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A New Method for the Assay of Tissue *S*-Adenosylhomocysteine and *S*-Adenosylmethionine

EFFECT OF PYRIDOXINE DEFICIENCY ON THE METABOLISM OF *S*-ADENOSYLHOMOCYSTEINE, *S*-ADENOSYLMETHIONINE AND POLYAMINES IN RAT LIVER

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The hepatic synthesis and accumulation of *S*-adenosylhomocysteine, *S*-adenosylmethionine and polyamines were studied in normal and vitamin B-6-deficient male albino rats. A method involving a single chromatography on a phosphocellulose column was developed for the determination of *S*-adenosylhomocysteine and *S*-adenosylmethionine from tissue samples. Feeding the rat with pyridoxine-deficient diet for 3 or 6 weeks resulted in a four- to five-fold increase in the concentration of *S*-adenosylhomocysteine, whereas that of *S*-adenosylmethionine was only slightly elevated. The concentration of putrescine was decreased to half, that of spermidine was somewhat decreased and that of spermine remained fairly constant. The activities of L-ornithine decarboxylase, *S*-adenosyl-L-methionine decarboxylase, L-methionine adenosyltransferase and *S*-adenosyl-L-homocysteine hydrolase were moderately increased. *S*-Adenosylmethionine decarboxylase showed no requirement for pyridoxal 5′-phosphate. The major effect of pyridoxine deficiency on *S*-adenosylmethionine metabolism seems to be a block in the utilization of *S*-adenosylhomocysteine, resulting in the accumulation of this metabolite to a concentration that may inhibit biological methylation reactions.

The major metabolic functions of methionine are its utilization for protein synthesis, action as the main methyl-group donor and conversion into cysteine and its derivatives through the trans-sulphuration pathway. The latter is also the major route for methionine catabolism in mammals (Finkelstein & Mudd, 1967; Finkelstein, 1974). The methylation and trans-sulphuration pathways are linked with each other and involve the activation of methionine by a specific adenosyltransferase (Lombardini & Talalay, 1971). The product, *S*-adenosyl-L-methionine, then acts as the methyl-group donor in numerous transmethylation reactions, and also as the propylamine-group donor in the biosynthesis of polyamines (for references see Lombardini & Talalay, 1971). Most of the *S*-adenosylmethionine synthesized is utilized in transmethylation reactions, yielding *S*-adenosyl-L-homocysteine (Finkelstein, 1974). In mammalian liver the main catabolic route of *S*-adenosylhomocysteine probably involves cleavage to adenosine and L-homocysteine by a specific hydrolase (Lombardini & Talalay, 1971). The further catabolism of L-homocysteine involves two pyridoxal enzymes: cystathionine synthase and cystathionase (for references see Finkelstein, 1974). Pyridoxine deficiency is known to decrease the activity of cystathionase greatly, leading to a marked accumulation of cystathionine (Sturman *et al.*, 1969). This block would also be expected to disturb the catabolism of *S*-adenosylhomocysteine.

The rate-limiting steps of polyamine synthesis in mammalian tissues are catalysed by two decarboxylases, i.e. ornithine decarboxylase, which shows an absolute requirement for pyridoxal phosphate, and putrescine-activated *S*-adenosylmethionine decarboxylase (Raina & Jänne, 1975). At present, some controversy still exists as to whether pyridoxal phosphate also acts as the prosthetic group of the latter enzyme (Hannonen, 1976). Thus pyridoxine deficiency might be a useful tool to study the regulative aspects in the biosynthesis and metabolism of *S*-adenosylhomocysteine, *S*-adenosylmethionine and polyamines.

The quantitative analysis of *S*-adenosylhomocysteine and *S*-adenosylmethionine from tissue samples has been fairly cumbersome (Salvatore *et al.*, 1971). A significant improvement in the analysis of these compounds was recently reported by Hoffman (1975). In the present work we have developed a method for the analysis of *S*-adenosylhomocysteine and *S*-adenosylmethionine from tissues. Further, we have studied the effects of pyridoxine deficiency on

the synthesis and accumulation of *S*-adenosylhomocysteine, *S*-adenosylmethionine and polyamines in rat liver. Our results demonstrate a remarkable accumulation of *S*-adenosylhomocysteine in the livers of vitamin B-6-deficient animals.

Experimental

Radiochemicals

L-[*Me*-^{14}C]Methionine (sp. radioactivity 56mCi/mmol), *S*-adenosyl-L-[*Me*-^{14}C]methionine (55mCi/mmol), DL-[1-^{14}C]ornithine monohydrochloride (58mCi/mmol) and [8-^{14}C]adenosine (59mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. DL-[1-^{14}C]Methionine (8.69mCi/mmol) was from New England Nuclear Corp., Boston, MA, U.S.A. DL-[1-^{14}C]Ornithine was treated with HCl (Hölttä & Raina, 1973) before use. *Carboxy*-^{14}C-labelled and unlabelled *S*-adenosylmethionine were synthesized essentially as described by Pegg & Williams-Ashman (1969).

S-[8-^{14}C]Adenosylhomocysteine was synthesized and purified as follows. The reaction mixture, containing 0.1M-potassium phosphate (pH7.3), 3mM-L-homocysteine, 2mM-dithiothreitol, 25μM-adenosine deaminase inhibitor (see below), 16μCi of [8-^{14}C]-adenosine (59mCi/mmol) and 2mg of crude supernatant protein from rat liver, was incubated at 37°C for 12min. Under these conditions the reaction proceeded essentially to completion. The incubation was terminated by adding 25μl of 30% (w/v) $HClO_4$. Further steps were performed at 4°C. The acid supernatant was quantitatively transferred into a Cellex-P (H^+ form) column (1cm×5cm), and the column was washed with 100ml of 1mM-HCl and 30ml of 10mM-HCl to remove adenosine and other contaminants. *S*-[8-^{14}C]Adenosylhomocysteine was eluted in 2.5ml fractions with 0.1M-HCl. The radioactive fractions were pooled, adjusted to pH9 with 10M-NaOH and evaporated to dryness at 30°C with a vacuum evaporator. The residue was dissolved in water and stored at −20°C. The radiochemical purity of the product was 98.3%, as determined by paper electrophoresis with 50mM-glycine/NaOH buffer (pH11) (Kajander *et al.*, 1976).

Other materials

Dithiothreitol and putrescine dihydrochloride were purchased from Calbiochem, Los Angeles, CA, U.S.A., and ATP was from Boehringer, Mannheim, Germany. L-Homocysteine thiolactone hydrochloride was supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. L-Homocysteine was prepared from L-homocysteine thiolactone as described by Duerre & Miller (1966). Pyridoxal 5′-phosphate, L-ornithine monohydrochloride, L-methionine and glycylglycine were from Fluka A.G., Buchs SG, Switzerland. The inhibitor of adenosine deaminase, *erythro*-9-(2-hydroxy-3-nonyl)adenine, was prepared by Dr. H. J. Schaeffer (Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A.) and obtained through the generosity of Dr. Seymour S. Cohen, Denver, CO, U.S.A. Whatman phosphocellulose paper P81 was purchased from Reeve Angel, London E.C.4, U.K., and cation-exchange cellulose Cellex-P was from BioRad Laboratories, Richmond, CA, U.S.A. Other chemicals were of analytical grade and were supplied by Merck A.G., Darmstadt, Germany.

Animals

Male albino rats of the Wistar strain weighing 40g were divided in three groups, each maintained under regularly alternating 12h periods of light and dark and allowed to eat and drink *ad libitum*. The control group was fed on a standard diet (Hankkija Oy, Turku, Finland). One group received pyridoxine-deficient diet (Nutritional Biochemical Corp., Cleveland, OH, U.S.A.), and the other group was fed on the same diet supplemented with 50mg of pyridoxine hydrochloride/kg of dry diet. The rats were killed between 08:00h and 10:00h to minimize diurnal variation in enzyme activities. Food was removed just before the animals were killed. The dishes containing the food were changed daily. Special care was taken to avoid contamination of the food by the excreta of the animals.

Enzyme assays

The rats were decapitated under ether anaesthesia, and the livers were rapidly removed, washed in ice-cold 0.25M-sucrose and homogenized with a Potter–Elvehjem homogenizer in 2–3vol. of ice-cold 0.25M-sucrose containing 1mM-mercaptoethanol, 0.1mM-EDTA and 1mM-dithiothreitol (Eloranta *et al.*, 1976). The homogenates were centrifuged with an MSE Superspeed 50 centrifuge for 45 min at 105000$g_{av.}$ (42000rev./min) at 4°C in an MSE angle rotor (catalogue no. 59113). Samples of the supernatant fractions were dialysed overnight at 4°C against 200vol. of 25mM-potassium phosphate (pH7.2) containing 1mM-mercaptoethanol, 0.1mM-EDTA and 0.1mM-dithiothreitol. All the enzyme activities were determined in both the dialysed and undialysed supernatant fractions.

The activities of L-ornithine decarboxylase (EC 4.1.1.17) and *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) were determined essentially as previously described (Eloranta *et al.*, 1976). The assay mixture for ornithine decarboxylase contained, in a total volume of 0.5ml, 0.1M-glycylglycine (pH7.0), 4mM-dithiothreitol, 10mM-EDTA and 0.2mM-L-[1-^{14}C]ornithine (5.07mCi/mmol) with or without 0.4mM-pyridoxal 5′-phosphate. The assay mixture for *S*-adenosylmethionine decarboxylase contained 0.1M-potassium phosphate (pH7.4), 2mM-dithiothreitol, 2mM-putrescine, 10mM-EDTA and 0.1mM-*S*-adeno-

syl-L-[*carboxy*-^{14}C]methionine (2.0mCi/mmol) with or without 0.05mM-pyridoxal 5'-phosphate.

The assay of L-methionine adenosyltransferase (EC 2.5.1.6) was performed by the method of McKenzie & Gholson (1973), slightly modified as described previously (Eloranta *et al.*, 1976). *S*-Adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) activity was measured by the method of Kajander *et al.* (1976).

Assay of S-adenosylhomocysteine and S-adenosylmethionine

Portions of liver homogenates containing 2–3g of wet tissue were immediately mixed with an appropriate volume of 50% (w/v) trichloroacetic acid to give a final concentration of 8%. In each sample 30–40nCi of both *S*-[8-^{14}C]adenosylhomocysteine and *S*-adenosyl-L-[*Me*-^{14}C]methionine were added as internal standards. After centrifugation (at 10000*g* for 15min at 4°C), the supernatant was separated and extracted three times with an equal volume of ether to remove most of the trichloroacetic acid and to give a final pH of 2.5–3.0. The supernatant was then filtered through a filter paper and applied to a Cellex-P (H^+ form) column (1cm×7cm). The column was washed with 100ml of 1mM-HCl, followed by 60–80ml of 10mM-HCl to remove u.v.-absorbing contaminants. Thereafter, *S*-adenosylhomocysteine was eluted with 20ml of 50mM-HCl. After passage of another 60ml of 50mM-HCl through the column, *S*-adenosylmethionine was eluted with 15ml of 0.5M-HCl. Although no degradation of *S*-adenosylhomocysteine or *S*-adenosylmethionine was observed when the separation procedure was carried out at room temperature, the chromatography was performed at 7–10°C in a special refrigerated chamber (0.60m× 1.45m×1.85m) equipped with sliding glass doors (Pakkaskone, Helsinki, Finland). The amounts of *S*-adenosylhomocysteine and *S*-adenosylmethionine were determined by measuring E_{257} and using a molar extinction coefficient of 15000 litre·mol^{-1}·cm^{-1} for both substances. The recoveries were measured by counting the radioactivities of 500μl samples of the fractions in 10ml of Bray's scintillant (4g of 2,5-diphenyloxazole, 0.2g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 60g of naphthalene, 100ml of methanol, 20ml of ethylene glycol, made up to 1000ml with 1,4-dioxan) in a liquid-scintillation counter (LKB Wallac model 81000). Counting efficiencies in 50mM-HCl and in 0.5M-HCl were determined with ^{14}C-labelled toluene (Packard Instrument International S.A., Zurich, Switzerland), and were 78 and 71% respectively.

To determine the specificity of the method described above, the fractions containing *S*-adenosylhomocysteine or *S*-adenosylmethionine were evaporated to dryness at 25–30°C and dissolved in 250μl of water. *S*-Adenosylmethionine samples were analysed by low-voltage paper electrophoresis with 0.1M-sodium citrate buffer (pH3.5) and Whatman no. 1 3cm× 40cm strips (Raina, 1963). After electrophoresis for 3h (300V, 8V/cm), the strips were dried, examined under u.v. light and cut into 0.5cm pieces. Each piece was eluted overnight with 2ml of 10mM-HCl and then centrifuged. Clear supernatant was used for measurement of E_{257} and for determination of radioactivity as described below. All the u.v.-absorbing material and the radioactivity were recovered in the fraction having the same electrophoretic mobility as pure *S*-adenosylmethionine. No other u.v.-absorbing material was detectable. Also, the specific radioactivity of the isolated *S*-adenosylmethionine was the same whether determined from the electrophoretic fraction or from the 0.5M-HCl effluent. The purity of the *S*-adenosylmethionine in the 0.5M-HCl effluent was further confirmed by ascending paper chromatography with butan-1-ol/acetic acid/water (2:1:1, by vol.) and with the solvent containing 20ml of propan-2-ol in 1000ml of 0.1M-sodium phosphate buffer (pH6.8) saturated with $(NH_4)_2SO_4$ (Schlenk & Zydek-Cwick, 1969). The R_F values for *S*-adenosylmethionine with these two solvent systems were 0.15 and 0.30 respectively.

Similarly, samples containing *S*-adenosylhomocysteine were analysed by paper electrophoresis with 50mM-glycine/NaOH buffer (pH11), as described previously in detail (Kajander *et al.*, 1976). Only one u.v.-absorbing fraction was detected on paper strips, this containing more than 90% of the radioactivity applied on the strip and having the same mobility as commercial *S*-adenosylhomocysteine. The specific radioactivity of this fraction agreed well with that determined from the 50mM-HCl effluent. On paper chromatography with butan-1-ol/acetic acid/water (2:1:1, by vol.) the sample obtained by evaporating the 50mM-HCl effluent revealed only one u.v.-absorbing spot, which migrated like commercial *S*-adenosylhomocysteine (R_F 0.22) and contained 90–95% of the radioactivity of the samples. The rest of the radioactive material migrated like adenine on paper chromatography. Although practically no hydrolysis of *S*-adenosylhomocysteine was noted in 50mM-HCl during storage for several days at 4°C, some degradation (less than 10%) to adenine did occur during the evaporation of the acid effluent (cf. Duerre, 1962). No degradation of *S*-adenosylhomocysteine during evaporation was observed when 10M-formic acid was used for elution instead of 50mM-HCl. Because of the high u.v. absorbance of concentrated formic acid, however, this was not used for elution.

After hydrolysis in 1M-HCl at 100°C for 15min, the isolated *S*-adenosylhomocysteine was quantitatively degraded to adenine, as shown by paper chromatography (see above).

Assay of polyamines, nucleic acids and protein

Portions of homogenates were treated with cold 10% (w/v) trichloroacetic acid, and polyamines were extracted into alkaline butan-1-ol (Raina, 1963). Spermine and spermidine were separated by electrophoresis (Raina, 1963) and analysed by the ninhydrin method (Raina *et al.*, 1967). Putrescine was determined from $HClO_4$-treated homogenates by the dansylation method (Cohen *et al.*, 1969). The dansylputrescine spots were located under u.v. light, scraped out by the aid of suction and extracted with 3ml of dioxan (Seiler & Knödgen, 1971). After sedimentation of the silica gel by centrifugation (at 1000*g* for 10min), the fluorescence of the dioxan extracts was measured with a Perkin–Elmer fluorescence spectrophotometer (model MPF-3) at 500nm (fluorescence activation, 365nm).

Nucleic acids were extracted from the trichloroacetic acid precipitates into hot 5% (w/v) trichloroacetic acid, and DNA was then determined by the diphenylamine reaction and RNA by the orcinol method, both as described by Ashwell (1957).

Protein was determined from the 105000$g_{av.}$ supernatants by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Separation of *S*-adenosylhomocysteine and *S*-adenosylmethionine in a phosphocellulose column is shown in Fig. 1. Under standard assay conditions an amount of acid tissue extract corresponding to 2–2.5g of liver was applied to the column. The elution profile shows that the separation of *S*-adenosylhomo-

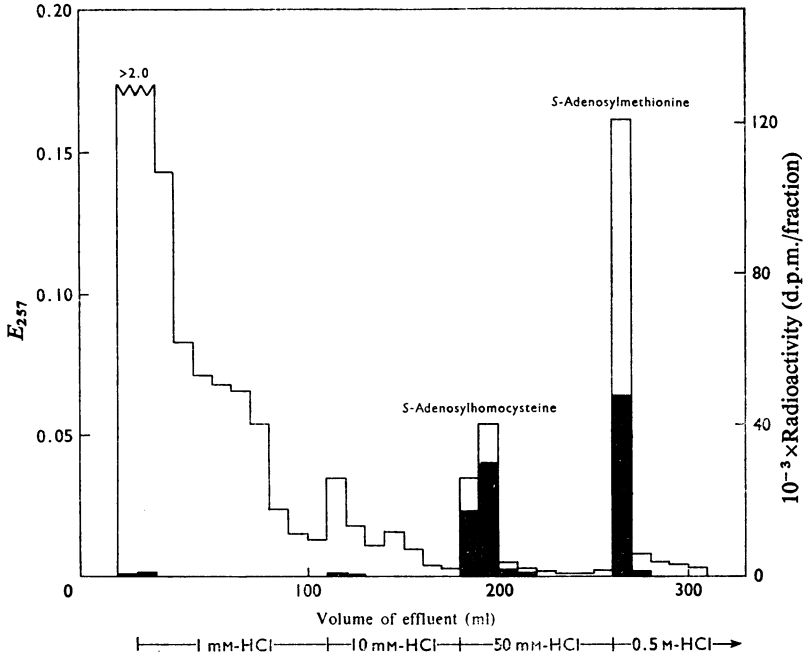

Fig. 1. *Separation of S-adenosylhomocysteine and S-adenosylmethionine from liver extract by phosphocellulose column chromatography*

A 12ml sample of homogenate (see the Experimental section), prepared from pooled livers of female rats (average weight 120g) kept without food for 12h, was added with *S*-adenosyl-L-[*Me*-^{14}C]methionine (82500d.p.m.) and *S*-[8-^{14}C]adenosylhomocysteine (87750d.p.m.) and immediately mixed with 2.5ml of ice-cold 50% (w/v) trichloroacetic acid. After centrifugation, the supernatant was extracted three times with ether. After filtration, a 9.0ml portion of the supernatant, corresponding to 1.86g of wet liver, was applied to a Cellex-P (H^+ form) column (1cm×7cm). The column was eluted with a stepwise HCl gradient by collecting 10ml fractions as shown in the Figure. Absorbance was read at 257nm, and radioactivities of samples of the effluent were counted. Radioactivity is shown by black and E_{257} by white columns. About 87% of radioactive *S*-adenosylhomocysteine present in the tissue extract was recovered in the first 20ml of 50mM-HCl effluent and 93% of radioactive *S*-adenosylmethionine in the first 10ml of 0.5M-HCl effluent. Adenosine, hypoxanthine and inosine were eluted with 1mM-HCl. To elute adenine from the column, 10mM-HCl was required.

cysteine and S-adenosylmethionine from each other and from contaminating material was satisfactory (Fig. 1). The fractions corresponding to S-adenosylhomocysteine and S-adenosylmethionine were uncontaminated by other u.v.-absorbing material, as determined by paper electrophoresis and paper chromatography (see the Experimental section).

Judged by the recovery of radioactivity from the internal ^{14}C-labelled standards, normally 80–90% of S-adenosylhomocysteine and 85–95% of S-adenosylmethionine present in acid tissue extract were recovered in the corresponding HCl effluents. To avoid overloading of the Cellex-P column (bed volume 1 cm × 7 cm) by various cations, the amount of tissue extract corresponding to 5 g of liver was not exceeded.

The effects of pyridoxine deficiency on the synthesis and accumulation of liver S-adenosylhomocysteine, S-adenosylmethionine and polyamines were studied in young male rats. Feeding with a pyridoxine-deficient diet rapidly resulted in marked growth retardation (Table 1), and typical deficiency symptoms (Wagner & Folkers, 1964; Sturman *et al.*, 1969) were observed. Also, increases in liver weight and hepatic content of nucleic acids were totally prevented (Table 1). The rats fed on a vitamin B-6-supplemented diet maintained normal development.

The most pronounced effect of pyridoxine deficiency was seen in the concentration of S-adenosylhomocysteine, which, during the 24-day deficiency period, increased almost fivefold compared with control groups (Table 2). Although the hepatic content of S-adenosylhomocysteine was somewhat lower in both vitamin B-6-deficient and control groups 46 days after commencement of the feeding schedule, the difference between the groups was of the same order as that found after the 24-day deficiency period. These changes were accompanied by a 45–55% increase in the activity of S-adenosylhomocysteine hydrolase. The synthesis and accumulation of S-adenosylmethionine were also increased, but less drastically. After a 6-week deficiency period the activity of methionine adenosyltransferase was increased

Table 1. *Effect of pyridoxine deficiency on rat growth and hepatic content of nucleic acids*

The experimental diet was introduced at the age of 2.5 weeks (mean animal weight 40 g) and continued for 24 or 46 days. For details see the Experimental section. The values for rat and liver weights are means ± S.D. of six animals and those for nucleic acids are means ± S.D. of three pooled samples, each obtained from two livers.

Group	Rat wt. (g)	Liver wt. (g)	Nucleic acids (μmol/liver) DNA P	Nucleic acids (μmol/liver) RNA P
24-day diet				
Vitamin B-6-deficient	101 ± 9	5.4 ± 0.9	25 ± 1	155 ± 7
Vitamin B-6-supplemented	158 ± 16	8.1 ± 1.0	44 ± 4	251 ± 13
Standard	162 ± 9	8.2 ± 0.4	46 ± 2	243 ± 7
46-day diet				
Vitamin B-6-deficient	122 ± 14	5.4 ± 0.8	27 ± 2	135 ± 20
Vitamin B-6-supplemented	259 ± 16	9.6 ± 0.9	58 ± 1	264 ± 19
Standard	265 ± 24	11.2 ± 1.1	64 ± 5	297 ± 33

Table 2. *Effect of pyridoxine deficiency on the metabolism of S-adenosylhomocysteine and S-adenosylmethionine in rat liver*

The values for enzyme activities are expressed as nmol of product formed/min per mg of soluble protein at 37°C and represent means ± S.D. of six undialysed enzyme preparations. The values for S-adenosylhomocysteine and S-adenosylmethionine are means ± S.D. of three preparations, each derived from two livers. $^{**}P < 0.01$; $^{***}P < 0.001$, for the significance of the differences (based on Student's *t* test) as compared with the group fed on the vitamin B-6-supplemented diet.

	S-Adenosylhomocysteine (nmol/g of wet liver)	S-Adenosylhomocysteine hydrolase	S-Adenosylmethionine (nmol/g of wet liver)	Methionine adenosyltransferase
24-day diet				
Vitamin B-6-deficient	429 ± 79**	61 ± 5***	111 ± 9	4.45 ± 0.42**
Vitamin B-6-supplemented	90 ± 12	42 ± 6	120 ± 3	3.57 ± 0.35
Standard	87 ± 12	40 ± 1	82 ± 1	3.87 ± 0.08
46-day diet				
Vitamin B-6-deficient	242 ± 7***	68 ± 11***	116 ± 15	5.70 ± 0.85**
Vitamin B-6-supplemented	61 ± 16	44 ± 4	89 ± 9	3.97 ± 0.42
Standard	72 ± 2	46 ± 4	85 ± 8	4.56 ± 0.21

Table 3. *Effect of pyridoxine deficiency on the synthesis and accumulation of polyamines in rat liver*

Except for putrescine, all the values are means±S.D. of three pooled samples, each obtained from two livers. The concentration of putrescine was determined from a pooled sample obtained from six livers. The specific activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase are expressed as pmol of $^{14}CO_2$ formed from *carboxy*-^{14}C-labelled substrate/30min per mg of soluble protein at 37°C. Ornithine decarboxylase was assayed in the presence and *S*-adenosylmethionine decarboxylase in the absence of pyridoxal phosphate. For assay methods and detailed assay conditions see the Experimental section. *$P<0.05$; **$P<0.01$; ***$P<0.001$, for the significance of the differences (based on Student's *t* test) as compared with the group fed on the Vitamin B-6-supplemented diet. n.d., not determined.

Group	Ornithine decarboxylase (undialysed)	*S*-Adenosylmethionine decarboxylase (undialysed)	*S*-Adenosylmethionine decarboxylase (dialysed)	Putrescine (nmol/g of wet liver)	Spermidine (nmol/g of wet liver)	Spermine (nmol/g of wet liver)
24-day diet						
Vitamin B-6-deficient	12.3±4.2*	110.3±12.4***	160.6±11.2***	6.1	556±31*	920±30**
Vitamin B-6-supplemented	3.5±2.2	47.6± 0.7	66.3±10.8	11.4	749±84	1031±17
Standard	2.5±0.4	75.2±14.7	108.1±11.1	17.4	874±44	900±51
46-day diet						
Vitamin B-6-deficient	8.9±4.4	83.6±27.3	131.0±34.5	n.d.	539±64	1062±96
Vitamin B-6-supplemented	2.1±1.1	69.9±22.9	109.1±24.1	n.d.	535±67	1093±66
Standard	1.4±0.4	35.3± 7.5	57.1±12.4	n.d.	774±36	912±50

by more than 40%, and the concentration of *S*-adenosylmethionine by 30% (Table 2).

Feeding with pyridoxine-deficient diet also resulted in some significant changes in the synthesis and accumulation of polyamines in the liver. In agreement with the results of Sturman & Kremzner (1974), during the first 3 weeks of deficiency the concentration of putrescine was decreased by about 50% and that of spermidine somewhat less (Table 3). However, with prolonged deficiency the difference in spermidine concentration between vitamin B-6-deficient and vitamin B-6-supplemented animals disappeared. The decreases in putrescine and spermidine concentrations were accompanied by clear rises in the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase. The activities of these enzymes were determined from dialysed and undialysed crude supernatants both in the presence and in the absence of exogenous pyridoxal 5'-phosphate. In the absence of pyridoxal phosphate the activities of ornithine decarboxylase were too small to be measured accurately. In agreement with the results of Hannonen (1976), but contrary to the results reported by Sturman & Kremzner (1974), the same activities of *S*-adenosylmethionine decarboxylase were observed whether or not pyridoxal phosphate was included in the assay system. However, it must be emphasized that our routine assay mixture contains putrescine and dithiothreitol (see Hannonen, 1976). Dialysis had no effect on the activity of ornithine decarboxylase, but significantly increased both the total activity (results not shown) and the specific activity of *S*-adenosylmethionine decarboxylase (Table 3). This might indicate the presence of some low-molecular-weight inhibitors of *S*-adenosylmethionine decarboxylase in crude liver extracts. There was no difference in this respect between the vitamin B-6-deficient group and the control groups. The spermine concentration remained at the control value throughout the 6-week deficiency period.

Discussion

In the present work we describe a simple method for a simultaneous analysis of *S*-adenosylhomocysteine and *S*-adenosylmethionine from tissue samples. We further demonstrate that pyridoxine deficiency for 3–6 weeks leads to a marked hepatic accumulation of *S*-adenosylhomocysteine, accompanied by smaller increases in the concentration of *S*-adenosylmethionine and in the activities of methionine adenosyltransferase and *S*-adenosylhomocysteine hydrolase.

Previous methods for a simultaneous analysis of *S*-adenosylhomocysteine and *S*-adenosylmethionine from biological material have been based on the use of strong cation-exchange resins (Shapiro & Ehninger, 1966; Salvatore *et al.*, 1971). These methods involve several column chromatographies, alkaline hydrolysis and purification by precipitation (Salvatore *et al.*, 1971). This multi-step procedure is time-consuming and requires about 10g of tissue. Further, strong acids necessary for the elutions may partly hydrolyse *S*-adenosylhomocysteine during isolation (cf. Duerre, 1962). Cellex-P, a weak cation-exchanger used by us, has certain advantages, because it binds only loosely or not at all most of the contaminating u.v.-absorbing material present in the tissue extracts. *S*-Adenosylhomocysteine and *S*-adenosylmethionine were easily separated from

each other and from all u.v.-absorbing contaminants by the use of a stepwise HCl gradient. No hydrolysis of S-adenosylhomocysteine occurred during the procedure. The limited binding capacity of Cellex-P requires a careful technique to avoid fortuitous losses of S-adenosylhomocysteine during elution. However, this loss can be corrected for by the use of a radioactive internal standard. For routine assays 2–3 g of liver is adequate; but, by appropriate decreases in the column size and in the elution volumes, less than 1 g of tissue would be sufficient for analysis. The sensitivity of the present method is clearly superior to the previous column-chromatographic methods (Shapiro & Ehninger, 1966; Salvatore *et al.*, 1971) and favourably compares with the liquid-chromatographic method reported by Hoffman (1975). One person can analyse ten or more samples daily.

Our results demonstrate a remarkable accumulation of S-adenosylhomocysteine and also moderate increases in the concentration of S-adenosylmethionine and in the activities of methionine adenosyltransferase and S-adenosylhomocysteine hydrolase in rat liver during pyridoxine deficiency. It is known that vitamin B-6 deficiency is accompanied by marked changes in the catabolism of methionine via the trans-sulphuration pathway (see below), but its effects on the tissue concentrations of S-adenosylhomocysteine and S-adenosylmethionine have not been determined previously (cf. Sturman *et al.*, 1969). Owing to some differences in experimental techniques, a direct comparison of the present data with those published earlier by others on methionine metabolism in pyridoxine-deficient animals (Sturman *et al.*, 1969; Finkelstein & Chalmers, 1970; Okada & Suzuki, 1974) is somewhat difficult. We used control animals fed *ad libitum* and killed the animals without previous deprivation of food, whereas in other studies (Sturman *et al.*, 1969; Okada & Suzuki, 1974) food consumption by the pyridoxine-supplemented group was restricted to that by the vitamin B-6-deficient group and the animals were kept without food for 12–24 h before being killed. However, looking at the results of Sturman *et al.* (1969) it appears that the activities of liver methionine adenosyltransferase, cystathione synthase and cystathionase as well as the concentration of liver cystathionine of the pair-fed controls did not significantly differ from those observed in their controls fed *ad libitum*, and only somewhat higher methionine concentrations were observed in their control animals as compared with the pair-fed animals. We can therefore conclude that pair-feeding is not essential for the demonstration of changes in methionine metabolism during pyridoxine deficiency. As regards methionine adenosyltransferase, Sturman *et al.* (1969) noticed no change and Finkelstein & Chalmers (1970) reported a 35% decrease in this enzyme activity in the livers of vitamin B-6-deficient animals. The reason for these different results is not obvious. However, Sturman *et al.* (1969) performed the analysis after starving the animals for 24 h, which is known to decrease methionine adenosyltransferase activity (Pan & Tarver, 1967). Further, the specific activities of methionine adenosyltransferase reported by us are 3–4 times higher than those reported by the above two groups, perhaps reflecting differences in the assay methods too. In any case, relatively small changes in the activity of methionine adenosyltransferase probably do not have a significant effect on the rate of S-adenosylmethionine synthesis. The K_m value of the enzyme for L-methionine, i.e. 0.3–0.6 mM (Chou & Talalay, 1972; T. O. Eloranta & A. M. Raina, unpublished work), greatly exceeds the liver methionine concentration (40–80 μM; cf. Sturman *et al.*, 1969; Adibi *et al.*, 1973). Since methionine concentration remains fairly unchanged (Sturman *et al.*, 1969) and the enzyme is not inhibited by S-adenosylhomocysteine (Finkelstein *et al.*, 1974), the synthesis of S-adenosylmethionine might proceed at a normal rate during pyridoxine deficiency.

As described previously (see the introduction section), S-adenosylhomocysteine produced from S-adenosylmethionine in transmethylation reactions is further cleaved to adenosine and L-homocysteine by S-adenosylhomocysteine hydrolase. Although this reaction is reversible, it appears likely that physiological conditions favour the hydrolytic reaction (de la Haba & Cantoni, 1959; Cortese *et al.*, 1974). In mammalian tissues L-homocysteine can further be utilized in at least three other enzymic reactions, two of which lead to resynthesis of methionine. In the third route, i.e. the trans-sulphuration pathway, L-homocysteine is used for the synthesis of cystathionine in an irreversible reaction catalysed by cystathionine synthase (Finkelstein, 1974). The marked accumulation of liver S-adenosylhomocysteine observed in the present study in pyridoxine-deficient animals is obviously due to a metabolic block in the trans-sulphuration pathway, probably at the level of cystathionine synthase and/or cystathionase, which are both pyridoxal phosphate-requiring enzymes. In fact, it has been shown that pyridoxine deficiency results in a marked accumulation of cystathionine and a greatly diminished activity of cystathionase in rat liver (Sturman *et al.*, 1969; Finkelstein & Chalmers, 1970). Results obtained with human subjects (Shin & Linkswiler, 1974) and rats (Ohmori, 1975) during vitamin B-6-deficiency indicate that L-homocysteine and its metabolites may also accumulate in tissues, although this is difficult to demonstrate. Accumulation of L-homocysteine might then powerfully inhibit the cleavage of S-adenosylhomocysteine (cf. de la Haba & Cantoni, 1959), leading to accumulation of this compound. As it is a potent inhibitor of most methyltransferases (for references see Walker & Duerre, 1975), the elevated

concentration of *S*-adenosylhomocysteine may evoke severe metabolic disorders. Although the increased concentration of *S*-adenosylhomocysteine may inhibit its own production in most transmethylation reactions, the high activity of hepatic glycine methyltransferase, which is only slightly inhibited by *S*-adenosylhomocysteine (Kerr, 1972), may ensure the continuing production of *S*-adenosylhomocysteine. Under these conditions the utilization of L-homocysteine for the resynthesis of methionine may also be stimulated, since 5-methyltetrahydrofolate–homocysteine methyltransferase is not inhibited by *S*-adenosylhomocysteine and the inhibition of betaine–homocysteine methyltransferase is fairly small (Finkelstein *et al.*, 1974).

Our results indicating increased ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities and decreased putrescine and spermidine concentrations during pyridoxine deficiency closely agree with those reported by Sturman & Kremzner (1974). In agreement with the report by Hannonen (1976), we could not confirm the data of Sturman & Kremzner (1974) on the pyridoxal requirement of *S*-adenosylmethionine decarboxylase. Possible reasons for this inconsistency have been studied more thoroughly by Hannonen (1976).

We conclude that prolonged pyridoxine deficiency blocks the trans-sulphuration pathway, leading to an accumulation of *S*-adenosylhomocysteine to a concentration sufficiently high to inhibit most of the transmethylation reactions and to cause severe disturbances in metabolism. The net changes in the accumulation of polyamines are minor and mainly seen in the decreased concentration of putrescine.

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