The manner in which cobalamin deficiency produces its effects in humans has not been established. The methylfolate trap hypothesis proposed more than 20 years ago has been widely accepted despite the difficulty in testing the hypothesis in any meaningful way.

**The Methylfolate Trap Hypothesis**

The methylfolate trap hypothesis is based on a cobalamin requirement for the transfer of the methyl group of methyltetrahydrofolate (methylfolate, 5-CH₃-H₄PteGlu) to homocysteine to yield methionine (reaction 1 and Fig 1).

1. 5-CH₃-H₄PteGlu + cobalamin + homocysteine → H₄PteGlu + methionine.

In the absence of cobalamin the reaction is impaired, the methyl group remains on tetrahydrofolate (H₄PteGlu), and a critical lack of free tetrahydrofolate develops. As a result, single-carbon units such as formate and methylene cannot be taken up because of lack of a folate acceptor and synthesis of purines requiring formate, and that of thymidine requiring methylene, is impaired. The thermodynamics of the reaction by which methylfolate is formed (reaction 2) strongly favors the formation of methylfolate and oxidation of the methyl group to methylene is considered unlikely.

2. 5,10-CH₂H₄PteGlu → 5-CH₃-H₄PteGlu

Thus the methylfolate trap hypothesis proposes that in cobalamin deficiency tetrahydrofolate is trapped in the methyl form. In tissue culture in which the supply of cobalamin is limited, there is an accumulation of methylfolate, although other forms of folate do not disappear.

**Methylfolate “Trapping” In Vivo**

Cobalamin-deficient sheep and rats show a fall in liver folate concentration, and the rate of fall of methylfolate is slower than that for the nonmethylfolates. In humans, serum and red cell folates are almost all methylfolate derivatives. In untreated pernicious anemia, the serum folate may be elevated, but the red cell folate is often low. The low liver folate in animals and the low red cell folate in humans indicate that folate deficiency develops in primary cobalamin lack. The data also imply that methylfolates are better retained than nonmethylfolates, but do not help in establishing a methylfolate trap mechanism.

**Deoxyuridine Suppression Test**

The deoxyuridine (dU) suppression test, in which the methylation of deoxyuridine to form thymidine is assessed, offers an opportunity to test the effect of various folate analogues in this pathway. Both folate and cobalamin are required for this step, and the test is abnormal in their absence. Formyltetrahydrofolate (CHO-H₄PteGlu) corrected the impairment of thymidine synthesis in both cobalamin and folate deficiencies in marrow cells in humans (Fig 2). Methylfolate did not correct the defect in untreated pernicious anemia. Like so many similar observations, the data are compatible with a methylfolate trap. However, methylfolate was not used very well by folate-deficient cells. Significantly, tetrahydrofolate itself was not used normally by cobalamin-deficient cells. These data thus do not help in determining whether methylfolate is trapped, but do indicate that some data are not explained by the methylfolate trap hypothesis, namely, the failure to utilize “untrapped” tetrahydrofolate.

**Oxidation of the Methyl Group**

Several observations suggest that the methyl group in methylfolate may be oxidized, that is, that there may be an alternative way of using the methyl group other than transferring it to homocysteine. Thorndike and Beck reported that the methyl group of methylfolate was oxidized in an essentially similar manner both by lymphocytes from normoblastic subjects and the lymphocytes from one patient with untreated pernicious anemia.

Methylfolate can serve as the methyl donor in the methylation of biogenic amines, and the mechanism involve oxidation of the methyl group to formate, which in turn is transferred to the biogenic amine. The enzyme responsible...
The synthesis of methyl groups occurs by reduction to methyl of a single carbon taken up by tetrahydrofolate. The methyl group is transferred to cobalamin and then to homocysteine to form methionine. The enzyme mediating this methyl transfer is methionine synthetase.

for oxidation of methylfolate was methylenetetrahydrofolate reductase (reaction 2). This has been interpreted as an unphysiological situation, the biogenic amine providing an artificial acceptor enabling reaction 2 to go to the left in a manner similar to that found by Thorndike and Beck.

Recent evidence suggests that the methionine level regulates the rate of methylfolate oxidation. Raising the level of methionine produced rapid oxidation of the methyl group of methylfolate to carbon dioxide. This is discussed below. It is unlikely therefore that the shift to the right in reaction 2 by which methylfolate is formed applies to the in vivo situation.

A Suitable Animal Model

Patients with untreated pernicious anemia do not lend themselves readily to studies on mode of action of cobalamin and methylfolate trapping. No suitable animal model for cobalamin deficiency has been available until the last few years, although outstanding observations were made in sheep reared on cobalt-deficient wheat hay-chaff and in monkeys. Others have used rats maintained on cobalamin-deficient (usually soy-bean) diets, or fruit bats fed on clean, and hence cobalamin-free, fruit. The development of cobalamin deficiency in these species took months or even years. The extensive studies of Stokstad and his colleagues using cobalamin-deficient rats have been reviewed.

THE NITROUS OXIDE-EXPOSED RAT AS A MODEL FOR COBALAMIN "DEFICIENCY"

Inhalation of nitrous oxide (N\textsubscript{2}O) in humans produces megaloblastic hemopoiesis and, if continued for several days, has proved to be fatal. Interruption may be fatal. Intermittent inhalation of N\textsubscript{2}O over weeks produces a neuropathy similar to that encountered in cobalamin deficiency. In vitro, the impairment of thymidine synthesis by marrow cells from patients anesthetized with N\textsubscript{2}O is corrected by the addition of cobalamin to the incubation mixture, indicating that N\textsubscript{2}O is producing its effect through cobalamin.

Nitrous oxide is activated on contact with organometallic compounds, of which cobalamin is a prime example. The N\textsubscript{2}O molecule is cleaved, releasing free nitrogen, and the cobalt atom of cobalamin is oxidized to the inert cobalamin (reaction 3).

3. Cob\textsubscript{a}[I]alamin + N\textsubscript{2}O$\rightarrow$ cob\textsubscript{a}[III]alamin + H\textsubscript{2}O + N\textsubscript{2}.

The rapid diffusion of inhaled N\textsubscript{2}O through all cells ensures that cobalamin inactivation is complete. Nitrous oxide, in fact, specifically inactivates the enzyme methionine synthetase, of which cobalamin is a coenzyme. The rapidity and ease with which small animals can be exposed to N\textsubscript{2}O make it possible to perform detailed studies on the effects of loss of cobalamin function. Following cobalamin inactivation, hemopoiesis in the rat remains normoblastic. Megaloblastic hemopoiesis in cobalamin deficiency is unique to humans. However, the biochemical effects are very similar in both species.

The overall effects of nitrous oxide on cobalamin and folate metabolism, and on pathways that depend on these coenzymes, are listed in Table 1.
activity falls sharply within 30 minutes of administration of COBALAMIN-FOLATE INTERRELATIONSHIPS

Table 1. Effects of Cobalamin Inactivation

| Primary and persistent defect | 1. Impairment of methionine synthetase |
| Transient defects appearing within 24 h and disappearing within 4 d | 2. Impaired cellular uptake of 5-CH₂H₂PteGlu and other folic acids |
| 1. Fall in methionine concentration in plasma and liver | 2. Cessation in folate polyglutamate synthesis from tetrahydrofolate with rise in activity of folate polyglutamate synthetase |
| 3. Fall in GAR transformylase and rise in AICAR transformylase, both involved in purine synthesis | 4. Relative accumulation of methyltetrahydrofolate polyglutamate |

Defects still present 1 to 20 d later
1. Reduced serum and liver cobalamin levels
2. Increase in microbiologically inactive cobalamins (cobalamin analogues) in liver
3. Raised plasma folate
4. Low tissue folate
5. Low concentration of S-adenosylmethionine in liver
6. Impaired thymidylate synthesis (abnormal dU suppression test)
7. Rise in activity of 10-formyltetrahydrofolate synthetase and a fall in 5,10-methenyltetrahydrofolate cyclohydrolase
8. Rise in AICAR transformylase
9. Megaloblastic hemopoiesis in humans (still present at 7 d)
10. Neuropathy in humans, monkeys, and fruit bats

Pathways tested and found to be unaffected
1. Urinary methylmalonic acid excretion
2. Serine hydroxymethyltransferase (serine → glycine reaction)
3. 5,10-methyltetrahydrofolate dehydrogenase and reductase, 5-formiminotetrahydrofolate transferase
4. Brain levels of methionine and S-adenosylmethionine
5. Hemopoiesis in the rat and fruit bat

Pathways induced in response to cobalamin inactivation
1. Betaine homocysteine methyltransferase
2. Oxidation of the methyl group of 5-methyltetrahydrofolate
3. Folate polyglutamate synthetase
4. 10-formyltetrahydrofolate synthetase
5. Thymidylate synthetase

**Methionine Synthetase**

In the Sprague-Dawley rat, liver methionine synthetase activity falls sharply within 30 minutes of administration of N₂O. The enzyme remains inactive as long as exposure to N₂O is continued (Fig 3). Methionine synthetase activities in brain, testis, small gut (unpublished observations) and marrow are equally affected, as is fetal methionine synthetase, when pregnant rats are given N₂O. Similarly, nitrous oxide anesthesia in humans is accompanied by a fall in hepatic and marrow methionine synthetase activity. Marrow cells from patients with untreated pernicious anemia have low methionine synthetase activity.

**Methylmalonyl-CoA Mutase**

In cobalamin deficiency in humans or animals, there is impaired conversion of methylmalonic acid to succinic acid so that increased amounts of methylmalonic acid are excreted into the urine. When the urinary excretion of methylmalonic acid was used as an index of activity, no effect of N₂O in rats was detected even after the pathway was stressed by supplying propionic acid, a precursor of methylmalonic acid. Exposure of rats to N₂O for several weeks was accompanied by a fall in mutase activity in liver and a rise in MMA in serum, presumably due to the loss of cobalamin as the oxidized cobalamin is not replaced. There is an unconfirmed report of increased urinary excretion of methylmalonic acid in patients anesthetized with N₂O. A relatively nonspecific colorimetric method for assay of methylmalonic acid was used. The absence of any immediate effect of N₂O on the mutase is probably related to the likelihood that adenosylcobalamin coenzyme is not in the reduced state on the mutase and hence is not directly susceptible to the oxidative effect of N₂O.

**Cobalamin Levels**

The rat shows a steady fall in the plasma concentration of cobalamin while exposed to N₂O. There is, however, a marked increase in the plasma concentration of microbiologically inactive cobalamins (so-called cobalamin analogues) within the first 24 hours of N₂O exposure. The liver concentration of cobalamin falls markedly with an increase in the proportion of cobalamin analogues.

**EFFECT OF N₂O ON FOLATES AND ON FOLATE-DEPENDENT REACTIONS**

**Serum Folate**

As in untreated pernicious anemia, rats exposed to N₂O show a rise in the plasma folate level. There is impaired cellular uptake of methylfolate, the transport form of folate, by both cobalamin-deficient human lymphocytes and human marrow cells obtained from patients with pernicious anemia; this appears to be the explanation for the raised serum folate level in humans. In the N₂O-exposed rat, there is impaired hepatic uptake of labeled methylfolate from plasma. The impairment of folate uptake could be a direct effect of cobalamin deficiency on cell membranes, although this aspect remains to be explored.
Folate-Interconverting Enzymes (Table 2)

Folate is concerned in the transfer of single carbon units for the synthesis of purines, pyrimidines, and methionine. For purine synthesis, an oxidized carbon (—CHO, formate) is required; for methionine synthesis, a fully reduced carbon (—CH3, methyl) is required, and for thymidylate synthesis a carbon in an intermediate state of reduction (—CH2, methylene) is necessary. The state of reduction of the single carbon transferred by folate is altered by a series of enzymes.

Of the five enzymes, two are affected by N2O exposure (Fig 5). These two enzymes involve uptake of formate by tetrahydrofolate and the reduction of formate.53 Formyltetrahydrofolate synthetase attaches formate to tetrahydrofolate and its activity in liver increases on exposure to N2O. Methenyltetrahydrofolate cyclohydrolase reduces formate (CHO-) to methenyl (CH=). Its activity falls in the N2O-exposed rat. Other enzymes which further reduce methenyl to methylene and to methyl remain unaffected. The data are compatible with a lack of formate and a need to conserve formylfolate.

Folate-Mediated Pathways

The folate-mediated pathways are concerned with the transfer of single carbon units from serine, glycine, and histidine, and the synthesis of methionine, purines and thymidine (Table 2). Methionine synthesis has been discussed.

Histidine catabolism. Carbon-2 of the imidazole ring of histidine is transferred as a formimino (—CH=NH) group to tetrahydrofolate. In the N2O-exposed rat, there is an increased urinary excretion of formiminoglutamic acid, implying impaired transfer of —CH=NH to tetrahydrofolate although the enzyme formiminotetrahydrofolate transferase is unaffected.54

Purine synthesis. Carbons 2 and 8 of the purine nucleus are donated as formyl groups from 10-formyltetrahydrofolate. With N2O exposure, there is a decline in the activity of

\[
\begin{array}{|c|c|c|}
\hline
\text{Enzyme} & \text{Reaction} & \text{Effect of N}_2\text{O} \\
\hline
\text{Formyltetrahydrofolate synthetase} & \text{CHO} \rightarrow \text{10-CHO-H}_4\text{PteGlu} & \text{Increased} \\
\text{5, 10-methenyltetrahydrofolate cyclohydrolase} & \text{10-CHO-H}_4\text{PteGlu} \rightarrow \text{5, 10-CH==H}_4\text{PteGlu} & \text{Reduced} \\
\text{5, 10-methenyltetrahydrofolate dehydrogenase} & \text{5, 10-CH==H}_4\text{PteGlu} \rightarrow \text{5, 10-CH3-H}_4\text{PteGlu} & \text{None} \\
\text{5, 10-methenyltetrahydrofolate reductase} & \text{5, 10-CH3-H}_4\text{PteGlu} \rightarrow \text{5-CH2-H}_4\text{PteGlu} & \text{None} \\
\text{5-formiminotetrahydrofolate transferase} & \text{5-CH==NH-H}_4\text{PteGlu} \rightarrow \text{5, 10-CH==H}_4\text{PteGlu} & \text{None} \\
\text{Serine transhydroxymethylase} & \text{1-serine} \rightarrow \text{glycine} + \text{5, 10-CH2-H}_4\text{PteGlu} & \text{None} \\
\text{Glycinamide ribonucleotide transformylase} & \text{glycinamide ribonucleotide} \rightarrow \text{formylglycinamide ribonucleotide} & \text{Decreased} \\
\text{5-amino-4-imidazole carboxamide ribonucleotide transformylase} & \text{AICAR} \rightarrow \text{inosinic acid} & \text{Increased} \\
\text{Thymidylate synthetase} & \text{Deoxyuridine monophosphate} \rightarrow \text{deoxythymidine monophospate} & \text{Increased} \\
\text{Methionine synthetase} & \text{Homocysteine} \rightarrow \text{methionine} & \text{Decreased} \\
\hline
\end{array}
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COBALAMIN-FOLATE INTERRELATIONSHIPS

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tetrahydrofolate polyglutamate. Methenyltetrahydrofolate cyclodine as assessed in the dU suppression test, and the defect, as

group to deoxyuridine monophosphate. Despite increased

hepatic GAR transformylase (adding carbon 8) and a rise in hepatic AICAR transformylase (adding carbon 2).55

Thymidylate synthesis. There is a considerable increase in the activity of thymidylate synthetase in narrow cells in the N2O-exposed rat.56 This enzyme transfers a methylene group to deoxyuridine monophosphate. Despite increased enzyme activity, there is impaired methylation of deoxyuridine as assessed in the dU suppression test, and the defect, as in humans,26,27,34,57 is corrected by adding cobalamin.14,26,28-40

The data suggest a shortage of single carbon units for both purine and pyrimidine synthesis or an inability to accept and utilize such single carbon units.

Serine catabolism. Serine is a major source of single carbon units, and the enzyme transferring carbon-3 of serine to tetrahydrofolatepolyglutamate, serine hydroxymethyltransferase, is unaffected49 in N2O-exposed rats. Because there is no impairment in the serine pathway, which is believed to be the principal source of methylene groups for methylation of deoxyuridine, it is surprising that this pathway does not meet the requirements for methylene.

Folate Polyglutamate Synthesis

Carbon-unit transfers in vivo are mediated not by folate monoglutamate but by a form that has additional glutamic acid residues. Folate monoglutamates are the circulating forms in body fluids, and the folate polyglutamates are the intracellular coenzymes. Cell mutants that are unable to synthesize folate polyglutamate are also unable to synthesize methionine from homocysteine.43 The fall in the red cell folate in untreated pernicious anemia is due entirely to a fall in folate polyglutamates.64,65

The N2O-exposed rat is unable to form folate polyglutamate from pteroylglutamic acid,48,64 tetrahydrofolate,66 and methyltetrahydrofolate.66 However, polyglutamate formation is entirely normal in N2O-exposed rats when the substrate is 5-formyltetrahydrofolate or 10-formyltetrahydrofolate and 5,10-methylenetetrahydrofolate66 (Table 3). Thus, formylfolate appears to be the required substrate for the addition of glutamic acid residues, at least in cobalamin inactivation, and the role of cobalamin appears to be that of provision of formate for the formation of formyltetrahydrofolate.67

ROLE OF METHIONINE AND ITS DERIVATIVES

The primary defect resulting from cobalamin inactivation is impairment of the synthesis of methionine. Methionine reverses or ameliorates many of the effects of cobalamin deficiency in rats, sheep, and humans.

Methionine and S-Adenosylmethionine Levels

There is a transient fall in plasma and liver methionine after N2O exposure, which recovers after a few days following the induction of the alternative betaine methyltransferase pathway for methionine synthesis (Fig 3). S-adenosylmethionine in liver continues to fall.

Urinary Formiminoglutamic Acid

Daily oral methionine produces a temporary fall in urinary formiminoglutamic acid excretion in untreated pernicious anemia.71,72 A similar effect is obtained in cobalamin-deficient rats33,74 and N2O-treated rats.54 Methionine also reverses the impaired 14CO2 excretion from 14C-histidine in N2O-exposed rats.21,75

Formyltetrahydrofolate Levels in Liver

Noronha and Silverman2 showed that methionine given to rats produced a large increase in liver formylfolic acids at the expense of methylfolic acids.

This has been confirmed by others76-79 and most recently by Brody et al.21 Thirty minutes after an injection of methionine to N2O-exposed rats, liver methylfolic acid had fallen from about 38% to 2%. Formylfolic acids present in low concentration in rats not receiving methionine, rose to 34%, and tetrahydrofolatepolyglutamates increased from 35% to 55%.

S-adenosylmethionine, formed in increased amount following an injection of methionine,66 inhibits further synthesis of methylfolic acid. But the half time of the methyl group of methylfolic acid in N2O-treated rats is 43 hours, and it is

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<th>Table 3. Air-Breathing Rats and Rats Breathing N2O for 24 Hours Given a Dose of Labeled Folate Analogue</th>
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<td>Substrate</td>
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<tr>
<td>PteGlu</td>
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<tr>
<td>H4PteGlu</td>
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<tr>
<td>5—CH3—H4PteGlu</td>
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<td>10—CHO—H4PteGlu</td>
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<td>5,10—CH=H4PteGlu</td>
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Total uptake by liver and the percentage converted into folate polyglutamate was measured.
difficult to explain the rapid effect of methionine in producing disappearance of liver methylfolate on the basis of the cessation of synthesis of new methylfolate. It is far more probable that two events took place: first, methionine stimulated rapid oxidation of the methyl group on methyltetrahydrofolate to formate and CO₂ and, second, methionine supplied formate units (see below), facilitating the synthesis of formylfolate. Indeed, the level of methionine appears to determine rate of oxidation of the methyl group of methyltetrahydrofolate.

**Folate Polyglutamate Synthesis**

Methionine increased the levels of folate polyglutamates in liver²⁷⁻²⁹ and decreased the loss of labeled folates into urine.⁷⁴

Methionine reverses the impaired folate polyglutamate synthesis in N₂O-exposed rats (Fig 6).⁶² S-adenosylmethionine was less effective at an equivalent dose but was equally active at a higher dose, probably reflecting poorer cellular uptake of S-adenosylmethionine as compared with methionine.⁶⁰ The most effective compound for restoring folate polyglutamate synthesis in cobalamin-inactivated rats was 5-methylthioadenosine (5 MTA) (Fig 6). This is formed from methionine along the pathway leading to the synthesis of polyamines (Fig 7). Methylthioadenosine in turn is converted to methylthioribose. The latter is reconverted to methionine with release of formate.⁸³ It was concluded that formate derived from methionine was used to convert tetrahydrofolate to formyltetrahydrofolate, and the latter was used as substrate for folate polyglutamate synthesis.⁶² In addition, methionine yields formate by oxidation of its methyl group. Thus, sodium formate suppressed the conversion of the methyl carbon of methionine to CO₂ by liver homogenate to only 4% of control values but did not affect the percentage of carboxyl carbon of methionine recovered as CO₂.⁸² This transamination pathway involves conversion of methionine to α-keto-γ-methylbutyrate, 3-methylthiopropionate, methanethiol with production of a 3-carbon fragment, and formaldehyde with release of hydrogen sulfide and formate.⁸²

**Other**

Methionine partially protected N₂O-treated monkeys⁸³ and N₂O-treated fruit bats⁸⁴ from developing neuropathy. Methylthioadenosine prevented the fall in GAR transformylase following N₂O exposure.⁵³ Methionine, in appropriate dose, corrected the abnormal deoxyuridine suppression test in cobalamin-deficient marrow cells,⁶⁴ but larger doses of methionine had the opposite effect.⁶⁷

**Effect of Other Methyl Group Donors**

Methyl group donors other than methionine were effective in reversing cobalamin deficiency or inactivation. These include S-adenosylmethionine, choline, betaine, sarcosine,⁶² and ethionine.⁶⁴ However, serine and glycine, which contribute single carbon units as methylene groups, were ineffective.⁶²

**SIGNIFICANCE OF FORMATE**

Formate can arise from the metabolites of methionine, as well as from oxidation of methyl groups of sarcosine, methanol, and methylchloride, from glycine and serine, from tryptophan, and from other sources such as glycolate, acetone, and glyoxylate. All these compounds are unlikely to be of equal biological significance. Formate may be used either as single carbon units for synthesis or may be oxidized to CO₂.⁹² All these pathways require folate coenzymes.

Utilization of formate is impaired in cobalamin deficiency. There was reduced incorporation of [1⁴C]-formate into serine by lymphocytes from patients with cobalamin deficiency.⁹⁰⁻⁹¹ [1⁴C]-Formate, given to rats exposed to N₂O for two hours, disappeared from the blood at half the control rate with reduced oxidation to [1⁴CO₂].⁵² The reduced rate of [1⁴C]-formate oxidation in rats and monkeys was restored by methionine⁵¹⁻⁵³ as well as by ethionine. There was impaired formate incorporation into DNA in N₂O-exposed rats and increased urinary excretion of labeled formate.⁹⁴

Different tissues are not able to utilize all sources of formate to an equal degree. In N₂O-exposed rats, neither
serine nor sodium formate was effective in restoring folate polyglutamate synthesis in liver, but methionine and methylthioadenosine were effective. On the other hand, rat small gut used sodium formate, methionine, and methylthioadenosine to formylate tetrahydrofolate but not serine (unpublished observations). Impairment in GAR transformylase activity in rat marrow was overcome by methylthioadenosine but not by methionine.

**ADAPTATION TO COBALAMIN INACTIVATION**

Rats exposed to N$_2$O remain well. Mice have been kept in an N$_2$O environment through several generations over a period of two years by Eger in San Francisco (J.F. Nunn, personal communication). Clearly, any vital pathways that are impaired by the initial exposure to N$_2$O are bypassed. There are at least two mechanisms; the induction of an alternative pathway to methylate homocysteine, and the oxidation of methylfolate back to methylene and formate.

**Induction of Betaine Homocysteine Methyltransferase**

An alternative pathway for the methylation of homocysteine to form methionine is the induction of betaine homocysteine methyltransferase, betaine supplying the methyl group instead of methylfolate. The enzyme is present in mammalian liver but not in other tissues and, in particular, is absent in brain. There is a rise in the activity of liver betaine methyltransferase within 24 hours in rats exposed to N$_2$O (Fig 3), which is followed by a rise in methionine levels in plasma and liver. Hepatic S-adenosylmethionine, however, continues to fall both in the N$_2$O-treated rat and in dietary-induced cobalamin deficiency in sheep.

**Escape From the "Methylfolate Trap"**

Utilization of the methyl group in methylfolate was assessed by giving rats a mixture of [methyl-3H]-methylfolate and methyl tritiated folate. The half time of the methyl group in livers of air-breathing rats was two hours with no evidence of direct oxidation of the methyl. The methyl group was presumably passed on to homocysteine to form homocysteine.

There was no significant shift of the methyl group from methylfolate in rat liver for the first 72 hours after N$_2$O exposure. In rats exposed to N$_2$O for three days and six hours after an injection of double-labeled methylfolate, 40% of the methylfolate was unchanged, 39% of the methyl had been converted into folate polyglutamate, and 20% passed on to nonfolate compounds. The half time of the methyl group of methylfolate in N$_2$O-breathing rats was 43 hours in the absence of added methionine. The fall in activity of methylenetetrahydrofolate cyclohydrase on N$_2$O exposure, may shift reaction 2 to the left. There is no induction of methylenetetrahydrofolate reductase. The delay of three days in utilizing the methyl group of methylfolate in N$_2$O-treated rats corresponds to the time for recovery of methionine levels following induction of betaine methyltransferase.

Methylfolate is a less efficient substrate for folate polyglutamate formation in N$_2$O-treated rats than formyltetrahydrofolate, with only about 30% changed to polyglutamate in six hours as compared with about 50% in controls. These data in part explain the slow turnover of methylfolates in cobalamin inactivation, as the chain length required for further metabolism of the methyl group appears to be hexapolyglutamate and heptapolyglutamate. However, the main factor in determining the reduced rate of methyl folate oxidation is the reduced level of methionine.

**EFFECT OF COBALAMIN INACTIVATION ON THE CNS**

There is an interesting species susceptibility to the effects of cobalamin-inactivation or deficiency. Humans develop both megaloblastic hemopoiesis and neuropathy. Monkeys and fruit bats develop neuropathy but there is no effect on hemopoiesis, whereas in the rat and mouse neither system is obviously affected. Nevertheless, the close similarity between biochemical effects in the rat, and, where these pathways have been tested, in humans, suggests that the basic biochemical pattern is similar in all species but that the consequences depend on the severity of the defect and the extent to which compensatory pathways are induced.

Monkeys and fruit bats develop a neuropathy in response to N$_2$O exposure. The effects are considerably ameliorated by methionine. Nevertheless, fruit bats receiving N$_2$O and methionine have an unexpectedly high mortality, indicating that methionine does not restore the status quo at all. This may be related to the observation that methionine does not improve the cellular uptake of folates in cobalamin-inactivated animals to any significant extent.

Brain methionine synthetase in the rat is inactivated on exposure to N$_2$O in the same way as is the liver enzyme. Brain does not have a betaine homocysteine methyltransferase pathway. Nevertheless, the level of methionine and S-adenosylmethionine in rat brain does not fall over an 11-day period of N$_2$O exposure and was even elevated in fatal neuropathy in fruit bats. Studies with [methyl-3H]-methionine have shown that the maintenance of brain methionine levels was due to increased uptake of the amino acid by brain from plasma at the expense of its uptake by liver (unpublished observations). The absence of a fall in S-adenosylmethionine makes it unlikely that impairment of transmethylation in brain is the explanation of the neuropathy. Furthermore, the N$_2$O-treated rat does not show any impairment of methylation of arginine 107 of myelin basic protein (submitted for publication), which has been proposed as the lesion in cobalamin neuropathy.

Brain is able to maintain its folate concentration despite considerable falls in other tissues. There is an initial decline in brain folate concentration that is reversed after four days despite continued N$_2$O exposure.

**HOW TO EXPLAIN THE DATA**

A variety of hypotheses have been advanced to explain the effect of cobalamin deficiency on both hemopoiesis and on the central nervous system.

**The Methylfolate Trap Hypothesis**

The methylfolate trap hypothesis has been discussed. The failure to utilize tetrahydrofolate and the efficacy of formyl-
roles are not accounted for by this hypothesis. In the rat, there is an escape from the "trap," and unconfirmed data suggest that this may be so in humans. It cannot therefore be the only explanation of events in cobalamin deficiency, and whether a degree of methyl trapping contributes to the overall picture remains to be established.

**Failure of Transmethylation**

Scott and Weir\(^2\) proposed that the main consequence of methylfolate trapping was failure in the supply of methionine and S-adenosylmethionine and hence an impairment of transmethylation. A fall in S-adenosylmethionine occurs in the liver of cobalamin-inactivated rats\(^69\) and sheep\(^96\) but not in brain.\(^96\) Thus, a low S-adenosylmethionine level may have a role in the effect of cobalamin deficiency on hemopoiesis but does not appear to be involved in the neuropathy. There is a defect in bacterial killing and an impaired oxidative burst by neutrophil polymorphs from patients with severe cobalamin deficiency.\(^103,104\) Furthermore, impaired methylation of lipoproteins in neutrophil plasma membranes is accompanied by an impaired oxidative burst.\(^109,106\) Thus lack of S-adenosylmethionine in cobalamin deficiency may be the explanation.

Cycloleucine, which abrogates transmethylation by S-adenosylmethionine, produces a demyelinating lesion in rats similar to that found in cobalamin neuropathy and impaired methylation of arginine 107 of myelin basic protein.\(^100,101\) It was suggested that a similar process produced the defect in cobalamin deficiency. The raised level of S-adenosylmethionine in brain of fruit bats with fatal cobalamin deficiency in both cobalamin-inactivated rat brain and cobalamin-deficient bat brain did not appear to show any difference between control and test specimens, although brains from cycloleucine-treated rats showed the expected result (submitted for publication).

**Role for Cobalamin in Synthesis of Thymidylate Synthetase**

It was proposed that cobalamin is required for the synthesis of the apoenzyme of thymidylate synthetase.\(^107\) This is based on a finding of low activity in lymphocytes from cobalamin-deficient patients. However, marrow from pernicious anemia patients showed increased thymidylate synthetase activity,\(^108\) and this was also the case with marrow from rats exposed to nitrous oxide.\(^96\)

**The Role of Odd Chain and Branched Fatty Acids**

The growth of cobalamin-deficient animals is retarded by feeding odd chain fatty acids (C3, C5, C7, C9, etc.)\(^100\) which are metabolized to propionic acid. Propionic acid is normally converted to methylmalonyl acid and succinic acid and requires cobalamin. In cobalamin deficiency, methylmalonyl-CoA instead of malonyl-CoA is incorporated into fatty acids, leading to the formation of monomethyl-, dimethyl- and trimethyl-branched fatty acids.\(^109\)

Frenkel\(^111\) demonstrated C15 and C17 fatty acids in peripheral nerve obtained by biopsy in pernicious anemia. It has been proposed that the incorporation of these abnormal fatty acids is the cause of neuropathy. Against this interpretation is that although mental retardation is a common feature of methylmalonylaciduria and cobalamin deficiency in early life, patients with methylmalonylaciduria do not develop neurological changes of the type seen in cobalamin deficiency. Further, experimental cobalamin neuropathy due to N\(_2\)O exposure is ameliorated by methionine,\(^83,84\) indicating that the defect lies in relation to methionine and not in relation to defects in methylmalonic acid metabolism.

**Formate-Starvation Hypothesis**

The formate-starvation hypothesis was put forward on the basis of the data that have accumulated as a result of nitrous oxide studies.\(^9\) The prime defect in cobalamin deficiency is failure of methionine supply due to impairment of methionine synthetase of which cobalamin is a coenzyme. Methionine is the major source of single carbon units at the formate level of oxidation. The substrate for making folate enzyme (folate polyglutamate) is formyltetrahydrofolate, and the formyl is derived from methionine. Hence, there is general interference with folate function because of the fall in folate coenzyme levels. There is also a fall in thymidylate synthesis, since formate rather than serine is an important source of methylene to methylate deoxyuridine. This hypothesis explains the effectiveness of folic acid (formyltetrahydrofolate) in reversing cobalamin deficiency in the dU suppression test and in N\(_2\)O inactivation. Enzymes responsible for formyltetrahydrofolate synthesis respond directly to loss of cobalamin activity. In humans undergoing nitrous oxide anesthesia, parenteral formyltetrahydrofolate was largely effective in maintaining normoblastic hemopoiesis.\(^112\)

**WHY DO HUMANS UNIQUELY GET MEGALOBLASTIC ANEMIA?**

There are various possible explanations for the megaloblastic anemia experienced only by humans:

1. Humans appear to be more dependent on the synthetic pathway for formation of thymidylate and less on the salvage path than other species. The result in the deoxouridine suppression test using marrow in cobalamin "deficiency" is a measure of the loss of the synthetic pathway in thymidylate synthesis. In the bat, this is <3%,\(^113\) in the rat it may reach 23%,\(^14\) but in humans it may rise to 65%.

2. Are humans able to induce a betaine homocysteine methyltransferase enzyme to compensate for the failure of methionine synthetase? If not, this too might explain the susceptibility of humans to cobalamin deficiency. However, the maintenance of near-normal serum methionine levels in children with hereditary methylene tetrahydrofolate reductase deficiency,\(^114,115\) as well as the response of these\(^116\) and other patients with homocystinuria to betaine,\(^117\) suggests that the betaine pathway for methionine synthesis is active in humans.

3. Do humans oxidize the methyl group in methyl folate in response to cobalamin lack as does the rat? The observations of Thorndike and Beck\(^4\) and others\(^97\) suggest that this is so.
4. In the rat treated with N\textsubscript{2}O, the impairment of folate polyglutamate synthesis is transient. In untreated pernicious anemia, red cell folate polyglutamate levels remain low, with a shift into short-chain forms, but monoglutamates remain normal. If humans are unable to restore folate polyglutamate in cobalamin deficiency, this may represent a significant difference between humans and the experimental animal.

5. Capacity to use formate derived from sources other than methionine should protect a species against cobalamin deficiency. If human marrow is largely dependent on methionine for its formate, whereas marrow from other species can utilize other compounds, this could explain why humans alone experience cobalamin-responsive megaloblastic anemia.

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