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LABILE METHYL GROUPS AND THE PROMOTION OF CANCER

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INTRODUCTION

Lipotropes constitute a highly significant group of biologically active compounds, the major components of which are choline, methionine, vitamin B_{12}, and folic acid. These nutrients play a central role in cellular metabolism through their regulation of the transfer and utilization of one-carbon moieties (8). These are essential to the synthesis and methylation of DNA, the production of nucleoproteins and membranes, and the metabolism of lipids, all of which are required for cell proliferation and the maintenance of tissue integrity. Lipo-

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tropes interact extensively with each other and with other nutrients. Figure 1 illustrates the role of folate, vitamin B_{12}, and methionine in the transfer of one-carbon units (17). There are a variety of reactions in which methyl groups are transferred, including (*a*) the formation of the purine ring; (*b*) pyrimidine biosynthesis; (*c*) amino acid interconversions; and (*d*) formate metabolism. This review concentrates on certain aspects of lipotropes and their role in the promotion of carcinogenesis; included are some of their immunoregulatory properties, their influence on xenobiotic metabolism, and proposed mechanisms for their interactions in the promotion of cancer.

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DIETARY SOURCES OF LIPOTROPES

Choline and folic acid are plentiful in both animal and plant foods; however, plants are low in methionine and do not contain vitamin B_{12}. Animal products and microorganisms are the sole dietary sources of vitamin B_{12}.

Each of the four lipotropic nutrients (methionine, choline, folic acid, and

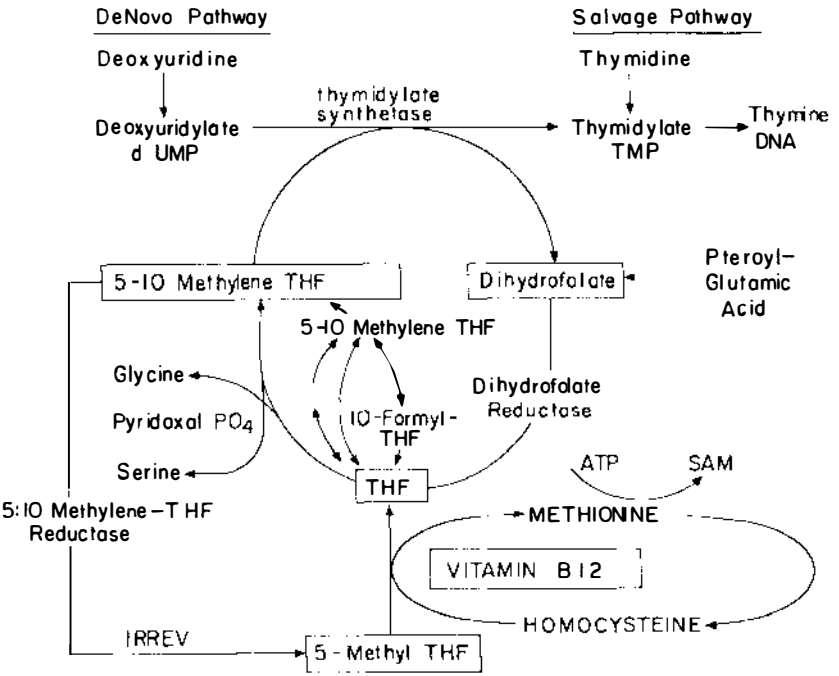

Figure 1 The biochemistry of one-carbon units indicates the fundamental role of folic acid in the metabolism of nucleic acids, protein, amino acids, and phospholipids, all essential to mounting an effective immune response and to cell proliferation in general. Vitamin B_{12} and methionine are integral components of the system as well as choline but the latter is less involved. From (17), with permission.

vitamin B_{12}) are stored in the body in given amounts in either pure or derivatized forms. They all turn over at specific rates. Vitamin B_{12} has the largest store relative to its daily requirement. Methionine is stored in tissue proteins that turn over constantly; it is also present as its t-RNA derivative and in an activated form, *S*-adenosylmethionine, essential to transmethylation. Free methionine is also present in plasma and in small amounts in tissues.

The main storage form of choline is in the choline phospholipids, which are widely distributed in all tissues. Choline is not essential in most animal species but is conditionally essential in the young rat. Folic acid is stored in red cells and in other tissues in significant amounts, but its turnover is rapid.

Stores of folic acid last no more than a few months after intake ceases, and choline, as such, lasts only a few days or at most a few weeks. Like methionine, choline can be derived from tissue breakdown since it is a major component of cell membranes and it can also be synthesized from other tissue components, including glycine and serine. Nevertheless, the diet is the major source of all lipotropes and a dietary deficiency of any or all of these nutrients is a potential threat to health. A decrease in the pool of methyl groups may contribute to susceptibility to many diseases, including cancer. It is to the latter chronic disease that this review is addressed.

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INTERRELATIONSHIPS OF METHIONINE, FOLATE, AND VITAMIN B_{12} METABOLISM

Before proceeding to the main subject relative to the influence of labile methyl groups and the promotion of cancer, a brief discussion of the interactions of lipotropes is appropriate.

Vitamin B_{12} and folate are essential for growth and proliferation of mammalian cells. The metabolism and participation of these cofactors in a variety of reactions requiring transfer of 1-carbon intermediates have been extensively reviewed (19, 20, 65). Rapid availability of nucleotide precursors is particularly important in the lymphatic system, which depends on proliferation and cell division in response to a foreign stimulus. The same is true for growth of all cancerous cells and serves as a basis for chemotherapy of cancer with antifolates (26). Moreover, the megaloblastic anemia precipitated by either folate or vitamin B_{12} deficiency appears to be caused by a common defect in thymidylate synthesis leading to a derangement of DNA synthesis. Menzies et al (36) found alterations in the chromosomes and abnormal DNA synthesis in bone marrow cells from patients with megaloblastic anemia. Other workers have reported a decrease in the number of megaloblasts synthesizing DNA, with some cells failing to exhibit any evidence for DNA synthesis (75).

Vitamin B_{12} is involved in a number of reactions in bacteria, only two of which have been demonstrated in mammalian systems. These are (*a*) the

isomerization of methylmalonate to succinate, which forms a link between carbohydrate and lipid metabolism, and (*b*) the methylation of homocysteine to methionine, the metabolic link between B_{12}, folate, and methionine metabolism.

A vitamin B_{12}–containing transmethylase is required for the conversion of homocysteine to methionine (Figure 1). In the process tetrahydrofolate (THF) is regenerated. Since methionine is available from other sources (diet and amino acid pools) it is thought that the importance of this reaction lies not in the synthesis of the amino acid but in the regeneration of THF.

As shown in Figure 1, folate coenzymes, carrying single-carbon units in different states of reduction, participate in a variety of reactions in which methyl groups are transferred. Cellular DNA synthesis depends on the availability of the four nucleotide precursors, and, although thymidylate can be formed directly from thymidine via a salvage pathway, most cells utilize the de novo path whereby *d*-uridine monophosphate (dUMP) is converted to TMP by the enzyme thymidylate synthetase. This is the rate-limiting step in DNA synthesis and requires 5,10-methylene THF as a cofactor, an observation made almost thirty years ago (12). In the process the cofactor is reduced to dihydrofolate (DHF), which can be further reduced via dihydrofolate reductase to THF.

An alternate way of regenerating THF is via the B_{12}-dependent methyl transferase reaction in which the methyl group is transferred from 5-methyl THF in the synthesis of methionine. As the folate-borne methyl groups go through successive stages of reduction, they are converted to 5,10-methylene THF, which can either serve as a cofactor for thymidylate synthetase or be further reduced via an irreversible reaction to 5-Me THF. This folate coenzyme must be converted to THF in order for the methyl group to reenter the methyl pool. In vitamin B_{12} deficiency this conversion cannot take place and 5-Me THF accumulates (21). Patients with vitamin B_{12} deficiency also exhibit an increase in the excretion of formiminoglutamic acid (64), formate (66), and 4(5)-amino-5(4)imidazole-carboxamide (35). These metabolites all require folate cofactors for further conversion and are restored to normal levels by the addition of dietary methionine (25).

Methionine, essential as a protein constitutent, also serves as a methyl donor in a large number of reactions. Mammalian liver systems have an enzyme for the direct methylation of homocysteine via betaine (8), but most cells utilize the B_{12}, 5-Me-THF-dependent methyl transferase reaction to synthesize methionine, which can then be further converted to *S*-adenosyl methionine (SAM). In addition to being an inhibitor of the 5-Me THF:homocysteine transmethylase reaction, SAM is a negative feedback inhibitor of 5,10-methylene THF reductase that reduces the amount of 5-Me THF formed and increases the availability of other folate cofactors (28).

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Methionine also accelerates the conversion of formate into CO_2 (25). This reaction is catalyzed by the enzyme formyl tetrahydrofolate dehydrogenase. The normal concentration of 10-formyl THF is far below the K_m of the enzyme (29); however, according to the scheme postulated by Krebs et al (25), an increase in dietary methionine increases SAM concentrations, causing a pile-up of 5,10-methylene THF that is at or near equilibrium with 10-formyl THF. Therefore, this latter intermediate would increase in concentration and thereby enhance the activity of the dehydrogenase. Krebs postulates that this pathway is important for disposing of excess one-carbon units and regenerating THF in the absence of vitamin B_{12}. This hypothesis is supported by the observations of others that added dietary methionine reduces the proportion of 5-methyl folate derivatives in rat liver and increases the 10-formyl monoglutamate (50) and polyglutamate (71) forms.

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EFFECT OF LIPOTROPE DEFICIENCY ON IMMUNE FUNCTION

Folic Acid

It is not surprising that the immune response, which requires rapid proliferation of sensitized cells, is affected by a deficiency of folic acid and other sources for methyl group synthesis. Despite the significance of folate to the immune system there have been only a few systematic studies of this important deficiency on immunocompetence in either animal or human models. Table 1 summarizes the alterations in humoral and cell-mediated immunity in patients with megaloblastic anemia due to folate deficiency as well as the results of studies in folate-deficient animals. A few comments on the reports represented by Table 1 are appropriate.

HUMAN STUDIES The stress of infection, superimposed on low reserves of labile methyl groups, through inadequate nutrition and vitamin loss (through diarrhea, vomiting, etc), along with increased cell turnover, places the patient at risk for deficiency of folate and other nutrients (18, 19, 61). In an isolated case of peripheral lymphocyte response to mitogenic (phytohemagglutinin A, PHA) stimulation from a patient with megaloblastic anemia due to folate deficiency, Das & Hoffbrand (6) found a depressed incorporation of ^{3}H-thymidine. Megaloblastic anemia was studied in Bantu patients in South Africa using dinitrochlorobenzene skin test and response of peripheral lymphocytes to PHA. These patients had normal levels of serum iron and vitamin B_{12} except for two subgroups that were studied for iron deficiency and combined folate-iron deficiency (18). The results are presented in Table 2.

Prior to treatment, the folate-deficient groups exhibited a significant depression in skin reactivity to DNCB, compared to the purely iron-deficient and

Table 1 Effects of folate deficiency on immune function[a]

I. Human studies
   A. Depressed peripheral lymphocyte response to PHA (6, 18)
   B. No change in neutrophil function (24)
   C. Increased incidence of folate deficiency in patients with hyperplastic candidosis (23)
   D. Delayed cutaneous hypersensitivity is depressed (18)

II. Animal studies
   A. Guinea pig
      1. Decreased WBC (70)
      2. Increased susceptibility to Shigella infection (40)
   B. Rats
      1. Decreased leukocytes and granulocytes (32)
      2. Decreased hemagglutination titers (53)
      3. Decreased number of antibody forming cells (27)
      4. Decreased number of T cells (77)
         Depressed cytotoxicity
         Depressed splenic PHA response
         Decreased delayed cutaneous hypersensitivity
      5. Increased susceptibility to parasitic infection (1)
   C. Chickens
      1. Decreased bacterial agglutination titers (31)
      2. Increased susceptibility to viral infection

[a] From Nauss & Newberne (39) by permission. Numbers in parentheses are references.

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control groups. Following therapy with folate supplements, 80% of the patients in the first three groups had a positive skin test response within 6–23 days. The PHA response of peripheral lymphocytes (as measured by ^{3}H-thymidine incorporation) was one third that of the control or of the iron-deficient subjects and returned to normal values 7–14 days after therapy. Vitamin B_{12} deficiency was ruled out in these patients by a negative Shilling test, which involves administration of a loading dose of the vitamin.

The remaining few studies in humans (10, 23, 24) have reported variable results.

ANIMAL STUDIES Selected nutrient deficiencies can be examined in animal models under rigidly controlled conditions. Impaired humoral (27, 32, 53) and cell-mediated (77) immune responses have been observed in rats on folate-deficient diets. Guinea pigs are particularly susceptible to folate deficiency (40, 70, 80).

Early studies showed that chicks raised on folate-deficient diets had decreased bacterial agglutination titers and increased susceptibility to viral infection (31). Folate-deficient rats were more susceptible to infection with *Trypanosoma lewisi* (1). The deficient animals had increased parasite levels in

Table 2 Dinitrochlorobenzene skin test and PHA response before treatment in folic acid–deficient, iron-deficient, and control patients[a]

Group[b]	Degree of dinitrochlorobenzene skin test reaction (no. of patients) 0	+	++	PHA stimulation of lymphocytes[b] (dpm)	Unstimulated lymphocytes[c] (dpm)
Folate-deficient					
nonobstetric	11	0	0	8,800±3,600 (3,091–13,995)	510 (219–1201)
obstetric	6	6	1	6,800±2,900 (3,691–12,323)	480 (176–1250)
Folate- and iron-deficient	5	0	0	6,000±3,280 (3,527–10,049)	330 (130–501)
Iron-deficient	0	2	3	23,300±5,470 (15,005–28,896)	390 (162–672)
Control	1	4	8	26,340±5,680 (18,740–33,725)	370 (130–719)

[a] From (18), by permission.
[b] Uptake of ^{3}H-thymidine; results are expressed in disintegrations per minute (dpm); mean ± S.D. and range.
[c] Uptake of ^{3}H-thymidine by lymphocytes in presence of saline; results are expressed as disintegrations per minute (dpm); mean ± S.D.; and range.

their blood compared to control animals and remained infected for longer periods of time.

Table 3 lists results from studies (16, 77) conducted in our laboratories using weanling Sprague-Dawley rats, kept on a folate-deficient diet for three months. They had a decreased number of T cells (as measured by ^{3}H-uridine labeling) in the spleen and peripheral blood. The cytotoxic activity of splenic lymphocytes of folate-deficient animals exposed to Brown Norway rat thymocytes decreased

Table 3 Folic acid deficiency in rats[a]

Immune parameter tested	Control	Folate-deficient
Delayed hypersensitivity (skin test response to PHA)	3.8±0.4[b]	1.6±0.4
Lymphocyte-mediated cytotoxicity (% killing)	29.1±3.7[c]	5.2±1.5
Spleen transformation (PHA response)	19,398±1,014[d]	4,263±579
[^{3}H]-Uridine labeling of T cells		
Spleen	70±2.4[e]	42±1.9
Thymus	82±1.5	73±2.0
Blood	67±1.7	44±2.8

[a] From (77) and (16), by permission.
[b] Histological grading based on degree of mononuclear cell infiltration: 0, no response; 4, severe response.
[c] Results expressed as mean ± S.E.
[d] Results expressed as cpm ± S.E.
[e] Results expressed as percentage of cells labeled.

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significantly (percentage of killing was 29.1 ± 3.7 in controls compared to 5.2 ± 1.5 in folate-deficient rats) as did sensitivity to stimulation by the T-cell mitogen PHA (stimulation index of 14.8 in controls compared to 3.0 in folate-deficient rats). An in vivo measurement of cell-mediated immune function (skin test sensitivity to intradermal PHA injection) showed a depressed response in the deficient animals based on the degree of mononuclear cell infiltration.

Additional experiments demonstrated a profound defect in young animals (3 months) kept on a folate-deficient diet from weaning. Rats fed a folate-deficient diet from one month of age through a year exhibited interesting changes, with time, in the transformation response of lymphocytes from the spleen, thymus, and lymph nodes to a variety of mitogens (39). The results were quite similar to those observed in rats deficient in lipotrope (choline-methionine), described later. These deprivations during this period of rapid growth and development of the lymphatic system, even for short periods of time, caused a depressed mitogen transformation response in the first three weeks. However, after twelve months there were no longer significant differences between the experimental and control groups.

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Vitamin B_{12}

HUMAN STUDIES With the exception of autoimmune phenomena in pernicious anemia, studies relative to vitamin B_{12} and immunocompetence are limited. Using the lymphocyte transformation test, Tai & McGuigan (69) showed that peripheral lymphocytes from patients with pernicious anemia were sensitized to a variety of gastric antigens. Using the leucocyte migration test, others have demonstrated in vitro delayed hypersensitivity to these antigens (11, 15). MacCuish et al (33) measured the lymphocyte transformation response to the mitogen PHA in 20 patients with pernicious anemia and an equal number of age- and sex-matched controls. The mean transformation response (as measured by ^{3}H-thymidine uptake) of peripheral lymphocytes from patients with pernicious anemia was significantly lower than controls at the three doses tested. There were no significant differences in the percentages of B and T lymphocytes as measured by immunofluorescence and rosette techniques respectively.

ANIMAL STUDIES Rats and other laboratory animals do not develop clean-cut megaloblastic anemia when fed a vitamin B_{12}–deficient diet even though levels of serum B_{12}, methylmalonyl-CoA mutase activities, and tissue coenzyme levels may be reduced (20). This may explain why, with the exception of an early report on depressed complement-fixing antibodies in vitamin B_{12}–deficient rats (74), studies of vitamin B_{12} deprivation in rat models have not demonstrated any effect on immune function (20). We showed that rats fed a

B_{12}-deficient diet from weaning until three months of age responded to Salmonella infection in a manner similar to control animals (45). Infection caused an identical increase in spleen weights and serum and globulin levels in control and deficient animals and there were no differences in the histopathology of the infected organs. However, if the B_{12}-deficient diets were fed during gestation and weaning, then infection with Salmonella at the age of three months caused a higher mortality (71% at 30 days compared to 25% in controls) in the vitamin B_{12}–deficient group (48). The resistance of this group improved appreciably when vitamin B_{12} was added to the diet during the postweaning period. Other studies have focused on combined vitamin B_{12}/choline-methionine deficiencies and are discussed below.

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Methionine and Choline

Most clinical studies of nutrient modulation of immune function have dealt with patients suffering from kwashiorkor (protein-calorie malnutrition, PCM) or marasmus (starvation). Protein deprivation as well as protein-calorie imbalances, which are ubiquitous in Third World countries, play major roles in the immune response of individuals to infection. These studies (17) point to the importance of protein quality as well as quantity in the effective maintenance of the immune system. While not clearly established, these studies suggest the importance of amino acid balance and certain specific amino acids, particularly methionine, in the functional capacity of the immune mechanism. For an in-depth discussion of protein-calorie malnutrition, resistance to infection, and some of the perceived mechanisms, the reader is referred to (9, 17, 61, 68a). Briefly, it has been observed that all lymphoid organs, particularly the thymus, are reduced in size; active cell division is sharply decreased in these tissues; and the PCM patients are markedly susceptible to infection. There seems to be agreement that PCM does not appreciably diminish the B-cell population in number or function; in fact, humoral immunity may be enhanced. The major effect appears to be on the T-cell subset of lymphoid cells, especially the T-helper cells. Specific effects are described in (17).

Recent experiments have concentrated on the use of animal models to define the role of individual essential amino acids in modulating the cell-mediated or humoral immune response. Conflicting results with methionine deficiency on immune response are partially attributable to differences in species and time of initiation of the experimental diet.

The effect of a specific nutrient deficiency on the immune response depends on many parameters, including age of the animal, length of time on the diet, adequacy of other nutrients, and age at the time of challenge to the immune system. The importance of defining these parameters has been demonstrated in studies in our laboratories examining the effects of reduced methionine-choline

levels at varying stages of development on the immunocompetence of rats or mice.

We have studied rats that were littered to dams fed diets marginally deficient in methionine and choline during gestation (39). Some of the animals were switched to an adequate diet at birth, others were maintained on the same diet the mother had received. One hundred days postweaning the animals were infected with *Salmonella typhimurium*. As seen in Table 4, rats fed the marginal methionine-choline diet had reduced body weight at three months of age and a high mortality rate following infection with Salmonella compared to the normal controls. Although supplementation in the postweaning period improved weight gain, it failed to alter the response to infection.

The thymus glands of the prenatally malnourished pups were smaller at birth than those of the pups adequately nourished *in utero* (Table 5). If the low lipotrope diets were continued to three months of age, the differences became even more marked. In addition to its decreased size, histological evaluation of the thymus showed a marked hypoplasia of tissue from not only this organ, but also the lymph nodes and spleen (44, 49).

The above-noted observations led to further studies (76), using a battery of mitogens. Response to the T-cell mitogen Con A was found to be depressed in spleen cells from rats fed diets low in methionine and choline. Depressed responsiveness to PHA and pokeweed mitogen (PWM) was seen in thymus cells (Table 6). It remains unclear whether the gestation or lactation period is the most critical; however, Figure 2, taken from later studies in our laboratory (39), clearly shows that the early period in life is a very sensitive period for the development of immunity and the thymolymphatic system.

Different periods of development of the immune system, including in-

Table 4 Effect of lipotropes on response of rats to infection with *Salmonella typhimurium* three months postweaning

Dietary treatment during average gestation and lactation	Dietary treatment during average postweaning only	Weight at infection (g)	Mortality[a] (%)
Marginal methionine-choline − B_{12}	Marginal methionine-choline − B_{12}	233±6	100
Marginal methionine-choline − B_{12}	Control	240±8	100
Marginal methionine-choline + B_{12}	Marginal methionine-choline + B_{12}	248±5	91
Marginal methionine-choline + B_{12}	Control	285±7	90
B_{12}-deficient	B_{12}-deficient	260±3	71
B_{12}-deficient	Control	308±4	35
Control	Control	303±4	25

[a] Thirty days postinfection.

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Table 5 Lipotropes and development of the thymolymphatic system[a]

	Control	Marginal lipotrope
Birth[b]		
Body	6.0 ± 0.5	5.6 ± 0.4
Thymus	25.0 ± 4.0	15.0 ± 2.0
Spleen	4.0 ± 0.3	3.0 ± 0.2
Three months[b]		
Body	337.0 ± 12.0	292.0 ± 16.0
Thymus	590.0 ± 21.0	270.0 ± 8.0
Spleen	740.0 ± 27.0	420.0 ± 23.0

[a] Figures based on 20 animals per group. Taken from (44), by permission.
[b] Body weight given in g; thymus and spleen weights in mg.

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trauterine and postnatal periods, vary in sensitivity and reversibility to deprivation (39, 44, 76). Much of this work was conducted in our own laboratories and these data combined with the results of others clearly indicate that lipotropes, similar to if not identical in many respects to human PCM, have a profound effect on immunocompetence, particularly during selected periods of growth and maturation of the thymolymphatic system. This may have some bearing on susceptibility to cancer (see below).

LIPOTROPES AND XENOBIOTIC METABOLISM

In attempting to define how lipotrope deficiency promotes cancer, particularly of the liver, it is important to consider the influence of lipotropes on xenobiotic metabolism.

Table 6 Marginal methionine-choline deprivation in rats[a]

Assay	Control	Deprived during gestation and lactation
Splenic lymphocyte response to Con A (SI)	47.2±19.5[b]	18.7±16.0
PFC/10^5 lymphocytes	108.1±20.9	52.4± 9.3
Hemagglutinin titer	1:2560	1:640
Hemolysin titer	1:20560	1:640
PHA skin test[c] response (4 mo.)	3+	2+

[a] From 76.
[b] Results expressed ± S.D.
[c] Mononuclear cell infiltration scored from 0 (none) to 5 (severe).

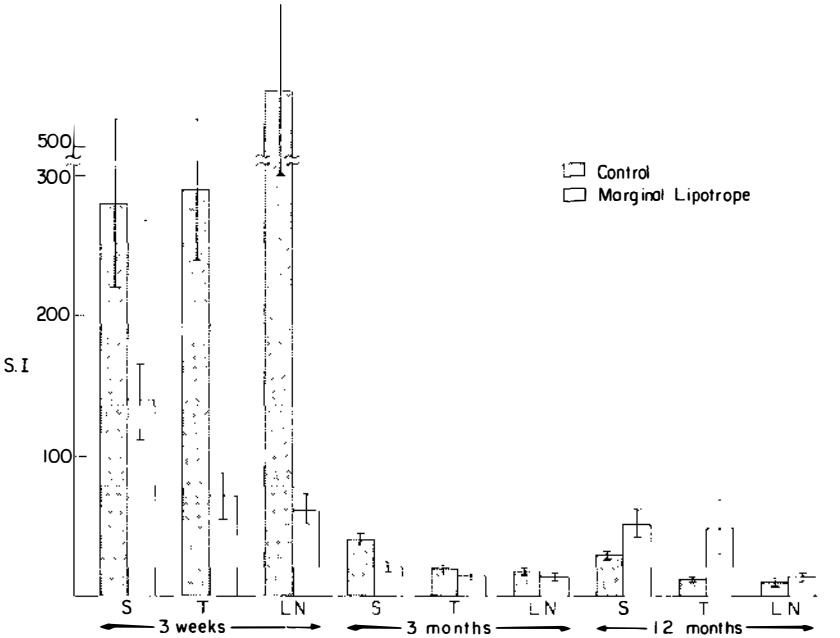

Figure 2 The response to concanavalin A in rats fed normal diets and diets marginal in supplies of methyl groups. *S* = spleen; *T* = thymus; *LN* = lymph node. From (39), with permission.

Microsomal oxidase activity of the liver is highly sensitive to lipotrope deficiency (Table 7) and its decrease may be responsible for modification of carcinogenesis there or at other sites (58, 59). However, we demonstrated that microsomal enzymes are inducible by a potent hepatocarcinogen, aflatoxin B_1 (AFB_1), in the liver of rats fed low lipotrope diets. We also showed in other studies (4, 58) that the metabolism of chemicals by the lipotrope-deficient rat liver is modified; the time at which metabolic activity is measured will produce variable results, however, as noted in Table 7. While AFB_1 and other hepatocarcinogenic chemicals induce enzymes that result in metabolic activation or deactivation, the magnitude of induction is reduced in lipotropic deficiency.

Others have shown a shifting of target organ to the liver by the choline-deficient diet (56), probably associated with local tissue metabolism, but this is yet to be documented.

Studies in vivo indicate that lipotrope deficiency alters carcinogen metabolism. Rats fed the low lipotrope diet are more sensitive to the toxicity of repeated doses of most hepatocarcinogens, including AFB_1 (Table 8) (59). They are, however, highly insensitive to toxicity of a single dose of AFB_1 (58).

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Table 7 Lipotropes and microsomal enzyme activity[a]

	Control[b]	Low lipotropes[b]
Microsomal protein		
90 days on diet	24.4 ± 1.5	18.7 ± 1.1
90 days on diet then 3 weeks AFB_1	28.4 ± 1.7	25.2 ± 1.8
Ethylmorphine *N*-demethylase		
90 days on diet	787 ± 79	572 ± 40
90 days on diet then 3 weeks AFB_1	1123 ± 93	1063 ± 104
Ethoxycoumarin *O*-dealkylase		
90 days on diet	0.350 ± 0.09	0.215 ± 0.03
90 days on diet then 3 weeks AFB_1	0.590 ± 0.14	0.509 ± 0.11
Cytochrome P450		
90 days on diet	1.8 ± 0.1	0.5 ± 0.1
90 days on diet then 3 weeks AFB_1	2.9 ± 0.4	2.1 ± 0.3
Cytochrome C reductase		
90 days on diet	66.2 ± 3.7	52.4 ± 3.5
90 days on diet then 3 weeks AFB_1	98.4 ± 5.2	88.6 ± 6.1

[a] Adapted from Campbell et al (4) and from additional studies conducted in our own laboratory). Details of diet composition are listed in Table 9 and references (41, 43, 58, 59).

[b] Microsomal protein measured in mg/kg; all others measured in nmole/mg protein.

This suggests that there is decreased metabolism of the chemical to its active, toxic form upon first exposure, but induction of metabolism by the carcinogen occurs following repeated exposures. This effect was not confirmed in the bacterial mutagenesis assays (68).

Other factors also impinge on the manner in which chemicals are activated or detoxified by biological systems. For example, it has been established that liver *S*-adenosylmethionine is decreased in lipotrope-deficient rats. GSH content of liver, however, does not appear to be affected (63). The reduction in SAM in rats fed the lipotrope-deficient diet is a direct result of the dietary treatment; this was confirmed by Mikol & Poirier (38) in rats fed diets deficient in one or more of the lipotropes. *S*-Adenosylmethionine may influence carcinogenesis by serving as a trap for electrophilic carcinogens or by playing a role in alkylation. Although the mechanisms of the interactions between methyl groups (lipotropes in general), xenobiotic metabolism, and neoplasia remain to be elucidated, the lipotropic regulatory role on metabolism of chemicals is likely of significance to cancer risk.

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Table 8 Chemical carcinogenesis in lipotrope deficiency[a]

Carcinogen[b]	Tumor site	Tumor incidence (%)	
		Control	Deprived
AFB_1	Liver	15	87
DEN	Liver	70	80
DBN	Liver	24	64
	Bladder	84	80
DMN	Liver	28	27
	Kidney	16	3
AAF	Liver	19	41
	Mammary	80	79
FANFT	Bladder	53	61
DMBA	Mammary	48	15
DMH	Colon	86	100

[a] From Rogers & Newberne (59), abridged.

[b] AFB_1, aflatoxin B_1; DEN, *N*-nitrosodiethylamine; DBN, *N*-nitrosodibutylamine; DMN, nitrosodimethylamine; AAF, *N*-2-fluorenylacetamine; FANFT, *N*-[4-(5-nitro-2-2furyl)-2-thiazolyl]formamide; DMBA, 7,12-dimetbylbenz[*a*]antbracene; DMH, 1,2-dimethylhydrazine.

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LIPOTROPES AND CANCER

Human Studies

There are numerous reports in the literature on the relationship of the lipotrope family to cancer in humans (26). A number of disorders in humans lead to modulation of lipotrope status and are associated with increased risk for cancer. The incidence of carcinoma of the stomach in patients with pernicious anemia is about three times that observed in the general population. Moreover, about 40% of patients with gastric cancer in one study had pernicious anemia (5). While it is not known that vitamin B_{12} deficiency in pernicious anemia is causally related to the increased incidence of stomach cancer, the association is significant. Mucosal atrophy and achlorhydria must also be considered.

A number of other reports have suggested that selected types of cancer may be associated with deficiencies of some of the lipotropes (e.g. leukemia and vitamin B_{12}; esophageal cancer and the B complex of vitamins, particularly folate). The associations have been put forth but the linkages have not been established (7, 67, 72, 81–83). The etiologies would appear to be much more complex than simple lipotrope deficits but the latter may have some important impact on the overall incidence of some types of cancer.

The countries where liver cancer occurs in highest incidence are also those areas where malnutrition (particularly protein, methionine, and vitamin B_{12},

and folate deficiencies) is common (52). These areas also have concomitant hepatitis B or other types of infection in high incidence, which suggests diminished immunocompetence (2). These liver cancer patients are predominantly male and have cirrhosis as well as hepatocellular carcinoma. The former is associated with protein-calorie malnutrition, and exposure to hepatitis B virus, as well as dietary contamination (2, 51, 52).

There are a number of immune defects in patients with chronic liver disease (52). For example, hyperglobulinemia and depressed cell-mediated immunity are common to most forms of chronic liver disease and probably secondary to liver damage. The anergic state seems to be related to increased activity of prostaglandin-secreting monocytes, which have suppressor functions, but it may also be due in part to a loss of T-suppressor cells.

It has been shown that many of the host defense systems are impaired when patients with chronic liver disease develop cirrhosis, effects insufficient in themselves to result in a significant rate of tumor development. However, when there are increased levels of carcinogenic stimuli such as environmental toxins, viruses, etc the tendency is for a more severe immunodeficiency to result. This then makes malignant transformation and evasion of immune surveillance more likely. The case for nutritional effects on xenobiotic metabolism and drug disposition in humans has been made by Vesell (73). This area of concern undoubtedly is associated with risks in human populations.

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Animal Studies

A deficiency of lipotropes associated with cancer was first reported from the laboratory of W. D. Salmon at Auburn University about thirty years ago (60). Rats maintained on choline-deficient diets (methyl group deficiency) for long periods of time developed hepatocellular carcinoma, an observation of far-reaching significance since it was a first instance in which removing something from the diet, instead of adding something (a carcinogen), resulted in cancer. The lipotrope deficiency caused a series of alterations in liver structure and function, including fatty liver, parenchymal cell hyperplasia, fibrosis, cirrhosis, and ultimately hepatocellular carcinoma in some of the animals. Accompanying these changes, as noted above, are alterations in xenobiotic metabolism and immunocompetence.

The discovery made by Salmon and co-workers was largely ignored for more than thirty years. Our work (41, 43) with diets using amino acids and intact proteins free of aflatoxin and other carcinogenic contaminants confirmed the work of Salmon and co-workers (see 47, 59 for more complete references). Others have duplicated our studies with results that document a diet-induced malignancy in lipotrope-deficient animals without a carcinogen superimposed (13a, 37). It is clear, however, that a diet low in lipotropes enhances experimental liver cancer in rats and mice (41, 43, 59). The following summary of

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experimental carcinogeneis in lipotrope-deficient animals is not encyclopedic because of space limitations. However, some of the more salient features of results are described. Our apologies go to the dedicated scientists working in the area in recent years whose important papers could not be included. Most of the following data are drawn from our own experiences.

Table 9 lists the diets most often used for lipotrope studies. The casein–peanut meal diet, assayed and determined to be free of AFB_1, is an excellently balanced diet and is useful because the methionine level is at the lower limits of a range satisfactory for normal growth of the rat and mouse. This then permits manipulation of the other three important lipotropes (choline, folate, vitamin B_{12}) and allows for gradations in the severity of the deficiency from severe (cirrhogenic) to mild. A mild deficiency results in no histologic evidence for injury; the only major measurable biochemical change is a small increase in liver lipid content.

A number of strains of outbred and inbred rats have been used for investigations of lipotrope deficiency–induced cirrhosis and its relationship to sensitivity of the liver to carcinogenesis. Rats of all strains, fed a diet low in lipotropes, develop fatty liver and cirrhosis similar if not identical to alcoholic or Laennecs cirrhosis in humans, who are at high risk for liver cancer. Figure 3 illustrates the appearance of a human liver with cirrhosis and hepatocellular carcinoma; compare this with the photograph of a rat liver in Figure 4. Mice of the inbred strains are also susceptible (43). Generally, the rodents have been fed a choline-free diet from weaning until cirrhosis develops, a period ranging from 200 to 500 days. In this animal model it is important that the rats or mice

Table 9 The diets most often used in lipotrope studies[a]

	Feed content (g/kg)	
	Diet 1 (control)	Diet 2 (deficient)
Casein	60	60
Peanut meal	250	250
Sucrose	467	470
Vitamin mix[b]	20	20
Mineral mix	50	50
Fat	150	150
Choline	3	0
Vitamin B_{12}	50 μg	0

[a] Amino acid diets have also been used for some studies (37, 46). The diet listed in Table 1 is a particularly good one because the combination of casein and peanut meal provides about 0.3 methionine, allowing for normal growth even in the choline-deprived group.

[b] Vitamin mix complete except for folic acid (in folate deficiency studies) and choline and vitamin B_{12}, which were added at time of mixing the diet.

consume the diet continuously from weaning until the study is terminated; starting the diet at a later period does not induce cirrhosis even though the liver is sensitized.

Using this regimen some rats succumb to the hemorrhagic kidney syndrome induced by choline deficiency, usually between 8 and 10 days following initiation of dietary treatment; beyond that time the clinical course is uneventful, although renal injury usually persists in most of the survivors. Mice (B6C3F1 strain), while resistant to the renal injury, do develop fatty liver, fibrosis, and hepatocellular carcinomas (43).

The development of cirrhosis in the acutely deficient rat or mouse proceeds through a series of histologically identifiable changes described in other publications (43, 46, 47). The earliest lesion in the liver is an accumulation of lipid in the centrilobular zone that occurs within a few days after initiating the diet. Lipid continues to increase and within 2–3 weeks has filled most cells of the lobule. This is accompanied by the appearance of bizarre nuclei, many of which contain intranuclear inclusions, and there is widespread single-cell necrosis throughout the lobe. It is at this point that mitotic figures and ^{3}H-thymidine labeling increases rapidly (Figure 5), as do focal accumulations of hyperchromatic (basophilic) proliferating parenchyma. The progression of le-

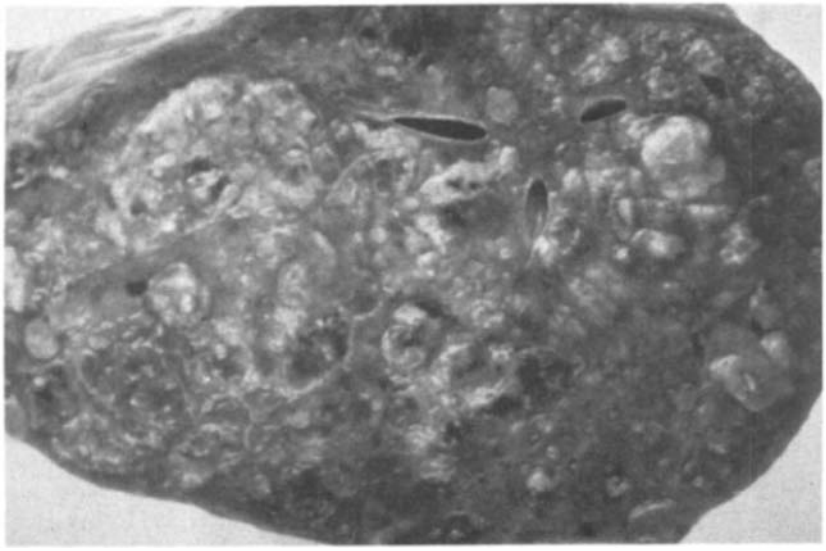

Figure 3 Photograph of human liver cancer accompanied by cirrhosis, which coexist in a large majority of such neoplasms. This type of cancer is associated with malnutrition, especially a deficit of protein and calories, with dietary contamination (AFB_1), and with infection, particularly hepatitis B virus.

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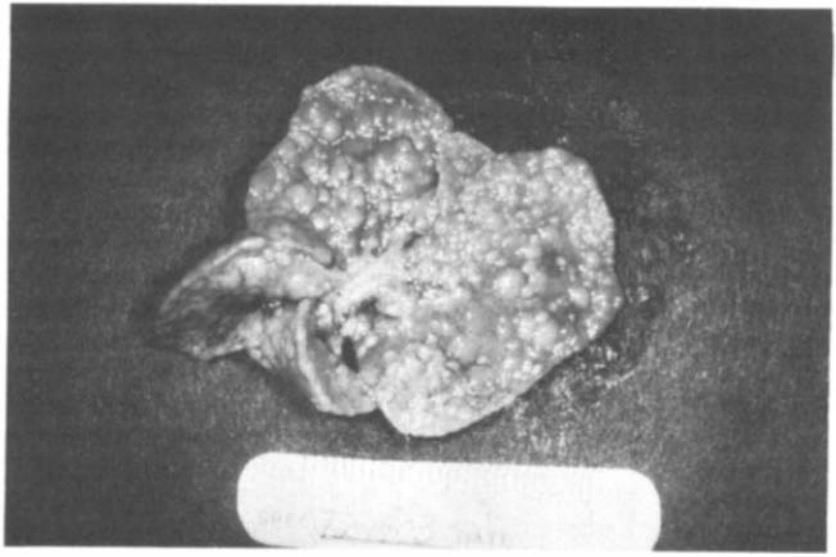

Figure 4 Liver from a rat fed a diet low in methyl groups and given aflatoxin B_1, a carcinogen contaminating a high percentage of diets in areas of the world where human liver cancer is the most common form of neoplasia. Note the similarity between Figures 3 and 4.

sions then follows a predictable series of changes including a pale, nodular liver with marked fibrosis and cirrhosis and with nodules that may or may not contain fat. Some of the lesions progress through atypical nodular hyperplasia, usually without lipid, to hepatocellular carcinoma (Figure 4) and then often metastasize to the lungs. These changes have been documented and illustrated in much greater detail in many publications. Table 8 lists the types of responses observed in some of our studies.

We have conducted additional investigations in attempts to determine mechanisms for injury and development of neoplasms. We have documented (41) that a diet low in lipotropes is sufficient to induce neoplasms, without superimposing a chemical carcinogen, although such injured livers are more sensitive to a number of hepatocarcinogens, as noted in Table 8.

In the choline-deficient liver as fat increases the number of cells labeled by [^{3}H]-thymidine also increases (Figure 5). In addition, we found that choline deficiency alone causes a sharp increase in cell death, as others have also reported (13). Despite the lipotrope deficiency, there is increased DNA synthesis and cell turnover, both of which are essential components of hyperplasia of the liver parenchyma. This implies the synthesis of defective genetic material, cell membranes that are imperfect, and other aberrant factors that can interfere with normal cell proliferation.

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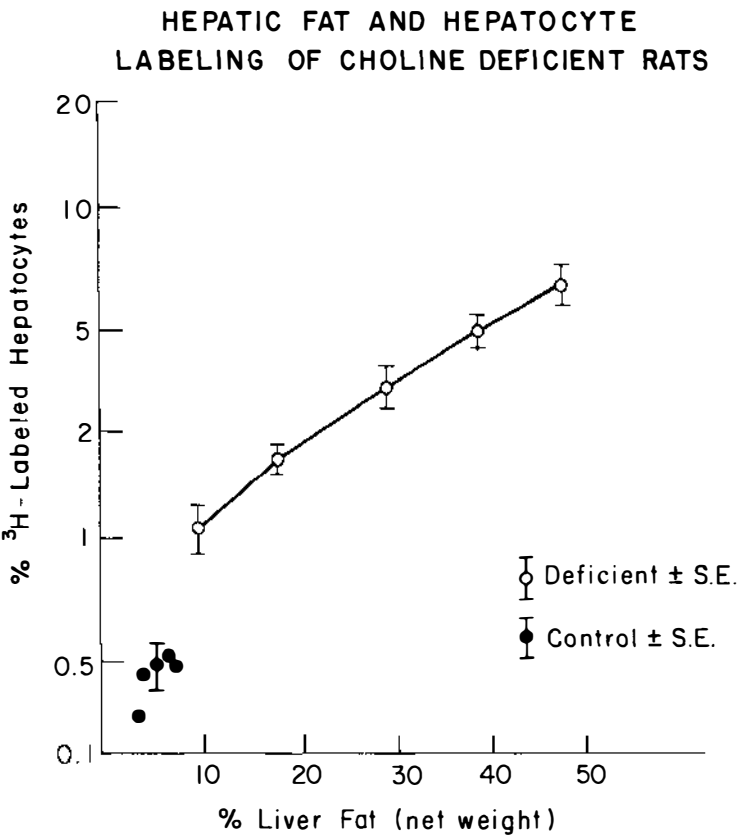

Figure 5 A deficiency of methyl groups results in lipid accumulation in the liver, parenchymal cell death, and progressive increase in DNA synthesis, an indication of cell proliferation. Liver cell necrosis has been confirmed by microscopy.

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DISCUSSION

Chemical induction of hepatocarcinoma is governed in part by the level of DNA synthesis in hepatocytes at the time of carcinogen exposure, but also following exposure. Several models have been described and are being used to explore biochemical and morphological aspects of this relationship. While mechanisms are unclear, the lipotrope deficiency lesions leading to hepatocarcinoma may in some way be related to the hepatic proliferation inhibitor, a chalone expressed by adult rat liver (22), or to the hepatic stimulator substance expressed by fetal and neonatal rat liver (30). Both of these substances have been described recently and are now under intense study.

The significance of cell division to proliferative liver lesions and hepatocarcinoma in lipotrope-deficient rats has been questioned by other investigators

(62). Shinozuka and colleagues found that an increase in preneoplastic changes in the deficient liver did not correlate with cell division. The level of cell proliferation in deficient livers was decreased by feeding phenobarbital, but appearance of preneoplastic lesions was unchanged. In addition, by reducing the amount of fat in the deficient diet the number of preneoplastic lesions was decreased without altering hepatocyte hyperplasia. Both alterations of the model must be confirmed in carcinogenesis studies, since the significance of the putative preneoplastic lesions to liver cell cancer is not clear and a confirmed linkage is yet to be established.

The above-referenced study, in which lower fat content was fed, is particularly interesting because lower levels of dietary fat reduce the severity of lipotrope deficiency and would be expected to decrease both hepatic fat and DNA synthesis; neither occurred in the Shinozuka studies. Further, using the same carcinogen, *N*-nitrosodiethylamine (DEN), we have found that lipotrope and lipid effects interacted but in a complex way not as yet fully elucidated.

Carcinogenesis may be increased where DNA synthesis is increased. This can be achieved by partial hepatectomy, by necrogenic chemicals, and by choline deficiency. In the choline deficiency model, partial hepatectomy had no effect on tumor incidence when the carcinogen AFB_1 was administered at different times with respect to the partial hepatectomy (57). Both partial hepatectomy and the choline-deficient diet increase DNA synthesis. This may be a factor in the enhancement of liver cancer by choline deficiency but the mechanism for enhancement is unclear. We feel that the significance of cell division to carcinogenesis requires further study before attempting to draw sweeping conclusions.

Lipoperoxidation has been considered by some to be related to the initiation of transformation of hepatocytes and promotion of hepatocarcinogenesis (14). Our laboratory published such a relationship more than 15 years ago (42) and confirmed the observations with more sophisticated techniques a few years later (79). The synthetic antioxidants BHA and BHT protected the kidney and liver from choline deficiency and largely returned serum and tissue lipids nearly to control values. Furthermore, the free radical index (FRI) and TBA values of the lipotrope-deficient liver clearly demonstrated the presence of lipoperoxidation in livers sensitized to hepatocarcinogens by the diet (79).

The laboratory of Poirier has published important data relative to the effect of lipotrope deficiency on fundamental cellular metabolism (37, 38). An effect of lipotrope deficiency on the concentration of *S*-adenosylmethionine, the obligatory source of methyl groups essential to methylation of important bases in DNA, suggests that lipotropes are concerned with control of cell proliferation. Deprivation of choline, methionine, or both resulted in decreased liver concentrations of SAM and an increased ornithine decarboxylase (ODC) activity,

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the latter essential to polyamine synthesis and important in regulation of cell proliferation. Generally, there was an inverse relationship between SAM and ODC; this bears on the induction of liver injury and, perhaps, promotion of carcinogenesis.

Additional data from Poirier's laboratory (78) and from our own investigations (Table 10) point toward hypomethylation. Wilson et al observed a 10–15% decrease in 5-methyldeoxycytidine in the deficient liver, but only after about six months on diet. Our studies (P. Punyarit, P. M. Newberne, unpublished, 1985) essentially confirm the data of Wilson et al, although our methods for examining effects on methylation were slightly different from theirs. We maintained rats on diet for up to six months, performed a partial hepatectomy at different time points to generate new DNA (and accompanying methylation), and two weeks later sacrificed the animals for DNA analyses. In agreement with Wilson et al (78), it was only after several months of continuous exposure to the lipotrope-deficient diet that we found significant hypomethylation of cytosine. This suggests hypomethylation may be a slow process; if it is involved in liver carcinogenesis, which occurs in this model, hypomethylation very likely requires chronic derangement of liver genetic material over long periods of time. Conversely, our analytical techniques may be too insensitive to detect hypomethylation in early stages.

The association of malnutrition, infection (hepatitis B virus), and dietary contamination with human liver cancer seems to be a realistic concept in attempts to understand mechanisms for this form of cancer. In those areas of the world where hepatocellular carcinoma is highest, liver disease, infection, and dietary contaminants are indigenous. There are some features of the liver disease and cancer seen in these areas that are similar to the choline-deficient animal model for cirrhosis and liver cancer. Fatty liver, fibrosis, increased hepatocyte turnover, nodular regeneration, cirrhosis, and, in some cases,

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Table 10 Lipotropes and 5-methylcytosine in liver DNA[a]

Time on diet	5-methylcytosine as % of cytosine	
	Control	Deficient
3 weeks	4.8 ± 0.09	4.5 ± 0.11
3 months	4.4 ± 0.13	4.7 ± 0.20
6 months	4.5 ± 0.10	3.3 ± 0.06

[a] From P. Punyarit and P. M. Newberne, 1985, unpublished. Two weeks prior to sacrifice a 2/3 partial hepatectomy was performed. We are indebted to Dr. Ronald Shank, University of California, Irvine, for some of the DNA analyses listed in this table.

hepatocellular carcinoma are common to the human and animal disease. Thus the choline-deficient animal model may provide a means for identifying factors that modulate or influence this important form of cancer (1a, 51).

In western countries, and in particular, the United States, chronic alcoholism seems to be the most common condition associated with the genesis of both cirrhosis and liver cell cancer. The choline deficiency rat liver model for cirrhosis is histologically similar if not identical to alcoholic cirrhosis in human patients and, as such, provides a convenient means for exploring mechanisms by which cirrhosis and hepatocellular carcinoma develop in humans. It should be pointed out here again that malnutrition (lipotrope deficiency), liver injury (i.e. hepatitis B infection), and dietary contamination with aflatoxins and other hepatotoxins coexist in areas with the greatest frequency of human hepatocellular cancer. Figure 6 indicates our unifying concept of how a deficiency of lipotropes, represented by malnutrition, may interact with other important factors in the complex, probable etiology of liver cancer. It is through a thorough study of the phenomena represented in Figure 6 that we may elucidate the mechanisms for lipotrope deficiency enhancing carcinogenesis.

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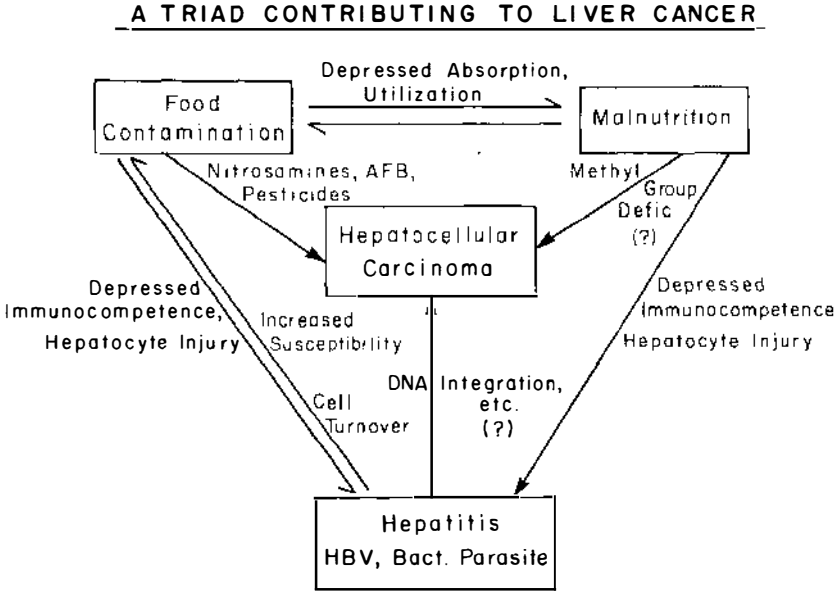

Figure 6 This figure depicts a unifying concept of a triad of conditions that contribute to liver cell cancer in many areas of the world: food contamination, malnutrition with methyl group deficiency, and infection (especially with hepatitis B virus). With increased cell turnover but without adequate materials (methyl groups) for synthesis of macromolecules and other components, errors in replication of genetic material are more likely, with loss of control over cell proliferation.

CONCLUSIONS

We do not know the mechanisms whereby lipotrope deficiency (and thus methyl group deficit) influences carcinogenesis. While the situation is unclear in human cancer, in animals the effects are more than promoting, however; at least three different laboratories have independently induced hepatocellular carcinoma in rodents with lipotrope deficiency alone. The deficient diets are characterized by marginal levels of certain essential amino acids, particularly methionine, in addition to deficiencies of choline, folate, and vitamin B_{12}. The relative importance of these factors in carcinogenesis varies with the carcinogen used to study them. Activity of *N*-2-fluorenylacetamide, a carcinogen used in many of the modeling studies cited above, is profoundly affected by choline and methionine but not detectably influenced by dietary fat content; AFB_1 and DEN results are affected by dietary fat content. Tumor induction varies considerably and yields complex results showing effects of lipotropes, amino acids, and fat, all of which interact in determining the final tumor incidence with these carcinogens.

Lipotropes influence immunocompetence and xenobiotic metabolism, and they are involved in the regulation of cell turnover in a number of tissues. A deficiency is also associated with a high incidence of liver disease, hepatitis B infection, and liver cancer in many areas of the world. We believe that the interactions between sensitive cells and carcinogens, resulting in the neoplastic change, are complex, not simple. Lipotropes impinge on a number of cell activities that can result in uncontrolled proliferation when lipotropes, and thus methyl groups, are in short supply.

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