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# THE RELATIONSHIP BETWEEN THE ACTIVITY OF METHIONINE SYNTHASE AND THE RATIO OF *S*-ADENOSYLMETHIONINE TO *S*-ADENOSYLHOMOCYSTEINE IN THE BRAIN AND OTHER TISSUES OF THE PIG

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**Abstract**—Using nitrous oxide to inactivate methionine synthase *in vivo*, the relationship of the activity of methionine synthase to the *S*-adenosylmethionine (AdoMet)/*S*-adenosylhomocysteine (AdoHcy) ratio was examined in neural and other tissues of the pig. Pigs were exposed to 15% nitrous oxide for varying intervals of up to 7 days or studied at varying intervals of recovery in air after 7 days nitrous oxide inhalation, and the rate of inactivation or resynthesis of methionine synthase was related to the corresponding AdoMet/AdoHcy ratios. The rate of inactivation of enzyme during nitrous oxide exposure was considerably faster in the liver and kidney than in the brain and spinal cord with activity levelling off between 10% and 20% of control values. The AdoMet/AdoHcy ratio fell in all tissues during nitrous oxide treatment, the fall being most marked in the brain and spinal cord where a 10-fold change occurred. This change was attributed mainly to a rise in AdoHcy levels. The recovery pattern of methionine synthase was broadly linear but was slower in the spinal cord ($0.10 \pm 0.03$% per hr; mean ± SEM) than in any other tissue examined including brain ($0.35 \pm 0.04$% per hr). Correspondingly, the recovery of the AdoMet/AdoHcy ratio was also significantly slower in the spinal cord. When values for exposure and recovery were combined there was a significant correlation between the activity of methionine synthase and the AdoMet/AdoHcy ratio in both the brain ($r = 0.90$; $P < 0.001$) and the spinal cord ($r = 0.92$; $P < 0.001$). These results support the concept that the AdoMet/AdoHcy ratio is closely related to the pathogenic process which produces the neurologic lesions associated with a reduction in methionine synthase activity.

Methyltransferase enzymes are present in all tissues and catalyse a wide variety of methylation reactions including the methylation of nucleic acids and certain amino acid side chains of proteins, the conversion of phosphatidylethanolamine to phosphatidylcholine, and the catabolism or synthesis of important intermediates. Except for the enzymes that synthesize methionine, all methyltransferases use *S*-adenosylmethionine (AdoMet§) as the methyl group donor and are regulated not solely by the availability of their substrates but more precisely by the relative levels of AdoMet to its product, *S*-adenosylhomocysteine (AdoHcy) [1]. The relative levels of these two compounds (AdoMet/AdoHcy), often called the "methylation ratio", are normally tightly regulated in cells with the former being maintained at concentrations more than 10-fold higher than those of the latter. Any circumstance under which this ratio decreases, either by reduction in AdoMet or by accumulation of AdoHcy, has a profound inhibitory effect on methyltransferase enzymes [2]. Maintenance of the correct ratio within cells is achieved by the conversion of AdoHcy to homocysteine and adenosine via the enzyme *S*-adenosylhomocysteinase (*S*-adenosylhomocysteine hydrolase, EC 3.3.1.1). Since the equilibrium of this reaction is towards synthesis of AdoHcy [3], a reduction in its level can only be achieved by the removal of one or both products. In the case of adenosine this is through adenosine deaminase (EC 3.5.4.4). Homocysteine can be removed either by catabolism to cystathionine or by remethylation to methionine. The cobalamin-dependent enzyme methionine synthase (5-methyltetrahydrofolate homocysteine methyltransferase, EC 2.1.1.13) plays a central role in methyl group metabolism and in the control of the methylation ratio, as it provides the only pathway in all tissues except the liver and kidney for the regeneration of methionine from homocysteine [4, 5]. This system is particularly important in neural tissues where the constant requirement for AdoMet is met from folate (and ultimately glucose)-derived methyl groups through methionine synthase [6] rather than through uptake from plasma methionine [7]. A second enzyme, betaine homocysteine methyltransferase (EC 2.1.1.5)

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§ Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine, TCA, trichloroacetic acid.

can also catalyse the formation of methionine from homocysteine using betaine, an oxidation product of choline. In some species, for example the rat, this latter enzyme is detectable only in the liver. In humans and pigs, however, it is found in both the liver and kidney but not in the brain [8].

During the past decade there has been considerable interest in artificially manipulating the intracellular AdoMet/AdoHcy ratio as a therapeutic antiviral or antitumour strategy. In the main, drugs have been produced which exert their effect by causing AdoHcy to accumulate, primarily by inhibiting the enzyme *S*-adenosylhomocysteine hydrolase [9]. An alternative approach is to decrease the activity of methionine synthase. This has recently been achieved *in vivo* by either using cobalt/cobalamin deficient diets [10] or using the anaesthetic gas nitrous oxide [11, 12].

Nitrous oxide has been shown to inactivate methionine synthase both *in vivo* [13–15] and *in vitro* [16, 17]. Recovery of enzyme activity takes some days [13, 15] as it requires synthesis of new enzyme. Chronic inhalation of nitrous oxide leads to a myelopathy indistinguishable from that seen in cobalamin deficiency in monkeys [18], fruit bats [19], pigs [11] and humans [20]. We have found a dramatic reduction of the AdoMet/AdoHcy ratio in the brain and spinal cord of pigs treated with nitrous oxide [11]. We have postulated that the myelopathy seen in these animals and also in cobalamin deficiency is a result of hypomethylation of essential brain components secondary to the unfavourable methylation ratio existing in these tissues.

We have reported recently that exposure of pigs to nitrous oxide for as little as 1 week produces marked changes in the methylation ratio of a wide variety of tissues [12]. In this study, we have used nitrous oxide inhalation as an agent to manipulate the activity of methionine synthase *in vivo* and we have examined the response in the AdoMet/AdoHcy ratio to decreasing and increasing methionine synthase activities. We present evidence that there is a close relationship in neural tissues between the activity of methionine synthase and the AdoMet/AdoHcy ratio.

## MATERIALS AND METHODS

*Chemicals and reagents.* 5-[$^{14}$C]Methyl-tetrahydrofolic acid, barium salt, sp. act. 57.5 mCi/mmol, was obtained from Amersham International (U.K.). *S*-Adenosyl-L-methionine, chloride salt, *S*-adenosyl-L-homocysteine, 5-methyltetrahydrofolic acid, barium salt, DL-dithiothreitol, cyanocobalamin and Dowex 1 × 8-400 were products of the Sigma Chemical Co. (Poole, U.K.). Methanol (Lab-Scan Ltd, Dublin, Ireland) and 1-heptanesulphonic acid, sodium salt (HiPerSolv, BDH, Poole, U.K.) were HPLC grade and were used as supplied. Radioactivity measurements were carried out using 'Ecolite' scintillation fluid (ICN Biochemicals Inc. NJ, U.S.A.). All other reagents were analytical grade.

*Animals.* Weanling Landrace pigs (5 weeks old) were used for all experiments and were fed *ad. lib.* with a standard diet containing an adequate amount of vitamins, trace elements and nutrients including methionine. In exposure studies, animals were placed in an atmosphere of 15% nitrous oxide for varying time intervals of up to 7 days. In recovery studies, animals breathed 15% nitrous oxide for 7 days and were then placed in air to allow recovery for varying time intervals of up to 7 days. Control animals breathed air throughout the experiment. At the end of each experiment the animals were killed by an intravenous pentabarbitone overdose. Six separate experiments (three exposure and three recovery) were carried out. In each experiment, one control animal was taken along with one treated animal for each of five to seven time points of exposure or recovery. Biochemical analyses were always performed in batches taking each experiment as a unit. Percentage alterations in enzyme activity were calculated with reference to the control animal of that experiment.

*Measurement of AdoMet and AdoHcy.* Aliquots (1 g) of tissue were homogenized with 2 mL of ice-cold 0.1 mol/L sodium acetate buffer, pH 6.0 and rapidly deproteinized with 1.5 mL of 40% trichloroacetic acid (TCA). In the case of the liver and kidney this procedure was always carried out within 3 min of death while for the brain (cortex) and spinal cord there was not more than a 10 min delay. Whole samples left on the bench and assayed at timed intervals of up to 180 min indicated that there were significant post-mortem changes within 30 min to 1 hr, particularly in the liver. Care was taken to ensure that samples were removed as quickly as possible and that there was no difference between animals in the time of removal of a particular tissue. Samples were then frozen to −20° for transport to the laboratory where homogenates were thawed, centrifuged and the supernatants washed three times with 4 vol. of peroxide-free diethyl ether to remove excess TCA. This method of extraction was described by Hyde *et al.* [21] who obtained quantitative recovery and no deterioration of AdoMet under these conditions. The method was later used to study AdoMet/AdoHcy ratios in a variety of rat tissues [22]. In our hands, the recovery of radiolabelled AdoMet and AdoHcy was (mean ± SD) 102 ± 8.7%; coefficient of variation = 8.5% (N = 6) and 95 ± 6.0%; coefficient of variation = 6.3% (N = 6), respectively. Aliquots (20 or 50 μL) were analysed by HPLC using a recently developed method [12]. Results were expressed as nmoles of AdoMet or AdoHcy per gram of tissue.

*Measurement of methionine synthase activity.* Samples of tissue, taken as described above at post-mortem, were snap-frozen in liquid nitrogen and then stored at −20° until assayed. Analysis of enzyme activity was carried out according to the method described by Koblin *et al.* [14]. Protein content was estimated using the Lowry method [23]. Results were expressed as nmoles methionine produced/hr/mg protein. Activity of the enzyme after nitrous oxide treatment was then converted to a percentage of the activity observed in control animals for that experiment.

*Statistical analyses.* The results from all six experiments were combined and subjected to computerized regression analysis using Enzfitter [24] to determine an estimate of the decay and recovery rates of enzyme activity and metabolite

concentrations. Correlations of enzyme activity and metabolite concentration were carried out using simple linear regression.

## RESULTS

### *Methionine synthase*

The methionine synthase activity of the six control animals was 10.00 ± 1.28, 6.47 ± 1.07, 7.41 ± 1.82 and 8.77 ± 1.61 nmol/hr/mg protein (mean ± SEM) in the liver, kidney, brain (cortex) and spinal cord, respectively. The rate of inactivation and subsequent resynthesis of methionine synthase following exposure to nitrous oxide inhalation, expressed as a percentage of control activity, is shown in Table 1. Exposure to nitrous oxide produced a decrease in measurable activity to between 10% and 20% of air control levels. Using the curve fitting program, the rate of decline in activity was best described by a double exponential decay curve. The initial decay constant indicated that loss of activity in the liver (−0.25 ± 0.02% per hr; mean ± SEM) and kidney (−0.17 ± 0.04% per hr) proceeded at a considerably faster rate than that in the cortex (−0.11 ± 0.03% per hr) or spinal cord (−0.12 ± 0.03% per hr). In all tissues the second exponential did not indicate significant further decline. The activity levelled off and remained between 10% and 20% of control values as exposure was continued.

Following nitrous oxide exposure, the recovery of methionine synthase in the same four tissues was broadly linear (Table 1). The best fit was obtained for the cortex which had a linear recovery constant of 0.35 ± 0.04% per hr (mean ± SEM). The rates of recovery in the liver and kidney approximated that of the cortex. Recovery in spinal cord was very slow (0.10 ± 0.03% per hr) and the activity remained well below control levels even after 7 days recovery in air. To examine this effect further, one animal was allowed to recover for 14 days after 7 days nitrous oxide exposure. The methionine synthase activity in the spinal cord was still only 54% of the control value for that experiment even though the recovery in liver, kidney and cortex of the same animal was 93%, 113% and 72%, respectively.

Table 1. Methionine synthase activity in pig tissues: inactivation by nitrous oxide and recovery in air

| Tissue | No. of pigs in exposure study | Exponential decay constant (% inactivation/hr) | Approx. time to reach lowest activity (hr) | Lowest activity % of air controls (N) | No. of pigs in recovery study | Linear recovery constant (% recovery/hr) | Calculated time to reach control activity* (hr) |
|---|---|---|---|---|---|---|---|
| Liver | 26 | −0.25 ± 0.02† | 20 | 13.03 ± 0.94† (16) | 22 | 0.30 ± 0.07† | 290 |
| Kidney | 26 | −0.17 ± 0.04 | 24 | 17.87 ± 1.51 (16) | 22 | 0.46 ± 0.08 | 178 |
| Brain | 25 | −0.11 ± 0.03 | 50 | 20.42 ± 1.71 (12) | 21 | 0.35 ± 0.04 | 228 |
| Spinal cord | 26 | −0.12 ± 0.03 | 50 | 19.23 ± 1.59 (13) | 22 | 0.10 ± 0.03 | 810 |

Pigs were placed in an atmosphere of 15% nitrous oxide for varying time intervals of up to 7 days or placed in 15% nitrous oxide for 7 days and then allowed to recover in air for up to 7 days. Control animals breathed air throughout the experiment.

Tissues were analysed for methionine synthase and the activity obtained was converted to a percentage of the activity of a control animal analysed at the same time. Rates of inactivation and recovery were estimated by subjecting the data to regression analysis.

* Values were calculated as the time taken to reach 100% activity from the mean lowest activity in a tissue using the mean linear rate of recovery in that tissue.

† Values are means ± SEM.

N, no. of pigs.

### *AdoMet*

There was some reduction of AdoMet in the liver, cortex and spinal cord over the 7-day period of nitrous oxide exposure although only the liver had significantly lower levels ($P < 0.02$) after 7 days when compared with air controls. No change was observed in the AdoMet content of the kidney (results not shown).

### *AdoHcy*

Nitrous oxide treatment produced a marked increase in the levels of AdoHcy in all tissues, especially in the cortex and spinal cord where there was a 4–6-fold increase after 7 days (Fig. 1). There was a sharp readjustment towards normal levels when the animals were replaced in air (Fig. 1) although complete recovery took more than 4 days. The recovery trend was similar in the liver and kidney although there was a much wider scatter.

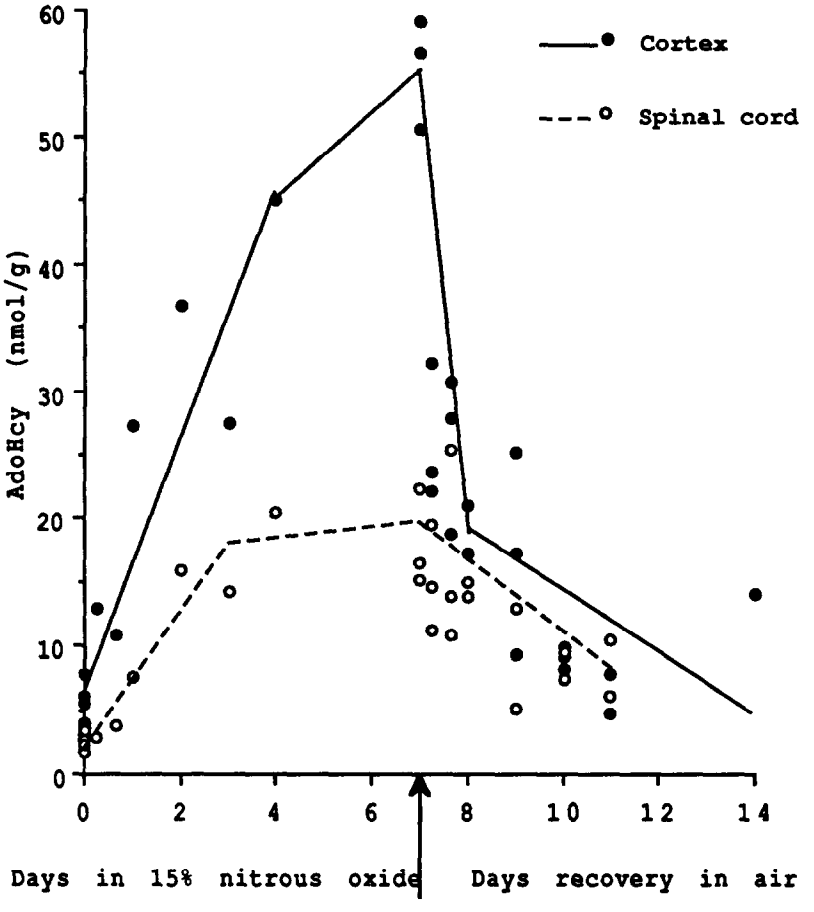


Fig. 1. Time course of alterations in the AdoHcy concentrations of the brain (cortex) (●) and spinal cord (○) in response to nitrous oxide. Pigs were placed in an atmosphere of 15% nitrous oxide for time intervals of up to 7 days or placed in 15% nitrous oxide for 7 days and then allowed to recover in air for up to 7 days. Control animals breathed air throughout the experiment. Tissues were extracted, deproteinized with TCA and analysed for AdoHcy concentrations using HPLC. Each point represents the value from one animal.

*AdoMet/AdoHcy ratio*

The effect of nitrous oxide treatment on the ratio of AdoMet/AdoHcy is summarized in Table 2. There was a marked reduction in the ratio in all tissues following 7 days exposure. This was most apparent in the spinal cord where there was a decrease from 12.50 ± 0.51 to 0.81 ± 0.24 (mean ± SEM). As with the methionine synthase activity, the ratio of AdoMet/AdoHcy returned to normal in a linear fashion in all tissues (results not shown). It was noted, however, that the calculated approximate time to reach control activity was somewhat shorter for the AdoMet/AdoHcy ratio than for the methionine synthase activity (Tables 1 and 2).

Using regression analysis, and combining the values for the exposure and recovery studies, the relationship of the change in AdoMet/AdoHcy ratio to percentage of control enzyme activity was examined. Table 3 summarizes the correlation coefficients obtained. There was a highly significant relationship between the relative activity of methionine synthase and the AdoMet/AdoHcy ratio in all tissues. This was most evident in the cortex and spinal cord which had correlation coefficients of 0.90 and 0.92, respectively. A scattergram for the correlation of the cortex AdoMet/AdoHcy ratio with respect to methionine synthase is given in Fig. 2.

## DISCUSSION

A wide range of studies have been carried out on the inactivation of methionine synthase by nitrous oxide in rats [13, 15, 25], mice [14], fruit bats [19], monkeys [26] and humans [27, 28]. These reports show that enzyme activity in the liver, kidney and brain drops within hours to between 10% and 30% of control values depending on the concentration of nitrous oxide administered. However, none of these studies has measured enzyme activity in the spinal cord, a tissue in which the histological lesions of cobalamin deficiency and nitrous oxide-induced neuropathy are most apparent [11, 18, 19]. This study has compared the rate of methionine synthase

Table 2. AdoMet/AdoHcy ratios in pig tissues: alteration by nitrous oxide and recovery in air

| Tissue | No. of pigs in exposure study | Highest ratio (air controls) (N) | Lowest ratio (7 days nitrous oxide) (N) | No. of pigs in recovery study | Calculated time to reach control activity* (hr) |
|---|---|---|---|---|---|
| Liver | 17 | 5.06 ± 0.82† (5) | 1.42 ± 0.62† (3) | 20 | 135 |
| Kidney | 17 | 5.20 ± 0.79 (5) | 0.88 ± 0.004 (4) | 21 | 288 |
| Brain | 15 | 6.16 ± 0.55 (5) | 0.52 ± 0.15 (4) | 21 | 152 |
| Spinal cord | 13 | 12.50 ± 0.51 (3) | 0.81 ± 0.24 (4) | 20 | 585 |

Pigs were placed in an atmosphere of 15% nitrous oxide for varying time intervals of up to 7 days or placed in 15% nitrous oxide for 7 days and then allowed to recover in air for up to 7 days. Control animals breathed air throughout the experiment.

Tissues were extracted and deproteinized with 40% TCA. Supernatants were analysed for AdoMet and AdoHcy concentrations using HPLC.

* Values were calculated as the time taken to reach the mean ratio for the air control from the mean lowest ratio in a tissue using the mean linear rate of recovery in that tissue.

† Values are means ± SEM.

N, no. of pigs.

Table 3. The relationship of changes in the activity of methionine synthase to changes in the AdoMet/AdoHcy ratio

| Brain | | Spinal cord | | Liver | | Kidney | |
|---|---|---|---|---|---|---|---|
| *r* | P | *r* | P | *r* | P | *r* | P |
| 0.90 | <0.001 | 0.92 | <0.001 | 0.48 | <0.003 | 0.66 | <0.001 |
| N = 33 | | N = 28 | | N = 34 | | N = 34 | |

*r*, correlation coefficient of the percentage methionine synthase activity with the AdoMet/AdoHcy ratio.

P, the significance of the relationship between the percentage methionine synthase activity and the AdoMet/AdoHcy ratio.

N, total number of pigs used to give measurements of methionine synthase activity and the corresponding AdoMet/AdoHcy ratio in that tissue. Values were combined from studies in which animals were exposed to 15% nitrous oxide for varying periods of time of up to 7 days and recovery studies in which animals were exposed to 15% nitrous oxide for 7 days and then allowed to recover in air for varying time periods. The percentage methionine synthase activity was determined by comparison of activity in treated animals with an untreated control for the same experiment.

inactivation and resynthesis in four organs of the pig, an animal that has been used previously as an experimental model for the neuropathy associated with nitrous oxide inhalation [11]. The rate of inactivation in each instance conforms to an exponential decay with the rates in the liver and kidney being similar and several-fold faster than those found in the brain and spinal cord. Thus, in the former two organs the administration of 15% nitrous oxide reduces the activity of the enzyme to approximately 15% of its starting value within 24 hr, and it remains at this level and is not inactivated further (Table 1). Slightly higher residual activities are found in the brain and spinal cord and the time taken to achieve these final levels is about double that of those in the liver and kidney (Table 1). The recovery pattern for the four organs is linear with similar rates in the liver, kidney and brain, and these being about three times as rapid as those found in the spinal cord. Thus, the time taken to reestablish normal activity is between 1 and 2 weeks for the former three organs while the spinal cord still shows greatly reduced activity after 2 weeks. Since the recovery of activity probably depends on new enzyme being synthesized [16], these results may reflect different rates of protein synthesis in these tissues. In this respect, intermittent nitrous oxide exposure may not have serious effects in any tissue other than the spinal cord where the ability to regenerate the enzyme is so low. This finding is consistent with the observation that long term intermittent nitrous oxide exposure in humans produces a myelopathy similar to that seen in cobalamin deficiency [20].

In this study, analysis of the tissue levels of AdoMet and AdoHcy in conjunction with methionine synthase activity has given further insight into the functional importance of methionine synthase particularly in the brain and spinal cord. Although there was no significant alteration of AdoMet levels in the time scale of this study the dramatic rise in AdoHcy in response to enzyme inhibition demonstrates the importance of remethylation of

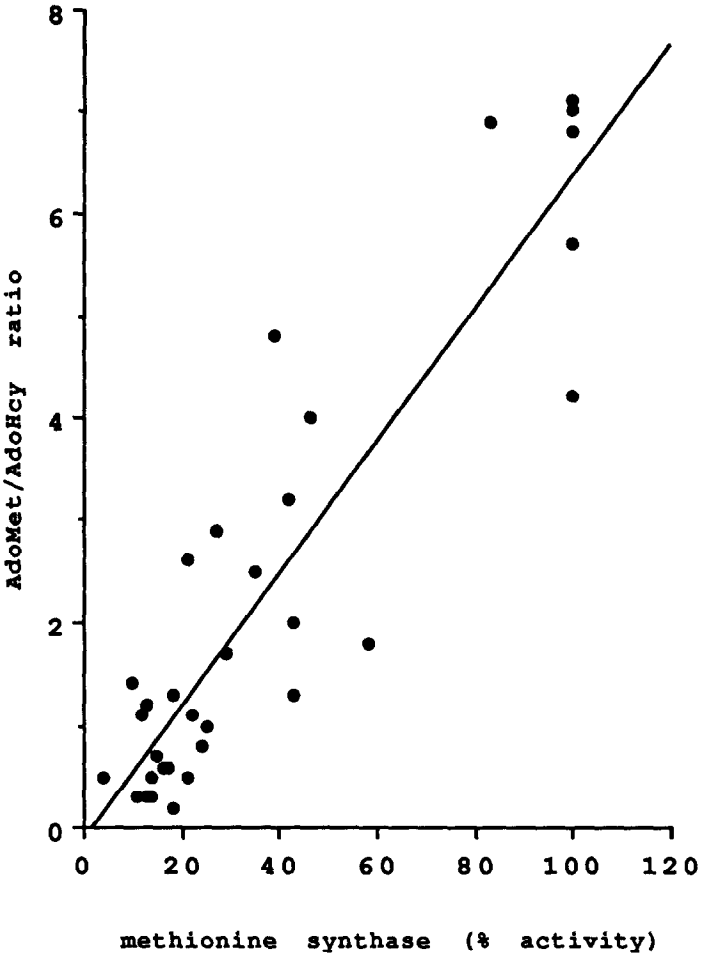


Fig. 2. The relationship between the relative activity of methionine synthase (expressed as a percentage of untreated air controls) and the AdoMet/AdoHcy ratio in brain (cortex). Regression analysis was carried out on values obtained from studies in which animals were exposed to 15% nitrous oxide for varying periods of time of up to 7 days and studies in which animals were exposed to 15% nitrous oxide for 7 days and then allowed to recover in air for varying time periods. Each point represents the results from one animal. The relationship was described by the equation $Y = 0.064X - 0.067$; $r = 0.90$; significance, $P \leq 0.001$; $N = 33$.

homocysteine as a means of maintaining the correct methylation ratio. The principal observation, however, was that both during inactivation and resynthesis of methionine synthase a very tight relationship existed between the activity of the enzyme and the AdoMet/AdoHcy ratio pertaining in the brain and spinal cord [correlation coefficients of 0.90 ($P < 0.001$) and 0.92 ($P < 0.001$), respectively; Table 3]. While similar analyses also showed significant relationships between the levels of enzyme activity and the methylation ratio in the liver and kidney, the correlations were not as good. This is presumably due to the fact that in the former two organs the sole means of regenerating methionine from homocysteine involves the enzyme methionine synthase whereas in the liver and kidney the alternative pathway involving betaine methyltransferase is also in operation [8]. The significance of these results is that inhibition of methionine synthase either through inactivation with nitrous oxide or because of cobalamin deficiency creates unfavourable conditions for methyltransferase activity. In addition, while all organs are affected to some extent the effects are more pronounced in neural tissue. This is consistent with the association between inactivity of methionine synthase however caused and the neuropathy seen under such circumstances in humans [20], monkeys [18], fruit bats [19] and pigs [11].

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