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METHIONINE BIOSYNTHESIS IN ISOLATED *PISUM SATIVUM* MITOCHONDRIA

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Abstract—Homocysteine-dependent transmethylases utilizing 5-methyltetrahydropteroylglutamic acid and *S*-adenosylmethionine as methyl donors have been examined using ammonium sulphate fractions prepared from isolated mitochondria of pea cotyledons. Substantial levels of a 5-methyltetrahydropteroylglutamate transmethylase were detected, the catalytic properties of this enzyme being found similar to those of a previously reported enzyme present in cotyledon extracts. The mitochondrial 5-CH_3-H_4PteGlu transmethylase had an apparent K_m of 25 μM for the methyl donor, was saturated with homocysteine at 1 mM and was inhibited 50% by L-methionine at 2·5 mM. At similar concentrations of methyl donor the mitochondrial *S*-adenosylmethionine methyltransferase was not saturated. Mitochondrial preparations were found capable of synthesizing substantial amounts of *S*-adenosylmethionine but lacked ability to form *S*-methylmethionine. Significant levels of β-cystathionase, cystathionine-γ-synthase, L-homoserine transacetylase and L-homoserine transsuccinylase were detected in the isolated mitochondria. The activity of the enzymes of homocysteine biosynthesis was not affected by L-methionine *in vitro*. It is concluded that pea mitochondria have ability to catalyze the synthesis of methionine *de novo*.

INTRODUCTION

PREVIOUS studies from this laboratory have demonstrated the association of formyl and methyl tetrahydropteroylglutamates* with mitochondria[1] and chloroplasts.[2,3] As both of these organelles were also found to contain key enzymes of serine and methionine biosynthesis[1,2,4,5] it was suggested that one-carbon metabolism of plant tissues may be to some extent compartmented. Recent studies of pea mitochondria[1] have supported this suggestion. Besides having ability to generate methyl and formyl groups from serine, glycine, formate and methylenetetrahydropteroylglutamate the mitochondria were also found capable of utilizing these one-carbon precursors for methionine biosynthesis. The reaction requirements for this latter synthesis clearly implicated the principal components of the mitochondrial pteroylglutamate pool.

It follows from these studies that pea mitochondria must contain a 5-CH_3-H_4PteGlu:-homocysteine transmethylase. Furthermore a sustained synthesis of methionine in this

* The abbreviations used for derivatives of tetrahydropteroylglutamic acid are those suggested by the IUPAC–IUB Commission as listed in the (1967) *Biochem. J.* **107**, 15, e.g. 5-CH_3-H_4PteGlu = N^5-methyltetrahydropteroylmonoglutamic acid.

[1] CLANDININ, M. T. and COSSINS, E. A. (1972) *Biochem. J.* **128**, 29.
[2] SHAH, S. P. J. and COSSINS, E. A. (1970) *FEBS Letters* **7**, 267.
[3] COSSINS, E. A. and SHAH, S. P. J. (1972) *Phytochemistry*, **11**, 587.
[4] SHAH, S. P. J. and COSSINS, E. A. (1970) *Phytochemistry* **9**, 1545.
[5] SHAH, S. P. J., ROOS, A. J. and COSSINS, E. A. (1970) in *Chemistry and Biology of Pteridines, Proc. IVth Inter. Symp. on Pteridines*, International Academic, Tokyo.

compartment would require a supply of the methyl group acceptor which might be generated by mitochondrial enzymes or be available from extra-mitochondrial pools.

The present paper describes experiments designed to examine the possibility that isolated pea mitochondria are capable of catalyzing a *de novo* synthesis of methionine.

RESULTS

It is evident from Table 1 that two homocysteine-dependent transmethylases were detected in mitochondrial protein precipitating between 20 and 60% of saturation with $(NH_4)_2SO_4$. Total enzyme activity with 5-CH_3-H_4PteGlu as the methyl donor was substantially greater than activity observed in the presence of *S*-adenosylmethionine (SAM) in both the cotyledon homogenate and the mitochondrial fraction. Approximately 45% of the 5-CH_3-H_4PteGlu transmethylase activity was recovered in the $(NH_4)_2SO_4$ fraction (Table 1) and a purification in excess of 10-fold was noted. The purification and recovery of transmethylase activity with *S*-adenosylmethionine as methyl donor were both substantially lower.

TABLE 1. FRACTIONATION OF EXTRACTS CATALYZING METHIONINE SYNTHESIS WITH [METHYL-^{14}C]-5-CH_3-H_4PteGlu AND SAM-[METHYL-^{14}C] AS METHYL DONORS

Fraction	Total act.* (units × 10^6)	Sp. act. (units/mg protein × 10^6)	Purification factor	Enzyme recovery (% of total units)
Cotyledon homogenate				
Methyl donor:				
5-CH_3-H_4PteGlu	21·5	8·4	1·0	100
S-adenosylmethionine	9·8	0·45	1·0	100
Isolated mitochondria (20–60% $(NH_4)_2SO_4$ fraction)				
Methyl donor:				
5-CH_3-H_4PteGlu	11·6	87	10·3	44·7
S-adenosylmethionine	0·13	0·47	1·05	1·3

Homocysteine-dependent methyltransferase activities were measured in reaction systems of 0·5 ml containing: 1 μmol L-homocysteine, 1·6 nmol [methyl-^{14}C]-5-CH_3-H_4PteGlu (2×10^5 dpm) or 1·6 nmol SAM-[methyl-^{14}C] ($1{\cdot}82 \times 10^5$ dpm) respectively, 50 μmol K phosphate buffer, (pH 6·9) and enzyme preparation (1 mg protein).

* Recovered from 1 g fr. wt of cotyledons.

The rate of product formation by both enzymes was found to be linear for over 1 hr and maximal activity occurred at pH 7·0. The mitochondrial 5-CH_3-H_4PteGlu and *S*-adenosylmethionine transmethylases were saturated with L-homocysteine at *ca* 1 and 1·2 mM respectively (Table 2). Dodd and Cossins[6] have shown that similar transmethylases, partially purified from cotyledon homogenates were saturated with L-homocysteine at 2 mM. The apparent Michaelis constants for 5-CH_3-H_4PteGlu and *S*-adenosylmethionine determined by the method of Lineweaver–Burk,[7] were found to be 25 and 50 μM, respectively.

Product inhibition of homocysteine-dependent transmethylases by L-methionine is well documented[6,8,9] and was displayed by the 5-CH_3-H_4PteGlu enzyme of pea mitochondria

[6] DODD, W. A. and COSSINS, E. A. (1970) *Biochim. Biophys. Acta* **201**, 461.
[7] LINEWEAVER, H. and BURK D. (1934) *J. Am. Chem. Soc.* **56**, 658.
[8] ABRAMSON, L. and SHAPIRO, S. K. (1965) *Arch. Biochem. Biophys.* **109**, 376.
[9] COSSINS, E. A., ROOS, A. J., CHAN, P. Y. and SENGUPTA, U. K. (1972) *Phytochemistry*, **11**, 2481.

(Fig. 1). The degree of inhibition in this latter case was similar to that observed earlier[6] for the 5-CH_3-H_4PteGlu transmethylase isolated from pea cotyledon homogenates. Fifty per cent product inhibition occurred at 2·5–3·0 mM L-methionine (Table 2).

TABLE 2. PROPERTIES OF HOMOCYSTEINE-DEPENDENT TRANSMETHYLASES FROM COTYLEDON HOMOGENATES AND MITOCHONDRIA

Preparations	Saturating concentration of homocysteine (mM)	Apparent K_m for the methyl donor (μM)	Concn of methionine producing 50% inhibition (mM)
Cotyledon homogenate after Sephadex G100 treatment*			
Methyl donor:			
5-CH_3-H_4PteGlu	2·0	26	3·0
S-adenosylmethionine	2·0	4	>10·0
Isolated mitochondria (20–60% $(NH_4)_2SO_4$ fraction)			
Methyl donor:			
5-CH_3-H_4PteGlu	1·0	25	2·5
S-adenosylmethionine	1·2	50	—

* Data from Dodd, W. A. (1969) Ph.D. thesis, Department of Botany, University of Alberta. See also Ref. 6.

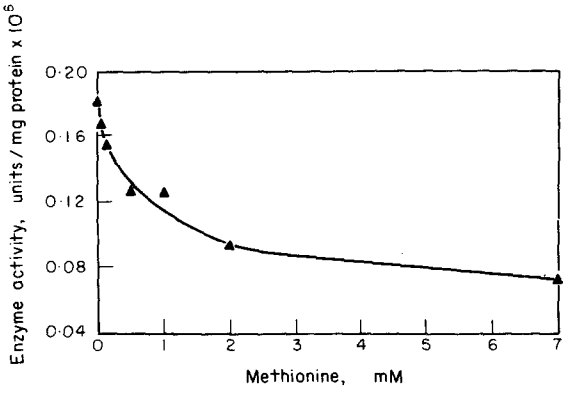


FIG. 1. INHIBITION OF 5-CH_3-H_4PteGlu TRANSMETHYLASE ACTIVITY BY L-METHIONINE. L-Methionine was added to standard reaction mixtures to give the final concentrations shown.

Dodd and Cossins[10] have suggested that *S*-adenosylmethionine in pea cotyledons is most likely formed by methionine adenosyltransferase activity rather than by methylation of *S*-adenosylhomocysteine. Isolated pea mitochondria were found to contain the former activity as shown by Table 3. Optimal synthesis of *S*-adenosylmethionine by these preparations was partially dependent upon ATP, Mg^{2+} and sulphydryl groups (Table 3). In contrast, no ability to synthesize *S*-methylmethionine could be detected.

Recent studies of germinating rape seed[11] have indicated that trans-sulphuration and sulphydration pathways are operative and well integrated in the biosynthesis of homocysteine. These studies by Ngo and Shargool have clearly shown the synthesis of cystathionine

[10] DODD, W. A. and COSSINS, E. A. (1969) *Arch. Biochem. Biophys.* **133**, 216.
[11] NGO, T. T. and SHARGOOL, P. D. (1972) *Biochem. J.* **126**, 985.

TABLE 3. MITOCHONDRIAL SYNTHESIS OF *S*-ADENOSYL-L-METHIONINE

Omission from reaction system	*S*-adenosylmethionine formed (μmol)	Omission from reaction system	*S*-adenosylmethionine formed (μmol)
None	1·920	2-Mercaptoethanol	0·760
ATP	0·432	Mitochondrial fraction	0·011
Mg^{2}	0·425		

The complete assay system (2 ml) contained: 8 μmol [methyl-^{14}C]-L-methionine (0·125 μCi/μmol), 10 μmol ATP, 5 μmol 2-mercaptoethanol, 100 μmol K-phosphate buffer (pH 6·9), 50 μmol $MgCl_2$ and 2 mg mitochondrial protein (20–60% $(NH_4)_2SO_4$ fraction). Reaction mixtures were incubated for 1 hr at 30°. The labelled product was recovered by column chromatography.

TABLE 4. LOCALIZATION OF β-CYSTATHIONASE, CYSTATHIONINE-γ-SYNTHASE, L-HOMOSERINE TRANSACETYLASE AND L-HOMOSERINE TRANSSUCCINYLASE

Cell fraction	Sp. act (nmol product/mg protein)	Total act. (nmol/g fr. wt)	Distribution (%)
Crude homogenate			
β-Cystathionase	2·62	127	100
Cystathionine-γ-synthase	0·403	19·2	100
Homoserine transacetylase	0·064	1·82	100
Homoserine transsuccinylase	0·062	1·76	100
Isolated mitochondria			
β-Cystathionase	3·20	1·7	1·34
Cystathionine-γ-synthase	0·435	0·24	1·25
Homoserine transacetylase	0·079	0·051	2·7
Homoserine transsuccinylase	0·027	0·018	1·1
Mitochondrial fraction (0–45% $(NH_4)_2SO_4$)			
β-Cystathionase	3·37	0·873	0·69
Cystathionine-γ-synthase	0·580	0·14	0·73

Data are expressed as the amount of pyruvate formed by β-cystathionase and the amount of α-ketobutyrate formed by cystathionine-γ-synthase, per 10 min at 37° under the reaction conditions described in the Experimental Section. Data for homoserine transacetylase and homoserine transsuccinylase are expressed as the amount of *N*-acetylhomoserine and *N*-succinylhomoserine formed after 30 min incubation at 37°.

TABLE 5. SYNTHESIS OF *O*-ACETYL-L-HOMOSERINE AND *O*-SUCCINYL-L-HOMOSERINE BY ISOLATED MITOCHONDRIA

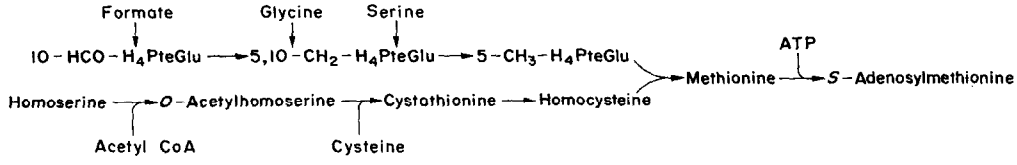
Omissions from complete reaction system	*O*-Acetylhomoserine produced (nmole)
None	0·55
Mitochondrial fraction	—
S-acetyl-CoA	0·001
Substituted *S*-succinyl-CoA for *S*-acetyl-CoA	0·19

The complete assay system (0·5 ml) contained: 50 μmol K-phosphate buffer (pH 7·4), 0·5 μmol *S*-acetyl-CoA, 50 nmol [U-^{14}C]-L-homoserine (1 μCi/μmol) and mitochondrial fraction (7 mg protein). Reaction mixtures were incubated at 37° for 30 min.

and suggested a possible product inhibition of its synthesis. From the enzyme assays summarized in Table 4 it is clear that β-cystathionase, cystathionine-γ-synthase, L-homoserine transacetylase and transsuccinylase activities were all detected in pea mitochondria. Of the total activities of these enzymes in the initial cotyledon homogenate only small percentages were recovered in the isolated mitochondria which suggests that these enzymes may be largely extra-mitochondrial in this tissue. However, the mitochondrial levels suggest that nanomolar quantities of homocysteine may be synthesized in this organelle. It is apparent from Table 5 that acetyl CoA was utilized more readily than succinyl CoA in the acylation of L-homosereine. The normal substrate for the mitochondrial cystathionine-γ-synthease reaction would therefore appear to be *O*-acetyl-L-homoserine. In other experiments the possible inhibition of these enzymes by L-methionine was examined. Using concentrations of this amino acid up to 1 mM no inhibition of L-homoserine transacetylase, cystathionine-γ-synthase or β-cystathionase could be demonstrated.

DISCUSSION

It was suggested earlier[1] that pea mitochondria had ability to catalyze the biosynthesis of amino acids related to one-carbon metabolism. The present studies support this contention with respect to methionine synthesis. Furthermore the data presented for enzymes of L-homocysteine biosynthesis show that these mitochondria can conceivably generate methionine and *S*-adenosylmethionine by the pathways shown in Scheme 1. It may also be concluded that the terminal reaction for synthesis of methionine *de novo* is largely located in the mitochondria of pea cotyledons. This conclusion is supported by, (a) the relatively large recovery of 5-CH_3-H_4PteGlu transmethylase activity in the mitochondria after density gradient centrifugation and ammonium sulphate fractionation of mitochondrial protein (Table 1), and (b) the similarity between the properties of this enzyme (Table 2) from isolated mitochondria and from aqueous extracts subjected to Sephadex chromatography.[6] A role for the mitochondrial *S*-adenosylmethionine:homocysteine transmethylase in pea mitochondria is more difficult to visualize. This enzyme clearly cannot represent an alternative route for synthesis of methionine *de novo* as *S*-adenosylmethionine is derived from methionine in this tissue[10] (Table 3). It is of interest to note that rat liver mitochondria[12] do not contain this enzyme but have a transmethylase utilizing 5-CH_3-H_4PteGlu$_3$ in the homocysteine-dependent synthesis of methionine. The possibility remains that the *S*-adenosylmethionine transmethylase of pea mitochondria may be an enzyme capable of transferring methyl groups to a number of endogenous acceptors and that such broad specificity results in some activity towards L-homocysteine. Clearly these possibilities which directly concern the metabolic fates of *S*-adenosylmethionine generated within the mitochondria are worthy of detailed study.



SCHEME 1. PATHWAY FOR BIOSYNTHESIS OF METHIONINE AND *S*-ADENOSYLMETHIONINE IN PEA MITOCHONDRIA.

[12] WANG, K. F., KOCH, J. and STOKSTAD, E. L. R. (1967) *Biochem. Z.* **346**, 458.

The presence of β-cystathionase, cystathionine-γ-synthase and L-homoserine transacetylase in the mitochondria (Table 4) implies that the transsulphuration pathway rather than a direct sulphydration of L-homoserine accounts for homocysteine formation. The presence and levels of these enzymes also argues for a sufficient endogenous synthesis of homocysteine to accommodate the mitochondrial homocysteine-dependent transmethylation reactions. The apparent lack of end-product inhibition of these enzymes by L-methionine raises the possibility that the pathway may be alternatively controlled by methylpteroylglutamate and *S*-adenosylmethionine as in *Neurospora crassa*.[13,14] Finally, if extensive recycling of sulphur[10] occurs in the mitochondria, a role for *S*-adenosylhomocysteine as a feedback inhibitor cannot be ruled out.

EXPERIMENTAL

Materials. 5-[Methyl-^{14}C]-CH_3-H_4PteGlu, [methyl-^{14}C]-L-methionine, [methyl-^{14}C]-SAM and L-homoserine-[U-^{14}C] were purchased from Amersham-Searle Corp., Des Plaines, Ill., U.S.A. DL-Homocysteine was prepared from the thiolactone[15] immediately before use. Other chemicals were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., Fisher Scientific Supplies Ltd., Edmonton, Canada and Sigma Chemical Co., St. Louis, Mo., U.S.A. Seeds of *Pisum sativum* L. cv Homesteader were germinated for 96 hr at 25°, as previously described.[1]

Preparations catalyzing methionine and S-adenosylmethionine synthesis. All operations were carried out at 2–4°. Mitochondria were isolated from cotyledon tissue and purified on a sucrose density gradient as previously described.[1] This material was used as a source of mitochondrial enzyme activity for further purification. After brief (45 sec) sonication of the mitochondrial fraction, diluted 2-fold in 0·05 M K-phosphate buffer (pH 6·9) containing 5 mM 2-mercaptoethanol, solid $(NH_4)_2SO_4$ was added to give 20% of saturation. The resulting suspension was stirred continuously for 30 min and then centrifuged (10000 *g* for 10 min). The 10000 *g* supernatant was adjusted to 60% of saturation by further additions of solid $(NH_4)_2SO_4$ and stirred continuously for 30 min. Precipitated protein was collected by centrifugation, dissolved in 2 ml of the diluting buffer and passed through a 1 × 5 cm column of Sephadex G25. The protein collected was used in studies of methyltransferase activity.

Preparations catalyzing homocysteine synthesis. The mitochondrial fraction was diluted 2-fold with 10 mM K-phosphate buffer (pH 7·3), sonicated for 45 sec and then centrifuged at 14000 *g* for 40 min. The supernatant was adjusted to 45% of saturation with solid $(NH_4)_2SO_4$ and stirred for 30 min. After centrifugation (10000 *g* for 15 min) the precipitated protein was dissolved in 4 ml 10 mM K-phosphate buffer (pH 7·3) and passed through a 1 × 5 cm column of Sephadex G25. The resulting soln was used in assays of β-cystathionase and cystathionine-γ-synthase. Assays of L-homoserine transsuccinylase and transacetylase employed the mitochondrial fraction without $(NH_4)_2SO_4$ or Sephadex treatments.

Enzyme assays. Homocysteine-dependent methyltransferase activity was assayed as described by Dodd and Cossins.[6] The reaction mixture (0·5 ml), contained: mitochondrial protein (1 mg protein), 2 μmol DL-homocysteine, 1·6 nmol methyl donor (2 × 10^5 dpm) and 50 μmol K-phosphate buffer (pH 6·9). After incubation at 30° for 1 hr the reaction was terminated by rapid cooling in an ice bath. An 0·1 ml aliquot of the chilled reaction mixture was then placed on a column (0·5 × 2·5 cm) of Dowex AG1-X10 (Cl^- form) when 5-CH_3-H_4PteGlu was the methyl donor and Dowex 50W-X8 (Li^+ form) when *S*-adenosylmethionine was the methyl donor. The [$^{14}CH_3$]-L-methionine formed was eluted with six 0·2 ml portions of H_2O. The column eluate was collected and counted by liquid scintillation spectrometry as previously described.[1] One unit of enzyme activity is defined as the amount of enzyme producing 1 μmol of methionine per min under the reaction conditions specified. Synthesis of *S*-adenosylmethionine by mitochondrial extracts was examined in reactions systems of 2 ml, containing: 8 μmol [methyl-^{14}C]-L-methionine (0·125 μCi/μmol), 10 μmol ATP, 5 μmol 2-mercaptoethanol, 50 μmol $MgCl_2$, 100 μmol K-phosphate buffer (pH6·9), and 2 mg mitochondrial protein. After incubation for 1 hr at 30°, the tubes were chilled and the labelled *S*-adenosylmethionine was separated by the column chromatographic method of Shapiro and Ehninger.[16] The method was modified[10] to include use of HCl instead of H_2SO_4 as the eluting acid. The possible synthesis of *S*-methylmethionine was examined in reaction systems of 1 ml containing: 2 mg mitochondrial protein (20–60% $(NH_4)_2SO_4$ fraction), 8 μmol L-methionine, 0·05 μmol [methyl-^{14}C]-*S*-adenosylmethionine (52 μCi/μmol), 5 μmol 2-mercaptoethanol and 100 μmol K-phosphate buffer (pH 6·9). After incubation at 30° for 90 min reaction products were examined by TLC and autoradiography using silica gel G thin layer plates and *n*-BuOH–HOAc–H_2O (60:15:25) and EtOH–HOAc–H_2O (65:1:34) as solvent systems. Assays

[13] KERR, K. and FLAVIN, M. (1969) *Biochim. Biophys. Acta* **177**, 177.
[14] SELHUB, J., SAVIN, M. A., SAKAMI, W. and FLAVIN, M. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 312.
[15] DUERRE, J. A. and MILLER, C. H. (1966) *Anal. Biochem.* **17**, 310.
[16] SHAPIRO, S. K. and EHNINGER, D. J. (1966) *Anal. Biochem.* **15**, 323.

of L-homoserine transacetylase and transsuccinylase were based on the conversion of the products to *N*-acetylhomoserine and *N*-succinylhomoserine respectively. These compounds were then readily isolated by use of Dowex 50W-X8 (H^+ form) columns.[17] The assay system (vol. 0·5 ml) contained: 50 μmol K-phosphate buffer (pH 7·5), 0·5 μmol *S*-acetyl-CoA (or *S*-succinyl-CoA), 50 nmol [U-^{14}C]-L-homoserine (1·0 μCi/μmol) and 3–4 mg protein of the mitochondrial fraction. Reaction mixtures were incubated at 37° for 30 min followed by addition of 50 μl 1·5 M TCA. After centrifugation 250 μl of the supernatant was mixed with 100 μl 1·0 M KOH, heated at 100° for 1 min, then cooled rapidly. Aliquots (150 μl) of this soln were transferred to 0·5 × 5 cm columns of Dowex 50W-X8 (H^+ form). The columns were washed with five 0·5 ml aliquots of H_2O, the eluate being collected directly in scintillation vials. Cystathionine-γ-synthase was assayed essentially by the method of Kaplan and Guggenheim.[18] α-Ketobutyrate, formed on hydrolysis of excess substrate, was measured using an excess of LDH. The assay procedure employed two stages, firstly reactions mixtures (0·5 ml) containing: 50 μmol K-pyrophosphate buffer (pH 8·2), 0·1 μmol pyridoxal-5′-phosphate, 2·5 μmol *O*-succinyl-DL-homoserine and 2 mg mitochondrial protein were incubated at 37° for 10 min. Control systems did not contain the substrate. The reaction was stopped by addition of 50 μl of 1·5 M TCA. After centrifugation aliquots of the supernatant were added to cuvettes containing: 100 μmol K-phosphate buffer (pH 7·3), and 0·15 μmol NADH, final vol. 1 ml. After ascertaining that the pH was above neutrality, the ϵ_{340} was measured before and after addition of an excess of cystalline LDH (20 μg). The amount of α-ketobutyrate was calculated using a molar extinction coefficient of 6200 (Ref. 18). β-Cystathionase activity was determined by the method of Guggenheim,[19] the production of pyruvate being assayed using LDH. The complete reaction system (1 ml) contained: 50 μmol K-pyrophosphate buffer (pH 8·2), 0·1 μmol pyridoxal-5′-phosphate, 4 μmol L-cystathionine and mitochondrial fraction (0·4 mg protein). Control systems lacked substrate. After incubation at 37° for 10 min the reaction was terminated by addition of 50 μl of 1·5 M TCA. Following centrifugation, aliquots of the supernatant were assayed for pyruvate using lactic dehydrogenase. Protein was determined colorimetrically.[20]

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