The methyl-deficiency model: History characteristics and research directions

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Introduction

Methyl donor research in progress today has its roots in some seemingly unrelated observations dating back to before the turn of the century. The impaired assimilation of fat and protein in the depancreatized dog was recognized soon after Mering and Minkowski produced diabetes mellitus in dogs by partial or complete pancreatic extirpation.[1] This technique permitted investigators to search for the etiology of diabetes, efforts that were rewarded with success in the laboratories of Banting, Best, and Macleod, in the summer, fall and winter of 1921.[2] Intense research in 1921 culminated in the successful treatment of a depancreatized dog on July 30. Following purification of the crude extract, human diabetics were treated; the first one, a 14-year-old boy, (Leonard Thompson) was injected on January 11, 1922 and immediately responded with a lowered blood sugar and improved clinical condition. Continuing into February, 1922, six additional diabetic patients were treated with pancreatic extract, all of whom responded favorably to its administration.[2] Incidentally, Leonard Thompson, the first human injected with insulin, lived another 13 years and died of pneumonia secondary to a motorcycle accident. Because much of what we know today about methyl donor metabolism was initiated by the insulin research, the highlights of that discovery are described here.

The discovery of insulin

Frederich G. Banting, a practicing surgeon in London, Ontario, Canada, first conceived the idea in 1920 of preparing a potent extract from the pancreas in an attempt to resolve the problems of diabetics. He took the idea to professor J.J.R. Macleod, Chairman, Department of Physiology at the University of Toronto (Canada), and discussed various aspects of his proposal with the learned physiologist. The hypothesis underlying the series of proposed experiments was first formulated by Banting in November 1920 while reading an article dealing with the relationship of the pancreatic islets of Langerhans to diabetes.[3] The article gave a resume of degenerative changes in the acini of the pancreas, but not the islets, following ligation of the ducts. Banting reasoned that advantage might be taken of this fact to prepare an active extract of islet tissue. Macleod was skeptical at first; in the recently revised version of his *Physiology Textbook* he stated that there was as yet no proof of the existence of an internal pancreatic secretion. However, despite some reservations in the beginning, Macleod was persuaded in the spring of 1921 to provide Banting with a room for surgery, some dogs as experimental subjects, and a graduate student, C.H. Best, as an assistant. In June 1921, after making significant suggestions to Banting regarding the experiments, Macleod went to Scotland for his summer vacation.

After laboring incessantly for 2 months, on Saturday, July 30, 1921, Banting and Best injected a depancreatized dog with a crude pancreatic extract. Within an hour, the dog's blood sugar level dropped 40%, and its clinical condition improved remarkably; an observation later described by Best as "the most exciting moment" in the life of either of the two investigators. On Monday, August 1, the experimenters gave an 8-mL injection of crude pancreatic extract to another depancreatized collie, then on the brink of death, and 1 hour later the dog came out of the coma, stood up, and walked around the room. Macleod returned from vacation in September 1921 and thereafter took an active part in the investigation. Following additional studies with dogs and more efficient purification of the extract, in January 1922 daily injections were given to a 14-year-old boy, the first diabetic human to receive the newly discovered insulin originally called "isletin."[2] Thus began the medical odyssey that led to the isolation, purification, and clinical use of "the elixir of life" for millions of human diabetics; the remainder is history. A summary of this remarkable series of early studies (1920–1922) is presented anonymously in the *Journal of Metabolic Research*.[4]

In 1923 Macleod shared the Nobel prize for Physiology of Medicine with Banting, a decision by the Nobel

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Committee that is still mired in controversy.[5] However, the brilliant achievements in such a short time bear witness to the genius and energy of the young investigators and the experience and skill of their older advisors and associates.

Nutritional biochemistry of methyl donors—early events

While the depancreatized dog played a central role in the discovery of insulin, simply replacing insulin did not completely restore the animal to normal health. Numerous reports from different laboratories noted that the depancreatized dogs did not survive for more than a few months on a meat and sugar diet, in spite of complete control of diabetic manifestations by the use of insulin.[6–8] The deterioration in physical condition of the depancreatized animals maintained on insulin was at first considered to be a result of digestive disturbances due to lack of the external secretion of the pancreas.[6] However, if the animals were fed raw pancreas, the physical debility was partially or totally prevented,[6] but as late as 1930[9,10] there was still doubt about the nature of the effect of raw pancreas. Some investigators did not feel that the effects of raw pancreas on protein and fat digestion was the complete answer. It seemed improbable that lack of digestion of the fat by lipase in the gut was responsible for the observed physical deterioration of the dogs.[10]

Attention then focused on alternative explanations, particularly the condition of the liver of the depancreatized dogs, which was always fatty, and the state of liver function with respect to fat metabolism. Signs of failure of liver function combined with post-mortem evidence of pathological hepatic changes suggested a disturbance in fat metabolism.[10] The report by Lombroso[11] had strongly suggested a relation between the pancreas and fat metabolism, an interesting concept seized by Hershey.[10] The relationship of the liver to fat metabolism was just gaining currency with some investigators; desaturation of fat by the liver was generally accepted by many. However, of equal interest was the role of phospholipids in liver fat metabolism and normal function.[10,12] Hershey[10] hypothesized that the problems in depancreatized dogs maintained on insulin was a result of failure of fat metabolism in the liver and related to phospholipids; and that the lecithin component might be substituted for pancreas in the diet. Experiments were designed to test this hypothesis.

Dogs were completely depancreatized by Hershey et al.[10] and insulin administration begun the following day. The animals were fed a diet of lean meat and sugar and given 15 units of insulin twice daily. In about 5–6 weeks the typical signs and symptoms appeared that had been observed previously (discolored urine, dark feces, positive test for jaundice). Lecithin, prepared from egg yolk, was given at this point, 5g at each meal, and within 3 days the urine and feces appeared normal and urine was negative for bile. Lecithin was discontinued and symptoms reappeared; after several cycles of administering and withholding insulin and exacerbation of symptoms, the effectiveness of lecithin seemed clear. Lecithin had successfully replaced raw pancreas in the diet. Seven dogs were used in this study, some of which were maintained for 1 year before detailed post-mortem examinations were performed. At autopsy, it was clear that insulin plus lecithin had prevented diabetes and fatty liver. In addition to demonstrating the effectiveness of lecithin (replacing raw pancreas), these experiments confirmed that the pancreatic enzymes were not essential to the life of the animal.

Following the report of Hershey and Soskin,[10] Best et al. conducted experiments that examined the effect of lecithin[13] and of the components of lecithin[14] on the deposition of fat in the liver of the normal rat. The conclusions from this series of experiments, documented by carefully controlled studies, were: (1) neither the fatty acid nor glycerophosphate of lecithin affected liver fat; (2) aminonitrogen of lecithin was negative; (3) choline, per os, consistently inhibited deposition of fat in liver; (4) choline did not increase fat excretion.

Prevention of fatty liver by choline

The discovery by Best et al. that choline prevented fat accumulation in the liver of rats[14] was rapidly expanded. These same investigators demonstrated that choline, under a variety of conditions, exercised both preventive and curative actions on fat deposition in the liver.[15] The doors were then opened for many investigators to join in the exciting new field of liver metabolism as influenced by nutrition and dietary factors. Channon et al.[16] found that an analogue of choline, with ethyl instead of methyl groups, had a preventive action on liver fat, but it was not as efficient as choline; they also found that methionine was effective, but only about 8–10% as effective as choline.[17] Cystine enhanced fatty liver, a finding in agreement with Tucker and Eckstein,[18] who also suggested that any preventive action of protein depended on the balance between its cystine and methionine content.

The many investigations in the early and mid 1930s regarding the metabolism of choline and related substances demonstrated that methionine could serve in lieu of cystine to promote the growth of animals on a cystine-deficient diet.[19] It was also shown that methionine, but not cystine, is an indispensable amino acid.[20]

During this period, the unique role of methionine as the only dispensable sulfur-containing amino acid was recognized. The critical data emerging from many studies (1930–1940) clearly suggested the importance of determining growth and the effects of other sulfur-containing compounds, notably homocystine, homocysteine, and cysteine.[21–23] Because of the prior preparation of homocystine in du Vigneaud's laboratory, it was possible to define some aspects of the metabolic importance of homocystine, even though at the time homocystine had not been isolated from natural sources.[22] The seminal work of Rose et al. at the University of Illinois[21] and du Vigneaud et al. at Cornell Medical College,[23] among many others, set the stage for the enormous

advancement in knowledge about methyl donors and the importance of transmethylation reactions. Aside from the major contributions that the Rose[21] and du Vigneaud[22,23] papers made to basic nutrition, there were two most interesting aspects of the work that the two investigations had in common. First, the experiments showed clearly that the animals could synthesize methionine from other compounds, but only if some unknown factor or factors were available as well. Second, the incredible amount of data derived from experiments using such a small number of experimental animals; Rose and Rice used six litters with a total of 55 offspring,[21] and du Vigneaud[22] used four litters, each of which had eight or nine pups. They extracted the maximum amount of data from a minimum number of animals by careful planning, keen observations, and astute interpretation of results.

The work of du Vigneaud and Rose and their colleagues pointed out that rats could survive without an "outside" dietary source of methyl groups if the diet contained homocystine plus a vitamin B complex. The elements in the B complex that permitted survival (methylation of homocystine) were identified by Mary Bennett[24,25] as folic acid and vitamin B_{12}, thus resolving this mystery. Bennett's studies also suggested that du Vigneaud's reports on transmethylation of homocystine actually illuminated an alternate pathway used by animals deficient in folic acid and vitamin B_{12}. In addition to Bennett's significant contributions, we also note the seminal work of Stokstad, who isolated and identified folic acid,[26] a key component in single carbon metabolism.

Hemorrhagic kidney and choline deficiency

A series of papers regarding organs other than the liver began to appear in the literature in 1939 from the laboratory of Wendell Griffith.[27–32] Other investigators soon joined Griffith in describing lesions in the kidneys, with limited reference to ocular hemorrhagic and atrophy of the thymus.[33–35] However, the main thrust of the studies was the hemorrhagic degeneration of the kidneys.[28–35] Griffith and Wade demonstrated that renal lesions and fatty livers were most readily induced within 7–10 days after placing 20–30-day-old rats weighing about 40 grams on the deficient diet.[28] Renal lesions developed over a 24–48-hour period between the 6th and 9th days after starting the deficient diet. There was a significant decrease in the incidence of renal hemorrhage in older, heavier rats. Hemorrhagic degeneration occurred invariably between the 8th and 10th days in another study.[34] Methyl deficiency caused a sharp drop in phospholipid concentration in the affected tissues (fatty liver and hemorrhagic kidneys). Renal hemorrhage was preceded by a decrease in phospholipid content in the kidney of the deficient rat, reaching a nadir on the 6th experimental day. This is a time of rapid synthesis of tissue and greatest demand for phospholipid. Methyl group requirements are less in rats over 35 days of age (compared to 25-day-old rats) because the rate of growth slows down, and needs for choline-containing phospholipid decreases; rats 35 days of age are more resistant to the deficiency compared to rats 25 days of age. The enormous significance of phospholipids and methyl metabolism in maintenance of the integrity of membranes and other components of the cell are described by Zeisel and Canty in a recent publication.[36] Griffith and Wade also reported on thymus atrophy associated with choline deficiency.[28] In addition,[29] it was first reported in 1940 by Griffith and Wade that cystine added to the deficient diet increased the severity of all lesions.

Influence of methyl deficiency on lymphoreticular tissue and immunocompetence

It was reported in some early experiments with choline deficiency that, in addition to fatty liver and hemorrhagic kidneys, the methyl deficiency was associated with enlargement of the spleen and regression of the thymus.[28] These lesions were intensified by addition of cystine and prevented by the addition of methionine to the diet.[29] Despite these interesting observations, the effects of methyl deficiency on immunocompetence was apparently ignored for more than two decades. In 1970,[37] our laboratory published results of studies indicating a role for lipotropes in resistance to infection. During the course of investigations into the role of dietary factors in resistance to infection we observed that rats deficient in vitamin B_{12} were more susceptible to *Salmonella* infection. In subsequent studies,[38] mother rats fed diets either severely or marginally deficient in lipotropes littered pups significantly more susceptible to infection in postnatal life, compared with controls. In addition, postnatal supplementation did not correct the effects of maternal deficits in methyl groups. Follow-up studies clearly confirmed these observations and identified the target tissues for the depressing effect of lipotrope deficiency as primarily the thymolymphatic system, with specific, severe depressing effects on the T-cell components of that system.[39,40]

Based on these observations, human patients with (1) megaloblastic anemia of pregnancy of folate deficiency; (2) megaloblastic anemia of pregnancy, or (3) iron-deficiency anemia were examined for cell-mediated immunity using dinitrochlorobenzene skin tests, phytohemagglutinin (PHA)-stimulated lymphocyte transformation, and rosette inhibition by antilymphocyte globulin.[41] These studies revealed that cell-mediated immunity was depressed in megaloblastic anemia due to folate deficiency; this was reversed by folate treatment. Iron-deficiency anemia was not associated with depressed cell-mediated anemia in these studies. Complementary studies in folate-deficient rats and mice[42,43] confirmed the effects on cell-mediated immunity observed in the folate-deficient human patients. Cellularity in both the thymus and thymic-dependent areas of spleen and lymph nodes was decreased, as was cytotoxicity and stimulation by the T-cell mitogen, in the folate-deficient animals.

Additional investigations[44,45] have shown that the age

at which the deficiency is examined can have a profound effect on the observed changes. The immunocompetence of weanling male Sprague-Dawley rats, maintained on a control, folacin-deficient, or marginal methionine-choline diet for 3 weeks, 3 months, or 12 months was determined by in vivo (response to infection with *Salmonella typhimurium*) and in vitro (lymphocyte transformation assay) methods. Young animals were most sensitive to *Salmonella* infection when deficient; this correlated with in vitro assessment of immune function. Histopathologic examination of spleens from *S. typhimurium*-infected rats maintained for 3 weeks on the experimental diets showed an overall decreased cellularity, especially in the inner follicular zone (T-cell zone) of the spleen, compared with the spleens of control animals. A short-term (3-week) lipotrope deficiency resulted in a depressed lymphocyte transformation response to concanavalin A (con A) in the spleen, thymus, and lymph nodes; depressed response to PHA was not observed in the thymus, but was present in the spleen and lymph nodes. After 3 months on the deficient diets, a depressed Con A-induced transformation response was still seen in the spleen, but the normal aging-induced immunosuppression resulted in a low response in all animals. Similar results were observed after 12 months. Detailed data on these studies can be found in the references.[36–45]

Teratogenicity of methyl deficiency

Richardson and Hogan[46] reported in 1946 that feeding an inadequate diet to mother rats resulted in the littering of pups with hydrocephalus and other congenital anomalies. The semipurified diet of weanlings contained all of the then-recognized water-soluble vitamins except vitamin B_c. B_c designates a substance that was later identified as a form of folic acid. Some females received, in addition, 5% of a liver extract fraction of 1% of an eluate of fullers earth adsorbate of liver extract. At maturity the females on the respective diets were mated with normal males from the stock colony. The pups born to the two groups of supplemented females were all normal, but the females fed the diet supplemented with known water-soluble vitamins (except B_c) littered 1786 young, of which 30 were hydrocephalic. Richardson and DeMottier[47] fed the control diet of Richardson and Hogan[46] to one group of females and fed another group the semi-synthetic diet without supplements. Their study confirmed the previous observations,[46] and reported that about 2% of offspring born to deficient mothers were hydrocephalic at birth or shortly thereafter.

These two reports clearly pointed to a nutritionally inadequate diet that was deficient in some unknown factor. Following up on these interesting observations, O'Dell et al.[48,49] designed experiments to test the effects of folic acid and vitamin A on the induced hydrocephaly.[48] Pteroylglutamic acid (folate) added to the semi-synthetic diet, 50 μg/100 g diet largely prevented the hydrocephaly, and lowering vitamin A in the diet had no further effect on the incidence of the anomaly. The addition of a folate inhibitor, crude methylpteroylglutamic acid (methyl PGA) raised the incidence of hydrocephaly to about 20%,[50] suggesting that folate deficiency, but not vitamin A, was an important component of the etiology of the congenital anomaly.

More recently, evidence has accumulated to clearly confirm that women consuming diets low or deficient in folate are at high risk for delivering infants with neural tube defects.[51,52]

During the period 1946–1950 there was considerable interest in the isolation, identification, and chemical synthesis of cyanocobalamin (vitamin B_{12}). When purified vitamin B_{12} became available, another door was opened to explore the enigma of hydrocephaly and other anomalies induced by maternal deficiency, apparently related to one-carbon metabolism. O'Dell et al.[53] confirmed in 1951 that vitamin B_{12} was a factor in the prevention of hydrocephalus in infant rats. With this established, efforts were initiated to determine mechanisms and detailed pathology of the hydrocephaly and other abnormalities associated with methyl deficiency. The nature of the hydrocephalus was described,[54,55] and some aspects of possible mechanisms for the B_{12}-deficit effect were elucidated.[56] There were defects, not only in the brain, but in the eyes, cardiovascular, and renal systems.

An interesting additional observation of some significance was the failure of peripheral nerves to properly develop the protective myelin sheath essential to proper functioning. A basic defect in the brain of the developing fetus born to vitamin B_{12}-deficient mother rats was identified as failure of normal development of the subcommissural organ, a specialized group of cells located on the anteroinferior surface of the posterior commissure of the midbrain where the third ventricle continues into the cerebral aqueduct uniting the lateral and third ventricles to the fourth ventricle.[57] The hydrocephalus in this system appears to be caused by stenosis of the cerebral aqueduct associated with aplasia of the subcommissural organ and other neural structures.[56] The subcommissural organ has been associated with fluid balance in the brain and the central nervous system.[58]

Choline deficiency liver cirrhosis and neoplasia

A number of investigators in the early 1940s reported the induction of liver cirrhosis in rats fed special diets.[59] Most diets were low in protein, high in fat, and in addition some used alcohol as a further stress. It was shown that the injury was increased by the addition of cystine and that the cirrhotic lesions could be prevented by choline, methionine, and casein; singly or in combination.[60] Lillie et al.[61] described in detail the histogenesis and repair of choline-deficiency liver cirrhosis. That paper was the first to describe the accumulation of a hyaline basophilic substance in the cirrhotic liver referred to by the authors as "ceroid." This substance was thought to be related in some way to peroxidation of liver fat combined with other tissue substances, including red cell membranes. During the several years following, a number of investigators further described

the lesions of choline deficiency and identified factors that influenced the development of hepatic damage. The descriptions of the cirrhogenesis process provided by Daft et al.[60] and Lillie et al.[61] have been amplified by many investigators with particularly good descriptions and illustrations by Hartroft,[62] Newberne and Rogers,[63] and, more recently, by Newberne.[64]

The basic series of changes in the liver begins with accumulation of lipids in the centrolobular hepatocytes. This progresses to involve the entire lobule and lobe, followed by hepatocyte necrosis and proliferation, fibrosis, and ultimately, cirrhosis. These fundamental changes are accompanied by varying degrees of bile duct and endothelial cell proliferation, along with inflammatory cell infiltration, interspersed with ceroid. These details, including evidence for peroxidation, are presented in another publication.[64]

One of the most interesting and significant observations of the methyl deficiency model was published by Copeland and Salmon in 1946.[65] This important discovery, the occurrence of liver cancer in choline-deficient rats, was quietly ignored by the scientific community and most cancer investigators. Salmon published an extension of the work 8 years later[66] and this, too, was lost to most oncologists. Thus, although largely ignored at the time, the choline-deficiency model for carcinogenesis was established. This was the first case to be described in the accessible literature in which taking something out of the diet (methyl groups) instead of adding something to it (chemical carcinogens) resulted in neoplasia. A part of the delay in recognizing the importance of results of this seminal research also came from Salmon's laboratory.[67] In a series of studies with methyl-deficient diets, it was observed that some of the choline-supplemented animals also developed liver tumors. It was subsequently determined[68] that some of the diets used in the choline-deficiency investigations were contaminated with what was identified later as aflatoxin, a potent hepatocarcinogen. These findings, combined with failure to induce liver cancer in rats with amino acid, methyl-deficient diets[69] led to the erroneous conclusion by Newberne that the tumors reported by Salmon and Copeland were actually a result of the contaminated diet. While this assumption temporarily derailed methyl-deficiency carcinogenesis, the delay was only temporary and other aspects of methyl deficiency were pursued instead for about two decades.[36–44] It seems likely that some of the liver tumors in the reports from Salmon's laboratory were associated with contamination of the diet complicated by methyl deficiency.

In 1982[70] the occurrence of liver cancer was again reported in methyl-deficient rats and mice without added carcinogen under conditions that precluded dietary contamination. These observations were quickly confirmed in other laboratories[71–73], and this remains an area of intense investigation today.

Cardiovascular disease in methyl-deficient rats

A little-noticed publication appeared in 1962 describing the cardiovascular effects of acute and chronic methyl deficiency in rats.[74] This was followed by sporadic reports that confirmed a peroxidation component in the development of liver, renal, and cardiovascular damage induced by methyl deficiency. The acute effects of the deficit were generally improved by synthetic antioxidants (BHA and butylated hydroxytoluene). Although the natural antioxidant, α-tocopherol (vitamin E), decreased cardiac and vascular lesions, ascorbic acid enhanced vascular damage.[75] These changes were associated with changes in tissue and serum lipid concentrations. In a subsequent study[76] using more refined analytical methodology, similar protective effects as noted earlier were observed. Free radical concentrations and thiobarbituric acid-reactive (TBA) material in the affected tissues were decreased by antioxidants in parallel with decreased tissue lipid concentration associated with reduced tissue injury.

In the long-term chronic deficiency studies,[74] the aorta and peripheral vessels accumulated lipids and ultimately developed typical arteriosclerotic damage. There were also atherosclerotic lesions in coronary arteries, some of which had thrombosis and myocardial infarcts. The renal and cardiovascular lesions, in particular, observed in methyl-deficient rats, suggest that these tissues are particularly vulnerable to inadequate methylation capabilities.

Future research directions

The fascination of the research related to methyl group metabolism resides in part in the attraction it has had for generations of investigators spanning the years from vitamin discovery and application to modern molecular biology (1920–1993). The remarkable tenacity of those dedicated individuals established a place in modern biology for methylation. Methyl group metabolism has provided a common bond between nutrition, biochemistry, and, more recently, molecular biology. Progress has sometimes been impeded by a lack of tools and methodologies. We now have the knowledge and technical capabilities to sharply increase incisive research dealing with methylation, extending from the whole animal to the level of the gene and its component parts.

The application of some modern approaches to methyl group research should include studies on methylation in the developing central nervous system and peripheral nerves, the immune system, pre-perinatal renal and cardiovascular systems, and, particularly, emphasis should be placed on understanding choline-deficiency liver cancer.

The work of many investigators, as described by du Vigneaud,[77] which firmly established the transmethylation concept, the nutritional components of carcinogenesis,[78] and the views and concepts set forth in a recent conference[79] on the health effects of vitamins, among many others, provide food for thought and suggest potential directions for research.

The role of methylation in biological processes is poised for a quantum leap, as indicated by the report by Bestor,[80] wherein methylation patterns in the vertebrate genome are described as an area for high priority in re-

search. Programmed changes in patterns of methylation may contribute to regulation of gene expression during development and help to explain some reported observations related to mammalian methyl group metabolism.

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