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The Regulation of Folate and Methionine Metabolism

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1. The isolated perfused rat liver and suspensions of isolated rat hepatocytes fail to form glucose from histidine, in contrast with the liver *in vivo*. Both rat liver preparations readily metabolize histidine. The main end product is *N*-formiminoglutamate. In this respect the liver preparations behave like the liver of cobalamin- or folate-deficient mammals. 2. Additions of L-methionine in physiological concentrations {or of ethionine [2-amino-4-(ethylthio)butyric acid]} promotes the degradation of formiminoglutamate, as is already known to be the case in cobalamin or folate deficiency. Added methionine also promotes glucose formation from histidine. 3. Addition of methionine accelerates the oxidation of formate to bicarbonate by hepatocytes. 4. A feature common to cobalamin-deficient liver and the isolated liver preparations is taken to be a low tissue methionine concentration, to be expected in cobalamin deficiency through a decreased synthesis of methionine and caused in liver preparations by a washing out of amino acids during the handling of the tissue. 5. The available evidence is in accordance with the assumption that methionine does not directly increase the catalytic capacity of formyltetrahydrofolate dehydrogenase; rather, that an increased methionine concentration raises the concentration of *S*-adenosylmethionine, thus leading to the inhibition of methylenetetrahydrofolate reductase activity [Kutzbach & Stokstad (1967) *Biochim. Biophys. Acta* **139**, 217–220; Kutzbach & Stokstad (1971) *Methods Enzymol.* **18B**, 793–798], that this inhibition causes an increase in the concentration of methylenetetrahydrofolate and the C_1 tetrahydrofolate derivatives in equilibrium with methylenetetrahydrofolate, including 10-formyltetrahydrofolate; that the increased concentration of the latter accelerates the formyltetrahydrofolate dehydrogenase reaction, because the normal concentration of the substrate is far below the K_m value of the enzyme for the substrate. 6. The findings are relevant to the understanding of the regulation of both folate and methionine metabolism. When the methionine concentration is low, C_1 units are preserved by the decreased activity of formyltetrahydrofolate dehydrogenase and are utilized for the synthesis of methionine, purines and pyrimidines. On the other hand when the concentration of methionine, and hence adenosylmethionine, is high and there is a surplus of C_1 units as a result of excess of dietary supply, formyltetrahydrofolate dehydrogenase disposes of the excess. When ample dietary supply causes an excess of methionine, which has to be disposed of by degradation, the increased activity of formyltetrahydrofolate dehydrogenase decreases the supply of methyltetrahydrofolate. Thus homocysteine, instead of being remethylated, enters the pathway of degradation via cystathionine. 7. The findings throw light on the biochemical abnormalities associated with cobalamin deficiency (megaloblastic anaemia), especially on the 'methylfolate-trap hypothesis'. This is discussed. 8. In the course of the present study it was found that crystalline ox liver glutamate dehydrogenase readily reacts with formiminoglutamate under the conditions used for the assay of glutamate.

The work reported in the present paper arose from an investigation of gluconeogenesis from histidine. That histidine is glucogenic in the mammalian body was first indicated by experiments of Dakin (1912) on phlorrhizin-diabetic dogs, and firmly established when Edlbacher (1926) showed that liver contains enzymes that can form one molecule of glutamate per molecule of histidine. Yet histidine fails to form glucose in the isolated perfused liver, although it is readily taken up and degraded (Cornell *et al.*, 1973). This observation provided the starting point of the present work. The follow-up was partly carried out on the perfused liver and partly on isolated hepatocytes. It showed that the pathway of histidine degradation in the isolated perfused liver and in isolated hepatocytes is largely blocked at the stage of *N*-formiminoglutamate, an intermediate that accumulates within the liver cell on loading with

histidine. Addition of methionine in physiological concentrations was found to diminish the accumulation.

It is already known that, in cobalamin or folate deficiency, a block of histidine degradation occurs at the stage of formiminoglutamate, and that this block can be overcome by increasing the concentration of methionine. Thus the isolated perfused liver and the isolated hepatocytes represent models for the study and analysis of the causes of the metabolic disturbances in cobalamin or folate deficiency. In cobalamin deficiency, methionine synthesis from homocysteine and methyltetrahydrofolate must be impaired, because the reaction requires cobalamin as a cofactor. The liver preparations have lost methionine, because together with other low-molecular-weight cell constituents methionine is washed out of the cells during the preparation of the perfused liver and of the hepatocytes. A likely common feature, then, of cobalamin-deficient liver and the tissue preparations used in the present work is a deficiency in methionine. The present paper throws light on the biochemical consequence of methionine and cobalamin deficiency and on the reasons for some of the disturbances of folate metabolism, and suggests a mechanism of action of methionine in overcoming some of the consequences of cobalamin or folate deficiency.

Materials and Methods

Tissue preparations

The liver was perfused by the method of Hems *et al.* (1966) and Krebs *et al.* (1969). Isolated liver cells were prepared by the method of Berry & Friend (1969) as modified by Cornell *et al.* (1973) and Krebs *et al.* (1974). Unless otherwise stated, livers of female rats deprived of food for 48h were used.

Analytical methods

Urea, NH_3, glutamate, glucose, lactate and pyruvate were determined by spectrophotometric methods as described by Cornell *et al.* (1974), and histidine was determined with histidine decarboxylase by the manometric method of Gale (1974). Urocanate (imidazol-4-ylacrylate) was measured by the increase in absorbance of $HClO_4$ filtrates at 277nm (Magasanik *et al.*, 1971). Glutamine was determined as described by Lund (1974). O_2 consumption was measured as described by Krebs *et al.* (1974).

Glutamate dehydrogenase was found to react quantitatively with formiminoglutamate at a rate only slightly less than with glutamate under the conditions of the spectrophotometric assay. This method was therefore not suitable for the determination of glutamate in the presence of formiminoglutamate. Methods for the determination of glutamate and formiminoglutamate were based on the finding that glutamate decarboxylase from *Escherichia coli* attacks glutamate, but not formiminoglutamate. Thus the sum of glutamate and formiminoglutamate can be determined by the spectrophotometric principle of Bernt & Bergmeyer (1974), and the concentration of formiminoglutamate is determined in a separate sample after treatment with glutamate decarboxylase.

The histidine decarboxylase of strain N.C.T.C. 6785 of *Clostridium welchii* was found to attack both glutamate and histidine, but not formiminoglutamate. For the determination of histidine, glutamate was removed by incubation with glutamate decarboxylase of *E. coli*. The quantities needed for the manometric determination of histidine are about ten times those needed for the spectrophotometric determinations of glutamate and formiminoglutamate.

For the quantitative removal of glutamate, 1ml of the neutralized $HClO_4$ extract was incubated with occasional shaking at 37°C for 1h with 0.1ml of 1M-acetate buffer, pH 5.0, and 1mg of glutamate decarboxylase (BDH Chemicals, Poole, Dorset, U.K.; activity 1.33μmol of CO_2 liberated/min per mg at 37°C and pH5) suspended in 0.1ml. At the end of the incubation, 0.1ml of 20% (v/v) $HClO_4$ was added, and, after neutralization and removal of the $KClO_4$, the supernatant was used for the determination of formiminoglutamate by the glutamate dehydrogenase method, and of histidine by the histidine decarboxylase method. Manometric tests showed that up to 10μmol of glutamate is quantitatively decarboxylated in 60min under these conditions. Usually much less glutamate was present in the sample.

The spectrum of amino acids was obtained with the J.E.O.L. amino acid analyser JLC-6AH as described by Jeppson & Karlsson (1972). Formiminoglutamate does not react readily with ninhydrin and therefore does not appear among the amino acids determined by the standard amino acid analyser, unless it is present in the sample in excess of other amino acids. In larger quantities it gives a very weak and broad-based peak after citrulline and before valine when the procedure of Jeppson & Karlsson (1972) is used.

Collection of $^{14}CO_2$

Incubations of cell suspensions were carried out in 50ml conical flasks fitted with a centre well (30mm high, 10mm diam.). After introduction of 4ml of cell suspension, a piece of pleated filter paper (20mm×30mm) was placed in the centre well, and,

after gassing the flask with O_2+CO_2 (95:5), it was closed with a Suba-Seal stopper. At the end of the incubation, 0.4ml of 20% (v/v) $HClO_4$ was injected with a syringe through the Suba-Seal into the suspension, and immediately afterwards 1 ml of 1 M-NaOH into the centre well. The flasks were shaken for 2h to allow for complete absorption of the CO_2. The filter paper was thoroughly broken up in the NaOH solution with a thin stirring rod and the contents of the centre well were transferred to a centrifuge tube with a Pasteur pipette. After centrifugation, a sample (0.1 ml) of the supernatant was counted for radioactivity in a Beckman LS 200 liquid-scintillation counter. The scintillation fluid consisted of 5.5g of Permablend III (Packard Instrument, Caversham, Berks, U.K.), 60g of naphthalene, 400ml of 2-methoxyethanol and 600ml of toluene.

Special chemicals

L-Histidine and L-methionine were from BDH. L-Ethionine [2-amino-4-(ethylthio)butyric acid], L-homocysteine thiolactone, DL-methionine, seleno-DL-methionine, methionine DL-sulphoximine and formiminoglutamate hemi-barium salt were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. The lactate used was obtained from crystalline L-lactic acid by neutralization with NaOH. Radioactive compounds were from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Glutamate dehydrogenase in glycerol, and lactate dehydrogenase were from Boehringer Corp. (London), Lewes, Sussex BN7 1LG, U.K. Urease powder (type VI) was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Presentation of data

The formation of a metabolite is expressed in the Tables by the '+' sign, removal by the '−' sign.

Results

Histidine metabolism in the perfused rat liver

Table 1 shows that histidine is rapidly removed by the perfused liver of rats deprived of food for 48h. The mean rate of removal for a 90min period was 0.79 μmol/min per g wet wt. when the initial histi-

Table 1. *Metabolic products of histidine in the perfused rat liver*

The concentration of histidine (added at 38min) was 10mM, and the perfusion was continued after addition of histidine for 90min. The values refer to the changes in the perfusion medium. They are means of three experiments±S.E.M. The mean weight of the livers was 6.08g. The volume of the perfusate was 150ml.

Metabolite	Total change in medium (μmol)	Rate of change (μmol/min per g)
Histidine	−435 ± 13	−0.79
Urea	+120 ± 13	+2.22
NH_3	+171 ± 27	+0.31
Urocanate	+273 ±110	+0.50
Glutamate	+ 30.0± 5.3	+0.055
Glutamine	+ 19.5± 1.9	+0.036
Glucose	+ 14.0± 6.9	+0.026

Table 2. *Products of histidine in isolated hepatocytes*

Cells (98mg) were incubated for 1h in 4ml of medium with 2mM-L-histidine. Results are expressed as μmol/h per g (means of triplicate analyses±S.E.M.). The calculation of the N and C balance was based on the established pathways of histidine metabolism, the formation of glucose occurring via phosphoenolpyruvate and involving the release of three molecules of CO_2. The assumption is made that addition of histidine does not affect the basal metabolism.

	Metabolite changes (μmol/h per g)		
		Histidine (2mM)	
Metabolite	Control (no substrate added)	Observed	Corrected for control
Histidine	—	−26.0 ±0.28	− 26.0
Urocanate	+ 0.7 ±0.02	+ 3.2 ±0.84	+ 2.5
Glucose	+ 7.68±0.1	+11.1 ±0.05	+ 3.43
Urea	+11.9 ±0.23	+30.1 ±0.95	+ 18.2
NH_3	+ 1.45±0.31	+ 6.85±0.75	+ 5.40
Formiminoglutamate	+ 0.3	+13.1 ±0.54	+ 12.8
Glutamate	+ 2.41±0.03	+ 6.17±0.41	+ 3.76
Histidine (N removed)			78.0
Histidine (N recovered)			76.2
Histidine (C removed)			156.2
Histidine (C recovered)			155.6

dine concentration was 10mM, but no extra glucose was formed, the rate of glucose production being even lower than the normal endogenous rate (0.14μmol/min per g; Hems *et al.*, 1966). The major products of histidine metabolism discharged into the perfusion medium were urocanate, NH_3 and urea. The amounts of glutamate and glutamine were only slightly above those observed in the absence of histidine. About 85μmol of the histidine removed was not accounted for. As will be seen below, this deficit is largely due to the accumulation of formiminoglutamate within the liver.

The results obtained with the perfused liver can be closely reproduced with isolated hepatocytes (Table 2). The only major difference concerns the accumulation of urocanate, which is lower in the hepatocyte suspension; in the perfused liver it is rather variable, as indicated by the large standard error in Table 1. The lower yield of urocanate may be due to the low histidine concentration (2mM) in hepatocyte experiments, as against 10mM in the perfusion experiments. As hepatocyte suspensions are much more convenient for serial experiments than the perfused organ they were used in most of the subsequent work. Table 2 lists formiminoglutamate in addition to the products given in Table 1. This metabolite is not mentioned in Table 1, because at that time a formation of formiminoglutamate was not expected. The clue leading to the detection of this intermediate came from the finding that hepatocyte suspensions when analysed for glutamate spectrophotometrically by the method of Bernt & Bergmeyer (1974) gave very high values for intracellular glutamate concentrations. An intracellular accumulation of this magnitude was surprising, because glutamate formed intracellularly, that is from glutamine, is known to form glucose readily. This led to doubts about the specificity of the glutamate assay, which is assumed to be very high when the crystalline enzyme is used. However, this assumption proved incorrect; glutamate dehydrogenase reacts quantitatively with formiminoglutamate, as already mentioned in the Materials and Methods section. In the light of this information it is probable that some of the glutamate listed in Table 1 was formiminoglutamate. The fact that the sum of glutamate plus formiminoglutamate concentrations found in the hepatocyte suspension was substantially greater (on a tissue weight basis) than that listed in Table 1 is the result of the intracellular location of formiminoglutamate. Thus it is included in the analysis of the hepatocyte suspension, but omitted when the perfusion medium only is analysed. When the formation of formiminoglutamate is taken into account in the calculation of the C and N balance of histidine metabolism, the recovery of C and N in the form of the six products listed in Table 2 is perfect, within the limits of error (Table 2). This implies that the amounts of any other intermediary metabolites were negligible.

Effect of methionine on histidine metabolism

Formiminoglutamate *in vivo* does not accumulate in the body, except in individuals with cobalamin or folate deficiency. Since the experimental animals received an adequate diet, the formation of formiminoglutamate could not be ascribed to nutritional deficiencies. The investigation of the reasons for the accumulation of formiminoglutamate was guided by the experience that the isolated perfused liver and isolated hepatocytes differ initially in one important feature from the liver *in vivo*. Both preparations lose low-molecular-weight constituents during the preparation because a semisynthetic medium containing no free amino acids is used for the perfusion of the liver and for the suspension of the hepatocytes. Under these conditions the liver sheds amino acids, lactate, pyruvate and other low-molecular-weight substances into the medium. Since the liver is perfused with 150ml of fluid, the extent to which the liver is washed out is considerable, and, since the isolated liver cells obtained by perfusing the liver with collagenase are further washed, the loss of low-molecular-weight constituents by the isolated hepatocytes may be expected to be even greater than the loss by the intact liver. Previous work on the rate of gluconeogenesis by hepatocytes (Cornell *et al.*, 1974) revealed a lag period of about 40min, during which the rate increases and normal amino acid concentrations are restored in the hepatocytes, a process that is much accelerated by the addition of lysine or NH_4Cl.

Quantitative information on the loss of amino acids incurred by hepatocytes during the preparation is provided by an experiment in which freshly prepared cells from a rat deprived of food were suspended in 30ml of medium. This was centrifuged, and the pellet weighing 2.0g was resuspended in 8.5vol. of medium, giving a total of 19ml. This was divided into three portions of 6ml each. The first sample was deproteinized with $HClO_4$ and prepared for the amino acid analyser. This sample indicated the amino acid content of the cells as prepared. A second sample was incubated without added substrate for 40min. It was then centrifuged and the supernatant and pellet were analysed separately. A third sample was incubated for 40min with 10mM-lactate, 1mM-oleate and 2mM-lysine. It was analysed in the same way as the second sample. In the calculation of the amino acid content of the cells, no correction was made for the contamination of the pellet with the supernatant. The values obtained for the cells are therefore slightly too low, because in every case the concentration of the amino acids in the supernatant was lower than in the cells.

In the freshly prepared cells all amino acids

Table 3. *Loss of amino acids from hepatocytes during preparation of cell suspension and recovery of amino acids on incubation*

For details of the procedure, see the text. The data show the absolute amounts of amino acids (μmol) in 6ml of suspension from the livers of rats deprived of food for 48h. The suspension contained 630mg of cells. The incubation was at 38°C with O_2+CO_2 (95:5). Each value *in vivo* is the mean of three analyses of freeze-clamped liver.

			Distribution of amino acids after 40min incubation without substrate; contents of			Distribution of amino acids after 40min incubation with 10mM-lactate/2mM-lysine/1mM-oleate; contents of:		
Amino acid	Amino acid content of 630mg of cells *in vivo*	Amino acid content of 630mg of freshly prepared cells	630mg of cells (μmol)	5.5ml of supernatant (μmol)	Sum of cells and supernatant (μmol)	630mg of cells (μmol)	5.5ml of supernatant (μmol)	Sum of cells and supernatant (μmol)
Asparagine	0.42	0.32	0.22	0.08	0.30	0.14	0.47	0.61
Threonine	0.26	0.14	0.32	0.29	0.61	0.24	0.29	0.53
Serine	0.50	0.23	0.34	0.19	0.53	0.28	0.42	0.70
Glutamic acid	2.32	0.33	0.52	0.34	0.86	0.85	3.52	4.37
Glutamine	2.19	0.19	0.18	0.38	0.56	0.57	0.27	0.84
Glycine	1.58	0.49	0.76	0.62	1.38	0.27	0.45	0.72
Alanine	0.25	0.11	0.10	0.11	0.21	0.54	0.36	0.90
Valine	0.07	0.11	0.11	0.56	0.67	0.49	0.11	0.60
Methionine	0.06	0.006	0.007	0.13	0.14	0.16	0.03	0.19
Isoleucine	0.04	0.060	0.072	0.39	0.46	0.33	0.06	0.39
Leucine	0.06	0.14	0.16	0.76	0.92	0.62	0.14	0.76
Ornithine	0.07	0.066	0.074	0.15	0.22	0.13	0.08	0.21

except those with branched chains were present at much lower concentrations than *in vivo* (Table 3). About 70% of the glutamate and glycine, and about 90% of glutamine and methionine, were lost. The loss was about 50% in the case of alanine, serine and threonine. On incubation without added substrates, the amino acid content of the suspension rose considerably in both cells and medium. As the volume of the medium was about 85 times that of the cells, it follows from the values in Table 3 that the concentration of amino acids in the cells was higher than in the medium except in the case of the branched-chain amino acids. As the increase in the amounts of amino acids on incubation included the essential amino acids (threonine, leucine, isoleucine, valine, methionine), proteolysis must have been the main source of the amino acids.

The largest percentage increase in the amino acid content on incubation was for the branched-chain amino acids (600–700%). This is accounted for by the fact that the degradation of branched-chain amino acids in the liver is negligible at low concentrations (see Krebs, 1972). Other amino acids that arise through proteolysis are liable to be degraded by the liver.

After the 40min incubation, the concentration of most amino acids within the cells had risen in the direction of normal values, but did not reach this concentration in all cases. This was not due to lack of amino acid formation, but largely to a discharge of amino acids into the medium. Addition of lactate, lysine and oleate accelerated the rate of recovery of intracellular non-essential amino acids, especially of glutamate, aspartate and alanine, and caused considerable amounts of glutamate to be discharged into the suspension medium.

These experiments suggested that the failure of the isolated hepatocyte preparation to convert histidine into glucose and the cause of the accumulation of formiminoglutamate may be connected with the loss of cell constituents in the course of the preparation. The effect of added amino acids on the fate of histidine was therefore systematically tested. This showed that the only protein amino acid that had an effect was methionine (Table 4). It increased glucose formation and the release of $^{14}CO_2$ from [*ring*-2-^{14}C]histidine. Already 0.02mM-methionine had a measurable effect and at 0.1mM-methionine the effect approached the maximum. In the normal rat liver *in vivo* the mean methionine concentration has been reported to be 0.09mM (range 0.08–0.1mM; Schimassek & Gerok, 1965). Freshly prepared hepatocytes were found to contain less than 0.01mM-methionine. The release of $^{14}CO_2$ from the ring carbon atom of histidine implies that formiminoglutamate has been metabolized. This is directly demonstrated in Table 5. In the absence of methionine, most of the histidine removed was accounted for as formiminoglutamate. On addition of methionine, the rate of removal of histidine was

Table 4. *Effect of methionine concentration on the metabolism of histidine in hepatocyte suspensions*

Hepatocytes from a rat deprived of food for 48h (76mg) were incubated in 4ml of 2mM-[*ring*-2-^{14}C]histidine for 60min with various concentrations of methionine.

Concentration of added methionine (mM)	Glucose found (μmol/h per g)	$^{14}CO_2$ formed (μmol/h per g)
0	9.7	2.2
0.02	11.2	3.15
0.05	14.7	6.60
0.1	18.2	10.8
0.5	18.6	12.7

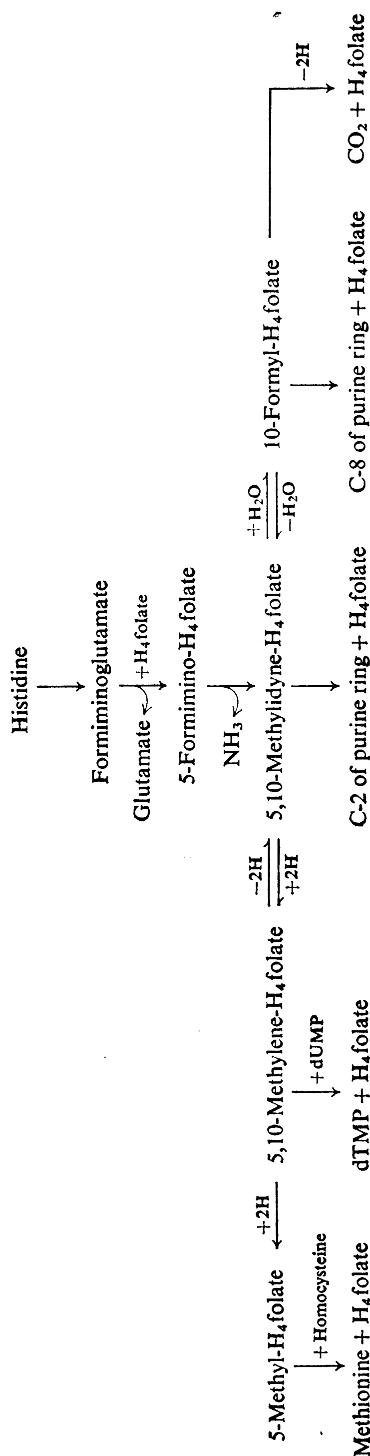
Table 5. *Effect of methionine on histidine metabolism in isolated hepatocytes*

Hepatocytes from a rat deprived of food for 48h (72mg) were incubated for 1h in 4ml of L-[U-^{14}C]histidine.

	Metabolic changes (μmol/h per g)	
Metabolite	With 2mM-histidine	With 2mM-histidine+ 0.1mM-methionine
Histidine	−30.1	−29.6
Glucose	+ 2.7	+ 7.4
Formiminoglutamate	+26.6	+12.7
Glutamate	+ 1.9	+ 2.2
NH_3	+ 4.6	+15.5
Urea	+16.3	+29.1
$^{14}CO_2$	+ 9.3	+36.1

unchanged, the formation of formiminoglutamate was greatly decreased, and the formation of glucose, NH_3 and urea was increased.

The absolute amounts of $^{14}CO_2$ released from [*ring*-2-^{14}C]histidine were lower than expected. In the experiment reported in Table 4 the expected yield of $^{14}CO_2$ is twice that of glucose, since two molecules of histidine are required for the formation of one molecule of glucose. In fact the yield of $^{14}CO_2$ was only about 30% at the higher concentrations of methionine and less at the lower concentrations of methionine. Two factors may be responsible for the low yield. First, some glucose is liable to be formed from sources other than histidine, and the proportion of glucose derived from endogenous precursors is greater when the methionine concentration is low. Secondly, with [*ring*-2-^{14}C]histidine, $^{14}CO_2$ can only be released after attachment of the labelled C_1 unit to tetrahydrofolate; but from that situation (see Scheme 1) it may react with acceptors, e.g. glycine, or exchange with C_1 donors, e.g. serine, thus diminishing the $^{14}CO_2$ yield.



Scheme 1. *Tetrahydrofolate (H_4folate) metabolism and its regulation*

The horizontal reaction scheme represents the different redox states of the C_1 derivatives of tetrahydrofolate (methyl, methylene, methylidyne or formyl). Vertically above this are the main donor reactions supplying C_1 units and binding free tetrahydrofolate. Vertically below are the main acceptor reactions of C_1 units, which regenerate free tetrahydrofolate. Important is the irreversibility *in vivo* of the reduction of methylene- to methyl-tetrahydrofolate, the ready reversibility of the reactions between 5,10-methylene- and 10-formyltetrahydrofolate, and the virtual irreversibility of methionine-dependent dehydrogenation of formyltetrahydrofolate to CO_2 and free tetrahydrofolate. The reactions denoting uptake of C_1 units do not necessarily balance with the reactions regenerating free tetrahydrofolate, i.e. the synthesis of methionine, thymidylate and purines. The flexible outlet for excess of C_1 units is formyltetrahydrofolate dehydrogenase.

With [U-^{14}C]histidine a yield of six molecules of $^{14}CO_2$ is expected per molecule of glucose formed. The actual yield approached this value; it was 4.9 in the presence of methionine and 3.4 in the absence of methionine.

Ethionine was as effective as methionine (Table 6). There was a slight effect with 1 mM-homocysteine, presumably because this substance is converted into methionine in the tissue. There was also a slight effect with selenomethionine.

To test whether formiminoglutamate also accumulates in the intact perfused rat liver (which as a routine is less washed by the perfusion medium than the isolated hepatocytes) a liver was freeze-clamped after 1 h perfusion under the conditions (10 mM-histidine) of the experiments of Table 1. The concentration of formiminoglutamate in the liver increased to 17.7 μmol/g, whereas the tissue glutamate concentration increased to 3.85 mM, only slightly above the normal concentration of about 3 mM. The concentrations of formiminoglutamate and glutamate in the medium were 0.053 and 0.43 mM respectively. The concentration gradient of formiminoglutamate between tissue and medium was thus very high (334).

Of the total formiminoglutamate formed (116 μmol), about 7% (7.8 μmol) appeared in the perfusion medium. Of the total glutamate present in the perfusion system at the end (88.5 μmol), more than 70% was in the medium. In another experi-

Table 6. *Effects of substances related to L-methionine on histidine metabolism in isolated hepatocytes*

Hepatocytes from a rat deprived of food for 48 h (83 mg) were incubated in 4 ml of 2 mM-[*ring*-2-^{14}C]histidine for 1 h in the conditions indicated.

Additional substrate (0.1 mM, except where stated otherwise)	Glucose formed (μmol/g)	Glutamate+ formiminoglutamate found (μmol/g)	$^{14}CO_2$ formed (μmol/g)
None	2.39	40.5	3.08
L-Methionine	7.73	20.3	9.51
L-Ethionine	7.14	19.8	9.56
D-Methionine	1.76	29.8	3.86
DL-Selenomethionine	3.01	24.0	6.10
L-Methionine sulphoximine	1.79	27.6	3.32
L-Homocysteine	2.30	29.0	3.21
L-Homocysteine (1 mM)	3.17	20.3	4.78

Table 7. *Distribution of formiminoglutamate and glutamate between cells and medium on incubation of hepatocytes with histidine*

The data refer to 4 ml of hepatocyte suspension containing 87.3 mg of cells. The suspension was incubated under standard conditions, and cells and medium were separated as described by Hems *et al.* (1975). A control (not shown in the Table) was incubated without added substrates. This contained no measurable amount of formiminoglutamate. In this control the glutamate concentration in cells was 0.504 μmol/g after 30 min and 0.882 μmol/g after 60 min. The glutamate concentration in the medium was 0.036 mM after 30 min and 0.055 mM after 60 min.

Substrates added ...	2 mM-Histidine		2 mM-Histidine+ 0.1 mM-methionine	
Incubation time (min) ...	30	60	30	60
[Formiminoglutamate] in cells (μmol/g)	8.76	14.1	3.27	5.13
[Formiminoglutamate] in medium (mM)	0.096	0.148	0.086	0.118
[Formiminoglutamate] in cells (μmol/g) / [Formiminoglutamate] in medium (mM)	91	95.5	38	43
[Glutamate] in cells (μmol/g)	0.847	0.939	1.34	1.94
[Glutamate] in medium (mM)	0.035	0.041	0.057	0.071
[Glutamate] in cells (μmol/g) / [Glutamate] in medium (mM)	24	23	23.5	27.3
Absolute amounts of formiminoglutamate in 87.3 mg of cells (μmol)	0.765	1.24	0.286	0.447
Absolute amounts of formiminoglutamate in 3.9 ml of medium (μmol)	0.382	0.593	0.344	0.472
Absolute amounts of glutamate in 87.3 mg of cells (μmol)	0.074	0.082	0.117	0.169
Absolute amounts of glutamate in 3.9 ml of medium (μmol)	0.139	0.165	0.226	0.282

Table 8. *Metabolism of urocanate in isolated hepatocytes*

The data shown are changes in metabolites in 4ml of cell suspension containing 69mg of cells (μmol/h per g) for 60min incubation. The values are corrected for control changes. The initial concentrations of histidine and urocanate were 2mM, and of methionine, 0.1mM.

Substrates added ...	Urocanate	Urocanate+ methionine	Histidine	Histidine+ methionine
Metabolic changes	−12.0	−12.3		
Urocanate	−12.0	−12.3		
Histidine			−41.0	−37.8
Glucose	+ 0.65	+ 3.7	+ 1.45	+ 8.95
Formiminoglutamate	+13.5	+ 7.2	+36.6	+24.6
Glutamate	− 2.8	+ 3.2	+ 7.0	+ 1.1

Table 9. *Effect of methionine on the formation of* $^{14}CO_2$ *from* [^{14}C]*formate*

About 90mg of cells were incubated for 1h with 2mM-[^{14}C]formate. The concentration of methionine (where added) was 0.1mM. The values are means±S.E.M. of seven observations.

Substrates added ...	Formate	Formate+ methionine
Rate of $^{14}CO_2$ formation (μmol/g)	12.2±1.0	24.6±2.9

ment, where 1mM-histidine was added under the same conditions, much less glutamate (14.4μmol) and only traces of formiminoglutamate were shed, and the concentration of formiminoglutamate in the liver increased to only 2.7μmol/g.

Formiminoglutamate is known to appear in the urine on histidine loading. It is apparent from these experiments that shedding of formiminoglutamate by the liver is relatively slow. This is also true for isolated hepatocytes (Table 7). High concentration gradients between cells and medium (about 90) developed when the cells were incubated with 2mM-histidine. The intracellular concentration increased to 14mM in 60min. When methionine was also added, the intracellular concentration of formiminoglutamate did not increase above 5.1mM, and the gradient was about 40. The gradient of glutamate was not affected by methionine, but the absolute amounts increased in both cells and medium. As the volume of the suspension medium was about 45 times that of the cells, the absolute amounts of formiminoglutamate in the medium were about one-half of those in the cells in the presence of histidine and almost equal in the presence of histidine plus methionine. Of glutamate about two-thirds was present in the medium, irrespective of the presence of methionine.

Addition of urocanate to hepatocyte suspensions in place of histidine also caused an accumulation of formiminoglutamate, which was decreased by methionine (Table 8). The rate of urocanate metabolism was much lower than that of histidine metabolism, which implies that added urocanate is metabolized less rapidly than urocanate generated intracellularly. Thus the entry of urocanate into the liver cell must be a rate-limiting factor.

Effect of methionine on formate metabolism

In attempts to establish the point of attack of methionine, the effect of methionine on $^{14}CO_2$ production from various donors of C_1 units to tetrahydrofolate was tested under conditions where methionine increased the rate of $^{14}CO_2$ release from histidine. Experiments with serine and glycine were negative, but the degradation of formate in isolated hepatocytes was also found to be accelerated by methionine (Table 9). The main pathway of formate oxidation in the liver involves tetrahydrofolate, the steps being the formation of formyltetrahydrofolate catalysed by formyltetrahydrofolate synthetase (EC 6.3.4.3) and formyltetrahydrofolate dehydrogenase (EC 1.5.1.6). The latter step is shared with the pathway of degradation of the formimino group of formiminoglutamate. Some formate can also be oxidized by peroxidation, catalysed by catalase (Chance, 1952; Keilin & Hartree, 1955), but the main pathway *in vivo* is that involving tetrahydrofolate. This follows from the fact that formate appears in the urine in folate deficiency (Stokstad *et al.*, 1966). The peroxidative degradation of formate is limited *in vivo* by the availability of NADPH.

The effects of methionine on CO_2 formation from formate and from [*ring*-2-C^{14}]histidine are brought about by the same low concentrations of methionine and are of the same order of magnitude (Table 10). In percentage terms the increase of formate oxidation is less than that of CO_2 release from histidine, perhaps because some formate is metabolized under the test conditions by the peroxidase pathway. There was a slightly increased rate of glucose formation in the presence of formate and methionine. This is not unexpected, because methionine is

Table 10. *Comparison of the effects of methionine on the formation of $^{14}CO_2$ and glucose in the presence of [^{14}C]formate or [ring-2-^{14}C]histidine*

Cells (62.2mg) were incubated in a 4ml volume for 1h. The amounts of $^{14}CO_2$ produced were as described in the Materials and Methods section.

[Methionine] (mM)	Formate (2mM) added		Histidine (2mM) added	
	$^{14}CO_2$ formed (μmol/g)	Glucose formed (μmol/g)	$^{14}CO_2$ formed (μmol/g)	Glucose formed (μmol/g)
0	13.8	16.6	2.6	23.0
0.05	21.8	22.8	4.4	33.6
0.1	27.8	24.2	12.0	60.5
0.5	42.0	25.0	13.0	60.5

Table 11. *Effect of formate on the release of $^{14}CO_2$ from [ring-2-^{14}C]histidine in isolated hepatocytes*

Hepatocytes (87mg) were incubated with 2mM-[*ring*-2-^{14}C]histidine for 1h. The concentration of added methionine was 0.1mM. The percentage of $^{14}CO_2$ formed from histidine was calculated on the assumption that formate did not affect the total CO_2 release.

Additional substrates ...	None	Methionine	Methionine+formate			
			0.5mM	1.0mM	2.0mM	4.0mM
$^{14}CO_2$ released (μmol/4ml suspensions)	0.46	1.31	1.19	1.06	1.02	0.92
Increase in $^{14}CO_2$ release caused by methionine (μmol)		0.85	0.73	0.60	0.56	0.46
Percentage $^{14}CO_2$ formed from histidine		100	85	71	66	54

Table 12. *Effect of histidine on the release of $^{14}CO_2$ from [^{14}C]formate in isolated hepatocytes*

The experiment was carried out in the same suspensions as those described in Table 11. Conditions were the same except that formate was labelled and histidine unlabelled. All samples contained 2mM-[^{14}C]formate. For further details, see the legend to Table 11.

Additional substrates ...	None	Methionine	Methionine+histidine			
			0.5mM	1.0mM	2.0mM	4.0mM
$^{14}CO_2$ released (μmol/4ml suspensions)	1.20	1.96	1.60	1.46	1.40	1.37
Increase in $^{14}CO_2$ release caused by methionine (μmol)		0.76	0.40	0.26	0.20	0.17
Percentage of CO_2 formed from formate		100	53	34	26	22

known to be glucogenic (via homoserine, α-oxobutyrate and propionyl-CoA). Methionine removal under the test conditions (measured by amino acid analyser) was 0.27μmol in 1h at 0.1mM-methionine, whereas the amount of cysteine increased by 0.21μmol. This rate of methionine disappearance was sufficient to account for the observed glucose formation. Ethionine increased formate oxidation to the same extent as did methionine (not shown in Table 10).

The sharing of the final stages of the pathway of degradation of the ring-2 carbon atom of histidine and of formate is further demonstrated by the fact that formate inhibits $^{14}CO_2$ release from histidine (Table 11) and that histidine inhibits $^{14}CO_2$ release from formate (Table 12). These observations (Tables 9–12) lead to the conclusion that the addition of methionine can increase the activity of formyltetrahydrofolate dehydrogenase.

Discussion

Effect of methionine

The main findings concern the effects of methionine on the degradation of formiminoglutamate and of formate in the isolated perfused liver and in isolated hepatocytes of normal rats. An effect of methionine on the degradation of formiminoglutamate is already known to occur in cobalamin- or folate-deficient mammals, including man. In cobalamin or

folate deficiency, formiminoglutamate is excreted in the urine after histidine loading, and Silverman & Pitney (1958) and Brown *et al.* (1960) have shown that supplementation of the deficient diet with methionine decreases the excretion of formiminoglutamate. Herbert & Sullivan (1963) made analogous observations on patients with megaloblastic anaemia. Since then, many observations demonstrating the involvement of methionine in the metabolism of formiminoglutamate have been reported (Noronha & Silverman, 1962; Kisliuk, 1964; McGeer *et al.*, 1965; Vitale & Hegsted, 1969; Kutzbach *et al.*, 1967; Stahelin *et al.*, 1970; Buehring *et al.*, 1972; Thenen & Stokstad, 1973; Smith *et al.*, 1974).

Results almost identical with the present findings have been reported by Buehring *et al.* (1972) for the perfused liver of cobalamin-deficient rats. In this material methionine also decreases the appearance of formiminoglutamate from histidine, and it increases the formation of $^{14}CO_2$ from [*ring*-2-^{14}C]histidine. What is new is that the liver of normal rats, when perfused with a semi-synthetic medium or handled in the form of hepatocyte suspensions, behaves in respect to histidine metabolism like the liver of cobalamin-deficient rats. Cobalamin tissues are taken to have an abnormally low methionine concentration, because cobalamin is an essential cofactor in the synthesis of methionine from homocysteine and methyltetrahydrofolate. In the liver preparations used in the present work, a lack of methionine was caused by the washing of the liver and the cells with the semi-synthetic medium devoid of free amino acids. Thus the likely common feature of cobalamin-deficient liver and the experimental liver preparations from normal rats may be taken to be a low methionine concentration in the tissue. Tissue preparations from normal animals could therefore be used for the investigation of the methionine effect. This led to one major new finding, namely an accelerating effect of methionine on the conversion of formate into CO_2. The concentrations of methionine that promote the degradation of formate and of formiminoglutamate are the same, and in both cases ethionine can replace methionine. Since 10-formyltetrahydrofolate is an intermediate in both the degradation of formiminoglutamate and of formate, the conclusion is justified that the effects of methionine on the degradation of formiminoglutamate and formate have a common basis.

Although the effects are brought about by the addition of methionine or ethionine, this does not imply that these substances act as such. Both can form *S*-adenosyl derivatives, and the evidence so far reported is compatible with the assumption that the effector is the adenosyl derivative. This idea can be tested on purified preparations of formyltetrahydrofolate dehydrogenase. M. C. Scrutton & I. Beis (Department of Biochemistry, King's College London; unpublished work) carried out such tests at the authors' suggestion and failed to find any direct effects of methionine or of *S*-adenosylmethionine in the purified enzyme. M. C. Scrutton (unpublished work) therefore suggests an alternative mechanism of action, based on the fact (Kutzbach & Stokstad, 1967, 1971) that *S*-adenosylmethionine is known to be an inhibitor of methylenetetrahydrofolate reductase (EC 1.1.1.68), the enzyme catalysing the (irreversible) formation of 5-methyltetrahydrofolate. It is further relevant that the tissue concentration of *S*-adenosylmethionine depends on the concentration of methionine (Lombardini & Talalay, 1971). An inhibition of the reductase would cause an increase in the concentration of methylenetetrahydrofolate. Since this intermediate is at near equilibrium with 10-formyltetrahydrofolate, the concentration of the latter would also increase. This increase would cause an increased activity of 10-formyltetrahydrofolate dehydrogenase (EC 1.5.1.6), provided that the substrate concentration is at or below the K_m value of the enzyme for this substrate. This is the case. The K_m value (for pig liver) is 8.2μM (Kutzbach & Stokstad, 1971); the total folate concentration in normal rat liver is between 10 and 20μM and about half this value in cobalamin deficiency (Williams & Spray, 1970). The concentration of 10-formyltetrahydrofolate is only a small fraction of the total, according to Buehring *et al.* (1972) and Shin *et al.* (1975) less than 10% in cobalamin-deficient rat liver. This concentration of 10-formyltetrahydrofolate in cobalamin deficiency is within the range where the enzyme activity is very much dependent on the substrate concentration.

By 1962, Noronha & Silverman (1962) found that dietary methionine causes a redistribution of the tetrahydrofolate derivatives in the livers of cobalamin-deficient rats, increasing the proportion of 10-formyltetrahydrofolate and free tetrahydrofolate. Striking increases in the relative concentrations of formyltetrahydrofolate and free tetrahydrofolate in livers of cobalamin-deficient rats on addition of methionine have been reported by Buehring *et al.* (1972) and Shin *et al.* (1975). Smith *et al.* (1974), studying cobalamin-deficient sheep, found analogous effects of methionine *in vivo*. Thus the present information is in accordance with the assumption that the increased activity of formyltetrahydrofolate dehydrogenase on addition of methionine is due to an increase in the concentration of the substrate caused by an inhibition of methylenetetrahydrofolate reductase by *S*-adenosylmethionine.

Physiological significance of the methionine effect

The effect of methionine on the formyltetrahydrofolate dehydrogenase reaction may be interpreted

as being a key factor in the regulation of the metabolism of folate and methionine. It is the function of the tetrahydrofolate system to serve as a carrier of C_1 units, as illustrated in Scheme 1. Tetrahydrofolate receives C_1 units from formiminoglutamate (derived from histidine) from serine, glycine and formate. On account of the low tissue concentration of tetrahydrofolate and its derivatives, free tetrahydrofolate must constantly be regenerated if it is to act as a carrier. The regeneration is achieved by disposing of the C_1 unit in the synthesis of purines (which utilizes methylidyne- and formyl-tetrahydrofolate), in the synthesis of pyrimidines (which utilizes methylenetetrahydrofolate), in the synthesis of methionine (which utilizes methyltetrahydrofolate) and by the formyltetrahydrofolate dehydrogenase reaction. The reactions by which the C_1 derivatives of tetrahydrofolate are formed do not necessarily balance with the reactions by which they are used in biosyntheses. Hence there must therefore be a flexible outlet either disposing or preserving C_1 units, in accordance with requirements. The demand for C_1 units in biosyntheses is not constant, but is determined by the requirements of purine, pyrimidine and methionine synthesis. The supply of C_1 units varies with the diet. The flexible outlet that disposes of the excess of C_1 units is the formyltetrahydrofolate dehydrogenase reaction, and the factor that controls the activity of this outlet is the concentration of methionine (or *S*-adenosylmethionine).

Methionine, it should be appreciated, is required, not only as a protein constituent, but also as a methyl donor for a large number of methylation reactions (the methylation, for example, of nicotinamide, noradrenaline, guanidinoacetate, acetylserotonin, histidine, phosphatidylethanolamine, glycine and many forms of tRNA). As long as methionine is utilized by these reactions its concentration will be relatively low. In this situation the activity of formyltetrahydrofolate dehydrogenase will be correspondingly low. When the requirements for methionine are fully met, its concentration will rise, and the activity of formyltetrahydrofolate dehydrogenase will be increased and thereby dispose of the excess of C_1 units.

Biochemistry of cobalamin deficiency and the 'methylfolate trap' hypothesis

In cobalamin deficiency the biochemical sequence of events may be visualized as follows. The primary event is the decrease in the rate of synthesis of methionine, because cobalamin is an essential cofactor in the resynthesis of methionine from homocysteine and methyltetrahydrofolate. In consequence the concentration of methionine in the tissue decreases. This decrease causes a loss of activity of formyltetrahydrofolate dehydrogenase. Thus the regulatory outlet of C_1 units is more or less closed, and the C_1 derivatives of tetrahydrofolate accumulate, that is unless the requirement for pyrimidine and purine synthesis happens to be identical with the supply of C_1 units. Whenever supply exceeds demand, the excess of C_1 units will be reduced to methyltetrahydrofolate, because the thermodynamic equilibrium favours the reduction and, because of lack of cobalamin methyltetrahydrofolate, cannot form methionine, nor can the reaction leading to the formation of methyltetrahydrofolate be reversed. Thus most of the total tetrahydrofolate will be 'trapped' as methyltetrahydrofolate. A consequence of the trapping as methyltetrahydrofolate is the decrease in the concentration of free tetrahydrofolate.

Lack of free tetrahydrofolate interferes with the normal metabolism of donors of C_1 units (see Scheme 1). Serine enters other pathways leading to glucose synthesis or yielding energy by complete degradation. Histidine degradation stops at the stage of formiminoglutamate; formate cannot be utilized. Hence both formiminoglutamate and formate appear in the urine as waste products (Stokstad *et al*., 1966).

The effects of methionine in cobalamin and folate deficiency, namely the decrease in urinary excretion of formiminoglutamate and formate, and a shift of the hepatic tetrahydrofolate derivatives away from methyltetrahydrofolate towards the less reduced C_1 derivatives and free tetrahydrofolate, can now be explained (as discussed under 'Physiological significance of the methionine effect') by a single primary action of *S*-adenosylmethionine: this is the inhibition of the reduction of methylenetetrahydrofolate to methyltetrahydrofolate, discovered by Kutzbach & Stokstad (1967). Its full significance could not be appreciated at the time when the inhibition was first observed, because an important consequence of the inhibition, namely the promotion of the formyltetrahydrofolate dehydrogenase reaction, was not yet known.

In the past, several hypotheses have been put forward to explain the decreased formiminoglutamate formation in cobalamin deficiency on administration of methionine (see Nixon & Bertino, 1970). One of these is the 'methylfolate trap' hypothesis by Noronha & Silverman (1962) and Herbert & Zaluski (1962). Up to now this concept has been controversial (see Beck, 1975; Editorial, 1975; Tisman *et al*., 1975; Lavoie *et al*., 1974; Hoffbrand, 1975), although the accumulation of methyltetrahydrofolate in cobalamin and folate deficiency had been directly demonstrated (Buehring *et al*., 1972; Smith *et al*., 1974; Shin *et al*., 1975). What remained controversial was the mechanism of trapping. The present work confirms the concept of a trap. The trap comes about by the low activity of formyl-

tetrahydrofolate dehydrogenase at low methionine concentrations, by the fact that the thermodynamic equilibrium favours the irreversible reduction of the C_1 units to the methyl stage, and by the weakness, in cobalamin deficiency, of the utilization of methyltetrahydrofolate in the cobalamin-dependent reaction, which regenerates methionine from homocysteine.

Regulation of methionine metabolism

In addition to playing a role in the regulation of folate metabolism, the controlling influence of the concentration of methionine (or *S*-adenosylmethionine) on the activity of 10-formyltetrahydrofolate dehydrogenase may also be visualized as regulating methionine metabolism. As methionine is an essential amino acid, its metabolism must be so regulated as to preserve adequate amounts for protein synthesis and for the methyl-carrier function, while at the same time disposing of dietary excess of methionine. The branching point where the alternative to degradation of methionine is decided is the stage of homocysteine, which may either be remethylated by methyltetrahydrofolate or react with serine to give cystathionine (see Finkelstein, 1974). The latter, via α-oxobutyrate and propionyl-CoA, then yields either energy or glucose. The signal deciding the fate of homocysteine in favour of degradation is a decreased supply of methyltetrahydrofolate. When the tissue is saturated with methionine methyltetrahydrofolate formation decreases owing to increased activity of formyltetrahydrofolate activity, so that homocysteine remains unmethylated and forms cystathionine.

Additional regulatory factors

The concept discussed here is only one aspect of the control mechanisms. Important additional components of regulation are already known. These concern the regulatory properties of other enzymes of folate metabolism. Thus *S*-adenosylmethionine is an essential cofactor, a primer, in the reconversion of homocysteine into methionine (Shapiro *et al.*, 1963; Buchanan *et al.*, 1964), an effect that may be looked upon as a positive feedback: when *S*-adenosylmethionine is formed the concentration of methionine must decrease stoicheiometrically and, by activating the synthesis of methionine, *S*-adenosylmethionine facilitates the replacement of methionine. 10-Formyltetrahydrofolate dehydrogenase is inhibited by free tetrahydrofolate (Kutzbach & Stokstad, 1971). Serine hydroxymethyltransferase (EC 2.1.2.1), which channels C_1 units from serine into the system, is inhibited by 5-methyltetrahydrofolate (Schirch & Ropp, 1967). In consequence, serine enters other pathways such as the conversion into glucose or complete oxidation. This inhibition regulates the amounts of serine used to supply C_1 units. Cystathionine synthase is activated by adenosylmethionine (Finkelstein *et al.*, 1975). It has been suggested that folate metabolism may also be regulated at the stage of the uptake of folate by cells and by the formation of polyglutamates from monoglutamates (see Beck, 1975; Shin *et al.*, 1975; Lavoie *et al.*, 1974; Thenen & Stokstad, 1973; Gawthorne & Smith, 1974). The isolated hepatocytes lend themselves to a study of the factors that control the transport of folates across the cell membrane. The technique may also prove useful in the study of the unsolved question of why cobalamin deficiency is usually associated with folate deficiency. As the folate deficiency occurs irrespective of dietary folate intake, it must be due to a failure of the uptake of folates by the tissues or a failure to retain folates in cobalamin deficiency. Since in this condition methyltetrahydrofolate is the predominant folate form, the transport or retention failure may concern specifically this form.

Methionine concentrations in animal tissues

The assumption has been made in the preceding discussion that the tissue concentration of methionine is low in cobalamin deficiency. Although it is to be expected, to our knowledge no direct demonstration of such a decrease is as yet on record. The tissue methionine concentration is expected to fall in cobalamin deficiency, because, without the cobalamin-dependent remethylation of homocysteine, the methionine requirements are liable to be greater than the dietary supply. Even when the dietary supply is ample to meet the needs for protein synthesis and spermidine synthesis (Williams-Ashman *et al.*, 1969), the additional requirements for methionine as a methylating agent may be a multiple of those of protein synthesis, in view of the large number of methyl aceptors. Creatine requirements alone, as measured by the daily excretion of creatine in the urine in man, may be about the same on a mol-per-mol basis as the methionine content of an average human diet of 60g of protein daily (though some of this may be covered by dietary creatine). Any extra requirements for methionine are met by the cobalamin-dependent regeneration of methionine from homocysteine.

A sub-optimal concentration of methionine in tissue is strongly indicated by the fact that supplementation with methionine decreases the excretion of formiminoglutamate and restores normality in the profile of tetrahydrofolate derivatives. Moreover a fall in the concentration of *S*-adenosylmethionine in the liver to one-half of its normal value (from the normal range of 0.01–0.02μM) has been demonstrated by Gawthorne & Smith (1974) for cobalamin-deficient sheep.

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