MINIREVIEW

Folate, Homocysteine and Neural Tube Defects: An Overview

NATHALIE M.J. VAN DER PUT,[1,*] HENNY W.M. VAN STRAATEN,† FRANS J.M. TRIJBELS,* AND HENK J. BLOM*

*Department of Pediatrics, University Medical Center Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands; †Department of Anatomy and Embryology, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands

Folate administration substantially reduces the risk on neural tube defects (NTD). The interest for studying a disturbed homocysteine (Hcy) metabolism in relation to NTD was raised by the observation of elevated blood Hcy levels in mothers of a NTD child. This observation resulted in the examination of enzymes involved in the folate-dependent Hcy metabolism. Thus far, this has led to the identification of the first and likely a second genetic risk factor for NTD. The C677T and A1298C mutations in the methylenetetrahydrofolate reductase (MTHFR) gene are associated with an increased risk of NTD and cause elevated Hcy concentrations. These levels can be normalized by additional folate intake. Thus, a dysfunctional MTHFR partly explains the observed elevated Hcy levels in women with NTD pregnancies and also, in part, the protective effect of folate on NTD. Although the MTHFR polymorphisms are only moderate risk factors, population-wide they may account for an important part of the observed NTD prevalence.

[Exp Biol Med Vol. 226(4):243–270, 2001]

Key words: homocysteine; folate; neural tube defects; MTHFR; metabolism

Neurulation

Principles of Neurulation. Neurulation comprises the process of the formation of the neural tube. The entire central nervous system and part of the peripheral nervous system are derived from this initially monolayered tube. Neurulation in humans occurs in two phases, a primary and a secondary neurulation phase. Primary neurulation refers to folding of an induced neural plate that occurs on the dorsal side of the embryo and results in the formation of the brain and spinal cord. Secondary neurulation refers to sequential processes of canalization and retrogressive differentiation of a massive neural cord, and results in the development of the most caudal part of the spinal cord. Thus, neural tube formation is initiated by neural folding and completed by canalization.

Primary Neurulation-Neural Folding. Primary neurulation concerns the transformation of a flat neural plate into the cylindrical neural tube and involves several processes that overlap both spatially and temporally. The involved morphogenetic processes have been reviewed elsewhere (1–4); they are illustrated in Figure 1 and will be briefly described below.

The first process regards the formation of the neural plate. The nervous system originates on the dorsal side of the embryo as a plate of tissue differentiating from the middle part of the ectoderm. Following induction by the underlying notochordal plate and prechordal mesoderm, the ectoderm transforms in a neural plate (5, 6) (Fig. 1A, top), while its cells increase in height and become pseudostratified. The earliest part of the neural plate consists only of tissue that will develop into the forebrain, mid-brain, and the most rostral part of the hindbrain, while later parts will become the remainder of the hindbrain and spinal cord.

The next process, shaping of the neural plate, takes place shortly after the neural plate is formed. The configuration of the plate changes, particularly due to extension in longitudinal direction and narrowing in transverse direction at the same time (Fig. 1B), a process that is referred to as convergent extension. This is the result of a combination of cellular processes: the epithelial cells increase in number, in cellular height, and in mutual positions (7, 8).

Funding was provided by prinses Beatrix Fonds 96–0104 and 98–0109 and by the Netherlands Heart Foundation D97021.

[1] To whom requests for reprints should be addressed at Department of Pediatrics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: N.vanderPut@ckslkn.azn.nl

0037-9727/01/2264-0243$15.00

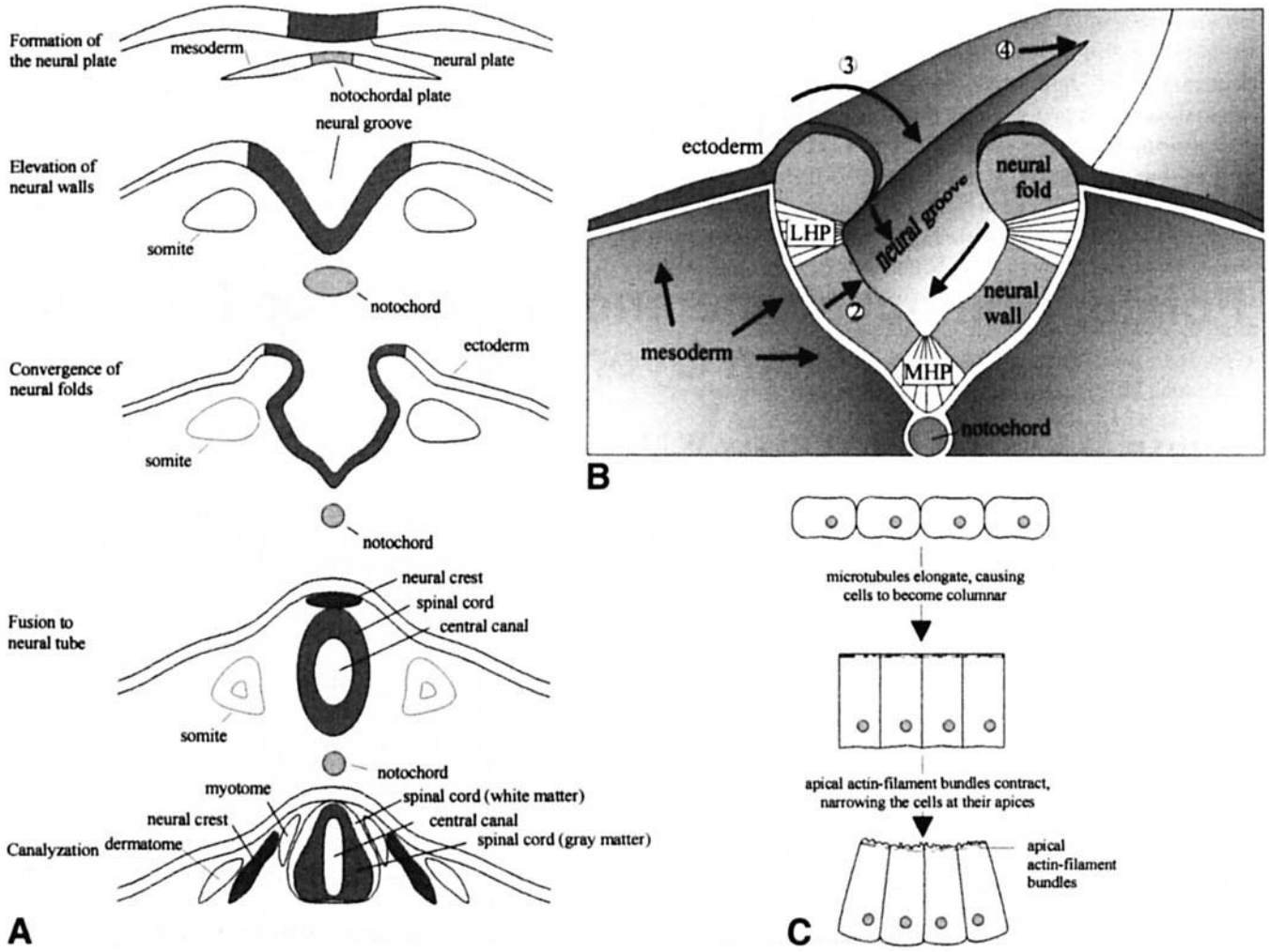

Figure 1. (A) Four phases of primary neurulation as seen in a transverse view. (B) Several processes during primary neurulation, which overlap both spatially and temporally. 1, the arrows in the neural groove indicate the narrowing and elongation, resulting in convergent extension; 2, elevation of the neural wall; 3, convergence of the neural fold; 4, fusion. The arrows in the mesoderm imply the mesodermal expansion. MHP, medial hinge point; LHP, dorsolateral hinge point. (C) Neuroepithelial transformation during primary neurulation; ultimately, the cells acquire a wedge-shape by contraction of the apically arranged microfilament bundles. (Modified with permission from Ref. 266).

Next, the neural plate starts to bend, which begins during the process of shaping. It involves the formation of hinge points, elevation of the neural walls, and the formation and convergence of the neural folds.

Due to the formation of the medial hinge point, a medial V-shaped depression in the neural plate arises over its length (Fig. 1B), as follows. The medial neural plate cells (floor plate) decrease in height and become markedly wedge-shaped in reaction to the underlying notochord (9–11). The mechanism of wedging is suggested to involve basal expansion by withdrawing cells from the cell cycle, resulting in basally positioned nuclei (12).

The next process is elevation, whereby the lateral neural plate halves move upward and form the walls of the neural groove (Fig. 1A). Several factors are proposed to generate or facilitate this process: (i) the medial hinge point is thought to at least facilitate bending of the neural plate in its midline; and (ii) the above mentioned process of convergent extension is suggested to force the lateral neural plate halves in an upward direction by buckling (3, 13) and, by contraction of microfilament bundles, which are arranged like a purse string around the cellular apex. All neural plate cells become slightly wedge-shaped (Fig. 1C) and as a result, the whole neural plate curls up (4, 14–19). Expansion of the mesoderm, which is underneath the neural walls, likely facilitates elevation as well (Fig. 1B) (18, 19).

The next process is convergence, or medial bending, of the neural folds. The neural folds comprise the dorsal extensions of the neural walls plus the ectoderm. In the region of the future brain and of the caudal spinal cord, hinge points arise at a dorsolateral position in both neural walls. These hinge points are the likely result of interaction between the neural wall and the adjacent ectoderm, and it is proposed that they are based on enhanced apical microfilament contraction. With the formation of the dorsolateral hinge points, two longitudinal furrows arise, allowing the neural folds to bend medially until they approach each other (Fig. 1B). This neural fold convergence is possibly facilitated by the expansion of ectoderm, as caused by changes in ectodermal cell number, shape and position (20, 21). Thus,

bending of the neural plate is facilitated by the median and dorsolateral hinge points around which the neural plate elevates and converges, respectively. In the somitic region, dorsolateral hinge points are not formed, and elevation alone allows the neural walls to clap shut like a book.

The last process of primary neurulation involves the closure of the neural groove with formation of the roof plate of the neural tube, the neural crest, and the overlying surface epithelium. When the neural folds have approached each other, they adhere and subsequently fuse. Such closure is effectuated by cell adhesion molecules, which are expressed at the neuroepithelial cell surface and by protrusive activity of the cells.

The initial fusion has long been proposed to occur according to the elevation-convergence-fusion concept, as depicted above. However, this sequence merely applies to progression of closure in longitudinal direction. Initial closure resembles the process as seen in between somites: the neural walls align parallelly, adhere, the adhesions release ventrally, allowing a lumen to arise, while the adhesion remains dorsally and changes into fusion (22). In this manner, no initial misalignment of the neural folds is possible, as would be by the process of convergence.

The first fusion of neural folds occurs in the high cervical region, leaving large neural tube openings called the anterior and posterior neuropore at both extremities. Previously, this fusion initiation point was considered to be the only one, and closure was thought to proceed until the anterior and posterior neuropore were closed. However, Golden and Chernoff (23, 24) showed in three mouse strains the presence of at least four different closure initiation sites plus five transient neuropores, and this was confirmed in other animal models (19, 25). In humans, such a multisite closure pattern was suggested to occur as well (26, 27) (Fig. 2). Closure 1 regards the cervical one mentioned above. Closure 2 takes place at the forebrain-midbrain transition and, like closure 1, proceeds bidirectionally, dividing the anterior neuropore in a forebrain and a mid/hindbrain neuropore. Closure 3 is unidirectional, beginning adjacent to the stomodeum and proceeding caudally to meet closure 2, thereby closing the forebrain neuropore. Finally, closure 4 takes place were it meets closure 2 to close the mid/hindbrain neuropore. The posterior neuropore is closed by caudal continuation of closure 1 and remains longest. Primary neurulation is completed after the closure of this neuropore in the sacral region (28–30).

Secondary Neurulation-Canalization. Closure of the neural tube is followed by secondary neurulation, which leads to formation of the most caudal portion of the spinal cord. The junction of primary and secondary neural tube occurs at the sacral level of the embryo (29, 31–35).

Caudally of the previous posterior neuropore the caudal eminence (tail bud) is formed. This eminence arises as a continuation of the organizer and is an ectoderm-covered mass of undifferentiated mesenchymal cells. This seemingly homogeneous tissue gives rise to various tissues including the tail hindgut, somites and their derivatives, neural crest cells, and a neural cord. This cord is transferred into a neural tube without the intermediate phase of a neural plate, and becomes subsequently connected to the primary neural tube, as follows. The peripheral cells of the neural cord arrange radially and differentiate from a mesenchymal into an epithelial form. In between the central undifferentiated cells, small caveoles begin to develop that coalesce and enlarge into a canal. Now the secondary neural tube has arisen. The lumen of the primary neural tube proceeds caudally and makes contact with the newly formed lumen of the secondary neural tube. The process of canalization continues until approximately the seventh week after conception, when retrogressive differentiation begins. At the end of the embryonic period the neural cord still reaches to the end of the vertebral column, but during the fetal period it ascends to the sacral and then to lumbar levels.

Figure 2. Schematic representation of the proposed multiple initial closure sites in the human embryo.

Neural Tube Defects (NTD)

Definition and Classification of NTD. After cardiac defects, isolated (nonsyndromic) NTD are the most common congenital structural defects worldwide. Congenital anomalies are abnormalities in structure or function that are inherited or acquired during the prenatal or perinatal period and that manifest before, at, or shortly after birth. Congenital anomalies have become more important causes of infant morbidity and mortality since the prevalence rates of infectious diseases and nutritional problems during child-

hood have decreased over the last decades in Western Europe and North America.

The term NTD applies to malformation of the embryonic brain and/or spinal cord. The various forms of NTD are characterized by incomplete development of the central nervous system and its closely related surrounding structures. As previously discussed, neurulation relies on many morphogenetic processes and both genetic and environmental factors are involved. Disturbances of any of these factors and processes may result in neural tube closure defects, which become manifest primarily as anencephaly or as spina bifida with myeloschisis and are accompanied by alterations of the axial skeleton, as well as the overlying meningovascular and dermal tissues (canalization). Although NTD are among the most common congenital malformations, little is understood about the underlying developmental mechanisms.

Clinically, the term spina bifida is used collectively and includes malformations with cord involvement. When NTD are subdivided according to their underlying defect, two main categories emerge: open NTD and closed NTD. In the former category, the neural tube is open due to nonclosure or to reopening of a closed tube. This defect results in the exposure of neural tissue at the surface and thus neurological damage to the child. This form of NTD is known as exencephaly if the cranial region is affected and it is known as myeloschisis (spina bifida aperta) if the caudal part is affected. Closed NTD are distinguished from the open NTD not only by their caudal locus, but also particularly by the presence of intact skin over the lesions. The medullary cone is usually prolonged and the terminal filum is thickened. Vertebral defects occur in 85% to 90% of cases with closed NTD and consist most commonly of laminar defects over several segments; other skeletal abnormalities include a widened spinal canal and sacral deformities (34, 36). About 80% of cases exhibit a dermal lesion in the lumbrosacral area, consisting of abnormal collections of hair, cutaneous dimples or tracts, superficial cutaneous abnormalities (e.g. hemangioma), or a subcutaneous mass. Although neurological deficits are unusual in the newborn, motor or sensory disturbances in the legs or feet, or sphincter abnormalities occasionally may be detected. The closed defects are known as spina bifida occulta and spina bifida cystica.

NTD

A total failure of neurulation will result in craniorachischisis totalis. In this case there is a neural plate-like structure present throughout, and there is no overlying axial skeleton or dermal covering (37, 38). Onset of this severe disorder is estimated to be no later than at the embryonic age of 20 to 22 days (34). This form of NTD is incompatible with life.

The essential defect of anencephaly is failure of anterior neural tube closure. The most common variety of anencephaly includes involvement of the forebrain and variable amounts of upper brain stem. The exposed neural tissue is represented by a hemorrhagic, fibrotic, degenerated mass of neurons and glia with little definable structure. The frontal bones above the supraciliary ridge, the parietal bones, and the squamous part of the occipital bone are usually absent. This anomaly of skull imparts a remarkable froglike appearance to the patient when viewed face on. Onset of anencephaly is estimated to be no later than at the embryonic age of 24 days (34). Most children die before or during delivery. If they are born alive they can survive only for a couple of weeks. This disorder is relatively common, and epidemiological studies reveal striking variations in prevalence as a function of geographical location, sex, ethnic groups, race, season of the year, maternal age and nutritional state, social class, and history of affected siblings (38–41).

The essential defect in myeloschisis is failure of posterior neural tube closure. A neural plate-like structure involves large portions of the spinal cord and appears as a flat, raw, velvety structure with no overlying vertebrae or dermal covering. Onset is no later than at 24 days of embryonic development (34). Most of these infants are stillborn and merge with the category of more restricted defects of neural tube closure, i.e., meningo(myelo)cele.

Encephalocele may be envisioned as a restricted disorder of neurulation involving anterior neural tube closure. This defect occurs in the occipital region in 70% to 80% of the cases (36, 42–45). Less common sites include the frontal region where the encephalocele may protrude into the nasal cavity. Least common lesion sites include the temporal and parietal region (46). The neural tissue in an encephalocele usually connects to the underlying central nervous system through a narrow neck of tissue. As many as 50% of cases are complicated by hydrocephalus (47). Anomalies of venous drainage occur in about one-half of the patients and must be considered in surgical approaches to these lesions (48). Onset of the most severe lesions is probably no later than the approximate time of anterior neural tube closure (26 days) or shortly thereafter. Later times of onset are likely for the lesions that involve primarily or only the overlying meninges or skull (38).

The essential defect in meningo(myelo)cele is restricted failure of posterior neural tube closure. The occurrence of approximately 80% of all lesions in the lumbar region may reflect the fact that this is the last part of the neural tube to close (36). The neural lesion is represented by a neural plate or abortive neural tube-like structure in which the ventral half of the cord is relatively less affected than the dorsal. The axial skeleton is uniformly deficient, and an incomplete, though variable, dermal covering is present. The defects of the spinal column consist of a lack of fusion or an absence of the vertebral arches resulting in bilateral broadening of the vertebrae, lateral displacement of pedicles, and a widened spinal canal. The caudal extent of the vertebral changes is usually considerably greater than the extent of the neural lesion. The large majority of the lesions is associated with dorsal displacement of the neural tissue, such

that a sac is created on the back. This kind of NTD is subdivided in two categories depending on the content of the sac. Saccular enlargements protruding through osseous defects of the vertebral column that contain anomalous meninges and spinal fluid but do not have neural elements affixed to their wall are called meningoceles. If the spinal cord or nerves are included in the saccular protrusion, one speaks of meningo(myelo)cele. Moreover, these structures frequently are "tethered" or fixed at their caudal end by fibrous bands, lipoma, extension of dermal sinus, or related lesions. This fixation is thought to impair normal mobility and functions such as bladder problems (incontinence).

Onset of meningo(myelo)cele is probably not later than at the embryonic age of 26 days, although this is a controversial point (34). Meningo(myelo)cele and its variants represent the most important examples of faulty neurulation since affected infants usually survive. The major clinical features relate primarily to the nature of the primary lesion, the associated neurological features, and hydrocephalus. Most patients with low lesions will be able to walk unaided, whereas those with higher lesions usually are wheelchair-dependent for at least a major portion of their activities.

Occult dysraphic states represent disorders of caudal neural tube formation, i.e. the development of the lower and sacral and coccygeal segments. The occult defects are limited to the posterior neural arches, laminae, and the spines.

If the anomaly is not visible externally, this defect is referred to as spina bifida occulta. Often this abnormality is so well concealed that it remains undetected for years, therefore the term "occult." With the occult dysraphic states, the neural lesion is often rather subtle and the major overt abnormality involves the vertebrae or the overlying dermal structures or both. The most common clinical presentations for occult dysraphic states later in infancy include delay in walking, asymmetry of legs or abnormalities of feet, and pain in the back or lower extremities. In the older child or adolescent, the major clinical features are gait disturbance, development of a foot deformity, and scoliosis.

The Aetiology Of NTD

Prevalence and Etiology of NTD. The birth prevalence is dependent on country and socioeconomic and ethnic groups. The numbers range from one in 2500 in Finland, one in 300 in Mexico, and one in 80 in South-Wales. According to a study in 1991, in the Netherlands a birth prevalence of one in 700 is observed (49), while a worldwide average of one in 500 is reported.

Spontaneous-aborted affected embryos are excluded from these numbers, although in these fetuses the frequency of NTD is about 10 times higher than observed at birth. The prenatal mortality rate of NTD-affected embryos was reported to be 98.4%, and most of them (93%) did not develop beyond embryonic stage (50). It is therefore likely that the prevalence of NTD after neurulation is 10 to 50 times higher than at term, which implies that immediately after neurulation, one in 50 up to one in 10 embryos are affected.

A prominent and steady decline in birth prevalence rates of NTD in both the United States and Great Britain has been observed (41, 51–54). One explanation for these observations is the introduction of prenatal diagnosis in developed countries. In Australia, the birth prevalence has declined by 84%, which is attributed entirely to prenatal diagnosis and termination of pregnancy (55). On the other hand, some authors reported an additional decline in prevalence of NTD that is independent of prenatal diagnosis (57), which is reminiscent of the peaks and troughs in frequency of NTD that occurred in the early decades of the 20th century (57). Furthermore, the decline is apparent also in the Republic of Ireland where there is neither prenatal diagnosis nor legal termination of pregnancy. Thus, for reasons poorly understood, NTD are occurring less frequently than before (58).

Neural tube formation is a multifactorial process determined by both extrinsic and intrinsic factors. Therefore, NTD will be of multifactorial origin, involving both genetic and environmental factors. As shown below there are now many recognized causes of NTD; some are genetic and others are environmental. However, over 90% of the NTD cases still have an unknown etiology. Therefore, further investigations in the etiology of NTD are necessary to come to an understanding of the underlying cause of NTD. This may result in better prenatal diagnosis and prevention of NTD.

Genetic Factors and the Etiology of NTD. Factors establishing the genetic role in several NTD forms include: (i) sex differences in the birth prevalence rates; (ii) ethnic differences that persist after geographical migration; (iii) increased prevalence with parental consanguinity; (iv) increased rate of concordance in monozygotic twin pairs; and (v) increased prevalence in siblings (as well as in second-degree and, to a lesser extent, third-degree relatives) and in children of affected patients (51, 52, 59–67).

Recognized causes of NTD include multifactorial inheritance, single-gene mutations (e.g. the autosomal recessively inherited Meckel's syndrome), and chromosomal abnormalities (e.g. trisomies 13 and 18) (52, 60, 68). Of these causes, the vast majority of cases are encompassed within the group in which NTD is the only major congenital abnormality, and inheritance is multifactorial, i.e., dependent upon a genetic predisposition that is polygenic and influenced by minor additive genetic variation at several gene loci (61). Upon this genetic background, environmental influences may play an important role.

Environmental Factors and the Etiology of NTD. Apart from folate/folinic acid status, which will be discussed later, some possible environmental influences are diabetes, hyperthermia, use of specific teratogens like aminopterin, thalidomide, valproic acid, and other antiepileptic medications, alcohol, and/or professional occupation. In addition, several environmental factors such as geography, month of conception, epidemic trends, maternal age, birth order, socioeconomic class, and maternal diet and

zinc status contribute to increased risk (69–71). Particularly potent data do suggest that environmental influences relate to long-term trends in incidence, i.e., in the northeastern United States an epidemic period could be defined between approximately 1920 and 1949, with a peak between 1929 and 1932 (72). In the Netherlands there was a peak in the prevalence of NTD-affected offspring after the Hunger Winter of 1944–1945; during this period in the Second World War there was a major shortage of food (73). This observation suggests that there is an involvement of a nutritional component in the etiology of NTD.

Prevention Of NTD

Primary Prevention of NTD by Periconceptional Folic Acid Supplementation. Numerous reports have suggested that nutritional deficiency in general, and folate deficiency in particular, can cause adverse birth outcomes. As an example of an anecdote report, a Dutch midwife who practice from 1693 to1745 found an increase in NTD in 1722 and 1732, two years that were linked with poor crops. She also noted that the children with NTD came from the poorest homes in urban areas (74). As pointed out above, a similar result was observed during the Second World War. In addition to a significant decrease in total births and birth weight of infants born during this period of severe food shortage, there was also a significant increase in the rate of NTD (75).

The possibility that folate was specifically linked to NTD in humans was first reported by Hibbard (76). In a retrospective study he observed that women who had pregnancies associated with fetal malformations had a higher incidence of aberrant folate metabolism. He used a formiminoglutamate- (FiGlu) function test, which is based on the increased urinary excretion of FiGlu after histidine loading, because folate deficiency leads to a decreased conversion of FiGlu into glutamic acid. Hibbard and Smithells subsequently repeated this finding in a separate group of mothers who had had a malformed child. From this study onwards, a key role of folate in preventing NTD has been suggested (77).

The early epidemiological studies were mostly focused on the etiology of NTD; however, over the last 10 to 20 years the emphasis of these studies has shifted toward an evaluation of the efficacy of periconceptional vitamin administration, in particular folate, in preventing the development of NTD. The incentive leading to this shift was a study by Smithells and coworkers (78). They measured the first trimester blood vitamin levels in women who had a baby with NTD, and observed that red blood cell folate and leukocyte ascorbic acid levels were significantly lower when compared to mothers without NTD-affected offspring (78). These women had reduced red blood cell folate levels, but no decreased serum folate levels. Red blood cell folate is used as an index of folate stores since red blood cells have a life span of 120 days, whereas serum folate reflects recent dietary intake (79). Therefore, Smithells (78) data suggest that folate stores were low in the women with NTD pregnancies, even though their recent meals may have contained a sufficient amount of the vitamin. This study resulted in the hypothesis that periconceptional intake of vitamins might protect against NTD offspring (80). This observation led to an increased interest in nutritional factors in the etiology of NTD and was the starting point of many studies involving the metabolic folate pathways in families with NTD offspring.

Studies on diet in relation to pregnancy outcome showed that, in general, poor diet in the early months of pregnancy was associated with NTD offspring (81). There are also reports of a higher birth prevalence of NTD in lower socioeconomic groups, as well as in infants conceived in early spring, when fresh foods are less available. In 1991 there was a report of an almost 3-fold increase in NTD after a hurricane in Jamaica which destroyed the island's vegetation (82).

Recurrence and Occurrence Studies: Prevention of NTD with Adequate Folic Acid Intake. Employing this background information, two different types of studies were carried out. One type intended to assess the specific effect of folate supplementation, and the other type intended to establish the effect of multivitamin supplementation. In the main, women who already had NTD-affected pregnancies were examined since they have an increased risk of another affected pregnancy. This risk is about one in 30, but if they have had two affected children, then the risk is further increased to one in 10, and after three, it is over one in five (83). In normal pregnancies this risk is about 1 to 2 in 1000.

Laurence and coworkers (84) studied the effect of a 4-mg folic acid supplementation before and during early pregnancy on the recurrence of NTD in a small-randomized trial. They showed a drop in NTD recurrences in folate users compared to nonusers. However, these studies have been criticized, mostly because of the small numbers (85, 86). The biggest criticism of the Laurence *et al.* (84) study was that two women in the folic acid treatment group had NTD-affected children. The study only became significant by retrospectively shifting them to noncompliant status, which was very unsound scientifically. The effect of multivitamin supplementation before and during early pregnancy on recurrence of NTD was studied by Smithells and colleagues (87–89) in women with a history of one or more NTD-affected pregnancies. The multivitamin supplement contained folate, riboflavin, ascorbic acid, and vitamin A. The obtained results were striking, and they were the first who reported primary prevention of NTD by periconceptional intake of a multivitamin preparation. This study has been criticized because of a selection bias; the case and control groups were not entirely comparable (85, 86). Several groups repeated these studies and showed a likewise effect of multiple vitamins (90, 91), or of folate (92) on the prevention for the recurrence of NTD.

Although the results of these early studies seemed

promising, there was still doubt about the accurateness of the data and the role of folate versus a multivitamin supplement in the observed protective effect. Therefore, the British Medical Research Council performed a multicenter study (93). This is the largest and most important randomized trial examining the effect of periconceptional folate and multivitamin supplements in about 1200 high-risk mothers who have already had a NTD-affected child. These women were randomly assigned to four groups allocated to receive the following regimens of supplementation. Group one received daily 4 mg of periconceptional folic acid; group two received the same, but additionally a multivitamin supplementation; group three received the vitamin supplementation without folate; and the final group received a placebo containing only minerals. This study showed that folic acid alone was as good at preventing recurrences of NTD as the multivitamin plus folate. The recurrence of NTD pregnancy was reduced by 72% if compared with placebo-treated women. Vitamins without folic acid and minerals alone were ineffective in preventing NTD. The results were decisive in demonstrating the preventive effect and the specific role of folate (versus other components of the previously used multivitamin preparations) in prevention of NTD.

Only 5% of all infants with NTD are born to women who have previously had an affected child. It is likely that folate therapy could also prevent a substantial part of the other 95% of NTD cases, which are the first occurrence of an affected member in a family. Therefore, occurrence studies have also been designed to evaluate the effect of maternal periconceptional folic acid supplementation in families without a previous child affected with an NTD. The largest and most important randomized-controlled study of prevention on the first occurrence of NTD by vitamins was performed by Czeizel and Dudas (94) in Hungary. This study lasted eight years and involved more than 4000 women contemplating a pregnancy. These women were subdivided in two groups: one received a multivitamin containing 0.8 mg of folic acid, 11 other vitamins, and trace elements, and the other group received a placebo multimineral containing only the trace elements. The results showed six children with NTD in the latter group and none in the first group. This study, also referred to as the Budapest trial, demonstrated a complete prevention of the first occurrence risk of NTD due to periconceptional folic acid use by the mother.

Several other groups studied the first occurrence of NTD and likewise showed an effect of multiple vitamins containing folate (95–98), or folate (96, 99, 100) on the prevention of the first occurrence of NTD. In contrast to these studies, one major study has failed to find a preventive effect of dietary multiple vitamins and folate. This study was based on a questionnaire concerning the pregnancy outcome and the use of periconceptional multivitamins (101). The negative outcome of this study could be due to misclassifications of the users and nonusers of multivitamins.

Both the MRC trial and the Budapest trial undoubtedly showed a reduction in the birth prevalence of NTD by folate (93, 94). Therefore, it is now generally accepted that periconceptional folate supplementation reduces the recurrence and occurrence risk of NTD. Since the last decade, the governments of several countries are encouraging women who are planning a pregnancy to take 0.4 to 0.5 mg of folic acid daily during the periconceptional period to prevent the occurrence, and even 4 to 5 mg of folic acid to prevent the recurrence of NTD (reviewed in Ref. 102). Nevertheless, public awareness of the benefits of folic acid is only very slowly increasing (103). Although the mean dietary folate intake in Europe is in line with the recommended daily folate intake, the desired dietary intake to prevent NTD is only attained by a small part of most European populations (104).

Several countries, like the Netherlands, advise a daily additional intake of 0.4 to 0.5 mg of folate during the periconceptional period until two to three months of pregnancy have passed. This additional folate intake can be achieved by intake of folate supplements, food fortified with folic acid, and consumption of folate-rich foods. The latter manner is, however, not as effective as the former two (105). In the United States, the government decided in 1993 to fortify cereals, grain, and flour with folic acid by adding 1.4 mg of folic acid/kg to increase folic acid intake in the general population. Although this fortification officially began on January 1, 1998, a lot of food manufacturers began fortifying foods before this date. The results of this food fortification on pregnancy outcome will become evident in coming years.

Folate

Folate Structure and Function. Folic acid is a water-soluble B vitamin. The name folic acid is derived from the Latin word folium, or leaf. It was first isolated from spinach leaves in 1941 and was synthesized in 1946. Its chemical name is pteroylmonoglutamic acid. The term folic acid is used for its synthetic form present in multivitamins, folic acid tablets, and fortified foods. Synthetic folic acid is relatively stable and exists as monoglutamate, a form that is rapidly absorbed without being processed.

The natural form is referred to as folate (folacin), which occurs naturally as polyglutamate derivatives with the glutamate moieties linked via γ-carboxyl peptide bonds. Several dietary sources rich in natural folate include a wide variety of fruits and vegetables, particularly green leafy vegetables such as spinach, brussel sprouts, and turnip greens. Other foods rich in folate include potatoes, oranges, beans, yeast, and organ foods such as liver. Natural folate mainly consists of 5-methyltetrahydrofolate (5-MeTHF) and 10-formyltetrahydrofolate (10-formylTHF) in their polyglutamate derivatives. Both compounds are readily oxidized and the rates vary directly with oxygen concentration, temperature, alkalinity, exposure to light, and concentration of cupric and ferric ions. Therefore, a considerable amount of folate can be destroyed in cooking, processing, and storage.

The generic term folate includes both naturally occur-

ring polyglutamates as the synthetic form, folic acid. The common feature of all folates is the p-aminobenzoicacid part of the molecule attached to the apterin-ring on the NH_2 end, and one or more glutamic acids in a γ-amide linkage at the carboxyl end (Fig. 3). The active center of folate is between the N^5 and the N^{10} site. Folate derivatives are formed by different one-carbon subgroups, which are added to the active site (Fig. 3). Essentially, tetrahydrofolate (THF) is carrying a one-carbon unit present as formate in its most oxidized form and as methyl in its most reduced form; all of these one-carbon subgroups can be converted into each other.

In mammalian tissues, folate functions as substrate in series of interconnected metabolic cycles involving thymidilate and purine biosynthesis (adenosine and guanine), methionine synthesis via homocysteine (Hcy) remethylation, serine and glycine interconversion, and the metabolism of histidine and formate (Fig. 4). Folate is also indirectly a methyl donor in many methylation reactions via S-adenosylmethionine (AdoMet), for example, in the regulation of gene expression. Thus, folate is directly or indirectly essential for cell function, division, and differentiation. Acquisition of folate, therefore, is critically important to the viability of proliferating cells. The shutdown of DNA synthesis and AdoMet synthesis arising from folate deficiency perturbs the cell cycle and could lead to premature cell death. Because eukaryotic cells are unable to synthesize the folate structure *de novo*, folate is an essential nutrient. Thus, mammals are dependent on transport systems for uptake of folate compounds from the environment.

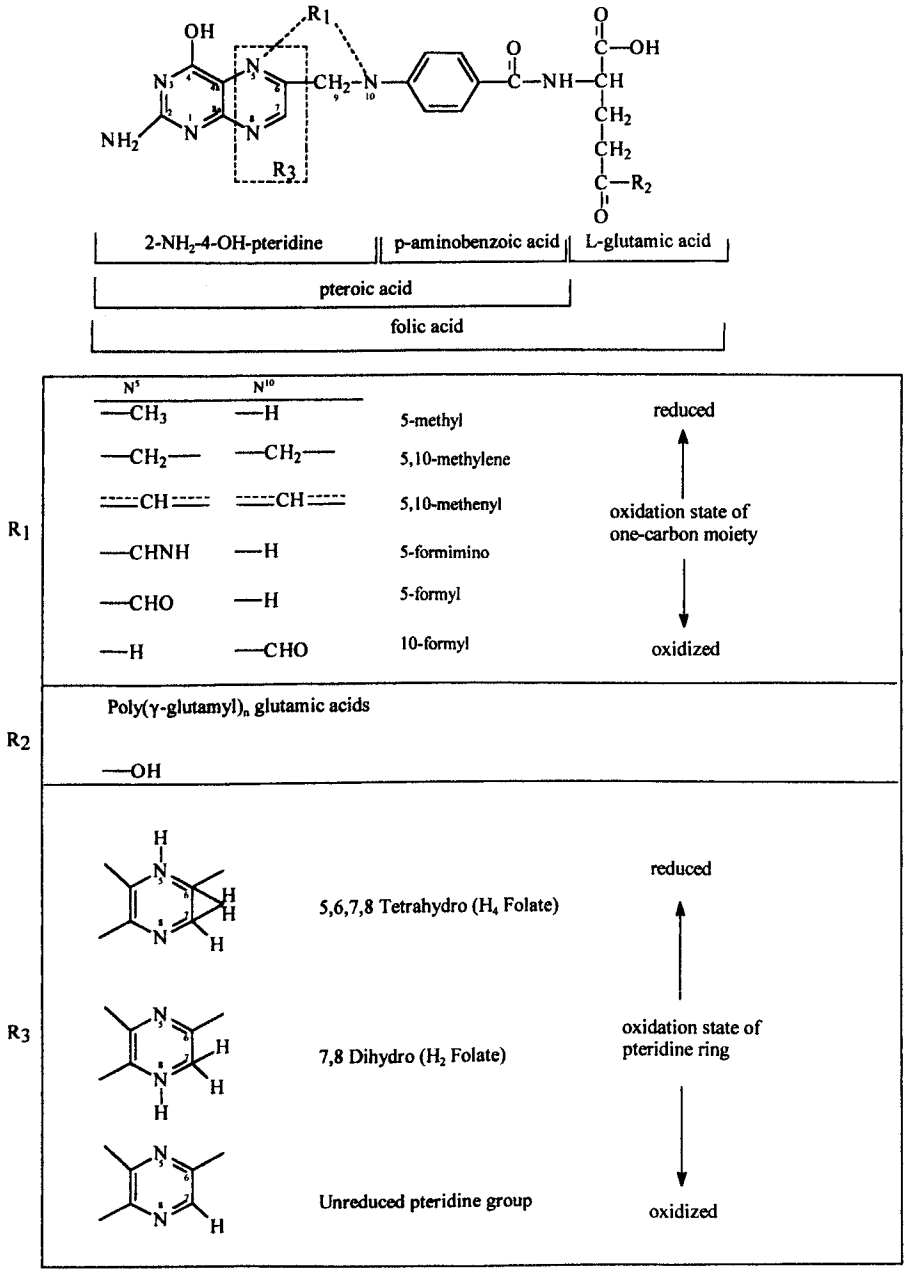

Figure 3. Folic acid structure and its derivatives. (Modified from Ref. 267).

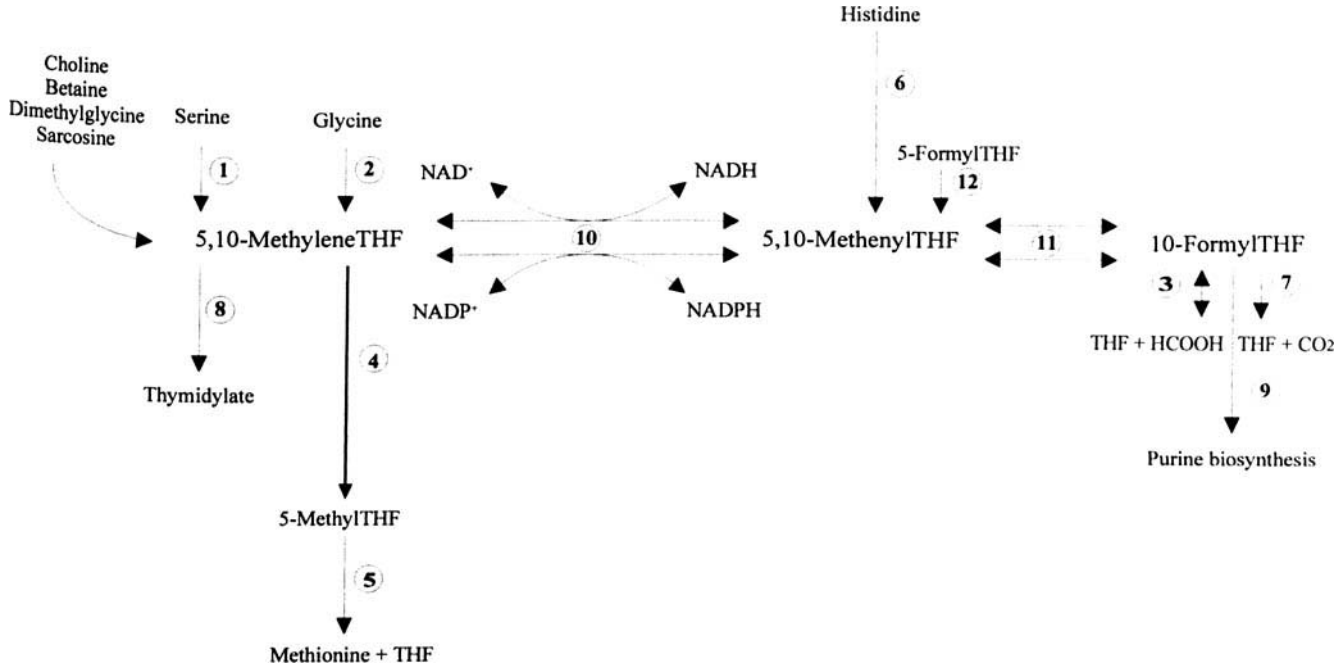

Figure 4. Simplified folate metabolism indicating its one-carbon donors and acceptors involved in methyl-group biogenesis, thymidylate synthesis and purine synthesis. 1, serine hydroxymethyltransferase; 2, glycine cleavage pathway; 3, 10-formyltetrahydrofolate synthase; 4, methylenetetrahydrofolate reductase; 5, methionine synthase; 6, glutamate formiminotransferase; 7, 10-formyltetrahydrofolate dehydrogenase; 8, thymidylate synthase; 9, 5-amino-4-imidazolecarboxamide ribonucleotide transformylase (AICAR) and glycineamide ribonucleotide transformylase (GAR); 10, methylenetetrahydrofolate dehydrogenase; 11, methenyltetrahydrofolate cyclohydrolase; 12, methenyltetrahydrofolate synthetase. THF, tetrahydrofolate.

Folate One-Carbon Metabolism. Vital cellular processes depend on folate-mediated one-carbon metabolism, i.e., the transfer of a carbon group. The major metabolic folate pathways are shown schematically in Figures 4 and 5. Folate acts as donor and acceptor of one-carbon units in a variety of critical enzymatic reactions involved in one-carbon metabolism. The one-carbon units are principally derived from the β-carbon of serine, but also from glycine, methyl- and dimethylglycine, formate, and histidine (106) (Fig. 4). One-carbon units are thus obtained from THF-mediated reactions, which are required for several major cellular processes like nucleic acid biosynthesis, protein biosynthesis, amino acid metabolism, methyl-group biogenesis, and vitamin metabolism (reviewed in Refs. 107 and 108).

One-carbon units derived from the third carbon of serine are transferred to THF in a reaction catalyzed by serine hydroxymethyltransferase (SHMT), generating 5,10-methylenetetrahydrofolate (5,10-MTHF) and glycine (Fig. 4, reaction 1). SHMT is the major provider of one-carbon units in the cell, particularly in replicating cells. Serine can enter the mitochondria, where it is converted to formate, which reenters the cytoplasm and acts as a primary carbon donor (109). The glycine-derived one-carbon units are generated from the second carbon of glycine by the glycine cleavage system, which results in the formation of 5,10-MTHF (Fig. 4, reaction 2). Formate-derived one-carbon units are formed by the ATP-dependent enzyme formyl synthetase, which activates formate to 10-formylTHF (Fig. 4, reaction 3). One-carbon units thus enter the active pool of one-carbon at the level of 5,10-MTHF and 10-formylTHF. 10-FormylTHF and 5,10-methenylTHF are substrates for purine biosynthesis, while 5,10-MTHF is involved in thymidine biosynthesis. Apart from this role in the biosynthesis of purines and thymidine and, thus, DNA and RNA, the folates provide a source of methyl-groups for over 100 methyltransferase-catalyzed reactions. This is done by converting 5,10-MTHF to 5-MeTHF, which is used to methylate Hcy to methionine by the vitamin B_{12}-dependent enzyme methionine synthase (MS) (Fig. 4, reactions 4 and 5). Methionine can be activated by ATP to AdoMet, the ultimate methyl-donor in the body.

An alternative one-carbon source is FiGlu (110). During the catabolism of histidine, a formimino-group is transferred to THF followed by the release of ammonia and by the generation of 5,10-methenylTHF by the two enzyme activities, glutamate formiminotransferase and formiminoTHF cyclodeaminase (Fig. 4, reaction 6 and Fig. 5). This pathway represents only a minor source of one-carbon and may exist only in liver and kidney. The enzymes seem to be absent in fibroblasts and blood cells. Excess one-carbon units are removed from the one-carbon pool by their oxidation to CO_2 by formylTHF dehydrogenase (Fig. 4, reaction 7). This enzyme also catalyzes the hydrolysis of 10-formylTHF to THF and formate. Activity of this bifunctional protein is restricted to the liver (111). The function of the hydrolase activity is yet unclear; it may represent an additional mechanism for regeneration of the unsubstituted THF under conditions in which utilization of substituted folate for biosynthetic purposes is impaired (107).

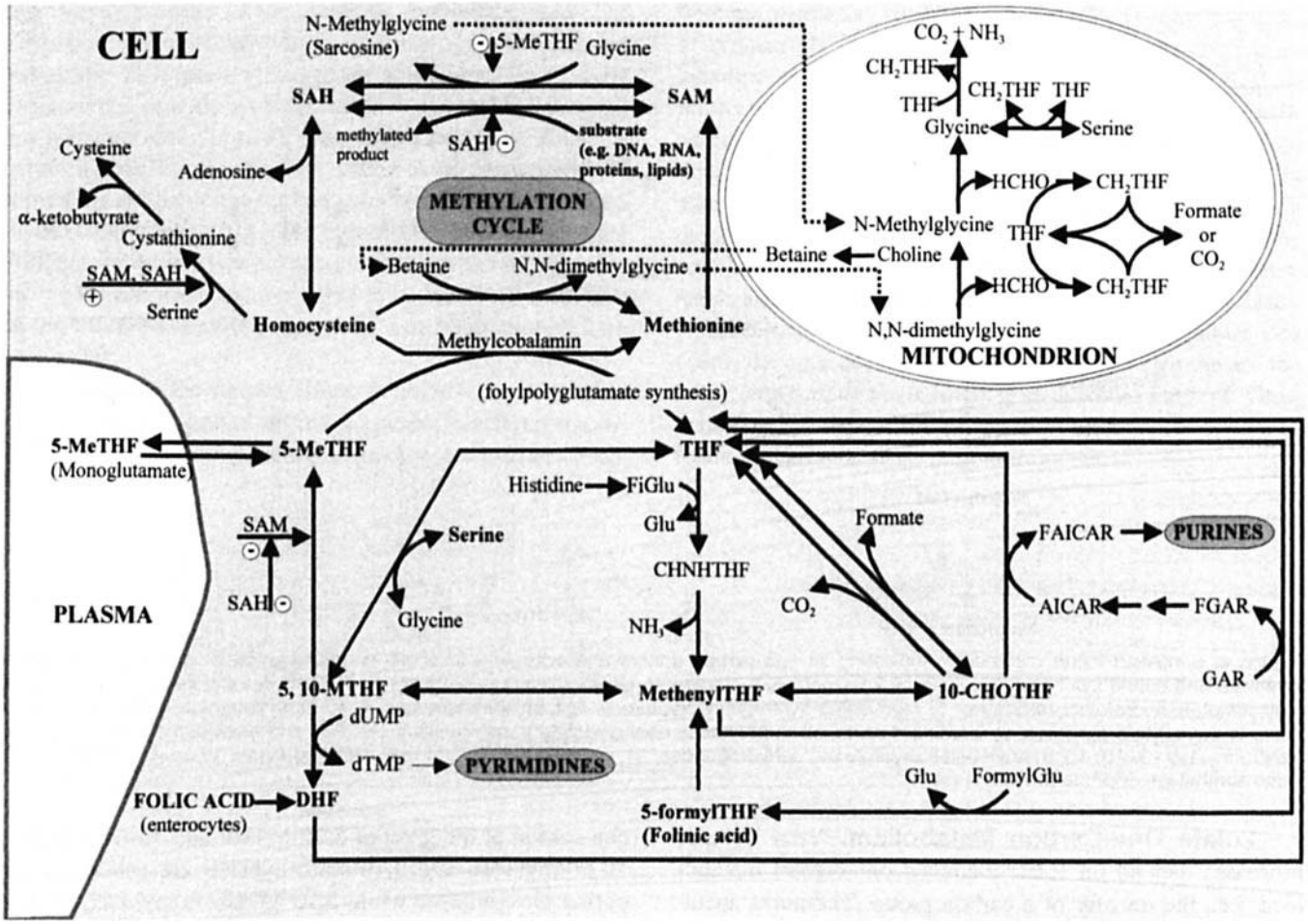

Figure 5. Extended folate metabolism, including compartmentation. (Modified with permission from Ref. 268). *MTHFR*, methylenetetrahydrofolate reductase; *SHMT*, serine hydroxymethyltransferase; *BHMT*, betaine homocysteine methyltransferase, *MAT*, methionine adenosyltransferase; *SAH-hydrolase*, S-adenosylhomocysteine hydrolase; *MT*, methyltransferase; *CBS*, cystathionine β-synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; and 5-MeTHF, 5-methyltetrahydrofolate.

Thymidine biosynthesis. Folate is required for the synthesis of thymidylate. This reaction is catalyzed by thymidylate synthase and involves the addition of formaldehyde group to the 5-position of deoxyuridylate. In this reaction the one-carbon group of 5,10-MTHF is transferred to deoxyuridine monophosphate (dUMP), resulting in the formation of deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF) (Fig. 4, reaction 8). Synthesis of deoxynucleotides, which is mediated by thymidylate synthase and ribonucleotide reductase, is considered to be the rate-limiting step in DNA synthesis. DHF formed in the thymidylate synthase reaction has to be reduced to THF before it can participate in one-carbon transfer reactions (Fig. 5). This reduction is catalyzed by dihydrofolate reductase (DHFR), an enzyme that also catalyzes the reduction of folic acid to DHF. Normally, folic acid is not found in nonsupplemented foods and the major role of DHFR appears to be to reduce DHF formed in the thymidylate synthase reaction.

The thymidylate synthase expression level is related to the replication state of a cell. A multi-enzyme complex called replitase is formed during the S-phase of the cell cycle. This replitase contains thymidylate synthase, DHFR, DNA polymerase, thymidine kinase, deoxycytidine monophosphate kinase, nucleoside diphosphate kinase, and ribonucleotide reductase (107).

Purine biosynthesis. One-carbon units at the oxidation level of formate are utilized in the *de novo* purine biosynthesis. The C_8 and C_2 positions in the purine ring are derived from the folate one-carbon pool from 5,10-methenylTHF in a reaction catalyzed by glycinamide ribonucleotide (GAR) transformylase to FGAR and from 10-formyl-THF by formylTHF 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase to FAICAR, respectively (Fig. 4, reaction 9 and Fig. 5). The 10-formyl needed for purine biosynthesis can be either directly derived from the 10-formylTHF synthetase-catalyzed reaction (Fig. 4, reaction 3), or can be derived from the oxidation of 5,10-MTHF to 10-formylTHF catalyzed by the NADP-dependent MTHF dehydrogenase and methenylTHF cyclohydrolase (112) (Fig. 4, reactions 10 and 11). Any 5-formylTHF (leucovorin) is converted to 5,10-methenylTHF by methenylTHF synthetase (Fig. 4, reaction 12). In mammalian tissues the dehydrogenase, cyclohydrolase, and synthetase activi-

ties are catalyzed by a single trifunctional protein, methylenetetrahydrofolate dehydrogenase (MTHFD) (113).

Methyl group synthesis. An important part of one-carbon utilization involves the reduction of 5,10-MTHF to 5-MeTHF by the enzyme methylenetetrahydrofolate reductase (MTHFR) (Fig. 4, reaction 4). This reaction is physiologically irreversible. The methyl group of 5-MeTHF is donated to Hcy resulting in methionine synthesis, which is catalyzed by MS (Fig. 4, reaction 5 and Fig. 5). This is the sole enzyme that is able to metabolize the methyl-group of 5-MeTHF. MS plays a major role in methyl-group metabolism, as it allows the reutilization of the Hcy backbone as a carrier of methyl-groups. This reaction channels the one-carbon units derived from formate and amino acids such as serine, histidine, and glycine into the methylation cycle, providing a methyl-group for the synthesis of AdoMet via methionine, and sequentially a methyl-group that is used by many essential methyltransferase enzymes.

Folate Absorption and Transport. Dietary folate polyglutamates need to be converted to monoglutamates before they can be absorbed (114, 115). In the jejunum, polyglutamates are converted to monoglutamates by a conjugase. Two distinct forms of human pteroylpolyglutamate hydrolase conjugase have been described, one in the intestine, which is present in the brush border and acts at a neutral pH, and one with a less clear function within lysosomes. At this point, luminal pH is critical (116) and absorption is optimal at a pH of 6.0, which significantly decreases at a pH of 5.0 or 7.0. During absorption in the jejunum, the different monoglutamyl folates are converted to 5-MeTHF; however, it is currently unclear whether the conversion takes place primarily in the intestine or the liver, or in both organs (116). 5-MeTHF monoglutamate is the principal circulating form of folate and is transported across the plasma membranes of cells, and thus the principal form by which the tissues are supplied with folate (117). Transcellular folate transport systems include transport across the placenta, renal tubular cells, and the blood brain barrier.

Three major pathways have been observed that mediate folate transport across mammalian cell membranes (118). Inward transport of folate and their analogues cells can occur by carrier-mediated (119), as well as receptor-mediated (120), mechanisms or by passive diffusion. These different folate transport systems may not be operational in all cell types. The pathway for entry of folate is likely to be distinct in different cells, depending on the relative efficiency of carrier-mediated and receptor-mediated mechanisms, as well as on the intra- and extracellular folate concentration.

Carrier-mediated folate uptake. Pteroylmonoglutamates can be transported by a carrier-mediated energy-dependent process. The carrier-mediated mechanism, or the reduced folate carrier (RFC), is an integral membrane protein that is primarily responsible for permeation of these compounds in tumor cells (119). This system has a markedly higher affinity for 5-MeTHF than for PteGlu and also accumulates methotrexate and other anti-folates. The RFC mainly mediates bidirectional fluxes of reduced folates and antifolates (121). Transmembrane α-helices of the RFC are believed to form channels through which substrates pass (122). The RFC operates at relatively high folate concentrations and has been referred to as the "reduced folate methotrexate," the "high capacity/low affinity," or the "micromolar" folate transport system. The RFC is driven by anion gradients, and anionic exchange may be the mechanism for the transport process (123).

Receptor-mediated folate uptake. The second system, the receptor-mediated folate transport, occurs via different isoforms of folate receptors (FRs) (118, 124–126). These FRs bind physiological levels of folate and have a high affinity for folate in the nanomolar range. Although these isoforms vary in their affinities for folate forms, in general they have a higher affinity for folic acid than for reduced folates like 5-MeTHF or 5-formylTHF. The FR was originally described as a folate-binding protein found in tissues and plasma, but it has been shown to be mainly a membrane protein anchored by glycosyl-phosphatidylinositol linkage. FRs are crucial to the assimilation, distribution, and retention of food folates and have been identified in various cells, extracellular fluids, and tissues in mammalians (127). FR expression is inversely regulated by the extracellular folate concentration. The receptor-mediated transport of 5-MeTHF appears to occur via typical endocytotic pathway involving endocytic vacuoles (128) or via a similar pathway involving caveolae, where the receptor recycles within the caveolae without dissociating from the plasma membrane (129). Caveolae are capable of transiently closing to form a membrane-bound compartment that protects FRs both from acid treatment and from antifolate receptor IpG. The FRs probably function to concentrate 5-MeTHF at the cell surface and deliver the vitamin to a vesicle (124). They are highly clustered on the cell surface of folate-dependent tissue cells.

The FRs can be separated into soluble and membrane-bound FR forms. These forms are structurally related, but differ in function (123, 127, 130). The soluble FRs likely serve multiple functions; they afford a convenient mechanism for concentrating folate compounds, they protect the bound, reduced folates from oxidation, and finally, soluble FRs could act as a storage protein to conserve folates. However, the precise function of the soluble FR is yet unknown (130). The membrane FR primarily accumulates folate at the cell surface and mediates the transport of folate compounds into cells.

FRs are encoded by a family of genes on chromosome 11q13.3 through 11q13.5, where four FR genes and a pseudogene were found within a 140-kb region (131). The human isoforms of the FR are known as FRα, FRβ, FRγ, and FR′γ which have a tissue-specific expression (132, 133). It is believed that the FRγ is a secretory FR, the soluble form of the FRs (134). The FR′γ is a truncated form of FRγ probably formed by alternative splicing (134) or results from gene polymorphism (133). Thus, there are several FR

isoforms with different relative affinities for folate and expression patterns, but their precise functions are still unclear

Maternal-to-fetal folate transport. Pregnancy is the most common cause of megaloblastic anemia in women worldwide. Folate requirements increase 5- to 10-fold compared with nonpregnant women for growth of the fetus, placenta, and maternal tissues (130). The FRs play a major role in transplacental folate transport, and the placenta is known to contain both FRα and FRβ (134, 135). The maternal-to-fetal folate transfer process consists of two steps. The first step is the concentrative component: circulating 5-MeTHF is bound to placental FR on the maternally facing chorionic surface. In the second step the folates are transferred to the fetal circulation along a downhill concentration gradient. This is a continuous process assuring unidirectional transplacental folate transport (130). However, one must keep in mind that during the process of neurulation the placenta is not yet developed. The exact mechanism of folate transport to the fetus previous to placental transport is unknown, but is thought to involve a similar mechanism.

Folate Polyglutamates and Polyglutamation. *Folate polyglutamates structure and function.* Cellular folates exist primarily as poly-γ-glutamate derivatives and should be considered as the normal substrates for the enzymes of one-carbon metabolism. Polyglutamate derivatives consist of a THF derivative, with from three to 11 glutamate residues linked as amides through the γ-carboxyl group. To describe accurately the number of glutamate residues, the nomenclature system is related to tetrahydropteroate (H_4Pte), which has no glutamate residues. Thus, THF is called H_4PteGlu, and the pentaglutamate form is $H_4PteGlu_5$. In addition to a varying number of glutamate residues, there are six different one-carbon derivatives of $H_4PteGlu_n$. Two of these derivatives, 5-formino- and 5,10-methenyl$H_4PteGlu_n$, exist in low concentration and probably do not contribute significantly to the total intracellular pool of folate. The major forms of reduced folate in the cell are 10-formyl-, 5-formyl-, 5-methyl-, and 5,10-methylene$PteGlu_n$ derivatives and $H_4PteGlu_n$ (136).

It was thought that polyglutamate derivatives were only storage forms of the vitamin. Now it is apparent that intracellular conversion of folates to polyglutamates is important for the normal function and regulation of the one-carbon metabolism. Folylpolyglutamates are as effective as, and in many cases even more effective than, pteroylmonoglutamates as substrates for the enzymes of one-carbon metabolism (107). Extension of the glutamate chain decreases K_m values, but also causes a decrease in V_{max} with chain lengths beyond the diglutamate level (137). Therefore, with most folates the diglutamate derivative is a more effective substrate than the monoglutamate, owing primarily to a decrease in K_m value. Polyglutamate folates of a chain length equal or greater than three have much lower K_m values for some of the folate-dependent reactions, allowing folate metabolism to progress at the low concentration of the folates present in the cells.

Polyglutamates are only poorly transported across cell membranes. Consequently, metabolism of pteroylmonoglutamates to polyglutamate forms allows the cell to concentrate folates at much higher levels than in the external medium. In tissues, a particular length usually predominates, but a distribution of different lengths is observed. The major polyglutamate species in human cells are hepta- and octaglutamates (137).

Folate polyglutamation by folylpolyglutamate synthetase. Intracellular metabolism of folates to polyglutamates is performed by the enzyme folylpolyglutamate synthetase (FPGS) (124, 138, 139). This enzyme is present in most mammalian tissues and adds glutamate residues one at a time to folate molecules (107). FPGS forms a peptide bond between the gamma-carboxyl of the glutamate already present on the molecule and the alpha-amino group of the glutamate to be added and requires ATP for this reaction (see below).

$$H_4PteGlu_n + MgATP + l\text{–glutamate} \rightarrow H_4PteGlu_{n+1} + MgADP + P_i$$

This gamma-glutamyl chain is resistant to digestion by common proteolytic enzymes and is hydrolyzed by specific conjugase enzymes. The chain length is influenced by the monoglutamate concentration in the cell, as well as the FPGS activity (140). The human FPGS is a monomeric low-abundance protein that utilizes THF and other folates as substrates with different affinities, but reacts poorly with folic acid and 5-MeTHF. Unsubstituted reduced folates are the preferred substrate for the mammalian FPGS. H_2PteGlu (DHF) is the most effective monoglutamate substrate, followed by H_4PteGlu (THF), while $H_4PteGlu_n$ derivatives are the most effective polyglutamate substrates (137).

Mammalian tissues contain cytosolic and mitochondrial FPGS isoenzymes (141), which are encoded by a single gene that is localized to chromosome 9q34.1 region. These isoenzymes are formed by alternative splicing (142, 143). Mitochondrial FPGS activity is required for folate accumulation by mitochondria. Specific activity of the mitochondrial enzyme is higher than the cytosolic enzyme, which may explain the longer folylpolyglutamate derivatives in this organelle. Monoglutamates are being transported into mitochondria before they are polyglutamated by cytosolic FPGS. There is an indication for competition for folate between folate transport into mitochondria and cytosolic FPGS activity. It has been suggested that oxidized monoglutamates are mitochondrial transport forms of folate and that reduced folates are not transported (144, 145). However, others suggested that reduced folates are also transported into mitochondria (146).

Regulation of the Folate One-Carbon Metabolism. Folate one-carbon metabolism is complex (Fig. 5). Regulation of the one-carbon flux through acceptors and donors is regulated by compartmentation of the substrates,

enzymes, and products. There are three types of intracellular compartmentation. One type is compartmentation within intracellular organelles. A second mechanism involves distribution of metabolites between free and protein-bound state. The third mechanism involves channeling of substrates through sequential enzyme reactions (108). Each of these serves to segregate the intermediates into distinct pools. At its simplest, folate exists as two pools: one small pool with rapid turnover that is composed mainly of the monoglutamyl forms of folate in plasma, and a larger slow-turnover pool of folates functionally trapped in tissues as polyglutamyl forms, many of which are noncovalently bound to proteins.

In addition, the one-carbon metabolism may be regulated by polyglutamation state by varying the glutamate chain length of folates in the cell under different physiological conditions such as infection, hormonal status, and nutritional conditions. Modulations of the glutamate chain length of folates have been observed in mammalian cells and tissues in response to folate, methionine, and vitamin B_{12} levels and to changes in growth rates (147). The lengths of the products are inversely dependent on the initial monoglutamate concentration. High H_4PteGlu concentrations lead to synthesis of shorter polyglutamates (148). In terms of metabolic efficiency, this is understandable because monoglutamates and short polyglutamates can be used in all mammalian folate reactions, although generally less efficiently than longer polyglutamates. Thus, under conditions of folate excess, formation of long polyglutamates would be wasteful of both ATP and glutamate. In folate-depleted conditions, not only does polyglutamate formation increase retention of folates once transported, but also each folate molecule is a more efficient carrier of one-carbon units (147).

Conversions of folates to polyglutamates are required for their cellular retention (148). Factors that affect the expression of FPGS will thereby also regulate the level of folate in the cell. Folylpolyglutamates with longer chain length retain a higher affinity for the enzyme, but have little or no activity. This suggests that they can act as end-product inhibitors of the enzyme and lead to a steady-state folate concentration in the cell. The changes would be expected to occur only at a slow rate, which would limit their physiological usefulness. According to a second hypothesis, many of the changes in the activity of the FPGS could also be explained by substrate availability for FPGS instead of regulation of the activity by polyglutamation. It has been proposed (149) that the regulation of folate metabolism by means of polyglutamate chain length is secondary to the classical alterations in folate metabolism characterized by the activation and inhibition of folylpolyglutamates of a number of folate-dependent enzymes involved in one-carbon metabolism.

Compartmentation within intracellular organelles. Intracellular compartmentalization of the folate pathway concerns the cytosol and mitochondria. Nuclei contain only very low levels of folates (108). It has been demonstrated that mammalian cells contain cytosolic and mitochondrial one-carbon donors and acceptors, generating one-carbon pools in both the cytoplasm and the mitochondria (Fig. 5). The folate-dependent enzymes SHMT, MTHFD, and FPGS are present in both the cytoplasm and the mitochondria. Three additional folate-dependent enzymes, the glycine cleavage system, dimethylglycine dehydrogenase, and sarcosine dehydrogenase are strictly mitochondrial (Fig. 5). This distribution of folates and folate-dependent enzymes in these compartments is consistent with the known roles of folate-mediated one-carbon metabolism in each (Fig. 5). In the cytoplasm, folate coenzymes participate in the biogenesis of purines and thymidylate and in methyl-group biogenesis. The interconversion of serine and glycine occurs in both compartments. In mitochondria, folate coenzymes are required for the formation of transfer RNA for organellar protein synthesis and catabolism of glycine, sarcosine, and dimethylglycine.

Compartmentation of metabolites between free and protein-bound state. In one-carbon metabolism both monoglutamates in the plasma and polyglutamates in the cells are distributed between free and protein-bound state. In the serum, two-thirds of the 5-MeTHF is loosely bound to a nonspecific folate binder with a low affinity, i.e., albumine. The folate-binding proteins are, importantly, involved in compartmentalization of folates by their uptake of free folates. Three folate-binding proteins have been found in the cytosolic fraction and two in the mitochondrial fraction. Four out of these five proteins have been identified as enzymes involved in one-carbon metabolism (108). One of these is the glycine N-methyltransferase, which is one of the major cytosolic folate-binding proteins in liver. This enzyme is inhibited by the binding of 5-MeTHF (150). Thus, binding of folate intermediates to one of these folate-binding proteins can inhibit or stimulate their activity or the activity of other enzymes. These folate-binding proteins bind about 60% of the total cytosolic folates and about 20% of the total mitochondrial folates in liver.

Compartmentation by channeling of folate substrates. Many enzymes of one-carbon metabolism form protein complexes that enhance channeling of substrates through sequential enzyme reactions (107, 108). Channeling of folate was demonstrated for the first time in the three-functional MTHFD protein. Substrate channeling through coupled-enzyme reactions provides a dynamic microcompartmentation in which reaction intermediates are functionally isolated from those in the bulk phase of the compartment. Substrate channeling offers a number of potential kinetic and regulatory advantages. High metabolic rates can be obtained at low substrate concentrations since reaction rates are independent of bulk phase substrates (108). Reaction intermediates are isolated from competing reactions and unstable intermediates may be protected. Folylpolyglutamate substrates are channeled from active site to active site in multifunctional proteins or protein complexes without release of the intermediate product, a phenomena that is

not observed with monoglutamate substrates. Substrate channeling of folate coenzymes in several folate-dependent reactions is greatly facilitated by the longer glutamate-chain length. Channeling by MTHFD was more efficient with the triglutamate substrate than with the monoglutamate. Thus, polyglutamates may play a role in maintaining specific protein-protein interactions (151).

Proposed Protective Mechanism of Folate on Preventing NTD. Folate intake is an important environmental factor in the etiology of NTD. However, it is not known how the administered folate works. It could be overcoming a folate deficiency or a metabolic disturbance in the folate metabolism. A folate deficiency can occur due to an inadequate dietary intake, malabsorption, altered hepatic and peripheral metabolism, or an increased elimination of folate (152). The preventive effects of additional folate intake could be working at the level of the mother, at the level of the embryo, or even at both.

In an Irish study, an inverse relationship between red blood cell folate levels and the risk of NTD offspring was found, and the lower the red blood cell folate levels the higher the risk (153). Estimates have been made on the effect of additional folic acid intake on the red blood cell folate levels (154). This study gives a model for estimating the risk of having an NTD-affected pregnancy on the basis of maternal red blood cell folate levels. It was estimated that women with red blood cell folate levels above 1292 nmol/L have a 48% reduction in the risk of having a NTD pregnancy. These folate levels can be achieved by an additional intake of 0.4 mg/d.

Studies on the folate status of women who have had NTD offspring are not conclusive. Some of these women have low folate levels (78). More recent studies report nondeficient folate levels in women with NTD-affected pregnancies, although some were in the lower normal range (155–158). Overall, there is a trend for such women having slightly lower folate levels than control women. These lowered folate levels could be due to a lower intake of folate via the diet. However, Yates et al. (159) demonstrated an association between NTD births and low levels of maternal red cell folate, which could not be attributed entirely to lower dietary intake of folate. Schorah et al. (160) hypothesized that the preventive effect of folate could be due to an increased folate requirement of women genetically predisposed to have an infant affected with a NTD. A related hypothesis is that the absorption of folic acid is reduced in women with a history of NTD-affected offspring (99). These women may be inefficient in converting dietary folate into blood folate stores and thus may secondarily have an increased requirement for folic acid (161). Several other studies, however, showed that there is no evidence for a defect in the absorption of folate in women with NTD-affected offspring (162, 163). Finally, it is suggested that folic acid may overcome a metabolic disturbance of folate-related metabolic pathways (164). They showed that the methyl group of 5-MeTHF is more slowly incorporated into trophoblast DNA of NTD-affected fetuses than from controls. Lucock et al. (165) showed that women with two NTD-affected pregnancies needed a higher dietary folate intake to achieve the same 5-MeTHF concentrations as controls. This indicates a disturbance in the uptake or utilization of 5-MeTHF and therefore in folate metabolism. Also an impaired regeneration of THF is observed in mothers of a child with spina bifida (166).

One of the functions of folate is to act as a substrate for some of the enzymes involved in the *de novo* DNA synthesis, a process that is essential to the developing embryo. Via Hcy to methionine remethylation folate is also involved in methylation reactions. Therefore, Hcy levels could function as a marker of an impaired folate metabolism. The developmental genes are transcription factors that are in part regulated by means of methylation. A defective folate metabolism or folate shortage in the mother may also lead to a shortage of folate in the developing embryo. This could result in a defective DNA synthesis or an impaired transcription of genes involved in the neurulation process. Additional folate intake may overcome an impaired DNA synthesis or DNA methylation, or impaired methylation of other molecules such as receptors, etc.

Homocysteine Metabolism

Folate-Related Homocysteine Metabolism. A brief overview of the Hcy metabolism (Fig. 6) will be given below. Hcy does not occur in the diet, but is an amino acid that is formed by the demethylation of methionine. Dietary methionine is necessary for the normal growth and development of mammals because methionine is an essential amino acid. Two reactions compete for the available amino acid: protein synthesis and the synthesis of AdoMet by means of methionine adenosyltransferase (MAT).

In the MAT-catalyzed reaction, methionine is linked to ATP, resulting in the formation of AdoMet. In this reaction a sulfonium bond is formed between the 5′-carbon atom of the ribose and the sulfur atom of the amino acid. This reaction occurs in most, if not all, tissues and there are two different isoenzyme forms of MAT: a liver-specific form, and extrahepatic tissues contain another form (167–169). The liver-specific form has a relative high K_m for methionine. The extrahepatic MAT has a low K_m for methionine. The liver MAT is activated by its product AdoMet and this, together with its high K_m for methionine, makes the liver capable of responding to excessive methionine (167).

Because of its sulfonium bond, AdoMet can be regarded as a high-energy compound. AdoMet is the universal substrate for methyltransferase reactions. Over 100 different methyltransferase reactions transfer the methyl group of AdoMet to a wide range of substrates, including proteins, RNA, DNA, and lipids (170). The methylation of DNA is an important regulatory mechanism in gene expression. AdoMet is, furthermore, a precursor of the polyamines spermidine and spermine (171). The transmethylation reactions produce S-adenosylhomocysteine (AdoHcy). AdoHcy is an

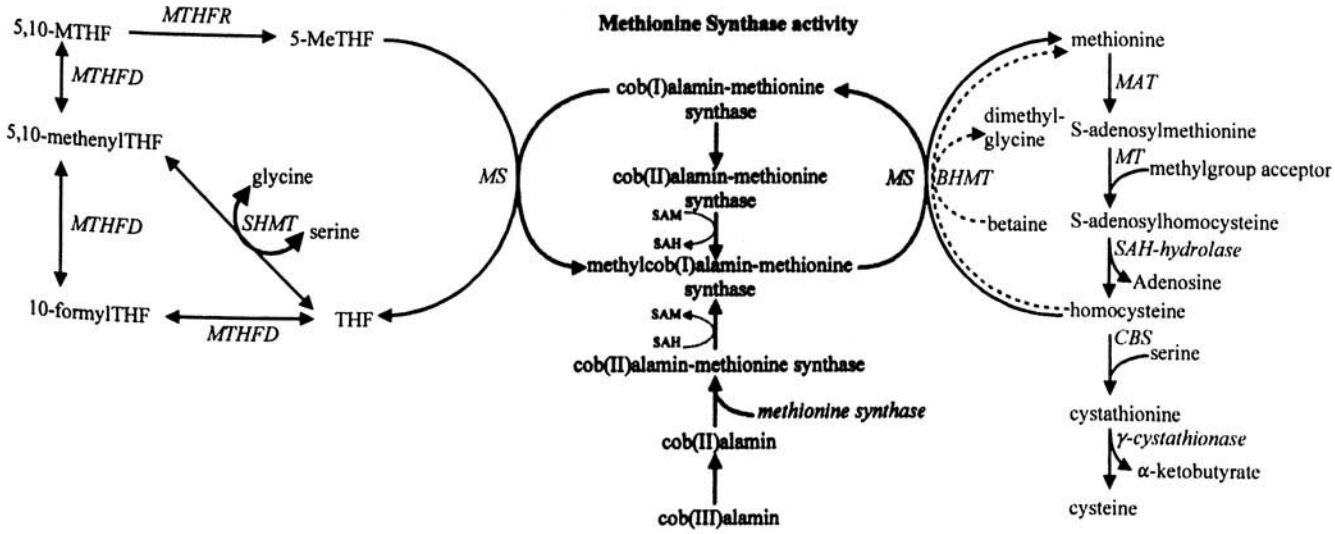

Figure 6. Key enzymes of homocysteine metabolism. Enzymes are presented in italics. *MTHFR*, methylenetetrahydrofolate reductase; *SHMT*, serine hydroxymethyltransferase; *BHMT*, betaine homocysteine methyltransferase; *MAT*, methionine adenosyltransferase; *SAH-hydrolase*, S-adenosylhomocysteine hydrolase; *MT*, methyltransferase; *CBS*, cystathionine β-synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; and 5-MeTHF, 5-methyltetrahydrofolate.

inhibitor of many methyltransferases; therefore, normal metabolism requires quick removal of AdoHcy, which is accomplished by S-adenosylhomocysteine hydrolase. SAH hydrolase, which is widely distributed in mammalian tissues, cleaves the thioether to Hcy and adenosine (172). The equilibrium of this reaction favors AdoHcy synthesis, and the rapid removal of the products Hcy, and adenosine ensures a quick AdoHcy conversion *in vivo*, which is essential to maintain flow through the Hcy/methionine cycle (172). The formed AdoHcy can also be removed by intracellular binding to proteins, or when the capacity of these proteins is exceeded, AdoHcy may partly be transported out of the cell.

Hcy lies at an important metabolic branch point. It can be catabolized to cystathionine in the transsulphuration pathway or remethylated to form methionine (Figs. 5 and 6). At least two alternative mechanisms exist in humans for Hcy remethylation. The methyl-group originates from 5-MeTHF or betaine. Hcy participates, therefore, in three essential reactions or sequences: (i) the transsulphuration pathway that leads via cystathionine to the formation of cysteine, which is used for protein synthesis and formation of glutathionine; (ii) the cycling of intracellular folates; and (iii) the catabolism of choline and betaine.

Transsulphuration pathway. The transsulphuration pathway converts the sulfur atom, originally derived from methionine to cysteine. This pathway is the main route of disposal of methionine and explains why cysteine is not an essential amino acid in humans. In this pathway Hcy condensates with serine to form the thioether cystathionine by the vitamin B_6 (pyridoxal phosphate)-requiring enzyme cystathionine β-synthase (CBS). The thioether cystathionine is cleaved by another vitamin B_6-dependent enzyme, γ-cystathionase, forming cysteine and α-oxobutyrate. Oxidation of the sulfur atom of cysteine into sulfate occurs through a number of enzymatic reactions. About 70% of the sulfur of methionine in the diet is excreted as inorganic sulfate, illustrating the major quantitative importance of this pathway in man (173).

Cycling of intracellular folates. The folate one-carbon metabolism is, as pointed out earlier, connected to the Hcy cycle by the synthesis of 5-MeTHF by MTHFR and the use of 5-MeTHF as methyl donor for Hcy remethylation. 5-MeTHF donates its methyl-group via catalysis by the cobalamin- (Cbl) containing MS to Hcy, whereby methionine and THF are formed (174). MS converts the circulating form of folate, 5-MeTHF, to THF, which can then support a variety of cellular reactions. MS is the only enzyme that is able to demethylate 5-MeTHF and thus occupies a strategic position in the control of folate metabolism. MS provides methyl-groups for the methylation cycle; in doing so, it recycles 5-MeTHF back to THF. After uptake from blood, demethylation of 5-MeTHF via MS is needed before it can be conjugated into a polyglutamate and retained by the cell. Intracellular remethylation of THF can occur by donation of one-carbon group from serine to form 5,10-MTHF, which can subsequently be reduced to 5-MeTHF by MTHFR (Fig. 6). Thus, cycling of folates is dependent on the MS and the MTHFR enzyme.

Catabolism of choline and betaine. Independent of folate and vitamin B_{12}, Hcy can also be methylated by betaine-homocysteine methyltransferase (BHMT). This enzyme is present in the liver and possibly in the kidney (175). Betaine (trimethylglycine) is converted to dimethylglycine by this enzyme, whereby Hcy is remethylated. This reaction is essentially irreversible. Betaine is derived from the catabolism of choline. In the mitochondria, the product of the BHMT reaction, dimethylglycine, can be further demethylated by dimethylglycine dehydrogenase to sarcosine, and

via sarcosine dehydrogenase to glycine (Fig. 5). The β-carbon of glycine can come available for the one-carbon metabolism by the glycine cleavage pathway. In these subsequent reactions one-carbon units are used for the conversion of THF to 5,10-MTHF (176). Theoretically, one betaine molecule can provide four one-carbon units for Hcy remethylation. The relative contribution of the overall betaine-dependent Hcy methylation is unclear (168). No mammalian mutants defective in betaine-dependent methylation of Hcy have yet been described.

Control of the Homocysteine Metabolism. The regulation of the Hcy metabolism is based on the disposition of substrate between competing reactions at three metabolic sites: the competition for methionine between protein synthesis and the synthesis of AdoMet; the use of AdoMet for the synthesis of spermine and spermidine or methyldonation, resulting in AdoHcy formation; and the disposition of Hcy between transsulphuration and transmethylation. Finkelstein and Martin (177) have demonstrated that the flux through the transmethylation and transsulphuration pathways is almost equal.

It is known that the levels of enzymes involved in the Hcy metabolism are influenced by protein or methionine content of the diet, hormones, and age. Regulation of Hcy metabolism includes up- and down-regulation of the relevant enzymes for remethylation versus transsulphuration (178). Methionine itself inhibits both MS and BHMT. AdoMet inactivates BHMT and extrahepatic MAT, and activates CBS and the liver isoenzyme of MAT. Furthermore, AdoMet inhibits MTHFR, thereby restricting the formation of 5-MeTHF and lowering the flux through MS. AdoMet thus plays a central role in the salvage or catabolism of methionine and its toxic product Hcy. Increased levels of AdoMet due to high methionine levels favors the Hcy degradation via the transsulphuration pathway, which takes place mainly in the liver. Extrahepatic cells most likely only have the remethylation pathway functional active (179). Liver is the only tissue that can respond to excess methionine since it is the only organ that contains CBS and a form of MAT that can respond to high levels of methionine, which underlines the importance of liver in the regulation of the Hcy metabolism.

Young's group obtains further evidence for the importance of the liver in the regulation of the Hcy metabolism by the use of stable isotopes (180, 181). They observed that dietary cystine can, due to a reduction in the rate of methionine transsulfuration, lower methionine requirements by about 50%. Dietary cystine did not profoundly affect the metabolism of methionine within the peripheral compartment. They hypothesized that a sparing effect of dietary cystine on methionine utilization occurs during the passage of these amino acids in the splanchnic region, especially the liver.

Many AdoMet-dependent methyltransferases are strongly inhibited by AdoHcy; an AdoHcy:AdoMet ratio of 1:4 decreases the activity of a variety of methyltransferases by 10% to 60% (182). Thus, the methylation cycle functions maximally only when the AdoMet-to-AdoHcy ratio is sufficiently high. Excess methionine is degraded via AdoMet, AdoHcy, and Hcy to cysteine. Excessive methylation of DNA is not desirable. In the liver, glycine N-methyltransferase is very abundant and uses AdoMet to methylate glycine to sarcosine. Glycine is a readily available methyl acceptor, and the produced sarcosine is a nontoxic product from which the substrate, glycine, can be regenerated. This enzyme converts excess AdoMet to AdoHcy to maintain an adequate AdoMet-to-AdoHcy ratio. Furthermore, the combined action of the glycine N-methyltransferase and sarcosine dehydrogenase reactions provides a route of AdoMet methyl-groups to reenter the folate one-carbon pool. The inhibition of MTHFR by high AdoMet concentrations and the inhibitory effect of its product 5-MeTHF on glycine N-methyltransferase provides control over the ratio of AdoMet and AdoHcy and balances the methylation needs of the cell with the availability of folate-mediated one-carbon units. This serves, physiologically, as a mechanism for linking the *de novo* synthesis of methyl-groups via the one-carbon folate pool to the availability of methionine in the diet (150).

Very recently, Taoka and colleagues (183) have suggested an additional regulating mechanism. The redox state might regulate the flux through the transsulphuration and transmethylation pathways. Both MS and CBS operating at the junction of Hcy have redox-active cofactors, Cbl in the former and heme in the latter. MS is redox sensitive since the intermediate Cob(I)alamin is labile to oxidation, which leads to MS inactivity. The activity of MS *in vitro* is enhanced by a lower redox potential.

Taoka et al. (183) suggest that the *in vitro* CBS activity is modulated by the redox state of the heme because the heme-group in the ferrous state could be less favorable than the oxidized ferric state for the binding of Hcy, or the reduction of the heme-group could cause less favorable changes in the conformation of the protein. Thus, both MS and CBS activity could be redox-regulated under *in vitro* conditions. A low redox stimulates the Hcy remethylation via MS activity and decreases CBS activity, whereas a high redox would result in increased Hcy degradation that would favor transsulphuration to cysteine, which is required for glutathione synthesis under conditions of oxidative stress and remethylation under more reducing conditions.

Disturbed Homocysteine and Folate Metabolism and NTD in Humans

Candidate Enzymes in the Etiology of Folate-Preventable NTD. There is little doubt that genetic factors are involved in the etiology of NTD. Steegers-Theunissen et al. (156, 184) showed increased fasting, as well as post-methionine load plasma Hcy concentrations, in women with NTD-affected offspring in comparison with women without a history of obstetric complications. The vitamin status of these women was not significantly differ-

ent from that of the control women, indicating a metabolic disturbance of the Hcy conversion; however, the number of patients and controls studied was relatively small and the women were not sampled in the index programming, which limits this conclusion. Steegers-Theunissen et al. (156) suggested that an increased level of Hcy may be harmful to the development of NTD. Additional studies confirmed the observation of elevated Hcy levels in women with NTD offspring (157, 158, 185, 186). A disturbance in the metabolism of Hcy may be overcome by an additional folate intake.

Folate-preventable NTD may be based on a folate-related genetic metabolic abnormality. These genetic aberrations could exert their influences through defects of storage, transport, or metabolism of folate.

Transport and Uptake of Folates and NTD. Folates and folic acid enter the cells by means of a receptor-mediated process. The FRs therefore play a critical role in intracellular folate internalization. Uptake of folate to the cells could be impaired due to defects in the RFC or the FRs. Mice lacking a functional FBP-1 failed to close their neural tube (187). Thus, a defective folate transport could be involved in the human etiology of NTD.

The genes FRα and FRβ code for the receptors that are involved in the cellular uptake of folate in humans. We observed no genetic aberrations in these genes of NTD patients or their mother's (188). The FRα was also examined in a large population of NTD patients in California; this study also could not detect any polymorphisms in this gene (189). There is no indication for a major involvement of a disturbed cellular folate uptake in relationship to NTD.

Folate-Related Homocysteine Metabolism and NTD. Several candidate enzymes could be involved in the etiology of NTD and, if deficient, can explain the observed protective effect by folate. Defects in the enzymes involved in the remethylation (MTHFR and MS) and the transsulphuration (CBS) of Hcy could result in the observed elevated Hcy levels in mothers with NTD-affected pregnancies. It is noteworthy that some of them also rely on other vitamins than folate, like vitamin B_6 and B_{12}.

Very recently, it was reported that MS activity is dependent on two additional components for its activity, the soluble cytochrome b5 (190, 191) and a new identified enzyme methionine synthase reductase (MTRR) (192). Defects in either of these enzymes could result, due to decreased MS function, in a reduced remethylation of Hcy to methionine and thus in elevated Hcy concentrations. Therefore, both enzymes should be regarded as candidate enzymes for folate-preventable NTD.

Disturbed homocysteine transsulphuration, cystathionine β-synthase, and NTD. CBS competes with the two Hcy methyltransferases (MS and BHMT) for Hcy conversion. CBS catalyzes the condensation of Hcy and serine to cystathionine. This is an irreversible step in the transsulphuration pathway. The overall reaction catalyzed by CBS involves β-replacement in which the hydroxyl group of the substrate serine is exchanged for thiolate of Hcy, resulting in the formation of cystathionine.

The CBS gene has been mapped to the subtelomeric region of chromosome 21 (21q22.3) and encodes a 63-kDa subunit consisting of 551 amino acids (193–195). CBS is a tetramer of identical subunits and is unique in being dependent on two cofactors, heme and vitamin B_6, for activity.

Recently, the complete sequence of the CBS gene has been identified (196). The gene contains 23 exons and has at least two alternatively used promoter regions. By *in situ* hybridization it has very recently been shown that the CBS is continuously expressed in tissues of the developing embryo (from the earliest stages studied, day 22 post-conception) during embryogenesis (197). In adults CBS activity could only be detected in liver and kidney.

The observed increased fasting, as well as post-methionine load plasma Hcy concentrations, in women with NTD-affected offspring may indicate an impaired transsulphuration (198). Steegers-Theunissen and colleagues studied the CBS activity in cultured skin fibroblast in 10 of these women with post-methionine load elevated Hcy levels. However, all the methionine-intolerant women had CBS activities within the reference range. Ramsbottom et al. (199) examined in women with NTD-affected offspring the most commonly found mutations in the CBS gene related to classic hyperhomocysteinemia in Ireland, and again no correlation between NTD and defects in the CBS gene was observed.

Disturbed homocysteine remethylation and NTD in humans. A disorder in the folate metabolism affects the remethylation of Hcy to methionine, and Hcy is known as a sensitive marker of the folate status. Supportive evidence linking impeded Hcy remethylation to increased risk for a NTD pregnancy was observed by studying the Hcy, folate, and vitamin B_{12} levels in an Irish population (157, 200). These studies showed that both mildly impaired folate metabolism leading to reduced availability of 5-MeTHF and mildly impaired vitamin B_{12} metabolism possibly in combination with a low folate or vitamin B_{12} status seem to be involved in the etiology of NTD. These studies also showed that plasma vitamin B_{12} and folate might be independent risk factors for NTD. Both studies support the hypothesis that subtle inborn errors of the Hcy metabolism, resulting in a reduced activity of MS or MTHFR, could be risk factors for NTD-affected pregnancies.

Methionine synthase and NTD. The MS enzyme catalyzes the transfer of a methyl-group from 5-MeTHF to the cob(I)alamin form of the cofactor to form methylcobalamin and THF, and then transfers the methyl-group from methylcobalamin to Hcy, forming methionine and regenerating the cob(I)alamin. Recently, the MS gene was mapped to chromosome 1q43, cloned, and the cDNA was sequenced (201, 202). The first genetic mutations in this gene in CblG patients have been reported (202–204). The enzyme has a modular construction, with distinct regions responsible for Hcy binding and activation, for 5-MeTHF binding, for vi-

tamin B_{12} binding and activation, and for AdoMet binding and reductive methylation. In vitamin B_{12} deficiency, or other forms of reduced MS function, for example, due to irreversible oxidation of Cbl by nitrous oxide (186, 205), Hcy remethylation decreases. Failure to regenerate methionine causes depletion of methionine and AdoMet and exposes the organism to increased levels of Hcy. The AdoMet deficiency increases MTHFR activity by abolishment of its inhibition by AdoMet, resulting in a depletion of the other forms of THF required for thymidine and purine synthesis. MTHFR shunts folates into the 5-MeTHF form, which can be processed due to reduced MS activity. This process is commonly referred to as the "methyl trap hypothesis" (206). Additionally, the rate of folate polyglutamate synthesis is decreased because 5-MeTHF is a poor substrate for the FPGS enzyme, and because 5-MeTHF is the circulating form of folate, this leads to a lower retention of folates by cells. The common denominator of a reduced substrate flow through the MS and MTHFR enzymes is increased Hcy levels and reduced methionine formation. Especially in combination with low folate or vitamin B_{12} status, this could affect the cell's ability to methylate important compounds such as DNA, proteins, lipids, and myelin-impairing cellular function, which could result in a disturbed neurulation.

Several studies mention that a defective MS could be involved in the aetiology of NTD (156, 157, 165, 166, 184, 186, 200, 207–214). The MS gene was recently cloned (201, 202), thereby enabling a genetic investigation of its role in NTD. We sequenced the coding region of MS of NTD patients with mild hyperhomocysteinemia, but no pathologic mutations were found (215). We observed a common polymorphism, the A2756G mutation, but this polymorphism gave no association between the Hcy levels of an individual or an association with an increased risk of NTD offspring. In another study the transmission test for linkage disequilibrium failed to detect a significant association between the mutated allele and NTD (216). Later studies also failed to find a relationship between a defective MS and NTD (217, 218). The A2756G mutation in the MS gene even tended to lower Hcy levels among homozygous mutated individuals (215, 217). However, one large study in which healthy males were studied reported a moderate but significant increase in Hcy levels due to the A2756G mutation (219). This increase in Hcy levels was independent of vitamin B_{12} levels.

In contrast, studies on the bacterial analogue of MS indicated that this common polymorphism does result in a 30% decreased enzyme activity (Matthews RG, personal communication). Low vitamin B_{12} levels, which are observed in some NTD populations, together with the A2756G mutation could theoretically result in a decreased methylation of Hcy to methionine.

Methionine synthase reductase and NTD. The MTRR enzyme activates MS, so a disturbed function of the MTRR could result in a decreased functional methionine synthase activity and thus in a decreased remethylation of Hcy to methionine. Mutation analysis of homocystinuric patients with severe MTRR deficiency led to the identification of a common polymorphism in this gene, the A66G mutation (192). This mutation replaces an isoleucine into methionine. Since there is no expression system available, the exact consequences of this mutation are unknown.

Thus far, only one study examined the prevalence of this very common polymorphism in relation to NTD and an increased prevalence of the homozygous mutated genotype in NTD patients and their mother's was observed (220). In combination with low vitamin B_{12} levels the relative risk almost reached significance and increased to a 2- to 5-fold risk in the NTD patients and their mothers, respectively. This may indicate that, especially when the cofactor of MS (vitamin B_{12}) is limited, a reduced activation of MS by MTRR could lead to a disturbed remethylation of Hcy to methionine. The study by Wilson, however, failed to find an association between the presence of the A66G mutation and Hcy levels (220).

Especially in countries where vitamin B_{12} intake is low, this polymorphism may be of importance. Thus far, no additional data on the prevalence of this polymorphism in other populations is available. Large study groups are necessary to examine the postulated relationship between a defective MTRR enzyme and NTD.

Methylenetetrahydrofolate reductase and NTD. MTHFR is a regulating enzyme in the folate-dependent remethylation of Hcy. MTHFR mediates the conversion of 5,10-MTHF to 5-MeTHF and probably utilizes only polyglutamates as substrates within the cell (221–224). The enzyme is a flavoprotein that is allosterically inhibited by AdoMet. The gene has been localized to chromosome 1p36.3 (225). Recently, most of the cDNA sequence has been identified (226). The cDNA contains 11 exons and shows a 90% amino acid sequence homology to mouse. The enzyme binds Flavin adenine dinucleotide (FAD) and utilizes NADPH as electron donor (223). *In vitro* the reaction is bidirectional, but *in vivo* it is essentially unidirectional in the direction of 5-MeTHF-synthesis (224). Methylene-THF binding by the enzyme is inhibited *in vitro* by DHF and dihydrobiopterin (227), providing a possible site of interaction between folate and pterin metabolic pathways.

Studies on vascular patients showed that a thermolabile variant of the MTHFR enzyme has a reduced activity, which appeared to be associated with higher plasma Hcy concentrations (228, 229). After isolation and localization of the MTHFR gene, a C677T mutation was identified (225, 229). This mutation is associated with decreased activity of MTHFR, with thermolabile properties, and elevated plasma Hcy concentrations. The effect of thermolabile MTHFR on Hcy can be reversed by an additional folic acid intake (230, 231). Therefore, a mutated MTHFR gene may be involved in the observed elevated Hcy levels in women with NTD pregnancies and may explain a part of the protective effect of folate on NTD.

We observed that the frequency of the C677T mutation in MTHFR gene is more prevalent in NTD patients and their mothers (185, 232–234). The C677T mutation was associated with a significant 2.9- and 3.7-fold increased risk in children with spina bifida and their mothers. Thus, we identified the first genetic risk factor for NTD. An Irish and American study confirmed this observation (235, 236). The C677T mutation is associated with decreased MTHFR activity, low plasma folate, and high plasma Hcy and red cell folate concentrations (185, 232).

The prevalence of this common polymorphism differs between populations (229, 233, 234, 237–243) and may not in every country be associated with an increased risk. Nevertheless, in meta-analysis of the worldwide data on the frequencies of the C677T mutation in the MTHFR gene in NTD studies, we demonstrated an overall statistically significant association of a 2-fold increased risk of NTD (234). This mutation, therefore, should be regarded as a genetic risk factor for spina bifida in some populations (234, 244, 245).

By studying the prevalence of the C677T mutation in families with sporadic, as well as familial NTD, we observed that the MTHFR genotype was associated with an increased risk in sporadic NTD offspring and not particular in familial NTD (233). This could indicate differences in the underlying mechanism of NTD in sporadic and hereditary forms. No transmission distortion of the 677T allele could be detected in British and American familial NTD cases (239, 245), which is in concordance with our study that indicated that the C677T mutation is not a strong determinant of the hereditary form of NTD (233).

The 677TT genotype is not associated with a certain type of NTD (238), and therefore does not seem to contribute to the observed differences in prevalence in different studies. The studies performed by De Franchis et al. (237, 238) underline the importance of large study populations. The authors observed in their first report, which included only 28 mothers of NTD-affected offspring, no association between the 677TT genotype and the risk of having a NTD pregnancy. Their second report, which included 203 NTD patients, did show a significant association between the MTHFR genotype and an increased risk of NTD.

It is unclear whether periconceptional folate intake overcomes a disturbed Hcy and folate metabolism in the mother or in her developing fetus. By studying the combined prevalence of the MTHFR genotype in the mother and her NTD child, we observed an additive increase in risk. This risk increased to 6- to 7-fold if both the mother and her child are homozygous for the C677T mutation (232, 233). This indicates that the genotype of the mother as well as the genotype of her unborn child is related to the risk on NTD. Our results are recently confirmed by a Canadian study, which also observed a 6-fold increased risk if both the mother and her child had the 677TT genotype (246). Other studies also reported, although to a lesser extent, an increased risk if both the mother and her child were homozygously mutated (247, 248).

We studied the plasma Hcy levels of mothers of a child with NTD and NTD patients after excluding individuals with a 677TT genotype and significantly elevated Hcy and decreased plasma folate levels were still observed (158). Red blood cell folate did not differ among patients, their parents, and controls, suggesting a normal dietary folate intake and uptake. This indicates the presence of other yet unrevealed defects in the Hcy remethylation to methionine.

Therefore, we sequenced the entire coding region of the MTHFR gene and identified a second common polymorphism in the MTHFR gene (249), the A1298C mutation. Homozygosity for this mutation does result in a decreased MTHFR activity, but is not associated with elevated Hcy levels or an increased risk for NTD offspring. However, combined heterozygosity for both the C677T and A1298C mutation might be another genetic risk factor for NTD and partly explains the observed elevated Hcy levels (249). Only two studies thus far have investigated the prevalence of this A1298C mutation in relation to NTD (250, 251). The study performed by Weisberg et al. (250) confirmed our results of a decreased enzyme activity, but observed no differences in prevalence of the combined heterozygotes for these mutations between controls and NTD patients and their mothers. This was a relatively small study of 141 mothers (controls plus mothers of a child with NTD) and 133 children (controls plus NTD patients). No data is given on the number of NTD patients, their mothers, and controls. The study performed by Stegmann et al. (251) did show an increased prevalence of the combined heterozygous genotype (677CT/1298AC) among NTD patients (24%) versus controls (16%). The authors did not find a selective transmission of either of the mutated alleles; from their data we could calculate odds ratios and we observed a significantly, almost 3-fold, increased risk for the combined heterozygous-mutated genotype among NTD cases compared with the 677CC/1298AA genotype.

The effect of the A1298C mutation on the MTHFR activity and Hcy concentration is lower than that of the C677T mutation, and thus probably also on the risk of NTD. Therefore, very large case/control studies are needed to confirm our tentative observation that the A1298A mutation may be a genetic risk factor for NTD. No additional data on the prevalence of this polymorphism in other populations is available yet.

Multifactorial Etiology of NTD. It has been suggested that NTD are of a multifactorial origin involving both genetic and environmental factors. The MTHFR C677T mutation is a perfect example of the interaction between nature and nurture in the risk of having a NTD pregnancy.

Both the observed A2756G mutation in the MS gene and the A66G polymorphism in the MTRR gene had no effect on Hcy levels in individuals homozygous for these polymorphisms (215, 220). It remains to be investigated if

these polymorphisms in the presence of low folate or vitamin B_{12} levels do result in mild elevations of Hcy.

The multifactorial origin of NTD is also likely due to aberrations in multiple alleles at multiple loci. The role of gene-to-gene interactions in NTD is still unclear. Interestingly, some studies (252, 253) showed a synergistic interaction between the C677T mutation in the MTHFR gene and a common 68-bp insertion in the CBS gene. Even though the CBS polymorphism itself showed no association with NTD, it increased the risk of the C677T mutation on NTD from 2- to 5-fold. This indicates that gene-to-gene interactions of seemingly neutral mutations might be related to the etiology of NTD.

MS needs, for its proper function, two activating proteins, the soluble cytochrome b_5 (190, 191) and the methionine synthase reductase (192). Mutations in these proteins, next to the A2756G mutation in MS itself, could disturb MS activity in such a way that it causes a disturbance in the catalytic activity of MS. The A2756G mutation in the MS gene and the A66G polymorphism in the MTRR gene could lead to an increased risk on NTD by gene-to-gene interactions with other mutations present in an individual.

Future studies should explore if gene-environment and gene-to-gene interactions are involved in the etiology of NTD. To examine these interactions, very large case-control groups are necessary; therefore, these studies will probably have to be performed in a multicenter trial setting.

Summary and Concluding Remarks

Folate-Preventable NTD. Mothers of children with NTD showed mildly elevated plasma Hcy levels (156–158, 184, 186). Since there were no pronounced folate deficiencies observed in these mothers (155–158), we hypothesized that defects leading to mild elevations in Hcy levels could be associated with the aetiology of NTD. Periconceptional folate supplementation may overcome such a relatively mild metabolic defect, and thereby this hypothesis is providing a rationale for the protective effect of folate in the developing child against NTD.

In view of these findings, genes involved in the folate-related Hcy pathway were examined by several laboratories including ours. We were the first to identify the first and possibly the second genetic risk factor of NTD (185, 232, 234). Both are common polymorphisms in the MTHFR gene and are associated with a decreased activity of the enzyme. The MTHFR genotype of the mother as well as that of her unborn child are involved in the etiology of NTD (232, 233).

The observed risk factors confirmed our earlier postulated concept of the involvement of a disturbed folate-related Hcy metabolism as a cause of NTD in mothers as well as their offspring. Although the MTHFR polymorphisms are only moderate risk factors, population-wide they may account for an important part of the observed NTD prevalence. The observed elevated Hcy concentrations in individuals with the MTHFR polymorphisms can successfully be lowered by folate, even in individuals with normal folate levels (230, 231).

In the study of Selhub et al. (254), it was observed that Hcy levels reached its nadir at folate intakes higher than approximately 400 μg per day. This indicates that 400 μg of folate per day is the minimal dose that should be taken periconceptionally, especially since an additional intake of this dose does not seem to have adverse side effects (255). This, together with the studies that indicated that the recurrence (93) and first occurrence (94) of NTD could be partly overcome by periconceptional folate use, is more than sufficient to warrant folate supplementation to women who want to become pregnant. An increase of folate consumption at a population-wide level may also prevent other obstetric complications like placental infarcts and recurrent spontaneous abortions, as well as common diseases like vascular diseases, cancer, and decreased cognitive function.

A reduced MTHFR activity can only explain in part the observed elevated Hcy levels in NTD patients and their mother's (158). Thus, other defects in the folate and Hcy metabolism may possibly cause mild hyperhomocysteinemia as well. At the present there is no compelling evidence to implicate defective FRs (188, 189), CBS (199), or MS directly (215–218) to the etiology of NTD.

To date, only one study has examined the MTRR gene (220). In this study a very common polymorphism in this gene, the A66G mutation, was observed. This polymorphism, together with decreased vitamin B_{12} levels, may lead to an increased risk in NTD. This finding will require confirmation of other studies and might result in an advisory to women to take not only additional folate, but also vitamin B_{12} when planning a pregnancy.

In addition, a defective transport of Cbl to the cells by the transcobalamine transport proteins could, due to a shortage of cellular vitamin B_{12}, also result in a decreased activity of the MS (210). Therefore, apo- and holotranscobalamin (active versus inactive) levels should be determined in NTD patients and their parents, and the genes coding for these transport proteins should be examined.

Mechanism of Defective Neural Tube Closure due to Defects in Folate and Hcy Metabolism. Taken together, our findings point to the significance of an impaired Hcy metabolism, which can partly be explained by a reduced MTHFR activity, in the etiology of NTD. The observed elevated Hcy levels in both NTD patients and their mothers still keep us in the dark in terms of the underlying mechanism(s) that result in a defective closure of the neural tube. A reduced substrate flow through MS, due to a decreased availability of 5-MeTHF levels, will result in increased Hcy levels and in reduced methionine formation. Research needs to focus on the biological mechanisms through which elevated Hcy or decreased methionine levels could cause NTD. Several hypotheses can be proposed: (I) Hcy itself is embryo-toxic during the process of neurulation; (ii) decreased levels of methionine due to a disturbed remethylation of Hcy to methionine will result in decreased

levels of AdoMet, which is the general methyl donor in the human body and in this way could disturb neurulation by an inadequate gene methylation and thus gene expression; and by an inadequate amino acid methylation which could disturb, for example, microfilament synthesis; and (iii) increased Hcy levels will lead to an increased concentration of AdoHcy, a competitive inhibitor of most methyltransferases, which will mimic the effects of AdoMet depletion.

Teratology of homocysteine during neurulation. It was initially hypothesized that Hcy itself is disturbing neurulation (156). If Hcy concentrations are causally related to NTD, one could expect that Hcy is disturbing processes that are involved in neural tube closure. Studies with rat embryos could not confirm an effect of Hcy on neurulation (256, 257). It was concluded that elevation of Hcy is not a causal factor in the etiology of NTD in those studies. Rather, these studies indicated that Hcy is necessary to serve as substrate for methionine synthesis and, therefore, Hcy was suggested to be a growth factor at low concentrations, as was seen in the rat embryo (257).

In the chick embryo, however, Hcy induced specifically NTD when applied before and during the period of neurulation (258). The authors suggested that the biological mechanism of Hcy toxicity is based on its cytotoxic properties in killing cells specifically affecting the neural crest, resulting in a closure defect. However, no description of the morphological and other aspects during the arisal of the neurulation defect has been given.

Methylation and gene expression. Folate metabolism is involved in Hcy remethylation to methionine and in purine and thymidine synthesis. The essential amino acid, methionine is, besides in protein synthesis, involved in formation of AdoMet, which is a substrate for many transmethylation reactions, including DNA methylation. A shortage in the supply of methyl-groups could result in an impaired methylation of DNA, which in turn, could lead to a disturbed gene expression. DNA methylation is undergoing rapid changes, especially during the first stages of the embryogenesis (259). Small delays in remethylation of genes could have major consequences during the neurulation phase in which gene expression of the different genes involved in the closure of the neural tube are following each other in a cascade event.

Folic acid intake prevents against all the different forms of NTD, since there is no special drop in a certain type of NTD (27). The different NTD types may result from different disturbed processes as pointed out earlier, but they all are prevented by additional folate intake. The pivotal role of folate in DNA synthesis and gene expression can explain why a simple vitamin influences a complex process like neurulation. By using differential display techniques, it would be possible to examine differences in gene expression brought about by elevated Hcy levels or decreased AdoMet and methionine levels.

Methionine depletion, decreased amino acid methylation and microfilament synthesis. Methionine supplementation prevents the arisal of NTD in rats (257) and possibly in humans (260). Methionine may act on neurulation by methylation of amino acids via the methylation cycle in at least two ways. First, mono- and dimethylarginine are found mainly on histone proteins. Hypermethylation is found to reduce mitotic activity, but reduced mitotic activity does not appear to be prerequisite for NTD (261). Second, trimethyllysine and 3-methylhistidine are the major methylamino acids in myosin and actin. It has been suggested that contraction of myosin/actin microfilaments is regulated by methylation of lysine and histidine residues. Thus, microfilament construction and functioning are dependent upon sufficient transmethylation.

In vitro experiments with rat embryos cultured in a methionine-deficient medium resulted in lowered mono- and dimethylarginine, trimethyllysine, and 3-methylhistidine (261). Moreover, a defective elevation of the cranial neural folds was seen, suggesting an impaired microfilament action. It has been suggested that contraction of these microfilaments generates an intrinsic force to the neuroepithelial cells, resulting in cell wedging. As a consequence, the neural plate bends inward, which in turn results in elevation of the neural plate and convergence of the neural folds. Many experiments have revealed that disruption of microfilaments results in NTD in rats and mice (reviewed in Refs. 15, 262, and 263) and the chick embryo (15, 16).

Elevated homocysteine levels and a disturbed AdoMet/AdoHcy ratio. Elevated Hcy levels reduce the AdoMet/AdoHcy ratio and thus reduce transmethylation, which subsequently alters gene expression and reduces methylation of amino acids, as described above. Therefore, elevated Hcy concentrations may simulate the effect of methionine depletion. Another possibility is that increased Hcy indicates a decreased intracellular pool of THF, as mentioned in the methyl-trap hypothesis, and reduces the availability of THF for nucleic acid synthesis. This could lead to an insufficient supply of material to the closing neural groove, which cannot close properly. By determining the AdoMet versus AdoHcy levels and the Hcy and methionine levels, more insight in the underlying cause of NTD may be revealed.

Non-Folate Preventable NTD. Folate supplements do not prevent all occurrences and recurrences of NTD. Some studies suggest that inositol (264) and methionine (257, 265) can reduce the prevalence of NTD. All of these studies are performed in animal models, but might be also applicable to man. Thus far, no harmful side effects of inositol and methionine have been established. In humans, a dietary questionnaire showed a positive effect of a diet rich in methionine on pregnancy outcome (260). This reduction in NTD prevalence was irrespective of folate intake.

The identification of non-folate-related Hcy elevations in NTD patients, next to the observed prevention of NTD by inositol and methionine in animal models, may have its repercussion on primary prevention on NTD. Extension of folate therapy with vitamin B_{12}, inositol, or methionine

might result in a lower occurrence and recurrence prevalence of NTD.

Conclusions. Future studies should focus on identification of the molecular basis of specific Hcy elevations and/or decreased plasma folate levels. Candidate genes, next to MTHFR and CBS, include SHMT, MTHFD, BHMT, genes involved in the synthesis of thymidylate, purines, regulatory proteins, or (co-)substrates involved in folate and Hcy metabolism. These candidate genes should be subjected to mutation analysis or linkages studies by using intra- and inter-gene markers. Molecular genetic studies should not only involve mutation analysis of coding regions of these genes, but also the quantification of mRNA levels, examination of the 3′- and 5′ UTR, intron-exon boundaries for alternative splicing, and test promoter functions.

It remains to be determined whether the preventive effect of folic acid on NTD, next to overcoming of reduced MTHFR function, is due to other defective enzymes of folate and Hcy metabolism. MTHFR catalyzes the reduction of 5,10-MTHF to 5-MeTHF, but 5,10-MTHF is also used in two alternative routes; 5,10-MTHF can also transfer its methylene-group to dUMP to give rise to dTMP or can be oxidized via 5,10-methenylTHF to 10-formylTHF. The latter is required for the synthesis of purines. This might indicate that even a disturbed thymidylate and purine synthesis could be involved in the risk of having a NTD pregnancy. Determination of purines and thymidylate in blood could be a useful tool to investigate this hypothesis.

The described animal models have only partly verified the underlying mechanism of an impaired Hcy metabolism in relation to a defective closure of the neural tube. Most of these studies only reported whether or not Hcy or methionine resulted in NTD in the studied animal model and still gave no real insight in the mechanisms that are affected during neurulation. To understand more about the underlying mechanism on the arousal of NTD due to a disturbed Hcy metabolism, processes such as DNA methylation and microfilament synthesis have to be examined. However, a role for Hcy as a cytotoxic agent should not be excluded. Hcy might be involved in cell loss, possibly at critical sites like the neural folds, and therefore result in NTD.

Research should also focus on the non-folate-preventable NTD, which likely represents 30% of the NTD cases. Theoretically, supplements containing, next to folate, also vitamin B_{12}, methionine, and inositol may lead to an extra reduction of NTD prevalence.

1. Karfunkel P. The mechanism of neural tube formation. Int Rev Cytol **38:**245–271, 1974.
2. Copp AJ, Brook FA, Estibeiro JP, Shum ASW, Cockroft DL. The embryonic development of mammalian neural tube defects. Prog Neurobiol **35:**363–403, 1990.
3. Schoenwolf GC, Smith JL. Mechanisms of neurulation: Traditional viewpoint and recent advances. Development **109:**243–270, 1990.
4. Smith JL, Schoenwolf GC. Neurulation: Coming to closure. Trends Neurosci **20:**510–517, 1997.
5. Nieuwkoop PD. The neural induction process: Its morphogenetic aspects. Int J Dev Biol **43 SI:**615–623, 1999.
6. Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J, De Robertis EM. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature **403:**658–661, 2000.
7. Jacobson AG, Gordon R. Changes in the shape of the developing vertebrate nervous system analyzed experimentally, mathematically and by computer simulation. J Exp Zool **197:**191–246, 1976.
8. Schoenwolf GC. Cell movements driving neurulation in avian embryos. Development **2:**(Suppl)157–168, 1991.
9. Van Straaten HW, Hekking JW, Wiertz Hoessels EJ, Thors F, Drukker J. Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo. Anat Embryol Berl **177:**317–324, 1988.
10. Smith JL, Schoenwolf GC. Notochordal induction of cell wedging in the chick neural plate and its role in neural tube formation. J Exp Zool **250:**49–62, 1989.
11. Placzek M, Dodd J, Jessell TM. The case for floor plate induction by the notochord. Curr Opin Neurobiol **10:**15–22, 2000.
12. Schoenwolf GC, Franks MV. Quantitative analyses of changes in cell shapes during bending of the avian neural plate. Dev Biol **105:**257–272, 1984.
13. Jacobson AG. Some forces that shape the nervous system. Zoon **6:**13–21, 1978.
14. Nagele RG, Hunter E, Bush K, Lee HY. Studies on the mechanisms of neurulation in the chick: Morphometric analysis of force distribution within the neuroepithelium during neural tube formation. J Exp Zool **244:**425–436, 1987.
15. Schoenwolf GC, Folsom D, Moe A. A reexamination of the role of microfilaments in neurulation in the chick embryo. Anat Rec **220:**87–102, 1988.
16. Ferreira MC, Hilfer SR. Calcium regulation of neural fold formation: Visualization of the actin cytoskeleton in living chick embryos. Dev Biol **159:**427–440, 1993.
17. Hildebrand JD, Soriano P. Shroom, a PDZ domain-containing actin-binding protein, is required for neural tube morphogenesis in mice. Cell **99:**485–497, 1999.
18. Hildebrand JD, Soriano P. Shroom, a PDZ domain-containing actin-binding protein, is required for neural tube morphogenesis in mice. Cell **99:**485–497, 1999.
19. Van Straaten HWM, Janssen HCJP, Peeters MCE, Copp AJ, Hekking JWM. Neural tube closure in the chick embryo is multiphasic. Dev Dynamics **207:**309–318, 1996.
20. Alvarez IS, Schoenwolf GC. Expansion of surface epithelium provides the major extrinsic force for bending of the neural plate. J Exp Zool **261:**340–348, 1992.
21. Hackett DA, Smith JL, Schoenwolf GC. Epidermal ectoderm is required for full elevation and for convergence during bending of the avian neural plate. Dev Dynamics **210:**397–406, 1997.
22. Van Straaten HWM, Peeters MCE, Szpak KWF, Hekking JWM. Initial closure of the mesencephalic neural groove in the chick embryo involves a releasing zipping-up mechanism. Dev Dynamics **209:**333–341, 1997.
23. Golden JA, Chernoff GF. Anterior neural tube closure in the mouse: Fuel for disagreement with the classical theory. Clin Res **31:**127a, 1983.
24. Golden JA, Chernoff GF. Intermittent pattern of neural tube closure in two strains of mice. Teratology **47:**73–80, 1993.
25. Peeters MCE, Viebahn C, Hekking JWM, Van Straaten HWM. Neurulation in the rabbit embryo. Anat Embryol **197:**167–175, 1998.
26. O'Rahilly R, Müller. Bidirectional closure of the rostral neuropore in the human embryo. Am J Anat **184:**259–268, 1989.
27. Seller MJ. Multi-site neural tube closure in humans and maternal folate supplementation. Am J Med Genet **58:**222–224, 1995.
28. Tam PP. A study of the pattern of prospective somites in the presomitic mesoderm of mouse embryos. J Embryol Exp Morphol **92:**269–285, 1986.
29. Müller F, O'Rahilly R. The development of the human brain, the closure of the caudal neuropore, and the beginning of the secondary neurulation at stage 12. Anat Embryol **176:**413–430, 1987.
30. Copp AJ, Brook FA. Does lumbosacral spina bifida arise by failure of neural folding or by defective canalization? J Med Genet **26:**160–166, 1989.

31. Griffith CM, Wiley MJ, Sanders EJ. The vertebrate tail bud: three germ layers from one tissue. Anat Embryol **185:**101–113, 1992.
32. Schoenwolf GC. Histological and ultrastructural studies of secondary neurulation in mouse embryos. Am J Anat **169:**361–376, 1984.
33. O'Rahilly R, Müller. Neurulation in the normal human embryo. Ciba Found Symp **181:**70–82, 1994.
34. Lemire RJ, Loeser JD, Leech RW, Alvord EC. Normal and Abnormal Development of the Human Nervous System. Hagerstown, MD: Harper & Row, 1975.
35. Catala M, Teillet MA, Ledouarin NM. Organization and development of the tail bud analyzed with the quail-chick chimaera system. Mech Dev **51:**51–65, 1995.
36. Matson D. Neurosurgery of Infancy and Childhood. Springfield, IL, Charles C. Thomas, 1969.
37. Larroche J-C. Malformations of the nervous system. In: Adams JH, Corsellis JAN, Duchen LW, Eds. Greenfield's Neuropathology. New York: Wiley & Sons, p385, 1984.
38. Lemire RJ, Siebert JR. Anencephaly: Its spectrum and relationship to neural tube defects. J Craniofac Genet Dev Biol **10:**163–174, 1990.
39. Nakano RK. Anencephaly: A review. Dev Med Child Neurol **15:**383–400, 1973.
40. Melnick M, Myrianthopoulos NC. Studies in neural tube defects. II. Pathological findings in a prospectively collected series of anencephalics. Am J Med Genet **26:**797–810, 1987.
41. Yen IH, Khoury MJ, Erickson JD, James LM, Waters GD, Berry RJ. The changing epidemiology of neural tube defects: United States, 1968–1989. Am J Dis Child **146:**857–861, 1992.
42. Ingraham FD, Scott HW. Arnold-Chiari malformations. N Engl J Med **229:**108, 1943.
43. Mealey J Jr., Dzenitis AJ, Hockey AA. The prognosis of encephaloceles. J Neurosurg **32:**209–218, 1970.
44. Brown MS, Sheridan-Periera M. Outlook for the child with an encephalocele. Pediatrics **90:**914–919, 1992.
45. Friede RL. Developmental Neuropathology. New York: Springer-Verlag, 1989.
46. Curnes JT, Oakes WJ. Parietal cephaloceles: Radiographic and magnetic resonance imaging evaluation. Pediatr Neurosci **14:**71–76, 1988.
47. Von Brandensky G, Klick A. Encephalocele und hydrocephalus. Z Kinderchir **7:**583, 1969.
48. Castillo M, Quencer RM, Dominguez R. Chiari III malformations: Imaging features. Am J Neuroradiol **13:**107–113, 1992.
49. Dolk H, Dewals P, Gillerot Y, Lechat MF, Ayme S, Cornel M, Cuschieri A, Garne E, Goujard J, Laurence KM, Lillis D, Lys F. Heterogeneity of neural tube defects in Europe: The significance of site of defect and presence of other major anomalies in relation to geographic differences in prevalence. Teratology **44:**547–559, 1991.
50. Shiota, K. Teratothanasia: Prenatal loss of abnormal conceptuses and the prevalence of various malformations during human gestation. Birth Defects **29:**189–199, 1993.
51. Myrianthopoulos NC, Melnick M. Studies in neural tube defects. I. Epidemiologic and etiologic aspects. Am J Med Genet **26:**783–796, 1987.
52. Lemire RJ. Neural tube defects. J Am Med Assoc **259:**558–562, 1988.
53. Stone DH. The declining prevalence of anencephalus and spina bifida: It's nature, causes and implications. Dev Med Child Neurol **29:**541–546, 1987.
54. Leech RW, Payne GG Jr. Neural tube defects: epidemiology. J Child Neurol **6:**286–287, 1991.
55. Chan A, Robertson EF, Haan EA, Keane RJ, Ranieri E, Carney A. Prevalence of neural tube defects in South Australia, 1966–1991: Effectiveness and impact of prenatal diagnosis. Br Med J **307:**703–706, 1993.
56. Roberts HE, Moore CA, Cragan JD, Fernhoff PM, Khoury MJ. Impact of prenatal diagnosis on the birth prevalence of neural tube defects, Atlanta, 1990-1991. Pediatrics **96:**880–883, 1995.
57. Janerich DT. Epidemic waves in the prevalence of anencephaly and spina bifida in New York State. Teratology **8:**253–256, 1973.
58. Kirke PN, Elwood JH. Anencephaly in the United Kingdom and Republic of Ireland. Br Med J **289:**1621, 1984.
59. Brown AS, Susser ES. Sex differences in prevalence of congenital neural defects after periconceptional famine exposure. Epidemiology **8:**55–58, 1997.
60. Holmes LB, Driscoll SG, Atkins L. Etiologic heterogeneity of neural-tube defects. N Eng J Med **294:**365–369, 1976.
61. Carter CO. Clues to the aetiology of neural tube malformations. Dev Med Child Neurol **16**(suppl 32):3–15, 1974.
62. Carter CO, Evans K. Children of adult survivors with spina bifida cystica. Lancet **2:**924–926, 1973.
63. Lorber J. The family history of spina bifida cystica. Pediatrics **35:**598, 1965.
64. Janerich DT, Piper J. Shifting genetic patterns in anencephaly and spina bifida. J Med Genet **51:**101–105, 1978.
65. Toriello HV, Higgins JV. Occurrence of neural tube defects among first-, second-, and third-degree relatives of probands: Results of a United States study. Am J Med Genet **15:**601–606, 1983.
66. Hall JG, Friedman JM, Kenna BA, Popkin J, Jawanda M, Arnold W. Clinical, genetic, and epidemiological factors in neural tube defects. Am J Hum Genet **43:**827–837, 1988.
67. Chatkupt S, Skurnick JH, Jaggi M, Mitruka K, Koenigsberger MR, Johnson WG. Study of genetics, epidemiology, and vitamin usage in familial spina bifida in the United States in the 1990s. Neurology **44:**65–70, 1994.
68. Martínez-Frías M-L. Valproic acid and spina bifida. Lancet **338:**196–197, 1991.
69. Campbell RL, Dayton DH, Sohal GS. Neural tube defects: a review of human and animal studies on the etiology of neural tube defects. Teratology **34:**171–187, 1986.
70. Gonzalez MJ, Schmitz KJ, Matos MI, Lopez D, Rodriguez JR, Gorrin JJ. Folate supplementation and neural tube defects: A review of a public health issue. P R Health Sci J **16:**387–393, 1997.
71. Shaw GM, Todoroff K, Velie EM, Lammer EJ. Maternal illness, including fever and medication use as risk factors for neural tube defects. Teratology **57:**1–7, 1998.
72. MacMahon B, Yen S. Unrecognised epidemic of anencephaly and spina bifida. Lancet **1:**31–33, 1971.
73. Stein Z, Susser M, Saenper G, Marolla F. Famine and Human Development: The Dutch Hunger Winter of 1944-1945. New York: Oxford University Press, 1975.
74. Michie CA. Neural tube defects in 18th century. Lancet **337:**504, 1991.
75. Stein SC, Feldman JG, Friedlander M, Klein RJ. Is myelomeningocele a disappearing disease? Pediatrics **69:**511–514, 1982.
76. Hibbard BM. The role of folic acid in pregnancy: With particular reference to anaemia, abruption and abortion. J Obstet Gynaecol Br Commonwlth **71:**529–542, 1964.
77. Hibbard ED, Smithells RW. Folic acid metabolism and human embryopathy. Lancet **1:**1254, 1965.
78. Smithells RW, Sheppard S, Schorah CJ. Vitamin deficiencies and neural tube defects. Arch Dis Child **51:**944–950, 1976.
79. Hoffbrand AV, Newcombe BF, Mollin DL. Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. J Clin Pathol **19:**17–28, 1966.
80. Smithells RW, Sheppard S, Schorah CJ, Seller MJ, Nevin NC, Harris R, Read AP, Fielding DW. Possible prevention of neural-tube defects by periconceptional vitamin supplementation. Lancet **1:**339–340, 1980.
81. Laurence KM, James N, Miller MH, Campbell H. Increased risk of recurrence of neural tube defects to mothers on a poor diet and possible benefits of dietary counseling. Br Med J **281:**1542–1594, 1980.
82. Duff EMW, Cooper ES, Danbury CM, Johnson BE, Serjeant GR. Neural tube defects in hurricane aftermath. Lancet **37:**120–121, 1991.
83. Seller MJ. Risk in Spina Bifida. Dev Med Child Neurol **36:**1021–1025, 1994.
84. Laurence KM, James N, Miller MH, Tennant GB, Campbell H. Double-blind randomised control trial of folate treatment before conception to prevent recurrence of neural-tube defects. Br Med J **282:**1509–1511, 1981.
85. Elwood JM. Can vitamins prevent neural tube defects? Can Med Assoc J **129:**1088–1092, 1983.
86. Wald NJ, Polani PE. Neural-tube defects and vitamins: The need for a randomized clinical trial. Br J Obstet Gynaecol **91:**516–523, 1984.
87. Smithells RW, Sheppard S, Schorah CJ, Seller MJ, Nevin NC, Harris R, Read AP, Fielding DW. Apparent prevention of neural tube defects by periconceptional vitamin supplementation. Arch Dis Child **56:**911–918, 1981.

88. Smithells RW, Nevin NC, Seller MJ, Sheppard S, Harris R, Read AP, Fielding DW, Walker S, Schorah CJ, Wild J. Further experience of vitamin supplementation for prevention of neural-tube defect recurrences. Lancet **1:**1027–1031, 1983.
89. Smithells RW. Neural tube defects: Prevention by vitamin supplementation. Pediatrics **69:**498–499, 1982.
90. Sheppard S, Nevin NC, Seller MJ, Wild J, Smithells RW, Read AP, Harris R, Fielding DW, Schorah CJ. Neural tube defect recurrence after partial vitamin supplementation. J Med Genet **26:**326–329, 1989.
91. Nevin NC, Seller MJ. Prevention of neural-tube defect recurrences. Lancet **1:**178–179, 1990.
92. Vergel RG, Sanchez LR, Heredero BL, Rodriguez PL, Martinez AJ. Primary prevention of neural tube defects with folic acid supplementation: Cuban experience. Prenat Diagn **10:**149–152, 1990.
93. MRC Vitamin Study Research Group. Prevention of neural-tube defects: Results of the Medical Research Council vitamin study. Lancet **338:**131–137, 1991.
94. Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med **327:**1832–1835, 1992.
95. Mulinare J, Cordero JF, Erickson JD, Berry RJ. Periconceptional use of multivitamins and the occurrence of neural tube defects. J Am Med Assoc **260:**3141–3145, 1988.
96. Millunsky A, Jick H, Jick SS, Bruel CL, MacLaughlin DS, Rothman KJ, Willett W. Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. J Am Med Assoc **262:**2847–2852, 1989.
97. Werler MM, Shapiro S, Mitchell AA. Periconceptional folic acid exposure and risk of occurrence neural tube defects. J Am Med Assoc **269:**1257–1261, 1993.
98. Shaw GM, Schaffer D, Velie EM, Morland KM, Harris JA. Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. Epidemiology **6:**219–226, 1995.
99. Bower C, Stanley FJ. Dietary folate as a risk factor for neural tube defects, evidence from a case-control study in Western Australia. Med J Aust **150:**613–619, 1989.
100. Berry RJ, Z Li, Erickson JD, Li S, Moore CA, Wang H, Mulinare J, Zhao P, Wong L-YC, Gindler J, Hong S-X, Correa A. Prevention of neural-tube defects with folic acid in China. N Engl J Med **341:**1485–1490, 1999.
101. Mills JL, Rhoads GG, Simpson JL, Cunningham GC, Conley MR, Lassman MR, Walden ME, Depp OR, Hoffmann HJ. The absence of a relation between the periconceptional use of vitamins and neural-tube defects. N Engl J Med **321:**430–435, 1989.
102. Scott JM, Weir DG, Kirke PN. Folate and neural tube defects. In: Bailey L, Ed. Folate in Health and Disease. New York: Marcel Dekker, pp 329–360, 1995.
103. Schaapveld K, Hirasing EA. Recente ontwikkelingen in de preventieve gezondheidszorg. Ned T Geneesk **141:**1909–1912, 1997.
104. De Bree A, Van Dusseldorp M, Brouwer IA, Van het Hof KH, Steegers-Theunissen RMP. Folate intake in Europe: Recommended, actual and desired intake. Eur J Clin Nutr **51:**643–660, 1997.
105. McNulty H, Cuskelly GJ, Ward M. Response of red blood cell folate to intervention: Implications for folate recommendations for the prevention of neural tube defects. Am J Clin Nutr **71:**1308S–1311S, 2000.
106. Pasternack LB, Littlepage LE, Laude DA Jr., Appling DR. 13C NMR analysis of the use of alternative donors to the tetrahydrofolate-dependent one-carbon pools in *Saccharomyces cerevisiae*. Arch Biochem Biophys **326:**158–165, 1996.
107. Shane B. Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. Vitam Horm **45:**263–335, 1989.
108. Appling DR. Compartimentation of folate-mediated one carbon metabolism in eukaryotes. FASEB J **5:**2645–2651, 1991.
109. Kastanos EK, Woldman YY, Appling DR. Role of mitochondrial and cytoplasmic serine hydroxymethyltransferase isozymes in de novo purine synthesis in *Saccharomyces cerevisiae*. Biochemistry **36:**14956–14964, 1997.
110. Shane B, Stokstad ELR. Folates in the synthesis and catabolism of histidine. In: Blakley RL, Benkovic SJ, Eds. Folates and Pterins, Vol 1. New York: Wiley & Sons, pp 433–456, 1984.
111. Krebs HA, Hems R, Tyler B. The regulation of folate and methionine metabolism. Biochemistry **158:**341–353, 1976.
112. Smith GK, Benkovic PA, Benkovic SJ. L-(−)10-formyltetrahydrofolate is the cofactor for glycinamide ribonucleotide transformylase from chicken liver. Biochemistry **20:**4034–4036, 1981.
113. Tan LU, Drury EJ, Mackenzie RE. Methylenetetrahydrofolate dehydrogenase-methenyl-tetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase: A multifunctional protein from porcine liver. J Biol Chem **252:**1117–1122, 1977.
114. Halsted CH. Intestinal absorption and malabsorption of folates. Annu Rev Med **31:**79–87, 1980.
115. Rosenberg IH. Folate absorption and transport. In: Cooper BA, Whitehead VM. Eds. Chemistry and Biology of Pteridines: Pteridines and Folic Acid Derivatives. Berlin, de Gruyter, p587, 1986.
116. Selhub J, Emmanouel D, Stavropoulos T, Arnold R. Renal folate absorption and the kidney folate binding protein. I. Urinary clearance studies. Am J Physiol **252:**F750–F756, 1987.
117. Shane B, Stokstad ELR. The interrelationships among folate, vitamin B12, and methionine metabolism. Adv Nutr Res **5:**133–170, 1983.
118. Fan J, Vitols KS, Huennekens FM. Multiple folate transport systems in L1210 cells. Adv Enzyme Regul **32:**3–15, 1992.
119. Sirotnak FM, Goutal LJ, Mines LS. Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis *in vivo*. Cancer Res **45:**3992–4000, 1985.
120. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. Proc Natl Acad Sci U S A **83:**5983, 1986.
121. Antony AC. The biological chemistry of folate receptors. Blood **79:**2807–2820, 1992.
122. Brigle KE, Spinella MF, Sierra EE, Goldman ID. Characterization of a mutation in the reduced folate carrier in transport defective L1210 murine leukemia cell line. J Biol Chem **270:**22974–22979, 1995.
123. Henderson GB. Folate-binding proteins. Annu Rev Nutr **10:**319–335, 1990.
124. Kamen BA, Wang MT, Streckfuss AJ, Peryea X, Anderson RGW. Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. J Biol Chem **263:**13602–13609, 1988.
125. Kamen BA, Smith AK, Anderson RGW. The folate receptor works in tandem with a Probenecid-sensitive carrier in MA104 cells *in vitro*. J Clin Invest **87:**1442, 1991.
126. Anderson RGW, Kamen BA, Rothberg KG, Lacey SW. Potocytosis: Sequestration and transport of small molecules by caveolae. Science **255:**410, 1992.
127. Selhub J. Folate binding proteins. Mechanism for placental and intestinal uptake. In: L Allen, J King, B Lönnerdal, Eds. Nutrient Relation during Pregnancy, Lactation, and Infant Growth. New York: Plenum Press, pp141–149, 1994.
128. Hjelle JT, Christensen EI, Carone FA, Selhub J. Cell fractionation and electron microscope studies of kidney folate-binding protein. Am J Physiol **260:**C338–C346, 1991.
129. Rothberg KG, Ying Y, Kolhouse JF, Kamen BA, Anderson RGW. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. J Cell Biol **110:**637–649, 1990.
130. Antony AC. Folate receptors. Annu Rev Nutr **16:**501–521, 1996.
131. Ragoussis J, Senger G, Trowsdale J, Campbell IG. Genomic organization of the human folate receptor genes on chromosome 11q13. Genomics **14:**423–430, 1992.
132. Prasad PD, Ramamoorthy S, Moe AJ, Smith CH, Leiback FH, Ganapathy V. Selective expression of the high-affinity isoform of the folate receptor (FR-alpha) in the human placental syncytrophoblast and choriocarcinoma cells. Biochem Biophys Acta **1223:**71–75, 1994.
133. Wang H, Ross JF, Ratnam M. Structure and regulation of a polymorphic gene encoding folate receptor type γ/γ′. Nucleic Acids Res **26:**2132–2142, 1998.
134. Shen F, Ross JF, Wang X, Ratnam M. Identification of a novel folate receptor, a truncated receptor, and receptor type β in hematopoietic cells: cDNA cloning, expression, immunoreactivity, and tissue specificity. Biochemistry **33:**1209–1215, 1994.
135. Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues *in vivo* and in established cell lines: Physiological and clinical implications. Cancer **73:**2432–2443, 1993.

136. Schirch V. Enzymatic determinations of folate pools. Methods Enzymol **281:**77–81, 1997.
137. Atkinson I, Garrow T, Brenner A, Shane B. Human cytosolic folylpoly-γ-glutamate synthase. Methods Enzymol **281:**134–140, 1997.
138. McGuire JJ, Bertino JR. Enzymatic synthesis and function of folylpolyglutamates. Mol Cell Biochem **28:**19–48, 1981.
139. Rosenblatt DS. Inherited disorders of folate transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Eds. The Metabolic and Molecular Bases of Inherited Disease, 7th edition. New York: McGraw-Hill, pp3111–3128, 1995.
140. Lowe KE, Osborne CB, Lin B-F, Kim J-S, Hsu J-C, Shane B. Regulation of folate and one-carbon metabolism in mammalian cells. II. Effect of folylpoly-γ-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-γ-glutamate specificity of metabolic cycles of one-carbon metabolism. J Biol Chem **268:**21665–21673, 1993.
141. Lin B-F, Huang R-FS, Shane B. Regulation of folate and one-carbon metabolism in mammalian cells. III. Role of mitochondrial folylpoly-γ-glutamate synthetase. J Biol Chem **268:**21674–21679, 1993.
142. Freemantle SJ, Taylor SM, Krystal G, Moran RG. Upstream organization of and multiple transcripts from the human folylpoly-gamma-glutamate synthetase gene. J Biol Chem **270:**9579–9584, 1995.
143. Chen L, Qi H, Korenberg J, Garrow TA, Choi Y-J, Shane B. Purification and properties of human cytosolic folylpoly-gamma-glutamate synthetase and organization, localization, and differential splicing of its gene. J Biol Chem **271:**13077–13087, 1996.
144. Zamierowski MM, Wagner C. Identification of folate binding proteins in rat liver. J Biol Chem **252:**933–938, 1977.
145. Cybulski RL, Fisher RR. Uptake of oxidized folates by rat liver mitochondria. Biochim Biophys Acta **646:**329–333, 1981.
146. Lin B-F, Shane B. Expression of *Escherichia coli* folylpolyglutamate synthetase in the Chinese hamster ovary cell mitochondrion. J Biol Chem **269:**9705–9713, 1994.
147. McGuire JJ, Hsieh P, Coward JK, Bertino JRx. Enzymatic synthesis of folylpolyglutamates. J Biol Chem **255:**5776–5788, 1994.
148. Osborne CB, Lowe KE, Shane B. Regulation of folate and one-carbon metabolism in mammalian cells. I. Folate metabolism in Chinese hamster ovary cells expressing *Escherichia coli* or human folylpoly-γ-glutamate synthetase activity. J Biol Chem **268:**21657–21664, 1993.
149. Priest DE. Folates in tissues and cells. In: Cooper BA, Whitehead VM, Eds. Chemistry and Biology of Pteridines: Pteridines and Folic Acid Derivatives. Berlin, de Gruyter, p479, 1986.
150. Yeo E-J, Wagner C. Tissue distribution of glycine N-methyltransferase, a major folate-binding protein of liver. Proc Natl Acad Sci U S A **91:**210–214, 1994.
151. Mackenzie RE. Summary. Pteroylpolyglutamate metabolism. In: Cooper BA, Whitehead VM, Eds. Chemistry and Biology of Pteridines: Pteridines and Folic Acid Derivatives. Berlin, de Gruyter, p767, 1986.
152. Sikka PK, McMartin KE. Determination of folate transport pathways in cultured rat proximal tubule cells. Chemico-Biological Interact **114:**15–31, 1998.
153. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM. Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. Q J Med **86:**703–708, 1993.
154. Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. Folate levels and neural tube defects: Implications for prevention. J Am Med Assoc **274:**1698–1702, 1995.
155. Scott JM, Kirke PN, Weir DG. The role of nutrition in neural tube defects. Annu Rev Nutr **10:**277–295, 1990.
156. Steegers-Theunissen RPM, Boers GHJ, Trijbels FJM, Finkelstein JD, Blom HJ, Thomas CMG, Borm GF, Wouters MGAJ, Eskes TKAB. Maternal hyperhomocysteinemia: A risk factor for neural-tube defects? Metabolism **43:**1475–1480, 1994.
157. Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, Scott JM. Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet **345:**149–151, 1995.
158. Van der Put NMJ, Thomas CMG, Eskes TKAB, Trijbels FJM, Steegers-Theunissen RPM, Mariman ECM, de Graaf-Hess A, Smeitink JAM, Blom HJ. Altered folate and vitamin B12 metabolism in families with spina bifida offspring Q J Med **90:**505–510, 1997.
159. Yates JRW, Ferguson-Smith MA, Shenkin A, Guzman-Rodriguez R, White M, Clark BJ. Is disordered folate metabolism the basis for the genetic predisposition to neural-tube defects? Clin Genet **31:**279–287, 1987.
160. Schorah CJ, Habibzadeh N, Wild J, Smithells RW. Possible abnormalities of folate and vitamin B12 metabolism associated with neural-tube defects. Ann N Y Acad Sci **778:**81–91, 1993.
161. Wild J, Schorah CJ, Sheldon TA, Smithells RW. Investigation of factors influencing folate status in women who have had a neural tube defect-affected infant. Br J Obstet Gynaecol **100:**456–459, 1993.
162. Bower C, Stanley FJ, Croft M, DeKlerk NH. Absorption of pteroylpolyglutamates in mothers of infants with neural tube defects. Br J Nutr **69:**827–834, 1993.
163. Davis BA, Bailey LB, Gregory III JF, Toth JP, Dean J, Stevenson RE. Folic acid absorption in women with a history of pregnancy with neural tube defects. Am J Clin Nutr **62:**782–784, 1995.
164. Habibzadeh N, Schorah CJ, Seller MJ, Smithells RW, Levene MI. Uptake and utilization of DL-5-[methyl]-14C]tetrahydropteroylmonoglutamate by cultured cytotrophoblasts associated with neural tube defects. Proc Soc Exp Biol Med **203:**45–54, 1993.
165. Lucock MD, Wild J, Schorah CJ, Levene MI, Hartley R. The methylfolate axis in neural tube defects: *in vitro* characterisation and clinical investigation. Biochem Med Metab Biol **52:**101–114, 1994.
166. Lucock MD, Daskalakis I, Lumb CH, Schorah CJ, Levene MI. Impaired regeneration of monoglutamyl tetrahydrofolate leads to cellular folate depletion in mothers affected by a spina bifida pregnancy. Mol Genet Metab **65:**18–30, 1998.
167. Finkelstein JD, Martin JJ. Methionine metabolism in mammals: Adaptation to methionine excess. J Biol Chem **261:**1582–1587, 1986.
168. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem **1:**228–237, 1990.
169. Akerman K, Karkola K, Kajander O. Methionine adenosyltransferase activity in cultured cells and in human tissues. Biochim Biophys Acta **1097:**140–144, 1991.
170. Scott JM, Weir DG, Molloy A, MacPartlin J, Daly L, Kirke P. Folic acid metabolism and mechanisms of neural tube defects. CIBA Foundation Symposium **181:**180–191, 1994.
171. Tabor H, Rosenthal SM, Tabor CW. The biosynthesis of spermidine and spermine from putrescine and methionine. J Biol Chem **233:**907, 1958.
172. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Eds. The Metabolic Basis of Inherited Disease, 6th edition. New York: McGraw-Hill, p693, 1989.
173. Fowler B. Disorder of homocysteine metabolism. J Inher Metab **20:**270–285, 1997.
174. Taylor RT, Weissbach H. N5-Methyltetrahydrofolate-homocysteine methyltransferases. In: Boyer PD, Ed. The Enzymes, 3rd edition. New York: Academic Press, p121, 1973.
175. McKeever MP, Weir DG, Molloy A, Scott JM. Betaine-homocysteine methyltransferase: Organ distribution in man, pig and rat and subcellular distribution in rat. Clin Sci **81:**551–556, 1991.
176. Allen RH, Stabler SP, Lindenbaum J. Serum betaine, N,N-dimethylglycine and N-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. Metabolism **42:**1448–1460, 1993.
177. Finkelstein JD, Martin JJ. Methionine metabolism in mammals: Distribution of homocysteine between competing pathways. J Biol Chem **259:**9508–9513, 1984.
178. Finkelstein JD. The regulation of homocysteine metabolism. In: Graham I, Refsum H, Rosenberg IH, Ueland PM, Shuman JM, Eds. Homocysteine Metabolism: From Basic Science to Clinical Medicine. Dordrecht, The Netherlands, Kluwer Academic Publishers, p3–9, 1997.
179. Van der Molen EF, Hiipakka MJ, Van Lith-Zanders, Boers GHJ, Van den Heuvel LPWJ, Monnens LAH, Blom HJ. Homocysteine metabolism in endothelial cells of a patient homozygous for cystathionine ß-synthase (CS) deficiency. Thromb Haemost **78:**827–833, 1997.
180. Ahiramatsu T, Fukagawa NK, Marchini JS, Cortiella J, Yu Y-M, Chapman TE, Young VR. Methionine and cysteine kinetics at different intakes of cystine in healthy adult men. Am J Clin Nutr **60:**525–533, 1994.
181. Fukagawa N, Yu Y-M, Young VR. Methionine and cysteine kinetics at different intakes of methionine and cystine in elderly men and women. Am J Clin Nutr **68:**380–388, 1998.
182. Cantoni GL, Richards HH, Chiang PK. Inhibitors of S-

adenosylhomocysteine hydrolase and their role in the regulation of biological methylation. In: Usdin E, Borchardt RT, Creveling CR, Eds. Transmethylation. New York: Elsevier/North-Holland, p155, 1979.
183. Taoka S, Ohja S, Shan X, Kruger WD, Banerjee R. Evidence for heme-mediated redox regulation of human cystathionine β-synthase activity. J Biol Chem **273:**25179–25184, 1998.
184. Steegers-Theunissen RPM, Boers GHJ, Trijbels FJM, Eskes TKAB. Neural-tube defects and derangement of homocysteine metabolism. New Engl J Med **324:**199–200, 1991.
185. Van der Put NMJ, Steegers-Theunissen RPM, Frosst P, Trijbels FJM, Eskes TKAB, Van den Heuvel LP, Mariman ECM, Den Heyer M, Rozen R, Blom HJ. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet **346:**1070–1071, 1995.
186. Lucock MD, Wild J, Lumb CH, Oliver M, Kendall R, Daskalakis I, Schorah CJ, Levene MI. Risk of neural tube defect-affected pregnancy is associated with a block in maternal one-carbon metabolism at the level of N-5-methyltetrahydrofolate: homocysteine methyltransferase. Biochem Mol Med **61:**28–40, 1997.
187. Finnell RH, Greer KA, Barber RC, Piedrahita JA, Shaw M, Lammer EJ. Neural tube defects and craniofacial defects with special emphasis on folate pathway genes. Crit Rev Oral Biol Med **9:**38–53, 1998.
188. Heil SG, Van der Put NMJ, Trijbels FJM, Gabreëls FJM, Blom HJ. Molecular genetic analysis of human folate receptors in neural tube defects. Eur J Hum Genet **7:**393–396, 1999.
189. Barber RC, Shaw GM, Lammer EJ, Greer KA, Biela TA, Lacey SW, Wasserman CR, Finnell RH. Lack of association between mutations in the folate receptor-alpha gene and spina bifida. Am J Med Genet **76:**310–317, 1998.
190. Gulati S, Chen Z, Brody LC, Rosenblatt DS, Banerjee R. Defects in auxiliary redox proteins leads to functional methionine synthase deficiency. J Biol Chem **272:**19171–19175, 1997.
191. Chen Z, Banerjee R. Purification of soluble cytochrome b5 as a component of the reductive activation of porcine methionine synthase. J Biol Chem **273:**26248–26255, 1998.
192. Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, Heng HHQ, Rommens JM, Scherer SW, Rosenblatt DS, Gravel RA. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A **95:**3059–3064, 1998.
193. Skovby FN, Krassikoff N, Francke U. Assignment of the gene for cystathionine β-synthase to human chromosome 21 in somatic cell hybrids. Hum Genet **65:**291–294, 1984.
194. Muncke M, Kraus JP, Ohura T, Francke U. The gene for cystathionine β-synthase (CBS) maps to the subtelomeric region on chromosome 21q and to the proximal mouse chromosome 17. Am J Hum Genet **42:**550–559, 1988.
195. Kraus JP, Le K, Swaroop M, Ohura T, Tahara T, Rosenberg E, Roper MD, Kozich V. Human cystathionine β-synthase cDNA: Sequence, alternative splicing and expression in cultured cells. Hum Mol Genet **2:**1633–1638, 1993.
196. Kraus JP, Oliveriusova J, Sokolova J, Kraus E, Vlcek C, de Francis R, Maclean KN, Bao L, Bukovsk, Patterson D, Paces V, Ansorge W, Kozich V. The human cystathionine β-synthase (CBS) gene: Complete sequence, alternative splicing and polymorphisms. Genomics **52:**312–324, 1998.
197. Quer PV, Rouillac C, Janbon C, London J, Demaille J, Kamoun P, Duffer JL, Abitbol M, Chass. Spatial and temporal expression of the cystathionine beta-synthase gene during early human development. Biochem Biophys Res Commun **254:**127–137, 1999.
198. Selhub J, Miller JH. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. Am J Clin Nutr **55:**131–138, 1992.
199. Ramsbottom D, Scott JM, Weir DG, Kirke PN, Mills JI, Gallagher PM, Whitehead AS. Are common mutations of cystathionine β-synthase involved in the aetiology of neural tube defects? Clin Genet **51:**39–42, 1997.
200. Scott JM, McPartlin J, Molloy A, McNulty H, Halligan A, Darling M, Weir DG. Folate metabolism in pregnancy. Adv Exp Med Biol **338:**727–732, 1993.
201. Li YN, Gulati S, Baker PJ, Brody LC, Banerjee R, Kruger WD. Cloning, mapping and RNA analysis of the human methionine synthase gene. Hum Mol Genet **5:**1851–1858, 1996.
202. Leclerc D, Campeau E, Goyette P, Adjalla CE, Christensen B, Ross M, Eydoux P, Rosenblatt DS, Rozen R, Gravel RA. Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. Hum Mol Genet **5:**1867–1874, 1996.
203. Gulati S, Baker P, Li YN, Fowler B, Kruger WD, Brody LC, Banerjee R. Defects in human methionine synthase in cblG patients. Hum Mol Genet **5:**1859–1865, 1996.
204. Wilson A, Leclerc D, Saberi F, Campeau E, Hwang HY, Shane B, Phillips JA III, Rosenblatt DS, Gravel RA. Functionally null mutations in patients with the cblG-variant form of methionine synthase deficiency. Am J Hum Genet **63:**409–414, 1998.
205. Borcsok E, Abeles RH. Mechanism of action of cystathionine β-synthase. Arch Biochem Biophys **213:**695–707, 1982.
206. Scott JM. Folate-vitamin B12 interrelationships in the central nervous system. Proc Nutr Soc **51:**219–224, 1992.
207. Shin YS, Buehring KU, Stokstad ELR. The relationship between vitamin B12 and folic acid and the effect of methionine on folate metabolism. Mol Cell Biochem **9:**97–108, 1975.
208. Schorah CJ, Smithells RW, Scott J. Vitamin B12 and anencephaly. Lancet **i:**880, 1980.
209. Gardiki-Kouidou P, Seller MJ. Amniotic fluid folate, vitamin B12 and transcobalamins in neural tube defects. Clin Genet **33:**441–448, 1998.
210. Magnus P, Magnus EM, Berg K. Transcobalamins in the etiology of neural tube defects. Clin Genet **39:**309–310, 1991.
211. Economides DL, Ferguson J, Mackenzie IZ, Darley J, Ware II, Holmes-Siedle M. Folate and vitamin B12 concentrations in maternal and fetal blood, and amniotic fluid in second trimester pregnancies complicated by neural tube defects. Br J Obstet Gynaecol **99:**23–25, 1992.
212. Adams MJ Jr., Khoury MJ, Scanlon KS, Stevenson RE, Knight GJ, Haddow JE, Sylvester GC, Cheek JE, Henry JP, Stabler SP. Elevated midtrimester serum methylmalonic acid levels as a risk factor for neural tube defects. Teratology **51:**311–317, 1995.
213. Dawson EB, Evans DR, Van Hook JW. Amniotic fluid B12 and folate levels associated with neural tube defects. Am J Perinatol **15:**511–514, 1998.
214. Steen MT, Boddie AM, Fischer AJ, Macmahon W, Saxe D, Sullivan KM, Dembure PP, Elsas LJ. Neural-tube defects are associated with low concentrations of cobalamin (vitamin B12) in amniotic fluid. Prenat Diagn **18:**545–555, 1998.
215. Van der Put NMJ, van der Molen EF, Kluijtmans LAJ, Heil SG, Trijbels FJM, Eskes TKAB, van Oppenraaij-Emmerzaal D, Banerjee R, Blom HJ. Sequence analysis of the coding region of human methionine synthase: Relevance to hyperhomocysteinaemia in neural-tube defects and vascular disease Q J Med **90:**511–517, 1997.
216. Morrison K, Edwards YH, Lynch SA, Burn J, Hol F, Mariman E. Methionine synthase and neural tube defects. J Med Genet **34:**958, 1997.
217. Christensen B, Arbour L, Tran P, Leclerc D, Sabbaghian N, Platt R, Gilfix BM, Rosenblatt DS, Gravel RA, Forbes P, Rozen R. Genetic polymorhisms in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. Am J Med Genet **84:**151–157, 1999.
218. Brody LC, Baker PJ, Chines PS, Musick A, Molloy AM, Swanson DA, Kirke PN, Ghosh S, Scott JM, Mills JL. Methionine synthase: High-resolution mapping of the human gene and evaluation as a candidate locus for neural tube defects. Mol Genet Metab **67:**324–333, 1999.
219. Harmon DL, Shields DC, Woodside JV, McMaster D, Yarnell JW, Young IS, Peng K, Shane B, Evans AE, Whitehead AS. Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations. Genet Epidemiol **17:**298–309, 1999.
220. Wilson A, Platt R, Wu Q, Leclerc D, Christensen B, Yang H, Gravel RA, Rozen R. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab **67:**317–323, 1999.
221. Kutzbach C, Stokstad ELR. Feedback inhibition of methylenetetrahydrofolate reductase in rat liver by S-adenosylmethionine. Biochim Biophys Acta **139:**217–220, 1967.
222. Matthews RG, Baugh CM. Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroyl polygluta-

mate substrates and with dihydropteroylpolyglutamate inhibitors. Biochemistry **19**:2040–2045, 1980.
223. Matthews RG. Are the redox properties of tetrahydrofolate cofactors utilized in folate-dependent reactions? Fed Proc **41**:2600–2604, 1982.
224. Lewis GP, Rowe PB. Methylenetetrahydrofolate reductase: Studies in a human mutant and mammalian liver. In: Blair JA, Ed. Chemistry and Biology of Pteridines. Berlin, de Gruyter, p229, 1983.
225. Goyette P, Sumner JS, Milos R, Duncan AMV, Rosenblatt DS, Matthews RG, Rozen R. Human methylenetetrahydrofolate reductase: Isolation of cDNA, mapping and mutation identification. Nat Genet **7**:195–200, 1994.
226. Goyette P, Pai A, Milos R, Frosst P, Tran P, Chen Z, Chan M, Rozen R. Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). Mamm Genome **9**:652–656, 1998.
227. Smith I, Hyland K, Kendall B. Clinical role of pteridine therapy in tetrahydrobiopterin deficiency. J Inherited Metab Dis **8**(suppl I):39–45, 1985.
228. Kang S-S, Wong PWK, Susmano A, Sora J, Nurusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: An inherited risk-factor for coronary artery disease. Am J Hum Genet **48**:536–545, 1991.
229. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk-factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. Nat Genet **10**:111–113, 1995.
230. Kang S-S, Zhou J, Wong PWK, Kowalisyn J, Strokosch G. Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet **43**:414–421, 1988.
231. Nelen WLDM, Blom HJ, Thomas CMG, Steegers EAP, Boers GHJ, Eskes TKAB. Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent miscarriages. J Nutr **128**:1336–1341, 1998.
232. Van der Put NMJ, Van den Heuvel LP, Steegers-Theunissen RPM, Trijbels FJM, Eskes TKAB, Mariman ECM, Den Heyer M, Blom HJ. Decreased methylenetetrahydrofolate reductase activity due to the C677T mutation in families with spina bifida offspring. J Mol Med **74**:691–694, 1996.
233. Van der Put NMJ, Trijbels FJM, Hol F, Eskes TKAB, Steegers-Theunissen RPM, van den Heuvel LPWJ, Mariman ECM, Blom HJ. Mutated methylenetetrahydrofolate reductase in sporadic and hereditary spina bifida offspring. In Mato JM, Caballero A, Ed. Proceedings of Workshop on Methionine Metabolism, Molecular Mechanism and Clinical Implications. Consejo Superior de Investigaciones Cientificas, Madrid, p186–191, 1996.
234. Van der Put NMJ, Eskes TKAB, Blom HJ. Is the common C677T mutation in the methylenetetrahydrofolate reductase gene a risk factor for neural tube defects? A meta-analysis Q J Med **90**:111–115, 1997.
235. Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Weir DG, Shields DC, Scott JM. A genetic defect in 5,10-methylenetetrahydrofolate reductase in neural tube defects. Q J Med **88**:763–766, 1995.
236. Ou CY, Stevenson RF, Brown VK, Schwartz CE, Allen WP, Khoury M, Oakley GP, Adams MJ. C677T homozygosity associated with thermolabile 5,10-methylenetetrahydrofolate reductase as a risk factor for neural tube defects. Am J Hum Genet **57**(suppl):A223, 1995.
237. De Franchis R, Sebastio G, Mandato C, Andria G, Mastroiacovo P. Spina bifida, C677T mutation, and role of folate. Lancet **346**:1703, 1995.
238. De Franchis R, Buoninconti A, Mandato C, Pepe A, Sperandeo MP, Del Gado R, Capra V, Salvaggio E, Andria G, Mastroiacovo P. The C677T mutation of the 5,10-methylenetetrahydrofolate reductase gene is a moderate risk factor for spina bifida in Italy. J Med Genet **35**:1009–1013, 1998.
239. Papapetrou C, Lynch SA, Burn J, Edwards YH. Methylenetetrahydrofolate reductase and neural tube defects. Lancet **348**:58, 1996.
240. Wilcken DEL, Wang XL. Relevance to spina bifida of mutated methylenetetrahydrofolate reductase. Lancet **347**:340, 1996.
241. Mornet E, Muller F, Lenvoise-Furet A, Delezoide AL, Col JY, Simon-Bouy B, Serre JL. Screening of the C677T mutation on the methylenetetrahydrofolate reductase gene in French patients with neural tube defects. Hum Genet **100**:512–514, 1997.
242. Shaw GM, Rozen R, Finnell RH, Wasserman CR, Lammer EJ. Maternal vitamin use, genetic variation of infant methylenetetrahydrofolate reductase, and risk for spina bifida. Am J Epidemiol **148**:30–37, 1998.
243. Ubbink JB, Christianson A, Bester MJ, Van Allen MI, Venter PA, Delport R, Blom H, Merwe A, Potgieter H, Vermaak WJ. Folate status, homocysteine metabolism, and methylenetetrahydrofolate reductase in rural South African blacks with a history of pregnancy complicated by neural tube defects. Metabolism **48**:269–274, 1999.
244. Wilcken DEL. MTHFR C677T mutation, folate intake, neural-tube defect, and risk of cardiovascular disease. Lancet **350**:603–604, 1997.
245. Johnson WG, Stenroos ES, Heath SC, Chen Y, Carroll R, McKoy V, Chatkupt S, Lehner T. Distribution of alleles of the methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism in familial spina bifida. Am J Med Genet **87**:407–412, 1999.
246. Christensen B, Arbour L, Tran P, Leclerc D, Sabbaghian N, Platt R, Gilfix BM, Rosenblatt DS, Gravel RA, Forbes P, Rozen R. Genetic polymorphism in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. Am J Med Genet **84**:151–157, 1999.
247. Koch MC, Stegmann K, Ziegler A, Schroter B, Ermert A. Evaluation of the MTHFR C677T allele and the MTHFR gene locus in a German spina bifida population. Eur J Pediatr **157**:487-492, 1998.
248. Shields DC, Kirke PN, Mills JL, Ramsbottom D, Molloy AM, Burke H, Weir DG, Scott JM, Whitehead AS. The thermolabile variant of methylenetetrahydrofolate reductase and neural tube defects: An evaluation of genetic risk and the relative importance of the genotype of the embryo and the mother. Am J Hum Genet **64**:1045–1055, 1999.
249. Van der Put NMJ, Gabreëls F, Stevens EMB, Smeitink JAM, Trijbels FJM, Eskes TKAB, van den Heuvel LP, Blom HJ. A second common mutation in the methylenetetrahydrofolate reductase gene: A risk factor for neural tube defects? Am J Hum Genet **62**:1044–1051, 1998.
250. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab **64**:169–172, 1998.
251. Stegmann K, Ziegler A, Ngo ETKM, Kohlschmidt N, Schroter B, Ermert A, Koch MC. Linkage disequilibrium of MTHFR genotypes 677C/T-1298A/C in the German population and association studies in probands with neural tube defects (NTD). Am J Med Genet **87**:23–29, 1999.
252. Botto LD, Mastroiacovo P. Exploring gene-gene interactions in the etiology of neural tube defects. Clin Genet **53**:456–459, 1998.
253. Speer MC, Nye J, McLone D, Worley G, Melvin EC, Viles KD, Franklin A, Drake C, MacKey J, George TM. Possible interaction of genotypes at cystathionine beta-synthase (CBS) and methylenetetrahydrofolate reductase (MTHFR) in neural tube defects: NTD Collaborative Group. Clin Genet **56**:142–144, 1999.
254. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. J Am Med Assoc **270**:2693–2698, 1993.
255. Herbert V, Colman N, Jacob E. Folic acid and vitamin B12. In: Goodhart RS, Shils ME, Eds. Modern Nutrition in Health and Disease. Philadelphia: Lea and Febiger, pp229–259, 1980.
256. Van Aerts LAGJM, Klaasboer HH, Postma NS, Pertijs JCLM, Peereboom JHJC, Eskes TKAB, Noordhoek J. Stereospecific *in vitro* embryotoxicity of L-homocysteine in pre- and post-implantation rodent embryos. Toxicol in Vitro **7**:743–749, 1993.
257. Van Aerts LAGJM, Blom HJ, Deabreu RA, Trijbels FJM, Eskes TKAB, Peereboom-Stegeman JHJC, Noordhoek J. Prevention of neural tube defects by and toxicity of L-homocysteine in cultured postimplantation rat embryos. Teratology **50**:348–360, 1994.
258. Rosenquist TH, Ratashak SA, Selhub J. Homocysteine induces congenital defects of the heart and neural tube: Effect of folic acid. Proc Natl Acad Sci U S A **93**:15227–15232, 1996.
259. Monk M, Boubelik M, Lehnert S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development **99**:371–382, 1987.

260. Shaw GM, Velie EM, Schaffer D. Is dietary intake of methionine associated with a reduction in risk for neural tube defect-affected pregnancies? Teratology **56**:295–299, 1997.
261. Coelho CND, Klein NW. Methionine and neural tube closure in cultured rat embryos: Morphological and biochemical analyses. Teratology **42**:437–451, 1990.
262. Morriss Kay G, Tuckett F. The role of microfilaments in cranial neurulation in rat embryos: effects of short-term exposure to cytochalasin D. J Embryol Exp Morphol **88**:333–348, 1985.
263. Matsuda M, Keino H. An open cephalic neural tube reproducibly induced by cytochalasin D in rat embryos *in vitro*. Zool Sci **11**:547–553, 1994.
264. Greene ND, Copp AJ. Inositol prevents folate-resistant neural tube defects in the mouse. Nat Med **3**:60–66, 1997.
265. Essien FB, Wannberg SL. Methionine but not folinic acid or vitamin-B12 alters the frequency of neural tube defects in Axd mutant mice. J Nutr **123**:27–34, 1993.
266. Peeters MCE. Embryonic axial curvature: Relationships to neuropore closure. In: Peeters MCE, Ed. Embryonic axial curvature: relationship to neuropore closure. Datawyse Maastricht, Universitaire Pers Maastricht, The Netherlands, pp9–25, 1998.
267. Rosenblatt DS. Inherited disorders of folate transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Eds.The Metabolic and Molecular Bases of Inherited Disease, 7th edition. New York: McGraw-Hill, pp2049–2064, 1989.
268. Van Aerts LAGJM. Investigation, using rat embryo culture, of the role of methionine supply in folic acid-mediated prevention of neural tube defects. Toxicol in Vitro **9**:677–684, 1995.