animal is realised to be of considerable importance and while the ideal model would mimic the human in its maternal metabolism and placentation, the choice of animal is ultimately made by practicability. Factors which govern this choice include the cost of the animal, its availability and also the ease with which it can be bred and maintained in good health. The rat meets all the criteria with the added advantages of short gestation, large litter size and low spontaneous malformation rate. (The Wistar rat, as used in this project, has a malformation rate of less than 1 in 1000.) Success in *in vitro* culture throughout the period of organogenesis has also been achieved with rat embryos. Mice have also been fairly extensively studied (New & Stein, 1964; Clarkson et al, 1969; Hernandez-Verdun & Legrand, 1971; Biggers et al, 1971), but their smaller size makes them less easy to handle than rat embryos. Guinea pigs were used in some very early work (Jolly & Lieure, 1938) but have not been employed since and experiments using hamster embryos were so disappointing that they too were not pursued as potential models (Givelber & DiPaolo, 1968).
Within the limits of the experimental criteria, the rat is therefore the best choice of test animal. However, despite the many advantages of post-implantation rat embryo culture, as with any *in vitro* system, there are limits to the value of results thus obtained. It is well recognised that any animal model or *in vitro* system does not behave as man. However, with careful evaluation, results obtained from such studies can yield valuable information which, although precluding direct extrapolation, can at least help predict the outcome of an environmental stress or agent in the human.

**Embryonic Nutrition**

During intrauterine development in the rat, two types of nutrition operate. These are histiotrophic and haemotrophic nutrition.

**Histiotrophic Nutrition.** While histiotrophic nutrition is functioning, the embryo obtains its nutrition via the extra-embryonic membranes from the breakdown of maternal macromolecules (histiotroph) (Beck et al, 1967; Freeman et al, 1980; Beck & Lowy, 1982). Histiotroph, derived from uterine secretions, extravasated maternal blood or endometrial cells (Amoroso, 1952; Gupta et al, 1982) is pinocytosed by cells of the visceral yolk sac (Beck et al, 1967). The macromolecules are digested by lysosomes in the vacuolar system of the cells and the soluble digestion products then diffuse into the embryo through a system of intercellular spaces (Beck et al, 1967; Williams et al, 1976; Beck & Lloyd, 1965; Freeman et al, 1980; Beck & Lowy, 1982). This is the sole source of nutrition for the embryo until the
chorioallantoic placenta develops around day 12. Although haemotrophic then becomes the major form of nutrition, histiotrophic nutrition also persists, and contributes to a lesser degree, until full term (Beck et al, 1967).

In \textit{in vitro} culture, the visceral yolk sac is explanted with the embryo and continues to function providing histiotrophic nutrition throughout the 48 hour culture period. This is the main reason for the success of \textit{in vitro} culture of the rat embryo between 9.5 and 11.5 days.

**Haemotrophic Nutrition.** Once the chorioallantoic placenta develops around day 12, haemotrophic nutrition takes over most of the embryos' nutritional requirements (Wild, 1974). For this to proceed the chorionic vesicle must be vascularised and the embryonic heart sufficiently developed to allow perfusion of solutes between the maternal and fetal circulations (Beck, 1976). As the embryo develops past day 12 its increased nutritive demands require the support of haemotrophic nutrition. However, as the chorioallantoic placenta does not develop \textit{in vitro}, embryonic growth past this stage becomes limited.

**Nutritional Studies**

The experimental creation of congenital malformations was first established about 50 years ago when Hale successfully produced eye defects in pigs by means of dietary vitamin A deficiency (Hale, 1933). Warkany and co-workers also demonstrated that dietary deficiencies could produce predictable types and severities of malformations in the offspring of rats exposed to a deficiency during pregnancy (Warkany & Nelson, 1941; Warkany et
al, 1942, 1943). Since then many studies have been carried out and this has helped advance basic knowledge of maternal nutritional failure and its relationship to congenital malformations.

More recently the introduction of superior methods for the *in vitro* culture of mammalian embryos (New et al, 1976a) has encouraged research into embryonic nutritional requirements independent of the maternal system. The major advantage of embryo culture compared with whole animal nutritional studies is its adaptability. It provides the opportunity to assess continuously the effects of nutritional factors directly on the embryo and offers much greater control of experimental conditions than could be attained *in vivo*. It makes it possible to discover precise details about the nature and time of onset of any deviation from normal development and allows continuous observation of the sequential progression and development of any abnormalities. In whole animal studies there is such a narrow range in which maternal dietary deficiency will result in congenital malformations, (rather than having no effect or inducing embryonic death), that the *in vitro* system offers a valuable alternative.

One major factor which must however be considered when employing *in vitro* culture in dietary studies, is that no account is taken of the potentially crucial influence of maternal factors in the prevention of fetal malformations. Maternal tissues may store nutrients and then act as "buffers" thus delaying, for as long as possible, exposure of the developing embryo to the deficiency. Thus as the ultimate goal is to understand the situation *in vivo*,

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both *in vitro* and whole animal studies must be considered as equally important sources of evidence in elucidating factors involved in the aetiology of nutritionally related embryonic malformations.

**Nutrition and Neural Tube Defects**

In man a correlation between maternal nutritional status and the incidence of fetal, and in particular, neural tube, defects have been demonstrated (Hibbard & Smithells, 1965; Hibbard & Hibbard, 1966; Elwood, 1976; Stein & Susser, 1976; Smithells et al, 1976, 1980, 1981a, 1981b, 1983; Laurence et al, 1980, 1981; Wild et al, 1986). A relationship between social class and neural tube defects (NTD) is suggested by the high prevalence in lower class communities and this has been linked with deficiencies of vitamins and other essential dietary constituents (Edwards, 1958; Record, 1961; Renwick, 1972; Smithells et al, 1976).

A possible relationship between NTD and a single vitamin deficiency was first suggested in 1965 when Hibbard and Smithells reported an apparent high incidence of folate deficiency in women with affected babies (Hibbard & Smithells, 1965). Accumulated data have both supported this finding (Fraser & Watt, 1964; Martin et al, 1965; Smithells et al, 1976; Cooper, 1976; Neiderwieser, 1979; Rosenblatt & Cooper, 1979) and countered it (Giles, 1966; Menon et al, 1966; Emery et al, 1969; Scott et al, 1970; Pritchard et al, 1970), but the most recent results are in favour of such an association (Laurence, 1982; Laurence et al, 1981, 1983; Rogozinski et al, 1983; Yates et al 1987). Also, treatment with aminopterin, a powerful folate antagonist, is
known to produce NTD in humans (Emerson, 1962). (Other congenital abnormalities including low infant birthweight have also been associated with maternal folate deficiency (Hibbard & Smithells, 1965; Martin et al, 1965; Hibbard & Jeffcoate, 1966; Hibbard & Kenna, 1974; Iyengar & Rajalakshmi, 1975; Zittoun et al, 1983; Navarro et al, 1984)).

The aetiology of an association between vitamin deficiencies and NTD has been sought by many workers and in 1981, evidence was produced which suggested that periconceptional administration of a multivitamin preparation (Pregnavite forte F, Bencard), markedly reduced the incidence of recurrence of NTD (Smithells et al, 1981a, 1981b). The mothers included in this study were classed as high risk as they had all previously given birth to at least one baby with NTD and therefore had significantly higher chance of having another affected child (Smithells et al, 1980, 1981a, 1981b). It was also proposed that dietary counselling might be of benefit to high risk women, as an improvement in the quality of their diet might well reduce the risk of recurrence (Laurence et al, 1980). Clinical studies confirmed that dietary counselling was indeed beneficial as the rate of recurrence in counselled mothers was markedly lower than in the uncounselled group (Laurence et al, 1980).

Although these results suggest that the aetiology of NTD may be related to dietary factors, it is important to emphasise that the origins of the defect are almost certainly multifactorial (Carter, 1974). The aetiology will have both genetic and environmental components and a threshold will probably exist,
below which the accumulated factors will result in NTD and above which the offspring will be normal. While attention to one single factor is unlikely to eliminate NTD completely it may be sufficient to shift the equilibrium towards normality thereby reducing the incidence of the defect.

Experimental Models

Experiments in rats and mice have demonstrated a wide variety of congenital malformations caused by maternal dietary folate deficiency. Warkany and Nelson (1940) described skeletal abnormalities, hydrocephalus was also reported (O'Dell et al, 1948; Nelson, 1957) and many groups have demonstrated a variety of single and multiple abnormalities including cleft lip, cleft palate, eye abnormalities, facial defects, cardiovascular defects, syndactyly, oedema and anaemia (Nelson & Evans, 1949; Baird et al, 1954; Monie et al, 1954; Nelson et al, 1955, 1956; Asling et al, 1955, 1960; Monie, 1957; Tuchmann-Duplessis et al, 1959; Asling, 1961). However, in many of these studies, folate antagonists were co-administered with a folate-free diet. As the mode of action of these compounds is complex (Kinney & Morse, 1964; Potier de Courcy, 1966; Chepenik et al, 1970), the aetiology of the observed defects must therefore be uncertain.
CHAPTER 3
FOLATE
Folates are a group of related compounds synthesised by higher plants and most microorganisms. They are widely distributed in foods and in particular are found in high concentrations in green leafy vegetables, liver, kidneys, nuts and beans.

History
In 1931, pernicious anaemia was remedied by a factor in yeast (Wills, 1931). Later Day et al (1938) found that yeast or liver extracts were active in treating anaemia in pregnant monkeys. The component was called vitamin M— the name being derived from its use in monkeys. However, at that stage the factor was neither isolated nor characterised. Shortly after this, Hogan and Parrot (1940) demonstrated that a substance in autolysed hog liver prevented macrocytic anaemia in chicks. They named the new factor vitamin Bc as they had established that it probably belonged to the vitamin B complex and because it had been demonstrated to be essential to chicks. Around the same time Snell and Peterson (1939) found that a component in liver was essential for the growth of the microorganism Lactobacillus casei. This factor was also found to be necessary for normal growth in chicks (Hutchings et al, 1941). These two independent findings were followed up and, in 1941, Mitchell et al isolated a similar bacterial growth factor from spinach leaves. They called the compound folic acid from the Latin "folium" meaning leaf. In 1943, a difference in activity between Lactobacillus casei growth factor isolated from liver, and the same substance isolated from
yeast, was reported (Wright & Welch, 1943). This was the first evidence for the existence of a group of related factors. In 1946, Angiers' group described how they had isolated a growth factor from the wing pigment of brimstone and cabbage white butterflies. The isolate was called "pteroperin" from the Greek word "pteron" meaning wing. It was characterised, the structure elucidated and the compound, pteroylmonoglutamic acid, synthesised (Angers et al, 1946; Boothe et al, 1948, 1949). Following this no other folates were synthesised until 1969 (Krumdieck & Baugh, 1969).

Structure and Metabolism

Folic acid, (or pteroylglutamic acid or pteroylmonoglutanic acid) is made up of three components. A pterin moiety (A) is linked by a methylene bridge to p-aminobenzoic acid (B) which is joined to glutamic acid (C) by an amide linkage.

In its pure state the compound exists as yellow spear-shaped crystals which are poorly soluble in water but very soluble in alkali. Its molecular weight is 441.4 daltons.

The term folic acid is now generally used to refer to
pteroylmonoglutamic acid and although folates can be detected in every organ in the body (Taylor et al, 1942) the monoglutamate form of the vitamin does not usually exist as such in natural materials (Hoffbrand, 1975). Instead, the majority of folates occurring in nature are polyglutamates i.e. have more than one glutamic acid residue. In fact most natural folates are penta, hexa or heptaglutamates. The polyglutamates, or folate conjugates as they are also known, are polypeptide chains which have glutamic acid polymers linked in peptide bond from the γ-carboxyl group of one glutamate to the amino group of the next. The term folate is used to describe any of this group of compounds including the reduced and conjugated forms.

Reduced compounds are named with numerals indicating the positions of the hydrogen atoms, i.e. a tetrahydro compound would be a 5,6,7,8-tetrahydrofolic acid. The polyglutamate forms of folic acid constitute the storage forms and in this state are biochemically inactive. Deconjugation to monoglutamate has to occur before absorption can take place. This process is catalysed by the deconjugating enzyme folate conjugase (or folate hydrolase) (Baugh & Krumdieck, 1971; Hoffbrand & Peters, 1969). The compounds are then reduced stepwise to the enzymatically active tetrahydrofolate derivatives. The absorbed monoglutamates are first reduced to the dihydrofolate form, 7,8-dihydropteroylglutamic acid, and then to the tetrahydro derivatives, 5,6,7,8-tetrahydropteroylglutamic acid. The reduction stages are catalysed by the enzyme dihydrofolate reductase. One-carbon adducts (R) are then substituted on the N5
and $N^{10}$ positions to yield enzymatically active polyglutamyl derivatives. Substitution groups include formyl (CHO-), methenyl (=$CH$), methylene (=$CH_2$), formimino (=$CHNH$) and methyl (=$CH_3$). Once reduction and substitution have occurred the compounds are metabolically active and act as coenzymes to donate, transport and accept single carbon units to and from different molecules, thus participating in synthetic reactions requiring one-carbon moieties (Stokstad & Koch, 1967; Hoffbrand, 1975). The major activities in which the folate coenzymes are involved, are (1) protein synthesis and (2) nucleic acid synthesis (fig 1).

FIG 1. Principal Reactions Involving Folate Coenzymes

- DHF - Dihydrofolate
- THF - Tetrahydrofolate
- dUMP - Deoxyuridine-5-monophosphate
- dTMP - Deoxothymidine-5-monophosphate
- DNA-Thymine
(1) Protein Synthesis
Folate coenzymes participate in protein synthesis by their involvement in 3 pathways, viz., amino acid conversions, biosynthesis of methionine and catabolism of histidine. The folate-dependent enzyme serine transhydroxymethylase catalyses the conversion of serine to glycine. Although this reaction is reversible the major product is glycine which generates methyl groups which are then used in the conversion of homocysteine to methionine. This latter reaction involves the vitamin B12-dependent enzyme, homocysteine-5-methyl tetrahydrofolate transmethylase. One of the most important uses for the methionine formed from the homocysteine is its conversion to S-adenosyl-L-methionine which is the major methyl donor in the mammalian system.

A folate-dependent enzyme is also involved in the catabolism of histidine:—formiminoglutamic acid acts as an intermediate enzymic degradation product of histidine. Glutamate formiminotransferase catalyses the further breakdown of this product to glutamic acid and the formimino groups are salvaged by attaching to tetrahydrofolate. Formiminitetrahydrofolate results and the formimino groups become available for further one-carbon reactions.

(2) Nucleic Acid Synthesis
Folates play a key role in nucleic acid synthesis and metabolism by governing purine metabolism and pyrimidine synthesis.
The introduction of carbon-2 and carbon-8 into the purine ring
involves, primarily, formylation of glycanamide ribonucleotide by 5,10-methyltetrahydrofolate (providing carbon-8) and then formylation of aminoimidazolecarboxamidine ribotide by 10-formyltetrahydrofolate (adding carbon-2). Closure of the purine ring is thus dependent on two folate coenzymes.

Folates are also involved in the methylation of deoxyuridine-5-monophosphate to deoxythymidine-5-monophosphate which is the rate limiting step for thymine-DNA synthesis in mammalian tissues. The enzyme thymidylate synthetase transfers a methyl group from 5,10-methylenetetrahydrofolate in the synthesis of deoxythymidine-5-monophosphate (dTMP) from deoxyuridine-5-monophosphate (dUMP). This reaction is the only de novo source of thymidylate (Daneberg, 1977). In the process 5,10-methylenetetrahydrofolate is not only demethylated but also reduced to dihydrofolate. The enzyme dihydrofolate reductase then reconverts the compound to tetrahydrofolate which can then participate in further one-carbon transfer reactions (Friedkin, 1963).

The amount of thymidine nucleotides in cells will only maintain synthesis of DNA for a few seconds (Prescott, 1976). DNA synthesis is therefore dependent on the constant replenishment of the folate coenzyme.
CHAPTER 4

FOLATE DEFICIENCY

Folate deficiency is one of the most common vitamin deficiencies in the world. The major cause of the deficiency is malnutrition, although other factors such as malabsorption and increased demand are also important aetiological factors.

Pathological conditions which exert an increased demand on folate include administration of certain drugs such as anti-convulsants or folate antagonists e.g. aminopterin which blocks the enzyme folate reductase (Lambie & Johnson, 1985). Tissue repair or restoration of blood cells after blood loss or haemolysis also increase folate requirements. Other conditions such as infancy and adolescence also draw heavily on folate supplies but, by far, the major physiological condition to increase demand for folate is pregnancy.

The tissues most dependent and therefore the first to be affected by a folate deficiency are those which have the most active cell turnover i.e. epithelial tissues of the skin and mucous membranes, bone marrow and red cells, where DNA replication normally proceeds at a rapid rate. In man and animals folate deficiency may be diagnosed from the blood picture (Baker et al, 1959; Stokstad, 1968; Chanarin, 1969; Chanarin et al, 1969; Scott et al, 1974; Shojania, 1980; Coleman, 1981). Abnormal development and maturation of the cellular components of the blood are first expressed in the erythroid and myeloid cells. The changes occurring can be attributed to decreased synthesis of DNA precursors, particularly thymidylate (Menzies et al, 1966).
Since DNA synthesis is dependent on folate (see Chapter 3), a folate deficiency will produce an inhibition of DNA synthesis in the developing erythrocyte. The cell continues to carry out RNA and protein synthesis which results in imbalanced cell growth which leads to macrocytic and giant polymorphonuclear cells (Beaulieu, 1979). In the most advanced stages of the deficiency a macrocytic megaloblastic anaemia, associated with neutropenia and thrombocytopenia results. An increased number of hypersegmented polymorphs and megaloblastic changes in bone marrow and epithelial tissues can also be observed (Van Niekerk, 1966). In earlier stages none of these overt symptoms may be apparent but a deficiency may still be detected by measurement of serum or red cell folate or by urinary formiminoglutamic acid (Figlu) excretion (which is usually elevated in folate deficiency).

Folate Deficiency and Pregnancy

During pregnancy maternal vitamin requirements are increased by the conceptus, the uterus, and by a marked rise in maternal blood volume. An increase in plasma volume of up to 50% is not uncommon (Hyttan, 1980) although the increase in red cell volume is not usually as great. Consequently, a physiological fall in haemoglobin, haematocrit and red cell count is often observed during pregnancy (Shojania, 1984).

The most common vitamin deficiency associated with pregnancy is a folate deficiency. Many factors may contribute to this including excess urinary loss and low dietary intake due to poverty, hyperemesis, food fads, etc. However, the main draw on folate
reserves is the production of placental tissues, red cells and, predominantly, embryonic tissues. There is a high demand for protein and nucleic acid during cell proliferation and the rapid rate of growth of embryonic cells can easily exhaust maternal folate reserves. During intrauterine growth fetal requirements are much higher than those of the mother, and the circulating folate concentrations in the infant are invariably much higher than the levels found in maternal blood (Baker et al., 1958; Grossowicz et al., 1960; Giles, 1966). Also, in order to protect against a deficiency, the placenta acts as a selective filter allowing an accumulation of folate on the fetal side of the placenta (Baker et al., 1981).

Although increased folate requirement is the major cause of maternal folate deficiency, other factors may also contribute. Inadequate utilisation and defective metabolism or absorption of folate have also been reported and are factors which could easily aggravate the deficiency (Forshaw et al., 1957; Gatenby & Lillie, 1960; Lowenstein et al., 1966).

Maternal folate deficiency is therefore common during pregnancy and although the effects on the maternal system may be slight or even unnoticed, if the developing embryo becomes exposed to the deficiency the consequences may be catastrophic. A folate deficiency will interfere with folate-dependent reactions which may cause disturbances in both mitotic and intermitotic stages of cell development. Abnormal differentiation and development resulting in malformations or impaired function of organs and tissues may ensue.
Congenital Abnormalities and Folate Deficiency

A number of human congenital malformations have been associated with nutritional folate deficiency. These include spina bifida, exencephaly, anencephaly, hydrocephalus, microphthalmia, harelip, cleft palate, club foot and syndactyly (Hillemann, 1961). Abnormalities of the immune system, reduced brain function, abruptio placentae, abortion, prematurity, accidental haemorrhage and low birthweight have also been reported (Youinon, 1982; Hibbard et al., 1965; Martin et al., 1965; Hibbard & Kenna, 1974; Hibbard & Jeffcoate, 1966; Gatenby & Lillie, 1960; Hibbard & Hibbard, 1963; Zittoun, 1985; Iyengar & Rajalakshmi, 1975). In spite of the many observations the evidence for this association is still only suggestive and the literature is full of conflicting reports.

Many studies investigating the effects of folate deficiency have been carried out using experimental animals. Rats have been the predominant model although other animal species have also been used.

Nelson (1960) noted a wide range and high incidence of congenital defects in rats fed a folate deficient diet. Her studies, however, included the use of folate antimetabolites whose complex mode of action, involving folate and non-folate related toxicity, makes it impossible to distinguish its influence on the induction of the observed abnormalities. Tagbo & Hill (1977) demonstrated a reduction in the total number of live births, average litter size, birth weight and percentage survival to weaning in rats receiving a folate deficient diet. Poor reproductive
performance, decreased weight gain during gestation and retarded structural development of embryos have also been reported (Nelson & Evans, 1947, 1949; Johnson et al 1963; Tagbo & Hill, 1977). Although a very broad spectrum of abnormalities have therefore been observed, their incidence and type have varied depending on the degree, duration and gestational stage during the establishment of the deficiency (Nelson et al, 1952 & 1955; Nelson, 1960; Chepenik & Waite, 1972; Jaffe & Johnson, 1973).

Folate Deficiency and Neural Tube Defects
There is much evidence from experimental animal studies and from clinical investigations to implicate folate deficiency in the aetiology of neural tube defects (NTD).

Using rats, Richardson & Hogan (1946) found that maintenance on a folate free diet for long periods, prior to and during pregnancy, resulted in the development of hydrocephalus in the offspring. Another group also reported central nervous system (CNS) abnormalities in animals born to dams fed a folate free diet during pregnancy (Whitley et al, 1951). A variety of defects including CNS malformations have also been observed in rat embryos in association with folate deficient diets and folate antagonists (Nelson et al, 1955; Nelson, 1960).

In the 1960's several clinical reports linked maternal folate deficiency with the birth of NTD babies (Hibbard & Watt, 1964; Hibbard, 1964; Hibbard & Smithells, 1965; Hibbard & Hibbard, 1966). In one study abnormal folate levels were observed in 69% of 35 mothers of infants with CNS malformations compared with 17% of 35 controls with normal babies. The controls were matched for
age, parity, time of conception and gestation. (Hibbard & Smithells, 1965). In another study 29% of women with megaloblastic anaemia (associated with folate deficiency) gave birth to infants with serious birth defects - 80% of which involved the neural tube (Fraser & Watt, 1964). Blood concentrations of folic acid have been shown to be lower in women with pregnancies ending in the birth of a NTD baby than among control mothers (Smithells et al, 1976 & 1980; Laurence et al, 1983).

An association between abnormal folate metabolism during pregnancy and NTD has been suggested. The profound effect which pregnancy has on folate metabolism (Rothman, 1970) (also see Chapter 4, Folate Deficiency and Pregnancy) implies that derangement of folate metabolism could increase the susceptibility of the infant to NTD.

Although the experimental evidence suggests that folate unavailability for the developing embryo might be a precipitating factor in the aetiology of NTD, it is widely accepted that folate deficiency is unlikely to be solely responsible (Schorah, 1983; Laurence et al, 1980). It is more probable that several factors such as other environmental elements and genetic characteristics, which may or may not influence folate metabolism, will also be involved. Accumulating evidence, does however imply that maternal supplementation with either folic acid alone or a multivitamin preparation containing folic acid, may result in a reduction in the expected incidence of NTD (Smithells et al, 1980, 1981a, 1981b & 1983; Smithells, 1982; Laurence et al, 1981 & 1983; Laurence, 1985; Schorah et al, 1983; Wild et al, 1986).
CHAPTER 5

THE PROBLEM AND AIMS OF THIS PROJECT

The evidence for an association between neural tube defects and a deficiency of either folate alone or in combination with other vitamins is accumulating, but as yet the results are far from conclusive.

The claim that NTD may be prevented by folate or multivitamin supplementation has caused much professional controversy. Until the evidence is clear, arguments for and against a supplementation regimen will continue to place at risk one or other sector of the population. If the hypothesis is proven, non-supplementation will increase the risk of NTD births and if the theory is untrue, supplementation will have resulted in the waste of a great deal of time and effort and possible damage to the fetus by unnecessary maternal vitamin supplementation. (At present there is no evidence to suggest that any of the vitamins included in the multivitamin supplementation studies will cause harm. There is also, however, no conclusive evidence that they are completely safe.)

The problem is therefore extremely important and resolution is urgently needed.

The best possible test system to confirm or disprove the supplementation hypothesis would be a well designed placebo-controlled double-blind clinical study. This would also include an analysis of the patient's general nutritional intake as a separate factor from additional vitamin supplements. Ethically this type of trial has many drawbacks and various ethical
committees have already rejected several study proposals. A placebo-controlled study is confronted by the persuasive argument that, as there is a great deal of evidence which suggests that multivitamin supplementation does reduce the risk of recurrence in high-risk mothers, it would be unethical to withhold supplementation from this group of subjects. This argument counters both the use of placebos for high-risk mothers and double-blind selection. Equally it has been argued that all studies to date have been carried out in high-risk areas and it is not yet clear whether the reported protection offered by folate and multivitamin supplements would be applicable to women without NTD history, i.e. whether prevention would work in low-risk as well as high-risk areas. It would be reasonable to expect first-time mothers to reject the use of an unproven remedy and it could also be argued that, as the evidence is still inconclusive, the cost of offering supplementation to all women is not justified.

The question of clinical trials and their design is therefore one of understandable controversy. However, there are presently at least four extensive multicentre trials either underway or planned.

In the U.K., the Medical Research Council is conducting a large multicentre randomised trial (Wald & Polani, 1984). This study is designed to test the hypothesis generated from the results of the earlier studies of Smithells and Laurence and will include 4 allocation groups, viz., (1) placebo, (2) multivitamins with folate, (3) multivitamins without folate, and (4) folate alone. Women included in this study will be high-risk i.e. have had a
affected pregnancy. Therefore, if any benefit is confirmed, it will still be uncertain whether the findings will apply to low-risk groups i.e. the general population.

A similarly designed study is also being conducted in Ireland (Elwood, 1983). However, as Ireland is a high-risk area, in view of the ethical issue, no placebo group has been included.

In the United States (which has lower rates of NTD than either Britain or Ireland) a retrospective (case-controlled) study has been organised (Rhoads & Mills, 1986). After pregnancy, neural tube defect cases will be identified and the mothers questioned, by telephone, about their periconceptional vitamin use. Incorrect recall could prove a major problem to the accuracy of results but this trial has the advantage of assessing the general population rather than a single highly-predisposed sector.

Hungary is also conducting a trial which is unique in that its major concern is primary prevention. Before embarking on their first pregnancy counselled couples are being allocated to either of 2 groups, viz., multivitamins including folate or placebo (Czeizel & Rode, 1984). This study again has the advantage of assessing the general population but the recruitment numbers will have to be very large in order to detect a significant reduction in the incidence of NTD.

These trials will obviously take a long time. However, while awaiting their results it may be possible to make some contribution to our knowledge of the NTD problem by using an animal system. By this approach it should be possible to circumvent some of the ethical difficulties of human studies.
Although direct extrapolation of animal data to man is not possible, it may offer some explanation for observations made in humans, or even indicate a potential therapy.

The purpose of this study is therefore to assess *in vitro*, the effect of exposing the developing embryo to a folate deficiency. By using whole embryo culture, it is proposed to culture rat embryos in folate deficient serum during the critical period of organogenesis which encompasses neural tube development and closure. If a relationship between folate deficiency and neural tube defect is confirmed it may then be possible to assess the protective effect of folate and the degree of reversibility of the maternal condition leading to the observed abnormalities.
SECTION II

MATERIALS AND METHODS

CHAPTER 6

WHOLE EMBRYO CULTURE

Over the past 20 years, largely as a result of the pioneering work of D.A.T. New and colleagues, techniques have been devised for growing post-implantation rodent embryos *in vitro*. Prior to this, much work had been carried out with pre-implantation eggs and blastocysts, whose greater availability, ease of handling and relative independance from the maternal system, resulted in more rapid development of *in vitro* culture techniques (for reviews see Mintz, 1967; Brinster, 1969; Whittingham, 1971 & 1975). The relevance of experiments using such early stage embryos for teratological studies is, however, limited because of their known resistance to teratogenic assault. The undifferentiated cells of the early cleavage embryo are able to reorganise to allow the continuation of normal development, even after extensive damage (Wilson, 1973b). On the other hand, disruptions during the post-implantation stages of development may subsequently result in embryolethality or gross dysmorphogenesis. In particular, during the period of major organogenesis, when extensive growth and differentiation is occurring, embryos are extremely sensitive to even subtle disturbances in the environment. A procedure enabling *in vitro* manipulation of this stage of embryogenesis is...
therefore more relevant for studying congenital abnormalities than a pre-implantation embryo culture system.

Procedures currently used for obtaining and culturing post-implantation embryos were first described by New in 1967. Since then the techniques have progressively been improved and refined (New, 1971; New et al, 1973) and now, simple and reproducible methods are available which allow rat embryos to be grown, for periods of up to 4 days, over a range of ages. (From the egg-cylinder stage to as much as 60 somites). Although embryos as young as 7.5 days have been successfully grown in vitro, rat embryos explanted at 9.5 days and cultured for 48 hours compare particularly well with in vivo embryos of the equivalent age. (Continuation of culture past 48 hours results in a gradual reduction in the comparable growth rate (New et al, 1976a & 1976b).)

The development of this system therefore provides a valuable tool for studying normal and abnormal development of mammalian embryos.

Preparation of Culture Serum
Serum was prepared by exsanguination of male or female Wistar rats. Blood, withdrawn aseptically from the dorsal aorta, was immediately centrifuged at 2000 rpm for 15 minutes in sterile plastic centrifuge tubes (Steele & New, 1974). In the supernatant, above the precipitated red cells, a fibrin clot rapidly formed. Once this started to contract naturally, its contained serum was extracted by squeezing the clot with sterile forceps. The remaining fibrin was removed and the tube
recentrifuged for a further 5 minutes. The clear straw coloured serum was then decanted and 0.02ml of antibiotics (Penicillin/Streptomycin, concentration 5000µg/5000I.U./ml) added per ml of serum. This could then be stored at -20°C for 6-8 weeks. Prior to use, the serum was heat-inactivated by incubation at 56°C for 30 minutes (Steele & New, 1974; New et al, 1976a).

**Culture Equipment**

Sterilised stainless steel dissecting instruments were used at all times. Glass Petri dishes and pasteur pipettes were heat sterilised prior to use. Heat sterilised pyrex reagent bottles with silicone rubber bungs were employed as culture vessels (fig. 2).

![Culture Bottle Diagram]

*Fig 2. Embryo culture bottle.*

Purpose built roller incubators were used throughout the study (fig. 3). These consisted of a number of horizontal rollers, driven at 30 - 60 rpm, housed in an incubator shell maintained at 37°C. The culture bottles were laid horizontally on the rollers. The continuous rotation promoted oxygenation of the medium by constantly exposing a fresh layer of medium to the gas phase.
This enhanced embryonic growth by assisting respiration (New et al, 1973).

Fig 3. Roller incubator.

Timing of Pregnancy
Wistar rats were housed under standard conditions and received food and water ad libitum. Breeding pairs were caged together in the late afternoon and the presence of vaginal plugs on the floor of the cage on the following morning indicated pregnancy. For purposes of estimating gestational age, conception was assumed to have occurred at midnight on the preceding night. At 11am on the 10th day of gestation the explantation procedure was started.

Explantation Procedure
Embryos were explanted using the methods described by New (1978). Firstly, the rats were anaesthetised by ether and the abdominal
area swabbed with 70% alcohol. Blood was removed via the dorsal aorta and the animals terminally anaesthetised. Following this the whole uterus was removed aseptically and placed in a Petri dish containing sterile Hank's balanced salt solution (Gibco Ltd., Paisley, Scotland) at 37°C. Each uterine horn was opened along the anti-mesometrial side using fine scissors and watchmaker's forceps. This revealed pear-shaped decidual swellings which were then gently teased away from their implantation sites (fig. 4a).

The decidual masses were transferred to a clean Petri dish
containing Hank's solution. Each was opened by making an angled incision in the broad end of the swelling using fine forceps (fig. 4b). One half of the tissue was then carefully torn away to expose the underlying cigar-shaped conceptus (fig. 5).

![Diagram of a Cigar-shaped conceptus surrounded by decidua.](image)

Using a dissecting microscope under low power magnification the remaining decidua tissue was teased away. The embryos and their yolk sacs with attached trophoblast and extraembryonic membranes were then placed in another fresh dish of Hank's solution. At one end of the conceptus the reddish coloured ectoplacental cone was evident and at the other end the Reichert's membrane projected beyond the underlying embryonic disc (fig. 5). As the Reichert's membrane will not develop in culture it had to be opened to allow the embryo and other membranes to grow (New, 1966). Using 2 pairs of watchmakers' forceps the Reichert's membrane was grasped and gently torn apart. The surplus membrane tissue was carefully trimmed back towards the ectoplacental cone as the adherent trophoblast could subsequently cause embryos to stick together in culture thereby restricting normal growth.
Throughout these procedures great care had to be taken not to strain or puncture the fragile underlying embryonic membranes. The 9.5 day egg-cylinders were then ready for culture (fig. 6).

As far as possible, embryos from each rat were equally allocated to the various experimental and control culture groups. Any damaged cylinders were disregarded.

Culture Procedure

9.5 day egg-cylinders were placed in sterile glass reagent bottles containing 1 ml of culture serum (pre-heated to 37°C) per embryo (New, 1978). 4 - 6 embryos were cultured in each bottle. The air in the vessel was replaced with a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen, the gas being introduced into the bottle via a glass pasteur pipette. The gassing procedure was carried out with the culture bottle in the horizontal position - the volume of medium being insufficient to allow spillage. (This volume also prevented contact between the embryos and the bottle neck where they could become stuck during
culture.) The flow of gas was regulated to give sufficient pressure to disturb the surface of the medium without severely agitating the embryos. Gassing was continued for approximately 2 minutes. The culture vessels were then sealed air-tight with sterile silicone rubber bungs and placed in a roller incubator at 37°C (figs. 2 & 3). The cultures were re-gassed with 20% oxygen, 5% carbon dioxide and 75% nitrogen after 24 hours of culture and then again after 42 - 44 hours with 40% oxygen, 5% carbon dioxide and 55% nitrogen. As the embryo develops, its oxygen requirements increase and the gassing schedule meets this demand (New et al, 1976b).

Embryonic Growth and Development During the Culture Period

At explantation, the 9.5 day rat embryo is at the pre-somite stage and the egg cylinder has divided into 3 cavities (fig. 6). These are the upper epamniotic cavity, middle extra-embryonic coelom and lower amniotic cavity. The cylinder is approximately 1.5mm long and 0.6mm in diameter. The dorsally concave embryo lies in the lower cavity and the bud of the allantois and the head folds are beginning to form.

At 10 days, the first few (occipital) somites have developed. Neural folds and the tubular endocardial rudiment are forming, the upper cavity has collapsed and the allantoic stalk is projecting into the extra-embryonic coelom.

After 24 hours in culture up to 12 somites have developed and the ectoplacental cone has fused with the epamniotic cavity wall forming the chorion. The neural folds are beginning to fuse and the S-shaped cardiac tube is beating. The optic sulci and otic
primordium are visible.

At 11 days, the upper thoracic somites (13 - 20) have formed and the anterior neuropore has closed. The prosencephalon, mesencephalon and rhombencephalon have developed as have the first 2 branchial arches. The cardiac tube has started to convolute and optic bulbs and otic pits have formed. The embryo has turned to become dorsally convex.

After 48 hours in culture i.e. at 11.5 days, the embryo has developed 26 - 28 somites and has completed many of the major processes of organogenesis. The posterior neuropore has closed and the third branchial arch and forelimb buds have developed. A vigorous heart beat provides good circulation to both the embryo and the yolk sac vessels. The crown-rump length of the embryo is now 3.0 - 3.5mm and it has a protein content of 120 - 200μg (fig. 7).
Assessment of Embryonic Growth

At the end of the 48 hour culture period the 11.5 day conceptuses were washed twice with Hank’s balanced salt solution at 37°C. Each embryo was then examined under a dissecting microscope and, with the aid of an eye-piece micrometer, the yolk sac diameter was measured (at the centre, horizontal to the ectoplacental cone). Embryonic growth and development were then quantitatively assessed using the morphological scoring system of Brown & Fabro (1981). This system assigns a numerical score (0-6), indicative of precise stages of development for each of 17 morphological features (table 1). (Table 1 details scores 0-4 which encompass development from 9.5 to 11.5 days. Scores of 5 and 6 apply to older embryos.) The scoring was carried out in 2 stages. Firstly, the yolk sac circulation, heart beat and fusion of the allantois with the chorion, were assessed. The yolk sac and
amniotic membranes were then carefully removed and each of the embryonic parameters listed in Table I scored. A half point was assigned to any feature at a point of development between 2 defined stages. The degree of development was also estimated by counting the number of somites in each embryo and measuring its crown-rump length. The total morphological score was then calculated as the sum of each of the scores for the 17 features. Occasionally, at this stage, an embryo was fixed and retained for photography. Otherwise, the embryonic protein content was determined using a modified version of the colourimetric method of Lowry et al. (1951) (see below). This involved solubilising the embryo for an hour in molar NaOH at 37°C. The resultant solution was neutralised and the total protein content of the embryo assessed, in duplicate, using phenol Ciocalteu's reagent and bovine serum albumin (Sigma Chemical Co. Ltd, Poole, Dorset) as a reference protein.

Determination of Embryonic Protein Content (by modified Lowry Protein Estimation).

The primary aim of this assay was to draw a standard curve of absorbance against µg of protein by assaying known amounts of bovine serum albumin (BSA) (Sigma Chemicals, Poole Dorset). The standard curve was then used to estimate the amount of protein in unknown samples from their measured absorbance (Δ).

To determine embryonic protein content, the embryos had to be solubilised in NaOH, prior to assay. In the standard protocol (Lowry et al., 1951), the various colour development steps are sensitive to pH changes and the presence of NaOH in the sample
<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>YOLK SAC CIRCULATORY SYSTEM</strong></td>
<td>No visible or scattered blood islands</td>
<td>Corona of blood islands w/o anastomoses</td>
<td>Vitelline vessels with few yolk sac vessels</td>
<td>Full yolk sac plexus of vessels</td>
</tr>
<tr>
<td><strong>ALLANTOIS</strong></td>
<td>Allantois free in amnion</td>
<td>Allantois free in amnion</td>
<td>Umbilical vessels</td>
<td>Separate aortic origins of umbilical and vitelline vessels</td>
</tr>
<tr>
<td><strong>FLEXION</strong></td>
<td>Ventrally convex</td>
<td>Turning</td>
<td>Dorsally convex</td>
<td>Dorsally convex with spiral torsion</td>
</tr>
<tr>
<td><strong>HEART</strong></td>
<td>Endocardial rudiment not visible or visible but not beating</td>
<td>Beating 's' shaped cardiac tube</td>
<td>Convoluted cardiac tube</td>
<td>Bulbus cordis, atrium commune and ventriculus communis</td>
</tr>
<tr>
<td><strong>CAUDAL NEURAL TUBE</strong></td>
<td>Neural plate or neural folds</td>
<td>Closing but unfused neural folds (groove)</td>
<td>Neural folds fused at level of somites 4/5</td>
<td>Posterior neuropore formed but open</td>
</tr>
<tr>
<td><strong>HIND BRAIN</strong></td>
<td>Neural plate</td>
<td>Rhombomeres A and B</td>
<td>Anterior neuropore formed but open</td>
<td>Anterior neuropore closed, rhombencephalon formed</td>
</tr>
<tr>
<td><strong>MED BRAIN</strong></td>
<td>Neural plate</td>
<td>Mesencephalic brain folds</td>
<td>Closing or fusing mesencephalic folds</td>
<td>Completely fused mesencephalon</td>
</tr>
<tr>
<td><strong>FORE BRAIN</strong></td>
<td>Neural plate or no visible prosencephalon</td>
<td>Prosencephalic brain folds</td>
<td>Completely fused prosencephalon</td>
<td>Visible telencephalic evaginations</td>
</tr>
<tr>
<td><strong>OTIC SYSTEM</strong></td>
<td>No sign of otic development</td>
<td>Flattened or indented otic primordium</td>
<td>Optic pit</td>
<td>Otocyst</td>
</tr>
<tr>
<td><strong>OPTIC SYSTEM</strong></td>
<td>No sign of optic development</td>
<td>Sulcus opticus</td>
<td>Elongated optic primordium</td>
<td>Primary optic vesicle with open optic stalk</td>
</tr>
<tr>
<td><strong>OLFACTORY SYSTEM</strong></td>
<td>No sign of olfactory development</td>
<td>Olfactory plate</td>
<td>Olfactory plate with rim</td>
<td>Distinct olfactory ridges</td>
</tr>
<tr>
<td><strong>BRANCHIAL BARS</strong></td>
<td>None visible</td>
<td>I visible</td>
<td>I and II visible</td>
<td>I, II and III visible</td>
</tr>
<tr>
<td><strong>MAXILLARY PROCESS</strong></td>
<td>No sign of maxillary development</td>
<td>Maxillary process demarcated, visible cleft anterior to bar</td>
<td>Maxillary process fused to nasal process</td>
<td></td>
</tr>
<tr>
<td><strong>MANDIBULAR PROCESS</strong></td>
<td>No sign of mandibular development from bar</td>
<td>First branchial bars fused and forming mandibular process</td>
<td>Fore limb bud</td>
<td>Paddle shaped fore limb bud</td>
</tr>
<tr>
<td><strong>FORE LIMB</strong></td>
<td>No sign fore limb development</td>
<td>Distinct evagination of wolffian crest at level of somites 9-13</td>
<td>Hind limb bud</td>
<td>Paddle shaped hind limb bud</td>
</tr>
<tr>
<td><strong>HIND LIMB</strong></td>
<td>No sign hind limb development</td>
<td>Distinct evagination of wolffian crest at level of somites 26-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOMITES</strong></td>
<td>0-6</td>
<td>7-13</td>
<td>14-20</td>
<td>21-27</td>
</tr>
</tbody>
</table>

**TABLE 1**
shifts the reaction pH away from its optimum value. The standard method therefore had to be modified by neutralising the excess alkali with acid.

Preparation of stock solutions and reagents:
(a) 1M NaOH : 40g/l.
(b) 3M HCl : 3.67ml conc. HCl plus 7.33ml distilled water.
(c) Sodium Carbonate (2%) : 2g anhydrous Na₂CO₃ in 100ml distilled water.
(d) Copper Sulphate (1%) : 1g CuSO₄·5H₂O in 100ml distilled water.
(e) Sodium Tartrate (2%) : 2g (CH(OH).COONa)₂·2H₂O in 100ml distilled water.
(f) Bovine Serum Albumin : 4.58mg BSA dissolved in 1ml 1M NaOH (Store at -20°C).
(g) Folin Ciocalteu's Reagent: BDH (Store at 4°C).

Folin A : Add 1ml of (d) and 1ml of (e) to 100ml of (c). (Must be freshly prepared.)
Folin B : Dilute (g) 1:1 with distilled H₂O.

Preparation of standard curve:
(1) 0, 10, 20, 30, 40 and 50µl aliquots of BSA stock solution were made up to 1ml with 1M NaOH.
(2) 0.145ml of 3M HCl was added and the samples mixed.
(3) 2 x 0.5ml aliquots were then taken into 9ml plastic tubes.
(4) To each tube 2.5ml Folin A was added and the samples left at room temperature for 20 minutes.

(5) 0.25ml Folin B was then added and mixed immediately. Optimum mixing was achieved by adding Folin B while mixing the tube contents on a whirlimixer. Note: At this stage of the assay, seconds delay in mixing diminishes the colour reaction.

(6) The samples were left at room temperature for 45 minutes and the absorbance then read at 750nm against a distilled water blank.

The resultant amounts of standard protein in the 0.5ml samples ranged between 0 and 100μg BSA. From this a standard curve was drawn as shown in fig. 8.

![Graph](image)

Fig 8. Typical standard curve for embryonic protein estimation.

Estimation of embryonic protein content:

(1) Each embryo was placed in 1ml NaOH in a sealed tube and
incubated at 37°C for 2 hours. The tubes were whirlimixed every 10-15 minutes.

(2) The assay procedures from stages (2)-(6) for the standard curve were then followed.

From the gradient of the standard curve, the embryonic protein content was estimated using the equation:

\[ y = a + bx \]

where \( y \) = corrected absorbance (\( \Delta c \)) of the sample (\( \Delta \)observed - \( \Delta \)blank).

- \( a = \) Intercept of curve = 0
- \( b = \) Gradient of curve.
- \( x = \) pg of protein in sample tube. Sample tube contains \( 0.5 / 1.145 \) of the total protein contained in the embryo.

Therefore, pg of protein in embryo = 2.29 \( x \)

**Folate Deficient Diet**

The composition of the diet was based on that used by Grossowicz et al (1964). The ingredients are detailed in Table 2. Vitamin-free casein (Fisons, Loughborough, Leics) was used as the protein source in the diet, while glucose, cornflour and vegetable oil formed the carbohydrate and fat sources. Succinylsulphathiazole was included to suppress folate synthesising gut micro-organisms.

The diet was prepared by adding each of the dry ingredients (starting with the smallest) to a large mixing bowl where they were thoroughly mixed before the next constituent was added. To ensure that the ingredients were completely and evenly distributed in the mixture they were first passed through a wire
**TABLE 2. COMPOSITION OF DIET**

(a) The Protein, Carbohydrate, Fat and Antibiotic Content of the Diet.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>per kg of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin free)</td>
<td>180g</td>
</tr>
<tr>
<td>Cornflour</td>
<td>600g</td>
</tr>
<tr>
<td>Glucose</td>
<td>200g</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>50g</td>
</tr>
<tr>
<td>Succinyl sulphathiazole</td>
<td>6g</td>
</tr>
</tbody>
</table>

(b) The Mineral Content of the Diet

<table>
<thead>
<tr>
<th>Mineral</th>
<th>per kg of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$</td>
<td>6.4g</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$</td>
<td>9.6g</td>
</tr>
<tr>
<td>Ca lactate</td>
<td>10.4g</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.4g</td>
</tr>
<tr>
<td>Na succinate</td>
<td>2.4g</td>
</tr>
<tr>
<td>KCl</td>
<td>1.8g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.6g</td>
</tr>
</tbody>
</table>

Trace Mineral

<table>
<thead>
<tr>
<th>Trace Mineral</th>
<th>per kg of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>KI</td>
<td>7mg</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>4mg</td>
</tr>
<tr>
<td>Fe citrate</td>
<td>400mg</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>117mg</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>40mg</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>28mg</td>
</tr>
</tbody>
</table>

(c) The Vitamin Content of the Diet

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>per kg of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>1mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>50mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>5mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>12mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>4mg</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>22mg</td>
</tr>
<tr>
<td>Inositol</td>
<td>33mg</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>20mg</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>52mg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.084mg</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2.7mg</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>6000 I.U.</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>1667 I.U.</td>
</tr>
</tbody>
</table>

**Supplemented diet only**

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>per kg of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid (Pteroylmonoglutamic acid)</td>
<td>2mg</td>
</tr>
</tbody>
</table>
mesh sieve. The vegetable oil and vitamin E (tocopherol oil) were thoroughly mixed together and then added to the mixture with distilled water.

The most practical and convenient means of presenting the diet was in tablet (cake) form. This vaguely simulated the type of pelleted food the animals normally receive and eliminated the problem of excessive tooth growth, which occurs in rats fed a paste diet which requires no gnawing. (The inclusion of lactate and glucose in the diet aided binding of the mixture to form the tablet.)

Once completely mixed, the white paste was transferred to a metal tray lined with grease proof paper. This was left overnight in an oven at 40°C. The white cake was broken and transferred to polythene bags where it was stored at -20°C until required.

Folate Supplemented Diet

As a control, a folate supplemented diet was also prepared. This had the same basal composition as the folate deficient diet (Table 2) but with the addition of 2mg/kg folic acid (Pteroylmonoglutamic acid, Sigma Chemical Company, Poole, Dorset).

(Standard rat chow contains 0.5-1.0mg/kg folate (Special Diet Services, Witham, Essex).)

Maintenance of Animals

Wistar rats (Allen & Hanbrough) were maintained in a temperature-regulated room at 20-22°C with a 12-hour light/dark cycle. They were caged in groups of 5 and had free access to food and water. All animals were inspected daily and their
folate status assessed fortnightly or monthly.

Monitoring Serum Folate

A monthly (in the initial stages of the study) or fortnightly sample of blood was collected from one rat in each experimental group. Samples were taken via the tail vein. The blood was immediately centrifuged and serum obtained as described previously (to maintain comparability throughout the study). The folate content of the serum was determined by radioimmunoassay (see next section). Each sample was analysed in duplicate.

Measurement of Serum Folate by Radioimmunoassay

Until fairly recently the only methods available for measurement of serum or red cell folate were various microbiological assays which used folate-requiring organisms. These assays tended to be laborious and time consuming and they were subject to certain problems (Kubasik et al, 1975). However, the discovery of a natural binder for several folates (Ghitis, 1967) and the availability of high-specific-activity radioisotopes of folates now provides a rapid and sensitive alternative to bioassays (Waxman, 1979; Waxman & Schreiber, 1980).

The $^{125}$Iodine Vitamin B$_{12}$/Folate dual radioassay kit used in this study (Amersham International plc, Amersham, U.K.) involved a two-stage procedure. This consisted of a sequential incubation in which the binder was first incubated with the unlabelled standards or serum samples. Tracer was then added which took up only the unoccupied binding sites. Dextran-coated charcoal was
used, followed by centrifugation, to separate the free folate from that which was bound. The iodine-labelled folates were then counted on a gamma counter (LKB RIA Gamma Counter). The radioactivity of samples was counted for at least 5 minutes to minimise the effects of counting error on the assay results. No serum blanks were necessary as the kit included standardised assay tubes containing the necessary samples to build a standard curve. This was in the range of 0–20μg/litre folate (fig. 9). Normal rat serum, which contains 40–100μg/l folate, had to be diluted 1 in 10 (with the included zero standard) before assay, to ensure that the measured value fell within the standard range. Serum from folate deficient animals did not require dilution as it usually contained less than 20μg/l folate.

Fig 9. Typical folate standard curve.

In this study it was postulated that it might be possible to create folate deficient serum by adding a folate antagonist directly to normal serum thus rendering the vitamin unavailable to the embryo in culture.

Folate Antagonists

Folic acid antagonists are a group of compounds which have a chemical structure almost identical to folic acid. Of the many anti-folate compounds which have been synthesised, the 3 most commonly used are aminopterin (4-amino folic acid), methotrexate (4-amino 10-methyl folic acid, also known as amethopterin) and 9-methyl folic acid.
Aminopterin and methotrexate are classic antimetabolites where the 4-hydroxyl group of folic acid has been replaced by an amino group (Baker, 1971). 9-methyl folic acid is characterised by the introduction of a methyl group on the methylene bridge in the 9-position (see Chapter 3).

Mechanisms of Action

Methotrexate and aminopterin are potent competitive inhibitors of folate coenzymes. The antagonists bind with dihydrofolate reductase preventing it from converting folates to the tetrahydrofolate coenzymes (Zakrzewski, 1966; Bertino, 1963;
Capizzi et al, 1971; Harrap, 1976). Because synthesis of the coenzymes is blocked, one-carbon transfers are inhibited and single carbons are not incorporated into the 2 and 8 positions of the purines. Deoxyuridylate is not methylated to thymidylate and DNA synthesis halts. Rapidly multiplying cells require the folate coenzymes in large amounts for mitosis and it is the disruption of this process which accounts for the chemotherapeutic value of folate antagonists in cancer therapy (Hitchings & Burchall, 1965; Karnofsky & Clarkson, 1963). Aminopterin and methotrexate have also been used in the treatment of severe psoriasis (Weinstein, 1983).

Unfortunately other normal proliferating tissues have a similar requirement for folate coenzymes thus accounting for the teratogenic properties of these compounds. Many folate antagonists have been identified as teratogens in both humans and laboratory animals (Nelson & Evans, 1947 & 1949; Thiersch & Philips, 1950; Giroud & Lefebvres, 1951; Thiersch, 1952; Nelson, 1960; Johnson, 1963; Kinney & Morse, 1964; Potier de Courcy, 1966; Potier de Courcy & Terroine, 1966).

**Experimental Aims**

By adding folate antagonists to rat serum prior to culture, it was postulated that the serum folate could be rendered ineffective. The serum folate would be prevented from performing its physiological role and any observed embryonic abnormalities would be a direct result of the unavailability of folate. The reversibility of abnormalities induced by this regimen could then be assessed by culturing embryos in antagonist supplemented...
serum to which excess folate had been added.

MATERIALS AND METHODS

Serum: Normal rat serum was obtained and prepared as described in Chapter 6).

Aminopterin solutions: Aminopterin (Sigma Chemical Company, Poole, Dorset) was made up in 7 different concentrations. The solutions were prepared by dissolving the powdered compound in sterile distilled water and then filter sterilising through a 0.45μm filter (Whatman Limited, Maidstone, Kent). The following concentrations were prepared - 1, 2, 3, 4, 5, 10 and 20μg/ml. By adding 5μl of the aminopterin solution per millilitre of serum, a final concentration of 5, 10, 15, 20, 25, 50 and 100ng/ml of aminopterin was included in the culture medium.

Folate solutions: 2 concentrations, 20 and 50μg/ml, of folate were prepared by adding the 5-methyl tetrahydrofolate form of the vitamin (obtained from Sigma Chemical Company, Poole, Dorset) to sterile distilled water. The final solutions were filter sterilised as for aminopterin. By adding 10μl of the folate solution per millilitre of culture serum, a final concentration of 200 or 500ng of folate per ml of serum was obtained.

Culture in serum with antagonist added: 9.5 day embryos were obtained from pregnant donor Wistar rats which had been maintained under standard conditions. These were explanted as detailed in Chapter 6.

Culture groups and procedure: Embryos were divided into 17 culture groups as follows;

Group (1) Normal rat serum (NRS).
Groups (2)-(8) NRS + 5, 10, 15, 20, 25, 50 or 100ng/ml aminopterin.

Groups (9)-(15) NRS + 200ng/ml folate + 5, 10, 15, 20, 25, 50 or 100ng/ml aminopterin.

Group (16) NRS + 200ng/ml folate.

Group (17) NRS + 500ng/ml folate.

The culture procedure described in Chapter 6 was followed.

Assessment of embryonic development: Embryonic growth and development were quantified as described in Chapter 6, i.e. the yolk sac diameter and crown rump length were measured, morphological features scored according to the protocol of Brown & Fabro (1981), and embryonic protein content estimated by the modified Lowry method (Lowry et al, 1951).

RESULTS

Table 3 summarises the results. Addition of either 5 or 10ng/ml of aminopterin induced no abnormalities but minor growth retardation was indicated by a slight reduction in embryonic protein content (fig. 10a).

![Fig 10a. 11.5 day embryo cultured in the presence of 10ng/ml aminopterin.](image-url)
<table>
<thead>
<tr>
<th>GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>YOLK SAC DIAMETER (mm)</th>
<th>CROWN RUMP LENGTH (mm)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/ RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Rat Serum (NRS)</td>
<td>15</td>
<td>42.07 ± 0.175</td>
<td>196.6 ± 10.72</td>
<td>3.79 ± 0.138</td>
<td>3.58 ± 0.083</td>
<td>0</td>
<td>0</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>NRS + 5ng/ml Aminopterin (Am.)</td>
<td>6</td>
<td>42.14 ± 0.245</td>
<td>140.8 ± 8.43</td>
<td>4.04 ± 0.120</td>
<td>3.46 ± 0.103</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NRS + 10ng/ml Am.</td>
<td>7</td>
<td>42.01 ± 0.365</td>
<td>142.6 ± 10.67</td>
<td>4.03 ± 0.071</td>
<td>3.38 ± 0.040</td>
<td>0</td>
<td>0</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>NRS + 15ng/ml Am.</td>
<td>6</td>
<td>38.25 ± 1.427</td>
<td>114.9 ± 3.43</td>
<td>2.96 ± 0.103</td>
<td>2.60 ± 0.055</td>
<td>2 (33%)</td>
<td>1 (17%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>NRS + 20ng/ml Am.</td>
<td>8</td>
<td>36.18 ± 0.979</td>
<td>103.6 ± 7.88</td>
<td>2.51 ± 0.083</td>
<td>2.44 ± 0.082</td>
<td>6 (75%)</td>
<td>3 (37%)</td>
<td>7 (87%)</td>
</tr>
<tr>
<td>NRS + 25ng/ml Am.</td>
<td>8</td>
<td>29.04 ± 1.873</td>
<td>88.6 ± 8.51</td>
<td>2.21 ± 0.119</td>
<td>1.97 ± 0.153</td>
<td>8 (100%)</td>
<td>3 (37%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>NRS + 50ng/ml Am.</td>
<td>7</td>
<td>21.93 ± 2.934</td>
<td>63.9 ± 9.25</td>
<td>2.17 ± 0.191</td>
<td>1.49 ± 0.200</td>
<td>7 (100%)</td>
<td>7 (100%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>NRS + 100ng/ml Am.</td>
<td>7</td>
<td>ND</td>
<td>33.4 ± 14.51</td>
<td>1.53 ± 0.119</td>
<td>1.00 ± 0.107</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NRS + 200ng/ml Folate (F)</td>
<td>6</td>
<td>42.40 ± 0.340</td>
<td>204.2 ± 8.74</td>
<td>4.02 ± 0.102</td>
<td>3.48 ± 0.097</td>
<td>0</td>
<td>0</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 5ng/ml Am.</td>
<td>6</td>
<td>42.0 ± 0</td>
<td>149.6 ± 6.01</td>
<td>3.38 ± 0.086</td>
<td>3.08 ± 0.037</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 10ng/ml Am.</td>
<td>7</td>
<td>42.0 ± 0</td>
<td>139.0 ± 5.16</td>
<td>3.20 ± 0.071</td>
<td>2.88 ± 0.037</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 15ng/ml Am.</td>
<td>6</td>
<td>41.11 ± 0.557</td>
<td>145.1 ± 8.89</td>
<td>3.08 ± 0.058</td>
<td>2.80 ± 0.071</td>
<td>1 (17%)</td>
<td>0</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 20ng/ml Am.</td>
<td>10</td>
<td>40.36 ± 0.211</td>
<td>136.8 ± 2.76</td>
<td>3.09 ± 0.070</td>
<td>3.05 ± 0.022</td>
<td>2 (20%)</td>
<td>2 (20%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 25ng/ml Am.</td>
<td>12</td>
<td>35.92 ± 1.867</td>
<td>131.9 ± 18.34</td>
<td>2.85 ± 0.136</td>
<td>2.50 ± 0.176</td>
<td>5 (42%)</td>
<td>6 (50%)</td>
<td>10 (83%)</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 50ng/ml Am.</td>
<td>7</td>
<td>30.86 ± 3.169</td>
<td>68.3 ± 13.43</td>
<td>2.41 ± 0.177</td>
<td>2.09 ± 0.199</td>
<td>4 (57%)</td>
<td>7 (100%)</td>
<td>6 (86%)</td>
</tr>
<tr>
<td>NRS + 200ng/ml F + 100ng/ml Am.</td>
<td>6</td>
<td>ND</td>
<td>47.2 ± 15.39</td>
<td>1.94 ± 0.194</td>
<td>1.73 ± 0.285</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NRS + 500ng/ml Folate</td>
<td>6</td>
<td>42.33 ± 0.211</td>
<td>189.3 ± 7.06</td>
<td>4.0 ± 0.058</td>
<td>3.55 ± 0.043</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the mean of the number of samples 'n' ± the standard error

ND = not done
Addition of 15 ng/ml gave a mixture of normal and slightly abnormal embryos and supplementation with 20 ng/ml aminopterin induced abnormalities and growth retardation in almost all embryos. The main defects were abnormal turning, retarded eye development and anaemia, with anaemic embryos and their yolk sacs exhibiting a distinct lack of colour in the corpuscular elements of the blood. Embryos also had reduced protein content, were smaller than normal and were generally underdeveloped (fig. 10b).

Addition of 25 ng/ml resulted in similar but more severe deformities and very reduced growth, and supplementation with 50 and 100 ng/ml aminopterin created an environment in which embryos would not grow. Morphological features were virtually...
indistinguishable in these embryos (fig. 10c).

Fig 10c. 11.5 day embryo cultured in the presence of 100ng/ml aminopterin.

Addition of 200ng/ml folate to the aminopterin treated cultures appeared to at least partially counter the effects of the antagonist. When added to the 15 and 20ng/ml aminopterin groups, improved growth and very few abnormalities were observed. Addition of folate to the 25 and 50ng/ml aminopterin treated cultures also resulted in better overall development although abnormalities were not completely eliminated and the protein content, particularly for the 50ng/ml aminopterin group, was still well below that observed in the normal rat serum control group. Gross abnormalities were still apparent in the 100ng/ml aminopterin folate supplemented cultures, although, even in this group, some improvement in embryonic protein was observed. An excess of 500ng/ml of folate did not appear to be embryopathic. (Normal rat serum folate levels range between 40 and 100ng/ml.)
Morphological scores, embryonic protein content, yolk sac diameter and crown rump length for the various groups are detailed in Table 3.

DISCUSSION

The abnormalities observed were similar to those reported by other workers who have studied the teratogenic nature of aminopterin (Nelson & Evans, 1947; Giroud & Lefebvres, 1951; Giroud et al, 1952; Tuchmann-Duplessis & Mercier-Parot, 1957; Nelson, 1957 & 1960; Johnson, 1963; Kinney & Morse, 1964; Potier de Courcy, 1966). However, the mode of action of folate antagonists is extremely complex (Kinney & Morse, 1964; Potier de Courcy, 1966; Chepenik et al, 1970). While it is known that they act as competitive inhibitors of folate coenzymes (Zakrzewski, 1966; Bertino, 1963; Capizzi et al, 1971; Harrap, 1976) they may also exert other non-specific toxic effects on the developing embryo. Because of this, it was impossible to conclude that the observed abnormalities were solely due to unavailability of enzymatically active folate and not to some other toxic effect of aminopterin. As the two confounding effects are inseparable the results remain inconclusive and the original experimental aims could not be achieved. An alternative means of obtaining a folate deficient culture serum therefore had to be sought.
DIETARY INDUCED FOLATE DEFICIENT SERUM

Numerous experiments have been carried out in the past in which a folate deficient state has been induced by dietary means. Most of these experiments have, however, combined a folate-free diet with folate antagonists to accelerate the development of the deficiency. But, as suggested in the previous chapter, the teratogenic effects produced cannot necessarily be equated with the possible pathological effects of the folate deficiency alone. To create embryonic abnormalities which were only related to a folate deficient state, another means of induction therefore had to be used.

In this study a folate-free diet containing an intestinal antibiotic, but no antagonist, was employed.

MATERIALS AND METHODS

Intestinal Antibiotic

Some animals, for example the chick and monkey, require an exogenous supply of folate for normal growth and development. For these species, the presence of adequate amounts of the vitamin in the diet is essential and a deficiency can readily be induced by excluding folic acid from the diet. In other animal species including the rat, the gut micro-organisms produce sufficient amounts of folic acid to meet normal requirements. Consequently deficiency symptoms do not develop by feeding a folate-free diet unless an intestinal antibiotic is included to depress bacterial growth (Wright & Welch, 1943; Giroud & Lefebvres, 1951; Hautvast & Barnes, 1974; Howard et al, 1974).
The antibiotic used in the present study was succinylsulphathiazole which is not absorbed into the bloodstream and does not interfere with normal metabolism, i.e. its presence had no effect other than depletion of gut microflora (Kodicek & Carpenter, 1950b; Klipstein et al, 1973; Tagbo & Hill, 1977). This was included in the composition of the folate deficient diet (see Chapter 6).

Dietary Studies

Female Wistar rats, approximately 6 weeks old and each weighing around 100 grams were randomly allocated to one of four dietary regimens. Group 1 rats received a normal rat chow diet and group 2 were fed a folate-free diet containing 0.6% succinylsulphathiazole (SST). Group 3 animals were given the folate-free diet with 0.6% SST and also supplemented with 0.2% folic acid and group 4 was maintained on the folate-free diet plus 0.2% folic acid and 3% SST.

4, 8 and 12 weeks after the start of the study, approximately 0.5 ml of blood was collected from the tail vein of 2 animals (randomly selected) from group 2, for determination of serum folate by radioimmunoassay. Thereafter, samples were collected at fortnightly intervals.

In an initial experiment, after 12 weeks, rats from each group were exsanguinated and serum used to culture embryos. No abnormalities were observed even though animals in group 2 exhibited up to a 20-fold decrease in serum folate (5-6 ng/ml
folate compared with 40-100ng/ml in normal rat serum).
The dietary regimen was therefore continued until 16 weeks, by which time the serum folate levels in group 2 animals had decreased to between 2 and 3.5ng/ml. When embryos were cultured in serum from this group malformations were observed. Maintaining rats on the folate deficient diet beyond 16 weeks resulted in only a marginal further decline in serum folate. By this stage the coats of the animals were beginning to show signs of piloerection and their eyes were becoming reddened and some rats had developed a reddish brown ocular discharge. This had previously been noted by others employing folate deficient dietary regimens although no explanation has as yet been offered (Darke & White, 1949; Stokstad, 1954; Tagbo & Hill, 1977). Otherwise the animals were in reasonable condition. In an attempt to further decrease the serum folate levels a new group of female animals were placed on the folate deficient diet and mated after 4 weeks. The Wistar males used were maintained on a normal rat chow diet until mating, when they were caged with individual females until pregnancy was confirmed by the presence of vaginal plugs. During mating, both animals had free access to only the deficient diet. After pregnancy was established, the males were removed and the females re-grouped and continued on the folate-free diet. Towards the end of gestation the dams were transferred to single cages containing nesting materials. When the litters had been weaned (3-4 weeks), 0.5ml blood samples were collected from the mothers and assayed for folate. Most animals had serum folate levels of 2.0-3.5ng/ml. The pregnancy had
therefore accelerated the decline of serum folate but
continuation on the diet did not appear to markedly further
decrease their folate status.
Serum samples from the weaned offspring demonstrated a fairly low
folate content (less than 3.0 ng/ml). The pups were single-sex
grouped (19 females and 23 males) and continued on the deficient
diet until sexually mature (10-12 weeks). They were then placed
in individual pairs for mating. Pairs were kept together either
until pregnancy was established or for a maximum of a week. The
folate deficiency appeared to have rendered many of the animals
infertile as only about 20% (4/19) females became pregnant.
(Mating the non-pregnant females with males maintained on a
normal rat chow diet also failed to induce pregnancy.) All of
these first generation animals, both male and female, had reduced
growth rates and as the diet progressed some became sick and had
to be destroyed. Only 2 pregnant females reached gestation and
the litters were very much smaller than normal with only 2 and 5
pups. Although the pups were initially fairly healthy their
weight gain was very much reduced. Both rats in the smaller
litter also developed an abnormal pattern of hair growth (fig.
11a & 11b) and the other litter retained their soft fluffy
primary coat with no signs of development of the sleeker
secondary fur (fig. 12). Because of their reduced weight gain,
weaning, which normally takes place around 3-4 weeks, was
postponed. However, by about 4 weeks of age one pup died and
over the following 2 weeks the remaining pups became so sickly
that all had to be destroyed.
Fig 11a. Normal 4 week old rat pup.

Fig 11b. Second generation folate deficient rat pup aged 4 weeks. Note reduced size and abnormal pattern of hair growth.

Fig 12. (see over) Second generation folate deficient rat pup aged 4 weeks. Note much reduced size and abnormal coat texture.
The surviving first generation animals had, by this stage, been maintained on the deficient diet for about 6 months and their serum folate levels had plateaued at around 1.5-2.5ng/ml (fig. 13).

Fig 13. Progressive depletion of serum folate with increasing time on folate deficient diet.
Serum was collected and prepared for embryo culture (see Chapter 6).

Following these studies it was decided that the production of serum with low enough levels of folate to produce embryonic malformations in \textit{in vitro} culture would be most rapidly and economically achieved by one of 3 methods. The first and simplest involved using serum from non-pregnant animals which had been maintained on the folate deficient diet for 4-6 months. The second and third alternatives were more rapid, using serum from pregnant animals post-partum or from their offspring post-weaning. All three methods achieved serum folate levels below the critical level necessary to induce embryonic malformations in culture.

\textbf{Culture of embryos}

9.5 day donor embryos, from rats maintained on a normal rat chow diet, were used in all experiments and embryos from different rats were randomly distributed between the various experimental groups. Serum was collected and prepared, and embryos explanted, cultured and assessed according to the procedures detailed in Chapter 6.

\textbf{Experiment 1}

After 12 weeks on the diet. Four groups of embryos were cultured as follows:

Group 1 - serum from animals receiving a normal rat chow diet. 
Serum folate = 86ng/ml.

Group 2 - serum from animals maintained on a folate-free diet
containing 0.6% SST. Serum folate = 5.1ng/ml.

Group 3 - serum from animals fed the folate-free diet supplemented with 0.6% SST and 0.2% folic acid. Serum folate = 102ng/ml.

Group 4 - serum from rats receiving the folate-free diet plus 0.2% folic acid and 3% SST. Serum folate = 97ng/ml.

Experiment 2
After 16 weeks on the diet. Culture groups were as detailed in experiment 1 with serum folate contents as follows:

Group 1 - 72ng/ml.
Group 2 - 2.9ng/ml.
Group 3 - 89ng/ml.
Group 4 - 94ng/ml.

Experiment 3
Animals maintained on the diets for 20 weeks. Groups as described in experiment 1 with the following serum folates:

Group 1 - 97ng/ml.
Group 2 - 2.7ng/ml.
Group 3 - 78ng/ml.
Group 4 - 66ng/ml.

Experiment 4
First generation rats (born to folate deficient mothers) maintained on the diet for 6 months. Groups as above with the following serum folate contents:

Group 1 - 75ng/ml.
Group 2 - 2.1ng/ml.
Group 3 - 78ng/ml.
Group 4 - 108ng/ml.

Experiment 5

To further reduce the amount of folate available to the embryos in culture, serum with folate content greater than that known to produce abnormalities was diluted. Hank's balanced salt solution was added to the serum, prior to culture, to give a 30% dilution. The aim of this experiment was to make use of serum which may otherwise have been of little value.

Four culture groups were included. These were groups 1-3 as detailed in experiment 1 except that the serum from each was diluted by 30% with Hank's solution, and the fourth group was the group 2 serum of the present experiment, undiluted. The serum folate content of the 4 groups was as follows:

- Group 1 - 53.4ng/ml.
- Group 2 - 3.08ng/ml.
- Group 3 - 61.2ng/ml.
- Group 4 - 4.4ng/ml.

RESULTS

Experiment 1: No embryonic malformations were observed in any of the 4 culture groups although embryos in group 2 did show a slight reduction in their protein content. The results are detailed in Table 4.

Experiment 2: In this experiment, results of which are given in Table 5, embryos in groups 1, 3 and 4 were normal with no apparent malformations and with normal protein contents. Group 2 animals, however, maintained on the folate deficient diet for 16
### TABLE 4

Experiment 1: After 12 weeks on the diet

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (μg)</th>
<th>YOLK SAC DIAMETER (mm)</th>
<th>CROWN RUMP LENGTH (mm)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1^a</td>
<td>15</td>
<td>42.30 ± 0.238</td>
<td>169.5 ± 4.76</td>
<td>3.63 ± 0.075</td>
<td>3.22 ± 0.088</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>GROUP 2^b</td>
<td>14</td>
<td>41.29 ± 0.194 (p&gt;0.1(NS))</td>
<td>155.1 ± 5.04 (p&lt;0.1 &gt;0.01)</td>
<td>3.51 ± 0.061 (p&gt;0.1(NS))</td>
<td>3.14 ± 0.071 (p&gt;0.1(NS))</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GROUP 3^c</td>
<td>10</td>
<td>42.10 ± 0.179 (p&gt;0.1(NS))</td>
<td>163.3 ± 5.55 (p&gt;0.1(NS))</td>
<td>3.45 ± 0.085 (p&gt;0.1(NS))</td>
<td>3.20 ± 0.056 (p&gt;0.1(NS))</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>GROUP 4^d</td>
<td>10</td>
<td>42.01 ± 0.149 (p&gt;0.1(NS))</td>
<td>165.5 ± 4.54 (p&gt;0.1(NS))</td>
<td>3.55 ± 0.058 (p&gt;0.1(NS))</td>
<td>3.12 ± 0.083 (p&gt;0.1(NS))</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.
p values calculated by Student's T-test indicating the difference from culture in normal rat serum (Group 1).

^a Serum from animals receiving a normal rat chow diet.
^b Serum from animals maintained on a folate-free diet containing 0.6% succinylsulphathiazole (SST).
^c Serum from animals fed the same folate-free diet but with a supplement of 0.2% folic acid.
^d Serum from animals fed the folate-free diet plus 0.2% folic acid and 3% SST.
NS = Not significant


<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>YOLK SAC DIAMETER (mm)</th>
<th>CROWN RUMP LENGTH (mm)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>42.30 ± 0.229</td>
<td>162.0 ± 3.95</td>
<td>3.72 ± 0.087</td>
<td>3.36 ± 0.098</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GROUP 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44</td>
<td>36.85 ± 0.553</td>
<td>120.9 ± 3.28</td>
<td>3.34 ± 0.058</td>
<td>2.97 ± 0.044</td>
<td>19 (43%)</td>
<td>15 (34%)</td>
<td>22 (50%)</td>
</tr>
<tr>
<td>GROUP 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30</td>
<td>42.02 ± 0.126</td>
<td>155.3 ± 4.03</td>
<td>3.46 ± 0.050</td>
<td>3.13 ± 0.058</td>
<td>0</td>
<td>0</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>GROUP 4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>41.98 ± 0.110</td>
<td>160.1 ± 5.88</td>
<td>3.45 ± 0.047</td>
<td>2.96 ± 0.093</td>
<td>0</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum (Group 1).

<sup>a</sup> b <sup>c</sup> c <sup>d</sup> d as for Experiment 1, Table 4.

NS = Not significant
weeks, produced serum which grew abnormal embryos. The most striking malformation was abnormal axial rotation which gave the embryos a twisted or kinked appearance (fig. 14).

Fig 14. 11.5 day rat embryo cultured in folate deficient serum. Embryo in yolk sac (A) and with extraembryonic membranes removed (B).
Anaemia was also frequently observed with affected embryos and their yolk sacs exhibiting a distinct lack of colour in the corpuscular elements of the blood. The yolk sac vessels also appeared to be much less well developed than those of the control embryos grown in normal rat serum (fig. 15).

Fig 15. 11.5 day rat embryo cultured in normal rat serum. Embryo in the yolk sac (A) and with extraembryonic membranes removed (B).
Other minor defects included poor development of the mid and forebrain which although closed, was often not as well advanced as group 1 (control) embryos. Slightly retarded development of the optic vesicles and branchial arches was also occasionally evident and the combination of these defects resulted in a significant reduction in the average morphological score for group 2 compared with the other groups (Table 5). The embryonic protein content at 120µg was significantly less than control embryos (p<0.00001).

Experiment 3: Results from this experiment were very similar to those from experiment 2. The group 2 animals produced only marginally more severely folate depleted serum and this in turn grew embryos which were not significantly different from those grown in serum from animals maintained on the deficient diet for 16 weeks. These results are summarised in Table 6.

Experiment 4: The group 2 serum used to culture embryos in this experiment had been obtained from 1st generation rats maintained on the folate deficient diet from birth. Culture in this serum, which was very folate deficient, resulted in embryonic malformations and very low protein content (Table 7). The embryos showed similar types of developmental defects to those shown in experiments 2 and 3, but more severe growth retardation which was marked by their smaller size and reduced development of many morphological features. Embryos grown in the three other experimental groups were essentially normal with no reduction in protein content and no apparent morphological defects.

Experiment 5: Table 8 summarises the results from this experiment. Even after 30% dilution, serum from groups 1 and 3
### TABLE 6
Experiment 3: After 20 weeks on the diet

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (μg)</th>
<th>YOLK SAC DIAMETER (mm)</th>
<th>CROWN RUMP LENGTH (mm)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/ RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1(^a)</td>
<td>22</td>
<td>42.14 ± 0.201</td>
<td>156.0 ± 4.26</td>
<td>3.54 ± 0.059</td>
<td>3.12 ± 0.038</td>
<td>0</td>
<td>0</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>GROUP 2(^b)</td>
<td>71</td>
<td>35.95 ± 0.874</td>
<td>122.9 ± 4.42</td>
<td>3.21 ± 0.045</td>
<td>2.90 ± 0.056</td>
<td>36 (51%)</td>
<td>32 (45%)</td>
<td>47 (66%)</td>
</tr>
<tr>
<td>GROUP 3(^c)</td>
<td>35</td>
<td>41.98 ± 0.131</td>
<td>158.5 ± 4.11</td>
<td>3.56 ± 0.050</td>
<td>3.06 ± 0.044</td>
<td>7 (20%)</td>
<td>3 (9%)</td>
<td>13 (37%)</td>
</tr>
<tr>
<td>GROUP 4(^d)</td>
<td>10</td>
<td>42.15 ± 0.259</td>
<td>168.0 ± 6.55</td>
<td>3.80 ± 0.097</td>
<td>3.38 ± 0.095</td>
<td>3 (30%)</td>
<td>1 (10%)</td>
<td>3 (30%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum (Group 1).

\( a \ b \ c \ d \) as for Experiment 1, Table 4.

NS = Not significant
### TABLE 7

Experiment 4: First generation rats maintained on the diet for 6 months

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (μg)</th>
<th>YOLK SAC DIAMETER (mm)</th>
<th>CROWN BUMP LENGTH (mm)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/ RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1⁴</td>
<td>20</td>
<td>42.09 ± 0.132</td>
<td>162.4 ± 3.67</td>
<td>3.36 ± 0.147</td>
<td>3.14 ± 0.042</td>
<td>0</td>
<td>0</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>GROUP 2⁵</td>
<td>53</td>
<td>34.24 ± 0.627</td>
<td>103.3 ± 3.94</td>
<td>3.13 ± 0.059</td>
<td>2.81 ± 0.053</td>
<td>27 (51%)</td>
<td>25 (47%)</td>
<td>31 (58%)</td>
</tr>
<tr>
<td>GROUP 3⁶</td>
<td>42</td>
<td>41.99 ± 0.177</td>
<td>157.1 ± 3.98</td>
<td>3.41 ± 0.064</td>
<td>2.92 ± 0.051</td>
<td>6 (14%)</td>
<td>2 (5%)</td>
<td>14 (33%)</td>
</tr>
<tr>
<td>GROUP 4⁷</td>
<td>9</td>
<td>42.12 ± 0.312</td>
<td>164.6 ± 2.86</td>
<td>3.42 ± 0.057</td>
<td>3.11 ± 0.093</td>
<td>1 (11%)</td>
<td>1 (11%)</td>
<td>4 (44%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

⁴ b c d as for Experiment 1, Table 4.

NS = Not significant
### Table 8

**Experiment 5: Diluted Serum**

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>YOLK SAC DIAMETER (mm)</th>
<th>CROWN RUMP LENGTH (mm)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETADED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1a</td>
<td>15</td>
<td>42.03 ± 0.206</td>
<td>151.3 ± 5.34</td>
<td>3.39 ± 0.076</td>
<td>3.02 ± 0.054</td>
<td>0</td>
<td>0</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>GROUP 2b</td>
<td>26</td>
<td>36.86 ± 0.420</td>
<td>129.3 ± 3.39 (p&lt;0.0001)</td>
<td>3.22 ± 0.088 (p&gt;0.1(NS))</td>
<td>2.91 ± 0.048 (p&gt;0.1(NS))</td>
<td>6 (23%)</td>
<td>5 (19%)</td>
<td>10 (38%)</td>
</tr>
<tr>
<td>GROUP 3c</td>
<td>15</td>
<td>41.97 ± 0.256</td>
<td>152.0 ± 8.45 (p&gt;0.1(NS))</td>
<td>3.47 ± 0.058 (p&gt;0.1(NS))</td>
<td>3.09 ± 0.051 (p&gt;0.1(NS))</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GROUP 4d</td>
<td>20</td>
<td>39.57 ± 0.786</td>
<td>141.3 ± 9.21 (p&gt;0.1(NS))</td>
<td>3.36 ± 0.086 (p&gt;0.1(NS))</td>
<td>2.95 ± 0.052 (p&gt;0.1(NS))</td>
<td>4 (20%)</td>
<td>5 (25%)</td>
<td>6 (30%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

*p* values calculated by Student's *T*-test indicating the difference from culture in normal rat serum.

a b c as for Experiment 1, Table 4, except that the serum was diluted by 30%.

d = undiluted serum from animals maintained on a folate free diet containing 0.6% SST.

NS = Not significant
experiment. Even after 30% dilution, serum from groups 1 and 3 supported completely normal embryonic growth. Embryos grown in diluted folate deficient serum (group 2), did however, show a slightly increased incidence of malformations and a decreased protein content when compared with undiluted folate deficient serum (group 4).

DISCUSSION

The data from these experiments show that animals must be maintained on the folate deficient diet for periods in excess of 12 weeks before their serum becomes sufficiently depleted of folate to induce embryonic malformations in vitro.

The results also suggest the existence of a critical serum folate level, below which malformations will be induced and above which embryos will be morphologically normal. This level appeared to be around 3ng/ml, and induction of a deficiency to this extreme took about 16 weeks. This theory of a threshold level was supported by the observation that after 12 weeks on the diet, even though serum folate levels had been significantly reduced (Table 4), no frank malformations were apparent. The embryos grown in this serum did, however, show a reduction in protein content when compared with embryos grown in normal rat serum. Embryos cultured in serum from animals maintained on the diet for periods of at least 16 weeks (i.e. serum folate content below the critical level), displayed abnormal turning (fig. 14), anaemia and reduced development of many morphological features, and this was paralleled by a significant reduction in their protein
content (Tables 5 & 6).

Animals fed the deficient diet from birth, and born to dams maintained on the same diet, demonstrated an even greater reduction in serum folate. Embryos grown in this serum had the same types of morphological malformations and were severely growth retarded. These first generation rats were, however, difficult to maintain and showed a marked decline in their general health including poor weight gain, infertility, lethargy and eye problems. Although their serum was sufficiently depleted of folate to consistently produce abnormal embryos, on consideration of the animals health problems and also the substantial time involved, this was not a particularly practical means of producing folate deficient serum.

Diluting the folate deficient serum with Hank's balanced salt solution to further reduce its folate content, resulted in a decrease in both embryonic protein content and a slight reduction in the morphological scores (Table 8). Although no significant changes were observed in the similarly diluted normal rat serum group, this was rather an artificial means of enhancing the folate deficiency as dilution of other critical growth factors could not be eliminated as possible aetiological factors in the observed embryonic abnormalities.

From the group 4 results in the first 4 experiments it was apparent that the inclusion of succinylsulphathiazole in the diet had no adverse effects on the growth or development of cultured embryos. It can therefore be assumed that the presence of the antibiotic had no effect other than promotion of the folate deficiency by elimination of folate producing gut microorganisms.
embryos grown in serum from rats fed the folate supplemented diet grew and developed normally. This confirmed that the deficient diet was adequate in everything except folate. As the dietary studies progressed, reduced reproductive performance was demonstrated in rats maintained on the folate-free diet, and by the time optimum serum folate levels of less than 3ng/ml had been achieved (16 weeks on the diet) there was complete reproductive failure. It was therefore concluded, that the most convenient and practical means of obtaining serum which was sufficiently depleted of folate, was to place post-weaning animals on the deficient diet for 16 weeks. Although pups born to mothers maintained on the diet, and also the dams themselves, both produced slightly more severely depleted serum, the practical problems involved in maintaining these animals outweighed the advantages of using serum which was only marginally more deficient.

The approximate time course of the development of the folate deficiency is shown in fig. 13. In the first 12 weeks, more than 90% of the serum folate was lost, but thereafter the level appeared to plateau with a much more gradual decrease over the following weeks. This possibly suggests a developing economy for the use of folate as the animals' stores fell below a critical level.

An interesting relationship became apparent during the course of the in vitro experiments. This being an approximately linear correlation between the folate status of the culture serum and
the embryonic protein content (fig. 16).

Surprisingly there was no such relationship between serum folate and morphological score even though, as previously discussed, the results indicated a critical level of folate depletion in association with the induction of embryonic malformations.
SECTION IV

REVERSIBILITY OF ABNORMALITIES INDUCED BY FOLATE DEFICIENCY

CHAPTER 9

IN VITRO SUPPLEMENTATION WITH FOLATE

Culture of embryos in serum which was sufficiently depleted of folate (<3ng/ml) induced embryonic abnormalities (see Chapter 8).

In an attempt to reverse the observed abnormalities, the deficient serum was supplemented with folate.

In an initial study, the use of pteroylmonoglutamic acid (as used in the diet) was investigated. This was, however, very insoluble in aqueous solution and although soluble in alkali, the resultant solution proved unfavourable for normal embryonic growth. A commercially available solution of this form of the vitamin (Boots Company plc, Nottingham, England), which is used clinically in the treatment of folic acid deficiency, also contained fairly high concentrations of alkali and this too was unsuitable to add to the culture serum.

Dihydrofolate and 5-methyl tetrahydrofolate both proved to be more soluble in aqueous solution and non-teratogenic in culture and were therefore used to supplement the culture serum.

MATERIALS AND METHODS

Preparation of serum and explantation of embryos: As detailed in Chapter 6.

Preparation of folate solutions: Dihydrofolate and 5-methyl
tetrahydrofolate were obtained from the Sigma Chemical Co., Poole, Dorset.

Dihydrofolinic acid

5-methyl tetrahydrofolinic acid

2.5, 5.0, 7.5, 10.0 and 15.0 μg/ml solutions of the two folates were prepared by adding the powdered form of each to sterile distilled water. The final solutions were filter sterilised by passage through a 0.45 μm membrane filter (Whatman Ltd., Maidstone, Kent). By adding 10 μl of the folate solution per millilitre of culture serum, a final concentration of 25, 50, 75, 100 or 150 ng of folate, per ml of serum, was obtained.

Culture of embryos in folate supplemented folate deficient serum:

The folate deficient serum used in all experiments was obtained from rats maintained on a folate-free diet (see Chapter 6) for a minimum of 16 weeks.

Experiment 1

Embryos were randomly divided between 7 culture groups as
follows:

(1) Normal rat serum.

(2) Folate deficient serum (FDS).

(3) - (7) FDS plus 25, 50, 75, 100 or 150ng/ml dihydrofolate.

The folate deficient serum used in this experiment contained an average of 2.72ng/ml folate.

Experiment 2

As in experiment 1, embryos were distributed between 7 experimental groups. Groups (1) and (2) as above and groups (3) - (7) were supplemented with 25, 50, 75, 100 or 150ng/ml of 5-methyl tetrahydrofolate acid.

In this experiment, the deficient serum had an average folate content of 2.94ng/ml.

Experiment 3

8 experimental culture groups were included in this experiment. These were:

(1) Normal rat serum (NRS).

(2) Folate deficient serum (FDS).

(3) - (7) FDS plus 25, 50, 75, 100 or 150ng/ml each of dihydrofolate and 5-methyl tetrahydrofolate.

(8) NRS supplemented with 150ng/ml dihydrofolate plus 150ng/ml 5-methyl tetrahydrofolate.

This last group was included to ensure that the mixture of the 2 highest concentrations of folate supplements was not embryopathic.

The average folate content of the deficient serum used in groups 2-7 was 2.55ng/ml.
RESULTS

Tables 9, 10 and 11 detail the results of the 3 experiments. In each, embryos cultured in folate deficient serum had significantly lower protein content (p<1x10^-7) and reduced morphological score (p<1x10^-5) than embryos grown in normal rat serum. Embryonic malformations were similar in type to those seen in previous experiments (see Chapter 8, fig 14), i.e. abnormal axial rotation, anaemia and reduced growth.

In all 3 experiments supplementation with folate eliminated the majority of embryonic malformations thereby increasing the morphological scores to approaching that observed in the normal rat serum control group. The protein content of the embryos was, however, only partially restored, and this observation was independent of the type or quantity of folate added.

A comparison between experiments 1 and 2 revealed very little difference between addition of dihydrofolate or supplementation with 5-methyl tetrahydrofolate. At all concentrations tested, both compounds gave similar results with morphological scores approaching the normal of 42 and protein content of around 130ng/embryo (compared with an average of 160ng in normal rat serum cultured embryos and around 110ng in those grown in unsupplemented folate deficient serum).

However, although not significantly different, those embryos cultured in 5-methyl tetrahydrofolate supplemented serum had marginally higher protein contents than those in the dihydrofolate culture groups (compare tables 9 & 10 and figs 17a,
<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (ug)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/ RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum (NRS)</td>
<td>42</td>
<td>42.31 ± 0.162</td>
<td>161.9 ± 4.11</td>
<td>0</td>
<td>0</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Folate deficient serum (FDS)</td>
<td>73</td>
<td>38.44 ± 0.402</td>
<td>108.1 ± 4.44</td>
<td>43 (59%)</td>
<td>34 (47%)</td>
<td>50 (68%)</td>
</tr>
<tr>
<td>FDS + 25ng/ml DHF</td>
<td>20</td>
<td>41.12 ± 0.386</td>
<td>128.3 ± 7.27</td>
<td>3 (15%)</td>
<td>4 (20%)</td>
<td>9 (45%)</td>
</tr>
<tr>
<td>FDS + 50ng/ml DHF</td>
<td>32</td>
<td>41.76 ± 0.261</td>
<td>122.7 ± 5.84</td>
<td>6 (19%)</td>
<td>4 (12%)</td>
<td>9 (28%)</td>
</tr>
<tr>
<td>FDS + 75ng/ml DHF</td>
<td>21</td>
<td>41.75 ± 0.151</td>
<td>126.9 ± 7.77</td>
<td>4 (19%)</td>
<td>2 (10%)</td>
<td>7 (33%)</td>
</tr>
<tr>
<td>FDS + 100ng/ml DHF</td>
<td>18</td>
<td>40.64 ± 0.258</td>
<td>127.7 ± 4.84</td>
<td>4 (22%)</td>
<td>3 (17%)</td>
<td>7 (39%)</td>
</tr>
<tr>
<td>FDS + 150ng/ml DHF</td>
<td>15</td>
<td>40.27 ± 0.852</td>
<td>121.1 ± 7.56</td>
<td>4 (27%)</td>
<td>2 (13%)</td>
<td>6 (40%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

p" values indicate the difference from culture in folate deficient serum.

NS = Not significant
### Table 10

**Experiment 2: Deficient serum supplemented with 5-methyltetrahydrofolate (THF).**

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum (NRS)</td>
<td>78</td>
<td>42.23 ± 0.124</td>
<td>157.8 ± 3.24</td>
<td>1 (1%)</td>
<td>0</td>
<td>8 (10%)</td>
</tr>
<tr>
<td>Folate deficient serum (FDS)</td>
<td>106</td>
<td>38.93 ± 0.314</td>
<td>113.2 ± 7.20</td>
<td>57 (54%)</td>
<td>45 (42%)</td>
<td>71 (67%)</td>
</tr>
<tr>
<td>FDS + 25ng/ml THF</td>
<td>20</td>
<td>41.47 ± 0.446</td>
<td>134.7 ± 7.88</td>
<td>9 (45%)</td>
<td>6 (30%)</td>
<td>13 (65%)</td>
</tr>
<tr>
<td>FDS + 50ng/ml THF</td>
<td>58</td>
<td>41.48 ± 0.202</td>
<td>126.5 ± 4.19</td>
<td>11 (19%)</td>
<td>3 (5%)</td>
<td>14 (24%)</td>
</tr>
<tr>
<td>FDS + 75ng/ml THF</td>
<td>22</td>
<td>41.09 ± 0.387</td>
<td>132.4 ± 5.45</td>
<td>3 (14%)</td>
<td>0</td>
<td>9 (41%)</td>
</tr>
<tr>
<td>FDS + 100ng/ml THF</td>
<td>20</td>
<td>40.89 ± 0.694</td>
<td>131.2 ± 6.52</td>
<td>5 (25%)</td>
<td>3 (15%)</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>FDS + 150ng/ml THF</td>
<td>20</td>
<td>41.55 ± 0.234</td>
<td>127.4 ± 3.82</td>
<td>3 (15%)</td>
<td>2 (10%)</td>
<td>6 (30%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

p" values indicate the difference from culture in folate deficient serum.
<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (μg)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum (NRS)</td>
<td>49</td>
<td>42.26 ± 0.156</td>
<td>160.5 ± 4.07</td>
<td>1 (2%)</td>
<td>0</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Folate deficient serum (FDS)</td>
<td>96</td>
<td>38.85 ± 0.412</td>
<td>109.7 ± 3.48</td>
<td>52 (54%)</td>
<td>39 (41%)</td>
<td>65 (66%)</td>
</tr>
<tr>
<td>FDS + 25ng/ml DHF + 25ng/ml THF</td>
<td>15</td>
<td>40.90 ± 0.589</td>
<td>138.6 ± 7.30</td>
<td>6 (40%)</td>
<td>1 (7%)</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>FDS + 50ng/ml DHF + 50ng/ml THF</td>
<td>14</td>
<td>40.88 ± 1.167</td>
<td>133.6 ± 9.38</td>
<td>3 (21%)</td>
<td>2 (14%)</td>
<td>5 (36%)</td>
</tr>
<tr>
<td>FDS + 75ng/ml DHF + 75ng/ml THF</td>
<td>16</td>
<td>41.16 ± 0.804</td>
<td>126.2 ± 7.65</td>
<td>3 (19%)</td>
<td>1 (6%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td>FDS + 100ng/ml DHF + 100ng/ml THF</td>
<td>20</td>
<td>40.66 ± 0.972</td>
<td>132.3 ± 7.58</td>
<td>5 (25%)</td>
<td>0</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>FDS + 150ng/ml DHF + 150ng/ml THF</td>
<td>15</td>
<td>40.28 ± 0.854</td>
<td>122.2 ± 9.48</td>
<td>4 (27%)</td>
<td>1 (7%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>NRS + 150ng/ml DHF + 150ng/ml THF</td>
<td>10</td>
<td>42.24 ± 0.549</td>
<td>160.6 ± 14.03</td>
<td>0</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

p'' values indicate the difference from culture in folate deficient serum.

NS = Not significant
Fig 17a. 11.5 day rat embryo cultured in dihydrofolate supplemented folate deficient serum.

Fig 17b. 11.5 day rat embryo cultured in 5-methyl tetrahydrofolate supplemented folate deficient serum.
Fig 17c. 11.5 day rat embryo cultured in normal rat serum.

DISCUSSION

From these results it was apparent that supplementation with 5-methyl tetrahydrofolic acid gave the best results. This is the physiologically active, extracellular and transport form of folate and is the form found in plasma and cerebrospinal fluid. It is the only form of folate presented to the body tissues by plasma (Perry & Chanarin, 1970; Hoffbrand, 1975; Chanarin, 1979) and as such was possibly more easily utilised by the embryos in culture.

The most surprising result from these experiments was, however, that while supplementation virtually eliminated morphological abnormalities, it appeared to only partially restore the embryonic protein content which remained significantly lower than
the normal value (p<0.01). The lack of difference between supplementing with the various concentrations and combinations of the 2 folate compounds indicated that the observed low protein could not be simply explained by the serum being deplete of folate alone. The fact that the embryos were affected by the folate deficiency was convincingly demonstrated by both the abnormalities observed in the deficient culture group and also by the fact that folate supplementation did eliminate morphological defects and at least partially restored embryonic protein content. The failure to completely reverse the effects must therefore have been due to some additional factor or factors. Results from the previous section confirmed that the diet was deficient in folate alone. This was proved by the culture of completely normal embryos in serum from animals maintained on a deficient diet which had been supplemented with folate. This implied that the secondary factor(s) was probably related to some physiological effect induced by the folate deficient state. Although the deficient diet was supplemented with all the essential vitamins except folate, it was thought possible that the folate deficiency may have interfered with the absorption or metabolism of another vitamin which in turn could obstruct normal embryonic growth. A further study was therefore undertaken to investigate this.
CHAPTER 10

SUPPLEMENTATION OF FOLATE DEFICIENT SERUM WITH MULTIVITAMINS

To test the hypothesis that maintenance on the folate deficient diet may also have induced a depletion of some other vitamin or vitamins (in addition to folate), a multivitamin mixture was added to the deficient serum, prior to culture.

MATERIALS AND METHODS

Multivitamin solution: The constituents and concentrations of the stock multivitamin solution (MEM Vitamin Solution (100x), Gibco Ltd, Paisley, Scotland) are detailed in Table 12. The amount of solution added to the culture serum was calculated from the amount required to give a folate concentration of either 50 or 100ng/ml serum. As the stock solution contained 100mg/l folate, by diluting x2 with sterile distilled water and adding either 5 or 10pl multivitamin solution per millilitre of culture serum, a final concentration of 50 or 100ng/ml folate was included.

TABLE 12

CONSTITUENTS OF MULTIVITAMIN SOLUTION

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>100</td>
</tr>
<tr>
<td>D-Ca Pantothenate</td>
<td>100</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>100</td>
</tr>
<tr>
<td>l-Inositol</td>
<td>200</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>100</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>100</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>10</td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>100</td>
</tr>
</tbody>
</table>

Preparation of serum and explantation of embryos: As detailed in Chapter 6.
Culture of embryos in multivitamin supplemented folate deficient serum: The folate deficient serum used was obtained from rats maintained on a folate-free diet (see Chapter 6) for a minimum period of 16 weeks.

Experimental groups: Embryos were randomly distributed to the following culture groups:

1. Normal rat serum (NRS).
2. Folate deficient serum (FDS).
3. FDS supplemented with multivitamin solution containing 50ng/ml folate.
4. FDS supplemented with multivitamins containing 100ng/ml folate.
5. NRS plus 100ng/ml multivitamin solution.

The folate deficient serum used in this experiment contained 2.83ng/ml folate.

RESULTS

Table 13 gives the results of this study. As in previous experiments there was a highly significant difference in the morphological scores and protein content of embryos grown in normal rat serum when compared with those grown in folate deficient serum.

Supplementing the deficient serum with either 5 or 10µl of multivitamin solution (i.e. 50 or 100ng/ml folate) produced embryos which were comparable with those grown in deficient serum supplemented with folate alone, i.e. morphologically normal embryos with reduced protein content. (Compare with table 10, Chapter 9.)

-78-
TABLE 13
Addition of multivitamin solution to folate deficient serum.

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (μg)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/ RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum (NRS)</td>
<td>19</td>
<td>42.43 ± 0.591</td>
<td>160.4 ± 2.51</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Folate deficient serum (FDS)</td>
<td>26</td>
<td>37.71 ± 1.066</td>
<td>117.0 ± 4.13</td>
<td>16 (62%)</td>
<td>13 (50%)</td>
<td>18 (69%)</td>
</tr>
<tr>
<td>FDS + 50μg/ml multivitamins</td>
<td>15</td>
<td>41.96 ± 0.420</td>
<td>139.8 ± 4.90</td>
<td>3 (20%)</td>
<td>1 (7%)</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>FDS + 100μg/ml multivitamins</td>
<td>16</td>
<td>41.65 ± 0.584</td>
<td>134.4 ± 8.02</td>
<td>4 (25%)</td>
<td>0</td>
<td>6 (37%)</td>
</tr>
<tr>
<td>NRS + 100μg/ml multivitamins</td>
<td>10</td>
<td>42.12 ± 0.462</td>
<td>158.0 ± 3.82</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.
p values calculated by Student's T-test indicating the difference from culture in normal rat serum.
p" values indicate the difference in folate deficient serum.
NS = Not significant
Embryos cultured in normal rat serum with the higher concentration of vitamins added, grew and developed normally, demonstrating that the multivitamin solution was not teratogenic.

DISCUSSION

The observations made in this experiment imply that, of the vitamins contained in the multivitamin supplement, only folate was deficient in the culture serum. The mixture added did not, however, contain vitamin B\textsubscript{12}. Although this was a constituent of the folate deficient diet (table 2, Chapter 6), folates and vitamin B\textsubscript{12} are known to have closely interrelated metabolic roles.

The synthesis of methionine from homocysteine is catalysed by vitamin B\textsubscript{12} and folate coenzymes. Vitamin B\textsubscript{12} participates in this reaction as a methylcobalamin (methyl-B\textsubscript{12}) enzyme (Buchanan, 1964). S-adenosyl-homocysteine-methyl-B\textsubscript{12} is converted to the S-adenosyl-methionine-methyl-B\textsubscript{12} enzyme by transfer of a methyl group from 5-methyl tetrahydrofolate. (The tetrahydrofolate generated then becomes available for participation in further 1-carbon unit transfer reactions.) The methyl-B\textsubscript{12} enzyme transfers its methyl group to homocysteine to form methionine (Weissbach & Taylor, 1970).

The physiological role of vitamin B\textsubscript{12} is therefore closely related to that of folate and the interactions of the two compounds are vital in the biosynthesis of essential constituents of the nucleic acids.

Because of this interrelationship it was thought possible that a deficiency of folate may have interfered with the metabolism of
vitamin B\textsubscript{12}. An assessment of the deficient serum's vitamin B\textsubscript{12} content and also a quantitative analysis of other serum constituents was therefore proposed.
CHAPTER II

SERUM PROFILE AND HAEMATOLOGICAL INVESTIGATIONS

To try to establish if the observed low protein content of embryos grown in folate supplemented deficient serum was due to an imbalance in other critical serum constituents, an analysis of various serum vitamins, enzymes and proteins was undertaken. A haematological profile was also carried out to investigate the alternative possibility that changes in the blood had been induced by the folate deficient diet.

Clearly it was not practicable to assay all known serum and blood constituents, however, a fairly comprehensive selection were analysed.

MATERIALS AND METHODS

SERUM CLINICAL CHEMISTRY

Vitamin B₁₂: The vitamin B₁₂ content of the folate deficient serum was determined using a Vitamin B₁₂/Folate dual radioassay kit (as used to assess serum folate) (Amersham International plc, Amersham, U.K.). The radioassay of serum vitamin B₁₂ was, in principle, as described for folate (see Section II, Chapter 6). Basically this involved competition between the serum B₁₂ and a quantity of radioactively labelled tracer for a limited number of specific binding sites. In the assay of folate, serum folate competed with ¹²⁵I-labelled folate for sites on folate binding protein, while for vitamin B₁₂, serum B₁₂ competed with ⁵⁷Co-labelled vitamin B₁₂ for sites on purified intrinsic factor. As with folate, serum vitamin B₁₂ was quantified by counting the protein-bound ⁵⁷Co on a gamma counter (LKB RIA) and interpolated
from a standard curve (fig 18) derived by plotting the proportion of $^{57}$Co-labelled vitamin B$_{12}$ bound in the presence of known reference solutions.

![Fig 18. Standard vitamin B$_{12}$ curve.](image)

**Vitamin E (Tocopherol):** For vitamin E determination, an adaptation of the standard oxidimetric colour reaction was used (Quaife et al, 1949). Tocopherol was first extracted into xylene and then a,a'-dipyridyl reagent added. A tocopherol-a,a'-dipyridyl complex formed. The addition of ferric chloride to the mixture resulted in a reduction from ferric to ferrous ions which formed a red colour with the a,a'-dipyridyl reagent. The maximum absorbance read at 520nm gave the concentration of vitamin E in
Sodium and Potassium: Serum sodium and potassium were determined by flame emission spectrometry. An I.L. 543 flame photometer was used and quantitation achieved by the standard method, i.e. the serum sample was first aspirated into the flame - this produced an electromagnetic radiation spectrum. As the intensity of the spectral line emitted by the analyte, was directly proportional to its concentration in solution, quantitation was made by reference to a prepared calibration curve.

The remaining serum constituents tested were assayed using a Cobas Fara Centrifugal Analyser (Roche Products Ltd, Welwyn Garden City, Herts). This automatic instrument employed test kits which included all the necessary reagents and instructions. The principles on which the test methodologies were based have been briefly outlined for each constituent.

Bilirubin: Bilirubin reacts with diazotised sulphanilic acid to form a purple coloured azo pigment - the colour intensity of which is directly proportional to the total bilirubin concentration. This can be determined photometrically by recording the increase in absorbance at 550nm.

Calcium: In alkaline solution calcium ions form a blue chelate with methylthymol blue solution. (Magnesium ions are simultaneously masked with 8-hydroxy quinoline.) The intensity of colour, which is proportional to the concentration of calcium, can be measured photometrically at 612nm.
Lactate Dehydrogenate (LDH): LDH is determined by its incorporation into the following reaction:

\[ \text{Pyruvate} + \text{NADH} + H^+ \overset{\text{LDH}}{\rightarrow} \text{L-Lactate} + \text{NAD}^+ \]

The rate of oxidation of NADH (Nicotinamide Adenine Dinucleotide, reduced form), is proportional to the catalytic activity of LDH. This can be determined by measuring the decrease in absorbance at 340nm.

**Protein:** Protein produces a coloured complex in alkaline solution with copper salts (biuret reaction). The colour intensity is proportional to the protein concentration and can be photometrically determined at 546nm.

**Glucose:** Glucose can be determined enzymatically with hexokinase (HK).

\[ \text{D-Glucose} + \text{ATP} \overset{\text{HK}}{\rightarrow} \text{D-Glucose-6-phosphate} + \text{ADP} \]

\[ \text{D-Glucose-6-phosphate} + \text{NAD}^+ \overset{\text{ALP}}{\rightarrow} \text{D-Gluconate-6-phosphate} + \text{NADH} + H^+ \]

The concentration of the NADH formed is directly proportional to the glucose concentration and can be determined by measuring the absorbance at 340nm.

**Alkaline Phosphatase (ALP):** The following kinetic reaction is used to assess ALP:

\[ 2 \text{-amino-2-methyl-1-propanol} + 4\text{-nitrophenylphosphate} \overset{\text{ALP}}{\rightarrow} 4\text{-nitrophenol} + 2\text{-amino-2-methyl-1-propanolphosphate} \]

The rate of formation of the 4-nitrophenol is directly proportional to the ALP concentration. This can be measured by recording the increase in absorbance at 405nm.
Creatinine: Creatinine reacts with picric acid in an alkaline medium to produce yellow-red 2,4,6-trinitrobenzidine. The intensity of the yellow-red colour is directly proportional to the creatinine and can be measured at 490 nm.

Urea: The principle of this method is a reaction between urease and glutamate dehydrogenase.

\[ \text{Urea} + 2\text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2 \]

\[ \text{NH}_4^+ + 2\text{oxoglutarate} + \text{NADH} \rightarrow \text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \]

The decrease in the NADH (Nicotinamide Adenine Dinucleotide, reduced form) concentration is directly proportional to the urea concentration and can be determined photometrically at 340 nm.

Aspartate Aminotransferase (AST): The kinetic determination of AST involves the following reactions:

\[ \text{L-aspartate} + 2\text{oxoglutarate} \rightarrow \text{oxaloacetate} + \text{L-glutamate} \]

\[ \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightarrow \text{malate} + \text{NAD}^+ \]

The rate of NADH oxidation is proportional to the catalytic AST activity and can be determined by measuring the decrease in absorbance at 340 nm.

Albumin: In this assay a spectral shift occurs in the absorbance maxima of bromocresol green (BCG) upon binding to albumin.

\[ \text{Albumin} + \text{BCG} \rightarrow \text{Albumin-BCG complex} \]

The change in absorbance at 630 nm is directly proportional to the albumin concentration.

Cholesterol: The principle involved in this test is an enzymatic colourimetric reaction with cholesterol esterase, cholesterol
oxidase and 4-aminophenazone (PAP).

Cholesterol ester + H₂O → Cholesterol esterase → Cholesterol + fatty acids

Cholesterol + O₂ → Cholesterol oxidase → Δ₃-cholest-3-en + H₂O₂

Due to the hydrogen peroxide formed, phenol and PAP are coupled oxidatively to a red quinoneimine derivative. The concentration of the quinoneimine formed is proportional to the amount of cholesterol and can be determined by measuring its absorbance in the range 480-550nm.

Alanine-amino Transferase (ALT): The following colorimetric test is employed to assay ALT:

2-oxoglutarate + L-alanine → L-glutamate + pyruvate

The pyruvate formed is mixed with the colour reagent 2,4-dinitrophenylhydrazine in alkaline media to yield hydrazone. The intensity of the colour of the hydrazone solution corresponds to the concentration of ALT activity and can be measured photometrically at 546nm.

All parameters were assessed in at least 3 serum samples which were obtained from animals maintained on the folate deficient diet for 16-20 weeks. For comparison, control samples, from rats fed standard rat chow, were also measured. Between 5 and 10 samples were assayed for each parameter and from this, a normal range defined.

Hematology

Red blood cell and white blood cell counts were performed on a
Coulter Counter model ZP (Coulter Electronics Ltd, Luton, Beds). With this instrument a known amount of blood, suspended in an electrolyte, was passed through an aperture of specific dimensions. A current path existed between two electrodes - one on either side of the aperture. As a particle passed through the aperture it displaced electrolyte, thus changing the resistance between the electrodes, producing a voltage pulse whose magnitude was proportional to the volume of the particle. The voltage pulses were passed into a threshold circuit which discriminated between them by passing count pulses for only the particles that exceeded a set threshold level, thus separately counting the number of white blood cells and red blood cells. The dilution ratio requirements for performing blood cell counts was 1:500 for white blood cells and 1:50,000 for red blood cells. The diluent used was Isoton II (Coulter Electronics Ltd, Luton, Beds), which is an azide-free filtered isotonic diluent based on saline. (Other solutions, including physiological saline, can change cell size and cause erroneous results.)

Mean cell volume (MCV) and packed cell volume (PCV) were also assessed using a Coulter Counter, with the additional assistance of the MCV/PCV accessory. This attachment used the voltage pulse produced by the main Coulter Counter. The pulse was passed to the MCV circuitry where the total pulse signals were added together and then divided by the number of pulses to produce the MCV in femtolitres (fl). The MCV was then multiplied by the red cell count to give the PCV (haematocrit) as a ratio.
Haemoglobin was measured on a Coulter Haemoglobinometer (Coulter Electronics Ltd, Luton, Beds) using the cyanomethaemoglobin method. This is based on the following principle:

Haemoglobin is defined as the oxygen-carrying pigment of the erythrocytes. The haemoglobin molecule contains four haeme groups and globin (haeme being a metal complex consisting of an iron atom in the centre of a porphyrin structure). The haemes constitute about 4% of the weight of the molecule and impart the red colour of the haemoglobin.

With the cyanomethaemoglobin method, the red cells were lysed with Zaponin (Coulter Electronics Ltd) and the native haemoglobin converted to cyanomethaemoglobin by a solution containing potassium ferricyanide and potassium cyanide. The amount of haemoglobin present in the original sample was calculated from the optical density of the cyanomethaemoglobin which was directly proportional to its concentration.

RESULTS

Table 14 gives the results of the serum constituent assays. Of the various enzymes, proteins and vitamins determined, none were significantly different from the normal values.

Similarly for the haematological parameters, a comparison of the average results for the deficient group with the normal control samples showed no significant changes (Table 15). Also, morphological examination of the cellular components of the blood showed no evidence of anaemia or megaloblastosis.

An interesting finding, which was not reflected in the 16 week results, was the apparent gradual decrease in white blood cell
Table 14

Levels of various vitamins, enzymes and proteins measured in folate deficient (animals maintained on the folate deficient diet for 16-20 weeks) and normal rat serum.

<table>
<thead>
<tr>
<th></th>
<th>Folate Deficient</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12</td>
<td>685 ± 35µg/l</td>
<td>475 - 1010µg/l</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>9.6 ± 3.3mg/l</td>
<td>4.8 - 11.7 mg/l</td>
</tr>
<tr>
<td>Sodium</td>
<td>139 ± 3.0mMol/l</td>
<td>140 - 155mMol/l</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.8 ± 1.7mMol/l</td>
<td>3.7 - 5.4mMol/l</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>10 ± 1.8µMol/l</td>
<td>6.2 - 9.8 µMol/l</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.43 ± 0.35mMol/l</td>
<td>2.4 - 3.1 mMol/l</td>
</tr>
<tr>
<td>Lactate Dehydrogenate</td>
<td>834 ± 71 I.U./l</td>
<td>236 - 1179 I.U./l</td>
</tr>
<tr>
<td>Protein</td>
<td>71 ± 6g/l</td>
<td>73 - 77g/l</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.8 ± 1.1mMol/l</td>
<td>5.9 - 11.5mMol/l</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>172 ± 21 I.U./l</td>
<td>204 - 386 I.U./l</td>
</tr>
<tr>
<td>Creatinine</td>
<td>67 ± 13µMol/l</td>
<td>59-80µMol/l</td>
</tr>
<tr>
<td>Urea</td>
<td>6.2 ± 1.0mMol/l</td>
<td>5.0 - 9.3 mMol/l</td>
</tr>
<tr>
<td>Aspartate Aminotransferase</td>
<td>109 ± 9 I.U./l</td>
<td>82 - 130 I.U./l</td>
</tr>
<tr>
<td>Albumin</td>
<td>29 ± 3g/l</td>
<td>22 - 37g/l</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.8 ± 0.2mMol/l</td>
<td>1.1 - 2.4mMol/l</td>
</tr>
<tr>
<td>Alanine Amino Transferase</td>
<td>42.5 ± 10.5 I.U./l</td>
<td>25.5 - 53.0 I.U./l</td>
</tr>
</tbody>
</table>

Folate Deficient - Values are the mean ± the standard error
TABLE 15

Haematological parameters measured in folate deficient* and normal rat serum.

* animals maintained on the folate deficient diet for 16 weeks.

<table>
<thead>
<tr>
<th></th>
<th>R.B.C.* (10^{12}/l)</th>
<th>W.B.C. ‡ (10^{9}/l)</th>
<th>M.V.C.§ (fl)</th>
<th>P.C.V. † (ratio)</th>
<th>Hb. ‡ (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum</td>
<td>6.38 ± 0.029</td>
<td>7.19 ± 1.119</td>
<td>59.01 ± 1.732</td>
<td>0.368 ± 0.0115</td>
<td>154.5 ± 1.44</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate deficient</td>
<td>6.34 ± 0.148</td>
<td>6.02 ± 0.087</td>
<td>55.64 ± 0.524</td>
<td>0.329 ± 0.0087</td>
<td>156.1 ± 2.73</td>
</tr>
<tr>
<td>rat serum (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Red blood cells  
‡ White blood cells  
§ Mean cell volume  
† Packed cell volume  
‡ Haemoglobin

Values are the mean of the number of samples 'n' ± the standard error.
count during the first 10 weeks of the dietary period (Fig. 19).

Fig 19. Change in white blood cell count with increasing time on the folate deficient diet.

This decrease could be attributed to reduced synthesis of DNA precursors (see Chapter 3), resulting in deficient production of the various types of blood cells. (Similar effects have been reported to occur in other renewal systems in the body, (Gardner, 1956; Van Niekerk, 1966).) The longer life span of the red blood cell (approximately 100 days) compared to the relatively short life of the white blood cell (approximately 10 days) could explain the earlier appearance of a reduction in the latter (Kodicek & Carpenter, 1950a; Hugh Jones & Cheney, 1961). There was no evidence of any trends in the other haematological components.

DISCUSSION

The results of the serum constituent investigations confirmed
that, of the parameters measured, none were significantly changed from the normal levels.

Although some of the constituents assessed were also ingredients of the folate-free diet and were therefore unlikely to be deficient, these were also assessed because of the possibility that the deficient state could have interfered with normal dietary absorption. From the results it was clear that, for these particular constituents no such interference had occurred.

The haematological studies confirmed that in rats receiving a folate deficient diet, the development of anaemia or megaloblastosis is very slow (Herbert, 1962; Chanarin et al, 1969; Siddons, 1974; Singh, 1975; Tagbo & Hill, 1977). The relatively rapid appearance of a reduction in white blood cell count was, however, not unexpected (Endicott et al, 1945; Stokstad, 1968; Tagbo & Hill, 1977) and could be attributed to decreased production of these cells due to the folate deficiency adversely affecting the synthesis of DNA precursors. The reduced white blood cell count also supported previous reports describing a much slower decline in red cell folate when compared to serum folate in animals maintained on a folate deficient diet (Chanarin et al, 1969; Thenen, 1979).

The apparent increase in white blood cell counts after 10 weeks (see Fig. 19) was difficult to explain. This may, however, have been a false reflection caused by biological variation in a very small group of animals (n=4). Increasing the sample size may have given a more accurate assessment of the general trend.

The results were disappointing in that none of the parameters
investigated were identified as possible factors in the aetiology of the reduced embryonic protein content. It was nevertheless obvious from previous results, that the folate deficient serum was unbalanced in some essential constituent, (other than folate), which rendered it unable to support normal embryonic growth.

In the event that the serum was only marginally deficient in some critical growth factor or nutrient, a possible remedy might be to provide more serum per embryo in culture.
CHAPTER 12

INCREASED VOLUME OF CULTURE SERUM

To evaluate the hypothesis that the dietary induced folate deficient rat serum might also be marginally deficient in some additional critical growth factor, an experiment was proposed in which embryos were grown in a greater volume of culture serum. If the low embryonic protein content was due to a partial deficiency of some essential factor, it was hoped that by increasing the volume of serum, the availability of all nutritive and growth elements would also increase and an improvement in embryonic protein content might be effected.

MATERIALS AND METHODS

Preparation of serum and explantation of embryos: As detailed in Chapter 6.

Preparation of folate solution: A solution containing $10\mu g/ml$ of 5-methyl tetrahydrofolate (Sigma Chemical Co., Poole, Dorset), was prepared by adding the powdered vitamin to sterile distilled water. The solution was then filter sterilised through a $0.45\mu m$ membrane filter (Whatman Ltd., Maidstone, Kent). By adding $10\mu l$ of this folate solution per millilitre of culture serum, a final supplementation of $100\mu g/ml$ was obtained.

Culture of embryos in folate supplemented folate deficient serum: The folate deficient serum used in this experiment was obtained from rats maintained on a folate free diet for a minimum period of 16 weeks (for details see Chapter 8). The culture procedure was as detailed in Chapter 6 except that each embryo was cultured in $2mls$ instead of $1ml$ of serum. Embryos were randomly
distributed to 5 experimental culture groups as follows:-

1. Normal rat serum (1ml serum/embryo).
2. Folate deficient serum (FDS) (1ml serum/embryo).
3. FDS (2mls serum/embryo).
4. Folate supplemented FDS (1ml serum/embryo).
5. Folate supplemented FDS (2mls serum/embryo).

The folate deficient serum used in this experiment contained an average of 2.74ng/ml folate.

RESULTS

The results from this study are detailed in Table 16. As previously, culture in folate deficient serum produced embryos which were markedly growth retarded, with abnormal axial rotation and a variety of minor morphological defects. Doubling the volume of serum failed to induce any improvement in either gross appearance or protein content.

Likewise in the folate supplemented culture groups. As in earlier experiments, morphological defects were virtually eliminated but embryonic protein content only partially restored to that measured in normal embryos. There was no apparent difference between the 1ml/embryo and the 2ml/embryo groups.

DISCUSSION

The results of this experiment show that if the serum was marginally depleted of some additional essential factor (other than folate), increasing the volume of culture serum did not counter the effect of this deficiency.

One possible explanation for this lack of effect was that
<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum 1ml/embryo</td>
<td>10</td>
<td>42.10 ± 0.233</td>
<td>155.5 ± 4.58</td>
<td>0</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Folate deficient serum (FDS) 1ml/embryo</td>
<td>11</td>
<td>38.04 ± 1.235</td>
<td>111.1 ± 10.26</td>
<td>7 (64%)</td>
<td>3 (27%)</td>
<td>8 (73%)</td>
</tr>
<tr>
<td>FDS 2ml/embryo</td>
<td>16</td>
<td>38.16 ± 1.131</td>
<td>100.9 ± 4.89</td>
<td>9 (56%)</td>
<td>7 (44%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>FDS + 100ng/ml folate 1ml/embryo</td>
<td>14</td>
<td>40.18 ± 0.554</td>
<td>126.3 ± 7.49</td>
<td>2 (14%)</td>
<td>0</td>
<td>6 (43%)</td>
</tr>
<tr>
<td>FDS + 100ng/ml folate 2mls/embryo</td>
<td>14</td>
<td>40.53 ± 0.702</td>
<td>129.0 ± 4.33</td>
<td>2 (14%)</td>
<td>0</td>
<td>5 (36%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

p" values indicate the difference from culture in folate deficient serum.

NS = Not significant
increasing the volume of culture medium did not result in the embryos being exposed to more nutritive/growth factors, as a finite exposure may be achieved dependent on other parameters such as surface area of the yolk sac rather than the volume of circulating medium. Likewise, if an equilibrium exists between materials passing from the culture medium to the embryo, unless the embryo has the ability to concentrate the depleted material, it would be unlikely that an increase in the serum volume would result in the embryo obtaining more of the factor in question. Another possible explanation for these results was that the dietary regimen had induced some embryopathic factor in the folate deficient serum. However, from previous results (Chapter 3), as embryos grew normally in serum from animals fed the folate supplemented folate deficient diet it was thought that this latter theory was unlikely.

As embryos consistently grew perfectly in normal rat serum, it was obvious that this contained all the essential growth factors. It was therefore postulated that by adding normal rat serum to folate deficient serum it would be possible to establish a level at which any depleted factor would become sufficiently available to eliminate all abnormalities.
CHAPTER 13

SUPPLEMENTATION OF FOLATE DEFICIENT SERUM WITH NORMAL RAT SERUM

As previous attempts to supplement the folate deficient serum with extra nutrients (as well as folate), had failed to completely restore the embryonic protein content, an experiment was planned in which normal rat serum was added to the deficient cultures.

From earlier studies (Chapter 8) it had been established that the serum folate had to be depleted to a level of less than 3ng/ml before morphological defects were observed. As normal rat serum usually contains more than 60ng/ml, it was expected that supplementation with very small amounts of normal rat serum would provide sufficient folate to eliminate gross abnormalities.

Other workers have reported that 50% normal rat serum alone will support normal embryonic growth (Huxham & Beck, 1985), therefore in this study, supplementation with lower percentages than this were proposed.

By supplementing with normal rat serum it was hoped that the amount required might indicate the nature or degree of the constituent responsible for the reduced embryonic protein, e.g. whether it was a completely depleted essential growth factor which required 50% normal rat serum present to restore normal growth, or whether it was a lesser deficiency of a more minor constituent which could be rectified by supplementation with a much smaller quantity of whole rat serum. Alternatively, if supplementation with normal rat serum did not fully restore the embryonic protein content, this might imply the presence of an
embryopathic factor in the folate deficient serum.

MATERIALS AND METHODS

Preparation of serum and explantation of embryos: As detailed in Chapter 6.

Folate deficient serum: The folate deficient serum was obtained from animals maintained on the deficient diet for a minimum period of 16 weeks.

Normal rat serum: Normal rat serum was obtained from Wistar rats (as used in the dietary studies) which were fed standard rat chow.

Explantation and culture of embryos: 9.5 day embryos, obtained from donor Wistar rats maintained on standard rat chow, were explanted and cultured as detailed in Chapter 6. The embryos were randomly distributed between 5 culture groups, viz.,

Group (1) Normal rat serum (NRS).
Group (2) Folate deficient serum (FDS).
Group (3) FDS + 10% NRS.
Group (4) FDS + 20% NRS.
Group (5) FDS + 30% NRS.

The folate content of the folate deficient serum and normal rat serum was 2.23 and 85ng/ml respectively.

RESULTS

The results of this experiment are detailed in Table 17 and are presented diagramatically in Figure 20. As shown, increasing the quantity of normal rat serum supplement resulted in a corresponding increase in both the embryonic morphological score and protein content, with embryos grown in the 30%
**TABLE 17**

Supplementation of folate deficient serum with normal rat serum.

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>SERUM FOLATE CONTENT (ng/ml)</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum (NRS)</td>
<td>10</td>
<td>8.5</td>
<td>42.40 ± 0.163</td>
<td>190.1 ± 11.55</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Folate deficient serum (FDS)</td>
<td>11</td>
<td>2.23</td>
<td>33.23 ± 1.875</td>
<td>108.3 ± 6.85</td>
<td>8 (73%)</td>
<td>8 (73%)</td>
<td>9 (82%)</td>
</tr>
<tr>
<td>FDS + 10% NRS</td>
<td>10</td>
<td>10.51</td>
<td>36.93 ± 8.497</td>
<td>126.6 ± 9.79</td>
<td>4 (40%)</td>
<td>5 (50%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>FDS + 20% NRS</td>
<td>9</td>
<td>18.78</td>
<td>39.60 ± 1.470</td>
<td>160.7 ± 14.34</td>
<td>2 (22%)</td>
<td>4 (44%)</td>
<td>3 (33%)</td>
</tr>
<tr>
<td>FDS + 30% NRS</td>
<td>11</td>
<td>27.06</td>
<td>42.13 ± 0.209</td>
<td>187.9 ± 6.73</td>
<td>0</td>
<td>0</td>
<td>1 (9%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

p" values indicate the difference from culture in folate deficient serum.

NS = Not significant
supplementation group being comparable in every respect with those grown in 100% normal rat serum.

Fig 20. Effect of adding normal rat serum to folate deficient culture serum.

In the 10 and 20% supplemented groups (particularly the 10%) there was a wide variation in the morphological appearance of the embryos ranging from normal, with scores around 42, to fairly
abnormal with defects similar to those seen in embryos cultured in unsupplemented folate deficient serum (see Chapter 8, fig. 14). This variation was not observed in the 30% normal rat serum supplemented group (see standard errors, Table 17).

DISCUSSION

Supplementing the deficient serum with normal rat serum gave a dose-related response (see fig. 20), with 30% supplementation completely restoring normal growth and embryonic protein content. The addition of 10% NRS produced embryos with protein contents comparable with those grown in folate supplemented FDS (Chapter 9). As the normal rat serum used in this experiment contained 85ng/ml folate, this observation may have been a direct result of the increased serum folate level (see Chapter 8). Although supplementation with 20% NRS achieved a significant improvement in embryonic growth, normal protein content was only fully restored by addition of 30% whole serum.

A possible explanation for these results was that the folate deficient serum was completely lacking in some essential growth factor(s) which was adequately available in 30% normal rat serum. Alternatively, the deficient serum might only have been partially depleted of the constituent and supplementation with 30% NRS was sufficient to restore the balance. (It is known that 50% normal rat serum will support normal embryonic growth (Huxham & Beck, 1985).) Another possible interpretation of the results might be that the folate deficient serum contained an embryopathic agent which was progressively diluted out by adding increasing amounts of whole serum. This latter explanation was, however, fairly
improbable as animals maintained on a deficient diet supplemented with folate produced serum which was capable of supporting completely normal growth. Also, the relatively small amount of NRS required to eliminate all abnormalities did not support the theory of an embryopathic factor being present.

As normal rat serum had effectively countered the adverse effects of culture in depleted serum, it was thought interesting to investigate the possibility of \textit{in vivo} reversal by restoring folate deficient animals to a normal diet.
CHAPTER 14

**IN VIVO REVERSAL OF THE EFFECTS INDUCED BY THE FOLATE DEFICIENT DIET**

From previous studies it had been established that maintenance on the folate deficient diet for periods in excess of 16 weeks rendered the animals' serum sufficiently depleted of folate to induce embryonic malformations when used for *in vitro* culture. Embryos grown in the deficient serum frequently displayed abnormal turning, anaemia and various other minor morphological defects, and they had substantially reduced protein content (see Chapter 8).

Attempts to reverse these effects *in vitro* by supplementing the deficient serum with folate resulted in the elimination of morphological abnormalities but the embryonic protein content was only partially restored.

Investigations into the possible aetiological factor(s) involved in this phenomenon indicated that the folate-free diet had probably induced a secondary deficiency of an essential growth factor.

In the present study, the feasibility of reversing the effects of the folate deficient diet by *in vivo* supplementation with folate were investigated, i.e. by restoring deficient animals to a fully supplemented diet.

**MATERIALS AND METHODS**

Dietary regimens: Female Wistar rats, approximately 6 weeks of age, were randomly distributed between 7 experimental groups and maintained on their allocated diet for the periods indicated:
(1) Standard rat chow (SRC) for 16 weeks.
(2) Folate deficient diet (FDD) (see Chapter 6), for 16 weeks.
(3) Folate supplemented FDD (see Chapter 6), for 16 weeks.
(4) FDD for 16 weeks followed by SRC for a further 16 weeks.
(5) " " " " " " " 12 "
(6) " " " " " " " 10 "
(7) " " " " " " " 8 "

After 16 weeks on the deficient diet, each rat in groups (4)-(7) was caged paired with a stock rat for the duration of the dietary study. This was done to facilitate, via coprophagy, the reinstatement of folate producing gut bacteria which would have been eliminated by the inclusion of succinylsulphathiazole in the composition of the folate deficient diet (Daft et al, 1963; Tagbo & Hill, 1977).

For each group, at the end of the scheduled period, animals were anaesthetised, exsanguinated and serum prepared for culture as described in Chapter 6. Serum folate levels were also assessed (see Chapter 6).

Explantation of embryos: Embryos were cultured in serum from the 7 experimental groups according to the standard explantation and culturing procedures (Chapter 6). The average serum folate level for each group was as follows:

(1) 83ng/ml.
(2) 2.87ng/ml.
(3) 73ng/ml.
(4) 74ng/ml.
(5) 69ng/ml.
RESULTS
The morphological scores and protein content of embryos grown in serum from animals in the various dietary groups is given in Table 18. The results were fairly dramatic. Even after only 8 weeks on the normal fully supplemented diet, rats which had been substantially depleted of folate, apparently regained normal nutritional status in all parameters including that which had been responsible for the observed reduction in embryonic protein content. Embryos cultured in all except the folate deficient serum, were morphologically normal with no signs of growth retardation.

DISCUSSION
This experiment demonstrated that the maternal system, restored to an adequate diet, has the ability to overcome the effects of a folate deficiency. The results also imply that the folate deficient diet had induced secondary effects which involved growth factors or complex metabolic factors (for example folate absorption), which could not readily be reversed \textit{in vitro}.

From previous studies it had been established that a fairly severe folate deficiency (less than 3ng/ml serum folate) was necessary before gross morphological abnormalities became apparent. To induce this severity of depletion animals had to be maintained on the deficient diet for periods in excess of 16 weeks. However, as these earlier studies had mainly been
### TABLE 18

Effects of restoring folate deficient animals (maintained on deficient diet for 16 weeks) to a fully supplemented standard rat chow diet for 16, 12, 10 or 8 weeks.

<table>
<thead>
<tr>
<th>CULTURE GROUP</th>
<th>n</th>
<th>MORPHOLOGICAL SCORE</th>
<th>PROTEIN CONTENT (µg)</th>
<th>ABNORMAL AXIAL ROTATION</th>
<th>CLINICALLY OBSERVABLE ANAEMIA</th>
<th>MINOR DEFECTS/RETARDED DEVELOPMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard rat chow (SRC)</td>
<td>20</td>
<td>42.15 ± 0.082</td>
<td>162.9 ± 4.39</td>
<td>0</td>
<td>0</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Folate deficient diet (FDD)</td>
<td>20</td>
<td>34.91 ± 0.972</td>
<td>101.5 ± 5.06</td>
<td>13 (65%)</td>
<td>10 (50%)</td>
<td>16 (80%)</td>
</tr>
<tr>
<td>Folate supplemented FDD</td>
<td>18</td>
<td>42.13 ± 0.201</td>
<td>158.8 ± 3.03</td>
<td>0</td>
<td>0</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>FDD + 16 weeks SRC</td>
<td>14</td>
<td>41.85 ± 0.244</td>
<td>160.8 ± 7.90</td>
<td>0</td>
<td>0</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>FDD + 12 weeks SRC</td>
<td>15</td>
<td>41.91 ± 0.315</td>
<td>157.0 ± 4.89</td>
<td>0</td>
<td>0</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>FDD + 10 weeks SRC</td>
<td>15</td>
<td>40.83 ± 0.789</td>
<td>158.8 ± 8.50</td>
<td>0</td>
<td>1 (7%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>FDD + 8 weeks SRC</td>
<td>8</td>
<td>41.88 ± 0.228</td>
<td>165.1 ± 7.31</td>
<td>0</td>
<td>0</td>
<td>1 (12%)</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error.

* p values calculated by Student's T-test indicating the difference from culture in normal rat serum.

* p" values indicate the difference from culture in folate deficient serum.

NS = Not significant
concerned with the production of morphological defects, the onset and progression of the reduction in protein content had not been monitored.

A further experiment was therefore planned to investigate the possible difference in time course between the induction of morphological abnormalities and reduced embryonic protein.
CHAPTER 15

RELATIONSHIP BETWEEN EMBRYONIC PROTEIN CONTENT AND THE DURATION OF DIETARY FOLATE DEPLETION

When serum from dietary induced, severely folate deficient rats was used for \textit{in vitro} culture of embryos, morphological defects and reduced embryonic protein content resulted. In earlier experiments, a relationship between serum folate level and embryonic protein content had been observed (see Chapter 8, fig. 16), but this had been established in association with the induction of embryonic malformations, i.e. the results were generated from animals in relatively advanced stages of folate depletion. At that point in the study, it had been assumed that embryonic malformations were the primary effects of the deficiency. However, following this, accumulated data indicated that an earlier and therefore better means of detecting the onset of the effects associated with a folate deficiency might be achieved by monitoring embryonic protein content. The purpose of this study was therefore to determine the relationship between the development of the folate deficiency and embryonic protein content, and to relate these factors to the established time course for the induction of morphological abnormalities.

MATERIALS AND METHODS

Dietary regimens: Female Wistar rats, approximately 6 weeks old and weighing around 100 grams, were randomly allocated to one of seven dietary regimens. The first group was fed standard rat chow to provide normal control serum. Group 2 animals were
maintained on a folate deficient diet (Chapter 6) for 4 weeks, and groups 3-7 received the deficient diet for 6, 8, 10, 12 or 14 weeks.

Preparation of serum: At the end of the scheduled period, rats were terminated and their serum collected and prepared as described in Chapter 6. Serum folate levels were measured (see Chapter 6).

Explantation and culture of embryos: 9.5 day embryos were obtained from donor Wistar rats maintained on standard rat chow. These were explanted and cultured, as detailed in Chapter 6, in serum from the 7 experimental groups. Culture serum folate levels were as follows:

- Group (1) 72ng/ml.
- Group (2) 48ng/ml.
- Group (3) 26ng/ml.
- Group (4) 21ng/ml.
- Group (5) 11.4ng/ml.
- Group (6) 7.8ng/ml.
- Group (7) 4.4ng/ml.

Assessment of embryonic development: Embryonic growth and development were quantified according to the method of Brown & Fabro (1981) and embryonic protein content estimated by the modified Lowry method (Chapter 6).

RESULTS

Table 19 gives the morphological scores and protein contents from the seven culture groups.

As expected, virtually all embryos in all groups were
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Morphological Score</th>
<th>Protein Content (g)</th>
<th>Abnormal Axial Rotation</th>
<th>Clinically Observeable Anemia</th>
<th>Minor Defects/Retarded Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>10</td>
<td>42.50 ± 0.166</td>
<td>164.9 ± 2.53</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(2) b</td>
<td>6</td>
<td>42.33 ± 0.211</td>
<td>162.9 ± 3.67</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(3) c</td>
<td>6</td>
<td>42.12 ± 0.307</td>
<td>153.7 ± 3.19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(4) d</td>
<td>6</td>
<td>41.83 ± 0.477</td>
<td>154.6 ± 4.31</td>
<td>2 (33%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(5) e</td>
<td>6</td>
<td>42.50 ± 0.224</td>
<td>145.6 ± 5.17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(6) f</td>
<td>6</td>
<td>41.58 ± 0.201</td>
<td>139.9 ± 4.35</td>
<td>2 (33%)</td>
<td>0</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>(7) g</td>
<td>6</td>
<td>41.05 ± 0.810</td>
<td>120.9 ± 4.99</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the mean of the number of embryos "n" ± the standard error. p values calculated by Student's t-test indicating the difference from culture in normal rat serum (Group (1)).

NS = Not significant
morphologically normal. Only in group (7), where the serum folate level approached that known to produce malformations (<3ng/ml), an occasional mild defect was observed.

Embryonic protein content, however, did not follow the same pattern. From a relatively early stage in the study, i.e. 6 weeks, a reduction in protein content was apparent. Thereafter, the progressive decrease followed a fairly linear pattern (fig. 21) and by week 8 (group (4)), the difference from culture in normal rat serum (group (1)) was statistically significant.

DISCUSSION
From the results of this experiment it was clear that embryonic protein content was affected by the folate deficiency well in advance of the appearance of morphological defects.
Fig. 22 demonstrates the relatively linear relationship between duration of diet and both serum folate and embryonic protein content and shows their much earlier correlation when compared with the induction of embryonic malformations and the associated decrease in morphological score.

Fig 22. The relationship between weeks on the folate deficient diet and serum folate levels and also between weeks on the diet and embryonic protein content and morphological score.
From previous studies, particularly the very early experiments (see Section III), morphological defects were presumed to be the primary adverse effect of culturing embryos in folate depleted serum. However, from accumulated data since then, a much clearer picture of the effects associated with the folate deficiency has emerged. This has indicated that the assessment of embryonic protein content would provide a much earlier and more accurate means of monitoring the effect, at embryonic level, of a folate deficiency.
CHAPTER 16

The findings presented in this study provide strong evidence for a correlation between folate deficiency and defective development of the rat embryo. The relevance of this investigation to the human situation is unknown but many of the observations noted in these experiments are comparable with reported clinical phenomena.

In this respect, probably the two most significant results were the occurrence of embryonic malformations and the marked reduction in embryonic growth. In the former, malformations including abnormalities of the neural tube were observed in embryos grown in severely folate depleted serum. This corresponds with clinical reports of folate deficiency in mothers who gave birth to babies with NTD (Hibbard & Smithells, 1965; Smithells et al, 1976; Laurence et al, 1981, 1983; Schorah et al, 1983; Yates et al, 1987). A major difference between the in vitro observations reported here and clinical findings is that a human infant with NTD would probably live whereas the malformed embryos seen in the present study would undoubtedly have died. This is because the in vitro morphological scoring system used in these experiments was fairly crude and would probably not be able to detect the types of abnormalities which would manifest as NTD in the human i.e. with the in vitro model, malformations...
comparable with human NTD would be very minor. To detect this type of abnormality histological examination would probably have been necessary. This may contribute to an explanation for the observed low embryonic protein in apparently normal embryos. Minor morphological defects may have been present but remained undetected.

A further correlation with clinical reports was the critical serum folate level. In culture, embryos grown in serum containing less than 3ng/ml folate tended to be malformed, while those cultured in serum containing more than 3ng/ml folate were morphologically normal. This critical level is comparable with that associated with human folate deficiency, that is, at or below 3ng/ml (Cooper & Lowenstein, 1961; Waters & Mollin, 1961; Davis & Kelly, 1962; Menon et al, 1966; Smithells et al, 1976; Schorah et al, 1983; Molloy et al, 1985; Coulibaly et al, 1987). This correlation is interesting considering the marked difference between normal human and rat serum folate levels which are 5-20ng/ml and 40-100ng/ml respectively.

The second significant similarity between the present study and human observations was the association between reduced folate levels and growth. In both the first and second generation rats born and maintained on the folate deficient diet, there was a marked reduction in normal post-natal weight gain (see Chapter 8, compare figs. 11a, 11b & 12). This was paralleled in the in vitro studies by a corresponding decrease in embryonic protein as culture serum folate declined (Chapter 8, fig. 16). These observations are comparable with many clinical reports.

Poor growth of rat pups born to folate deficient dams had been reported previously by Tagbo & Hill (1977) who suggested that this might relate to maternal inability to synthesise protein normally (see Chapter 3). If this was the case, lactating pups would have the additional problem of reduced nutritional intake as it is known that protein deficiency can reduce milk production (Turner, 1973). In the present study no frank congenital malformations were observed in any young born to folate deficient rats, but as none of the dams were actually monitored during birth it is quite likely that abnormal young were born but immediately disposed of by the mothers. It was clear, however, that the surviving offspring were not as healthy as normal pups. This compares with the suggestion that for every human baby affected by spina bifida there are probably many associated cases of mild handicaps and growth retardation (Edwards, 1982).

Malformations observed when embryos were cultured in the presence of low levels of aminopterin (Chapter 7) were comparable with those seen in embryos grown in dietary induced folate deficient serum. However, reversal of these effects by folate supplementation would probably have been unsuccessful due to the mode of action of aminopterin. Folate antimetabolites such as aminopterin and methotrexate act by inhibiting the enzyme folic acid reductase, thereby preventing the formation of active tetrahydrofolate from folic acid (see Chapter 3). The affinity
of folic acid reductase for the antagonist is so much greater than its affinity for the normal folate substrates that even excessive doses of folate will not reverse the toxic effects of the antagonists (Karnofsky & Clarkson, 1963). Therefore, although similar embryonic malformations could be induced by either aminopterin or by dietary depletion of folate, the use of antagonists would have been an unphysiological mode of producing folate deficient serum and would have made questionable the value of any extrapolation of results to the clinical situation. Also, the reversal studies (Chapter 15) would clearly have been unsuccessful. Although the use of antagonists proved unsuitable, an interesting coincidence emerged between the dietary induced folate deficient state of the present study and the clinical chemotherapeutic use of folate antagonists. When the dietary depletion was continued to second generation animals the folate deficiency appeared to adversely affect hair growth (see Chapter 8, fig. 11). Similarly, patchy alopecia is reported as one of the many toxicological consequences caused by the use of folate antagonists in man (Hitchings & Burchall, 1965).

In the studies investigating the reversibility of the effects induced by the folate deficiency, another result was noted which suggested some analogy to clinical observations. This was the beneficial effect of supplementing folate deficient rats with folate. In the in vivo experiments (Chapter 14), rats which had previously been so folate deficient that culture in their serum would have resulted in malformed embryos, after folate supplementation, produced serum which supported completely normal
embryonic growth. In comparison with this, clinical studies have demonstrated the benefits of giving mothers with high risk of having a NTD baby, either multivitamins containing folate (Smithells et al, 1980, 1981a, 1981b, 1983), folate supplements or dietary counselling to improve nutritive intakes (Laurence et al, 1980, 1981 & 1983). From the results of these clinical studies it has been suggested that increased availability of folate is the factor most probably responsible for the demonstrated benefits (Laurence, 1986).

However, in the \textit{in vitro} experiments (see Chapter 9), while supplementing folate deficient culture serum with folate provided clear evidence of a beneficial effect, it was apparent that complete restoration of normal embryonic growth was not achieved. This inability to completely reverse the effects suggested two facts. Firstly, inadequate availability of folic acid for the rat embryo during the critical period of 9.5 - 11.5 days was an important factor in the aetiology of the observed malformations, and secondly, depletion of folate was not the sole consequence of dietary folate deficiency. Coupled with this the comparative ease with which \textit{in vivo} reversal occurred implied that complex metabolic factors may be involved. These results can be more readily understood by considering the metabolic role of the vitamin. Vitamins act as prosthetic groups in enzymes which catalyse complex biochemical reactions. As these sequences are mediated only indirectly via the prosthetic groups their absence may lead to abnormal reactions. These reactions may not be readily reversed \textit{in vitro} by subsequent availability of the vitamin because of the absence of the supporting metabolic
system.

In comparison with these findings clinical reports have indicated that disordered maternal folic acid metabolism, possibly involving folate absorption, utilization or transport (or even a combination of these factors), may contribute to the occurrence of NTD (Hibbard & Smithells, 1965; Laurence et al, 1981 & 1983; Smithells, 1982; Navarro et al, 1984; Yates et al, 1987). The in vitro findings presented here could not, however, be compared to these clinical observations, as the in vitro system only examined the direct effect of folate supplementation on the embryo and could not assess indirect, maternally induced, metabolic effects. Nor could they confirm the hypothesis equating disordered folate metabolism with the characteristic clinical pattern of inheritance of NTD (Yates et al, 1987), as the embryos were cultured in serum from unrelated serum donors. It was nevertheless clear from both the in vitro and the in vivo experiments that some secondary factor in addition to inadequate folate had adversely affected embryonic growth. Therefore in conclusion, it appeared probable that both dietary and metabolic factors were involved in the aetiology of the observed embryonic abnormalities and that the dietary deficiency possibly potentiated disordered folate metabolism.

Another minor correlation between the present in vitro study and reported clinical studies was the demonstration of a beneficial effect by supplementation with doses of folate which increased the serum level from a common deficiency of around 3ng/ml to within the normal range for the species i.e. more than 50ng/ml in
the rat and more than 10 ng/ml in the human (Laurence et al., 1981).

While investigating the effect of the folate deficient diet on the various serum constituents, it was noted that although serum folate was significantly lower in animals on the deficient diet, none showed evidence of anaemia or megaloblastosis. The significance of this result to the human situation is not known as anaemia and megaloblastosis are fairly common symptoms in the clinical picture of folate deficiency (Beck, 1977; Chanarin, 1979; Cauchi & Smith, 1982; Shojania, 1984).

The observation of a reduced white blood cell count had been reported previously (Daft & Sebrell, 1943; Kronberg et al, 1943; Tagbo & Hill, 1977; Beaulieu et al, 1979) and its direct association with folate depletion had been demonstrated by complete restoration of leukocyte count following folate supplementation (Daft & Sebrell, 1943; Kronberg et al, 1943). The longer life span of the red blood cell (approximately 100 days) compared to the relatively short life of the white blood cell (approximately 10 days) could explain the earlier appearance of a reduction in the latter (Kodicek & Carpenter, 1950; Hugh Jones & Cheney, 1961).

Another result from the present study which could be compared with clinical observations was that of the serum vitamin B12 level, which although closely related, was not affected by the folate deficient diet. Correspondingly in the human, a significant relationship between quality of diet and folate levels but not with quality of diet and vitamin B12 levels has been reported (Laurence, 1986).
Although increasing the volume of folate supplemented serum per embryo in culture, apparently failed to fully restore embryonic protein content (Chapter 12), if more time had been available, it would have been interesting to carry out a further experiment. This would have involved replacing the serum repeatedly throughout the 48 hour culture period. Al Alousi (1983) demonstrated that for normal development the rat embryo requires traces of specific growth factors as well as vitamins, proteins, minerals and glucose. Therefore, if the folate supplemented serum had marginally insufficient levels of some specific energy supply or essential growth factor, repeated replenishment of the serum might provide a means of compensating for this.

The only supplement to the folate deficient serum which produced normal embryonic growth and development was whole rat serum. Although no specific factor was identified, this observation was compatible with the hypothesis that the folate deficient serum was also deplete of some essential growth factor. Also, the relatively small amount of supplement required (30%) indicated that the factor in question was very readily available in whole rat serum.

The present investigation has shown that embryonic growth retardation and the induction of frank malformations were the two main defects caused by culturing embryos in folate deficient serum. From this, probably the most valuable discovery was the relationship between the onset of the adverse effects and the severity of depletion. While reduced embryonic size and protein content were induced relatively early in the folate deficiency,
embryonic malformations appeared to be a secondary effect occurring at a much later stage (see Chapter 15, fig. 22). This earlier correlation could be of value because, if identified soon enough, a minor defect such as retarded growth may be amended or at least prevented from deteriorating. If however, the early indicator is not recognised and the deficiency is allowed to progress, the chances of correcting the much more serious secondary effect may be less hopeful.

Although the mechanisms by which the folate deficiency induced the observed malformations were not fully elucidated, the results provided strong evidence that adequate availability of folate is essential to the developing embryo. They also proved that, if provided with an adequate supply of folate, the rat’s metabolism has the ability to overcome a fairly severe deficiency. The results did not, however, determine either the stage or the extent to which correction of the maternal deficiency would affect the outcome of pregnancy. As the requirement for folate is much higher in the developing embryo than in the mother, it is likely that the embryo would be impaired by a folate deficiency before the maternal system was noticeably affected. Clinical results have suggested that pharmacological doses of folate may be beneficial to the outcome of high risk pregnancies (Laurence et al, 1981), even though there are no overt symptoms of folate deficiency in the mothers (Magnus et al, 1986). This implies that folate deficiency, and related maternal metabolism disorders, can be overcome by folate supplementation and results of the _in vivo_ supplementation studies in rats supported this (Chapter 14). Comparisons are, however, difficult to make.
because the present experiments only examined the *in vitro* effect of exposing the developing embryo to a folate deficiency. They did not investigate the *in vivo* outcome of folate deficiency in pregnancy. Also, the rat's metabolism and requirement for folate is probably very different from the human.

However, together with the clinical reports suggesting a reduction in the prevalence of NTD by folate and multivitamin supplementation, the findings of this study emphasise the importance of adequate folate in pregnancy. Although direct extrapolation of these results to man is clearly not possible, it is hoped that they may contribute to our understanding of observations made in humans.
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ABSTRACT

EFFECT OF FOLATE DEFICIENCY IN MAMMALIAN PREGNANCY

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The consequence of a folate deficiency during organogenesis has been investigated in the rat embryo. *In vitro* culture of 9.5 day embryos in serum from dietary induced folate deficient rats frequently resulted in abnormal embryos. Many were growth retarded and exhibited a defect in the turning mechanism which inverts the embryo from ventrally to dorsally convex. Affected embryos displayed abnormal twisting or kinking of the neural tube. Gross anaemia was also frequently observed and the protein content of the embryos was markedly less than that of embryos grown in normal rat serum. Supplementation of the deficient serum with folic acid improved growth and greatly reduced the occurrence of deformities. It virtually eliminated the incidence of gross anaemia but only partially restored the protein content to the level observed in embryos cultured in normal rat serum. The effects of the folate deficiency could not be reversed by supplementation with multivitamins or by increasing the volume of culture serum. They were, however, eliminated by supplementing the deficient culture serum with normal rat serum. The effects could also be overcome by *in vivo* folate supplementation; rats which had previously been so folate deficient that culture in their serum would have resulted in malformed embryos, after restoration to a folate supplemented diet produced serum which supported completely normal embryonic growth. The results indicate that embryos undergoing organogenesis require adequate folate in order for normal growth and differentiation to take place. They also suggest that some of the embryopathic effects of maternal folate deficiency are mediated by secondary effects. These may involve complex growth or metabolic factors which can be corrected *in vivo* but are not readily reversed *in vitro*. 