Detection of heterozygotes for homocystinuria

Study of sulphur-containing amino acids in plasma and urine after L-methionine loading

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Sardharwalla, I. B., Fowler, B., Robins, A. J., and Komrower, G. M. (1974). *Archives of Disease in Childhood*, 49, 553. **Detection of heterozygotes for homocystinuria: study of sulphur-containing amino acids in plasma and urine after L-methionine loading.** Twelve parents of patients with homocystinuria and 12 normal control subjects were given standard L-methionine loads. Determination of plasma concentrations of homocystine and cysteine-homocysteine disulphide, of urine concentration ratios of homocystine : cystine, cysteine-homocysteine disulphide : cystine, and homocysteic acid : cysteic acid allowed a distinction to be made between the two groups. The findings indicate the value of the L-methionine loading test for detection of heterozygotes for homocystinuria, particularly where facilities for fibroblast culture and cystathionine synthase assay are not available.

It has been shown that heterozygotes for homocystinuria have approximately half the normal cystathionine synthase activity in liver biopsy specimens (Finkelstein *et al.*, 1964). This reduced ability of heterozygotes to metabolize methionine has been tested by a number of investigators in order to devise a test for the carrier state in this condition.

Brenton, Cusworth, and Gaull (1965) loaded 2 parents of affected children and 3 normal adult male controls with L-methionine (0.1 g/kg body weight). Measurement of sulphur amino acids in blood and urine revealed no conclusive differences between the parents and normal subjects. Similar observations were made by Kennedy, Shih, and Rowland (1965) and White *et al.* (1964). Laster *et al.* (1965) administered a single oral dose of L-methionine to parents who had been shown to have reduced activity of cystathionine synthase in liver biopsy specimens. Measurement of inorganic sulphate excretion during the post-load period failed to distinguish between the parents and 5 control subjects, even when the dose was varied between approximately 0.1 and 0.5 mmol L-methionine/kg body weight. Dunn, Perry, and Dolman (1966) measured plasma methionine concentrations in 4

normal controls and 4 parents after an L-methionine load (0.1 g/kg body weight) after a 14-hour fast. Though there was no overlap of the range of methionine concentration in the two groups at 4 hours post load, the difference between ranges was insufficient to make this a reliable indication of the carrier state. Chase, Goodman, and O'Brien (1967) determined plasma methionine concentration 2 hours after an oral load of L-methionine in 4 parents and 8 normal subjects. Though a distinction between the two groups was noted, the number of heterozygotes studied was small and the value of this parameter for detection of heterozygotes is doubtful.

It is clear that up to now no conclusive distinction between heterozygotes and normal subjects has been shown except by enzyme assay of liver biopsy specimens.

In the present investigation the use of the methionine loading test for detection of heterozygotes for homocystinuria has been evaluated by loading 12 parents of affected children and 12 normal subjects. Part of the work has been reported (Fowler, Sardharwalla, and Robins, 1971).

Methods

Subjects fasted for 12 hours before administration of the oral load of L-methionine (0.1 g/kg body weight) suspended in orange juice and given at 9 a.m. to all subjects. Blood was taken immediately before ingestion

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of L-methionine and at 2, 4, 6, 9, 12, 24, and 30 hours post load. Urine was collected 12 hours before and 30 hours after the load. The latter collection was divided into four periods of which the first three were of 8 hours duration. Plasma was obtained from blood collected by venepuncture into tubes containing heparin, and plasma proteins were removed within 10 minutes by precipitation with sulphosalicylic acid (Hamilton, 1962). Urine was stored at 4 °C during collection, total volumes were recorded, and an aliquot of 2 ml was diluted with an equal volume of 20% (v/v) glycerol in 0.1 mol/l. HCl for analysis. Plasma and urine samples were stored at -20 °C before analysis. Normal meals were allowed and the dietary intake of protein was recorded during the 0- to 12-hour post-load period of the test on the first 6 heterozygotes and 6 normal subjects. The methionine intake was calculated by reference to McCance and Widdowson (1960). During the 0- to 12-hour period of the test on the remainder of the subjects, standard meals were taken containing 6 mg/kg, 9 mg/kg, and 12 mg/kg methionine at breakfast, lunch, and evening meal, respectively.

Sulphur amino acids were determined in plasma by ion-exchange chromatography of 0.4 ml plasma using manual loading with varigrad elution (Piez and Morris, 1960) or an automatic sampling and gradient elution device (Thomas, 1970). Cysteic acid was used as internal standard. Sulphur-containing amino acids were determined in 0.5 ml urine by varigrad elution ion-exchange chromatography using an automatic continuous flow analysis system in which the column effluent is split and monitored with iodoplatinate reagent ($\text{H}_2\text{PtCl}_6/\text{KI}$) and ninhydrin in parallel (Fowler and Robins, 1972). The identity of sulphur-containing amino acids analysed in this study was confirmed by reaction with iodoplatinate and by co-chromatography with the authentic compound. Quantitative determination of cysteic acid and homocysteic acid in oxidized

post-load urines (1/150 of the total volume) was carried out using an ion-exchange chromatographic technique with Amberlite CG-4B resin (Fowler and Robins, 1972). Urine samples were oxidized with performic acid (Schram, Moore, and Bigwood, 1954). Statistical analyses of plasma and urine sulphur amino acid concentrations were carried out using the 't' test.

Results

Plasma findings. Results of plasma methionine determinations are shown in Fig. 1 and Table I. Methionine values include small but variable amounts of methionine sulphoxide which may arise *in vivo* or during storage and/or analysis of plasma samples. A reduced ability of heterozygotes to metabolize methionine is indicated (e.g. $P < 0.001$ at 12 hours post load), though there is overlap of the normal and heterozygote ranges at all times post load.

Homocystine was detected in some plasma samples from heterozygotes at different times post load; 6- and 9-hour plasma samples from each heterozygote contained homocystine (Table I). Homocystine was not detected in any plasma sample taken at any time from the normal subjects.

Cysteine-homocysteine disulphide was detected in small amounts in the preload plasma of 5 heterozygotes. Marked differences in the plasma concentration of this amino acid were found between the two groups during the 24-hour post-load period. There was no overlap of the range of concentrations of each group and differences were highly significant (Fig. 2; Table I). However, 1 SD on either side of the mean gave a range almost as wide as the span.

TABLE I
Concentration of sulphur amino acids ($\mu\text{mol/ml}$)

Time (hr)	Methionine (range and mean $\pm$ SD)		Cysteine-homocysteine
	Heterozygotes (12)	Normals (12)	Heterozygotes (12)
0	0.020-0.051 (0.034 $\pm$ 0.01)	0.018-0.050 (0.035 $\pm$ 0.011)	0.000(7)-0.006
2	0.782-1.798 (1.107 $\pm$ 0.328)	0.685-1.208 (0.922 $\pm$ 0.150)	0.006-0.028 (0.016 $\pm$ 0.008)
4	0.806-1.526 (1.057 $\pm$ 0.262)	0.576-1.058 (0.771 $\pm$ 0.157)	0.011-0.037 (0.018 $\pm$ 0.008)
6	0.602-1.343 (0.939 $\pm$ 0.27)	0.480-0.813 (0.632 $\pm$ 0.118)	0.011-0.039 (0.020 $\pm$ 0.009)
9	0.450-1.288 (0.743 $\pm$ 0.221)	0.301-0.572 (0.484 $\pm$ 0.084)	0.008-0.037 (0.020 $\pm$ 0.010)
12	0.166-0.902 (0.553 $\pm$ 0.220)	0.207-0.397 (0.285 $\pm$ 0.067)	0.010-0.031 (0.017 $\pm$ 0.007)
24	0.039-0.581 (0.195 $\pm$ 0.195)	0.030-0.088 (0.053 $\pm$ 0.016)	0.006-0.026 (0.010 $\pm$ 0.006)
30	0.026-0.407 (0.105 $\pm$ 0.113)	0.019-0.093 (0.047 $\pm$ 0.020)	0.000(3)-0.023 (0.006 $\pm$ 0.006)

Note: The number of subjects with values of zero is indicated in parentheses.

FIG. 1.—Range of plasma methionine concentration after L-methionine loading in heterozygotes and normal subjects. 2 hours post-load, $P < 0.1$; 4 hours, $P < 0.005$; 6 hours, $P < 0.05$; 9 hours, $P < 0.001$; 12 hours, $P < 0.001$; 24 hours, $P < 0.05$.

FIG. 2.—Range of cysteine-homocysteine disulphide concentration after L-methionine loading in heterozygotes and normal subjects. At 2, 4, 6, 9, 12, and 24 hours post load, $P < 0.001$.

This observation may be related to the fact that the distribution was not Gaussian. The number of subjects is too small to allow for its correction. Plasma cystine concentration did not reveal any marked differences between the two groups though concentrations of this amino acid were generally higher in the heterozygotes than in normals. The methionine: cystine ratios in fasting plasma revealed no differences between the two groups (heterozygotes, range 0.40–1.00, mean 0.647, SD 0.185; normals, range 0.307–1.00, mean 0.535, SD 0.175).

plasma after oral loading with L-methionine

range and mean $\pm$ SD)	Cystine (range and mean $\pm$ SD)		Homocystine (range and mean)	
	Heterozygotes (12)	Normals (12)	Heterozygotes (12)	
None detected in any subject	0.036–0.084 (0.060 $\pm$ 0.016)	0.036–0.056 (0.047 $\pm$ 0.006)	None detected	
0.003–0.006 (0.005 $\pm$ 0.001)	0.036–0.078 (0.060 $\pm$ 0.014)	0.040–0.059 (0.049 $\pm$ 0.006)	0.000 (5)–0.010 –0.010	
0.003–0.009 (0.006 $\pm$ 0.002)	0.028–0.074 (0.055 $\pm$ 0.014)	0.038–0.057 (0.047 $\pm$ 0.006)	0.000(3)–0.009 (0.004)	
0.001–0.007 (0.005 $\pm$ 0.002)	0.028–0.076 (0.051 $\pm$ 0.015)	0.036–0.056 (0.045 $\pm$ 0.007)	0.001–0.010 (0.005)	
0.001–0.006 (0.005 $\pm$ 0.002)	0.033–0.088 (0.053 $\pm$ 0.016)	0.031–0.058 (0.043 $\pm$ 0.008)	0.002–0.011 (0.005)	
0.000(1)–0.007 (0.004 $\pm$ 0.002)	0.028–0.067 (0.047 $\pm$ 0.012)	0.031–0.060 (0.044 $\pm$ 0.009)	0.000(1)–0.012 (0.005)	
0.000(3)–0.005 (0.002 $\pm$ 0.002)	0.035–0.086 (0.058 $\pm$ 0.018)	0.027–0.053 (0.045 $\pm$ 0.007)	0.000(9)–0.004	
0.000(7)–0.002 —	0.035–0.078 (0.055 $\pm$ 0.017)	0.029–0.059 (0.043 $\pm$ 0.009)		

Urine findings. Analysis of preload urine samples did not reveal any differences in sulphur amino acid concentrations between the two groups. Urine excretion of cystine did not differ significantly between the two groups during the post-load period.

Cysteine-homocysteine disulphide excretion was generally higher in the heterozygotes than in the normal subjects (Table II). In the 0- to 8-hour and 16- to 24-hour post-load periods there was some overlap of the ranges of the two groups, with $P < 0.001$ and < 0.001 , respectively. In the 8- to 16-hour and 0- to 24-hour periods only 1 normal subject excreted an amount of cysteine-homocysteine disulphide which fell in the heterozygote range. This subject, however, ingested a larger amount of methionine in meals taken during the 0- to 12-hour post-load period, 43 mg/kg compared with a range of 25-38 mg/kg (mean 32 mg) for the other normal subjects and a range of 9-31 mg/kg (mean 21 mg) for the heterozygous subjects. For this reason subjects tested during the latter half of this investigation were given standard meals containing a total of 27 mg/kg methionine during the 0- to 12-hour post-load period. Homocysteine was excreted by each heterozygote and 2 of the normal subjects after the load (Table II). Distinct differences between the two groups are observed in the 16- to 24-hour and 0- to 24-hour periods. The normal subject falling in the heterozygote range at 8-16 hours is the subject whose dietary intake of methionine was high during the test period.

This variation in dietary intake of methionine appeared to be reflected by the excretion of cystine. Therefore, cysteine-homocysteine disulphide:cystine ratios and homocysteine:cystine ratios were determined (Table II). These ratios are more satisfactory parameters than concentrations of

cysteine-homocysteine disulphide and homocysteine for distinction between the two groups (Fig. 3 and 4). Homocysteic acid:cysteic acid ratios in oxidized post-load urines are shown in Table II. There are marked differences between the two groups. The normal subject in the heterozygote range at 0 to 8, 8 to 16, and 0 to 24 hours is the subject who had a high dietary intake of methionine

FIG. 3.—Ratios of sulphur-containing amino acids in urine of heterozygotes and normal subjects at 16 to 24 hours after L-methionine loading. A, cysteine-homocysteine disulphide/cystine ratios, $P < 0.001$. B, homocysteine/cystine ratios. (All the normal subjects had values of zero.) C, homocysteic acid/cysteic acid ratios in oxidized urine, $P < 0.01$.

TABLE
Range and mean of concentration and ratios of sulphur

Time (hr)	Cysteine-homocysteine concentration ($\mu\text{mol}/\text{specimen}$) (range and mean $\pm$ SD)		Homocysteine concentration ($\mu\text{mol}/\text{specimen}$) (range and mean $\pm$ SD)		Cysteine-homocysteine (range and mean $\pm$ SD)
	Heterozygotes (12)	Normals (12)	Heterozygotes (12)	Normals (12)	Heterozygotes (12)
0-8	2-14 (8 $\pm$ 4)	0(5)-10	2-20 (11 $\pm$ 6)	0(11)-3	0.151-2.4 (0.592 $\pm$ 0.602)
8-16	5-21 (11 $\pm$ 4)	0(2)-15 (3 $\pm$ 4)	3-32 (11 $\pm$ 9)	0(10)-4*	0.115-1.166 (0.596 $\pm$ 0.294)
16-24	2-23 (8 $\pm$ 6)	0(3)-6 (2 $\pm$ 2)	1-28 (7 $\pm$ 7)	None detected in any subject	0.137-1.277 (0.507 $\pm$ 0.302)
24-30	1-8 (3 $\pm$ 2)	0(10)-3	0(3)-6 (2 $\pm$ 2)	None detected in any subject	0.083-0.538 (0.267 $\pm$ 0.141)
0-24	15-42 (26 $\pm$ 9)	1-31 (7 $\pm$ 8)	13-77 (29 $\pm$ 18)	0(10)-7	0.244-1.00 (0.546 $\pm$ 0.260)

Note: The number of subjects with values of zero is indicated in parentheses.

*The values were 1 and 4 μmol . †These determinations were not carried out.

FIG. 4.—Ratios of sulphur-containing amino acids in urine of heterozygotes and normal subjects at 0 to 24 hours after L-methionine loading. A, cysteine-homocysteine disulphide/cystine ratios, $P < 0.001$. B, homocystine/cystine ratios. (All but 2 of the normal subjects had values of zero.) C, homocysteic acid/cysteic acid ratios in oxidized urine, $P < 0.001$.

during the post-load period. There is no overlap of the two ranges at 16 to 24 hours post load. Differences between the two groups are highly significant, e.g. $P < 0.001$ at 0 to 24 hours post load (Fig. 3 and 4).

Discussion

The results of the methionine loading tests on the normal and heterozygous subjects studied reveal

several parameters which reflect the reduced ability of the latter group to metabolize L-methionine. Plasma methionine results show overlap of the normal and heterozygote range at all times post load, though the mean values indicate a delayed removal of methionine from the plasma of heterozygous subjects. These findings are similar to those of Dunn *et al.* (1966), and also indicate the unsuitability of 2-hour post-load plasma methionine concentrations as an indication of the carrier state in this condition as suggested by Chase *et al.* (1967). Differences at 2 hours post load are not highly significant ($P = 0.1$), whereas differences at other times post load are more significant, e.g. $P < 0.005$ at 4 hours and < 0.001 at 12 hours, the 2-hour levels of methionine probably reflecting absorption of the oral load rather than metabolism of this amino acid.

The presence of homocystine in the plasma of heterozygotes but not in that of normals appears to indicate the carrier state. Homocystine was detected in 6- and 9-hour post-load samples from every heterozygote, though the concentrations were relatively low (0.001 – 0.011 $\mu\text{mol/ml}$), the smallest concentration detected being close to the limit of detection for the method.

Plasma cysteine-homocysteine disulphide concentrations give a clear-cut distinction between the normal and heterozygous subjects investigated. There was no overlap of the two ranges at any time during the 24-hour post-load period and differences were highly significant, e.g. $P < 0.001$ at 2, 4, 6, 9, 12, and 24 hours post load. This appears to provide the most reliable single parameter for distinction between the two groups and indicates a reduced ability of heterozygotes to metabolize homocysteine when the methionine-cystine pathway is stressed.

II

amino acids in urine after oral loading with L-methionine

Cysteine/cystine ratio (range and mean $\pm$ SD)		Homocystine/cystine ratio (range and mean $\pm$ SD)		Homocysteic acid/cysteic acid ratio (range and mean $\pm$ SD)	
Normals (12)	Heterozygotes (12)	Normals (12)	Heterozygotes (12)	Normals (12)	
0.00(5)–0.400	0.068–3.00 (0.826 $\pm$ 0.802)	0.00(11)–0.12	0.080–0.460 (0.220 $\pm$ 0.122)	0.034–0.131 (0.059 $\pm$ 0.024)	
0.00(2)–0.205 (0.117 $\pm$ 0.067)	0.16–2.46 (0.705 $\pm$ 0.682)	0.00(10)–0.054	0.084–0.625 (0.261 $\pm$ 0.176)	0.046–0.097 (0.060 $\pm$ 0.015)	
0.00(3)–0.200 (0.104 $\pm$ 0.070)	0.133–1.555 (0.524 $\pm$ 0.410)	—	0.073–0.785 (0.205 $\pm$ 0.199)	0.011–0.049 (0.029 $\pm$ 0.01)	
—	0.00(3)–0.461 (0.174 $\pm$ 0.164)	—	†	†	
0.021–0.233 (0.107 $\pm$ 0.060)	0.172–1.75 (0.684 $\pm$ 0.533)	0.00(10)–0.052	0.080–0.605 (0.229 $\pm$ 0.149)	0.030–0.083 (0.051 $\pm$ 0.013)	

The finding of higher mean levels of plasma cystine in heterozygotes than in normal subjects is unexpected and difficult to explain.

Urine findings revealed a number of parameters which allow distinction between the heterozygous and normal subjects. Urine excretion of homocystine was clearly higher in heterozygotes than in normal subjects. The concentrations were small and could not be determined with confidence using ninhydrin alone. The use of the iodoplatinate detection system facilitates such determinations since for the detection of small amounts of sulphur-containing amino acids in urine it is essential to use a more specific colorimetric reagent than ninhydrin. The iodoplatinate system used in this investigation is sufficiently sensitive and specific to fulfil this requirement. The conclusions of previous workers based on ion-exchange chromatography and ninhydrin detection should be evaluated with caution, though Brenton *et al.* (1965) used lability of homocystine to oxidation as a confirmatory technique. The appearance of 1 normal subject in the heterozygote range at 0 to 8 and 8 to 16 hours is probably due to the excessive intake of protein during the test period. It might be argued that this subject is a heterozygote for homocystinuria; against this he did not excrete homocystine in the 16- to 24-hour post-load period, there was no detectable homocystine in his plasma and the values of plasma cysteine-homocysteine disulphide fell within the range of normal subjects. The 0- to 24-hour excretion of homocystine was distinctly higher in heterozygotes than in the 2 normal subjects who excreted this amino acid. Homocystine excretion gives a good indication of the carrier state, particularly in the 16- to 24- and 0- to 24-hour post-load periods (Fig. 3 and 4). Homocystine:cystine ratios appear to be a more reliable parameter since they are affected less by variation in dietary intake of methionine during the post-load period. Indeed, the excretion of cystine in the normal subject who excreted homocystine at 0 to 8 and 8 to 16 hours post load was conspicuously higher than that of any other subject investigated. Also, cysteine-homocysteine disulphide:cystine ratios are a more reliable parameter since no overlap of the normal and heterozygote range was found at 8 to 16 and 0 to 24 hours post load, indicating that reliability of the methionine loading test is increased by consideration of ratios rather than concentrations of homocystine and cysteine-homocysteine disulphide.

Homocysteic acid:cysteic acid ratios were significantly higher in heterozygotes ($P < 0.001$ at 0-8, 8-16, and 0-24 hours post load) and at 16 to 24 hours there was no overlap of the heterozygote and

normal ranges (Fig. 3 and 4). Such differences are to be expected since they reflect the differences in homocystine and cysteine-homocysteine disulphide excretion found between the two groups. Oxidation of urine allows determination of total homocystine and cysteine as their sulphonic acids so that homocysteic acid:cysteic acid ratios adequately reflect ratios of homocystine:cystine and cysteine-homocysteine disulphide:cystine and also include any other compounds which may contain cysteine or homocysteine. This parameter can be determined in urine in the absence of an iodoplatinate detection system and could be used as part of a simplified test for detection of heterozygotes for homocystinuria.

This investigation has revealed several parameters which indicate that methionine metabolism is impaired in heterozygotes for homocystinuria, the number of subjects studied being large enough for conclusions to be made with confidence.

(i) Plasma concentrations of homocystine. The plasma of each heterozygote contained homocystine at 6 and 9 hours after the L-methionine load. *No homocystine was detected in the plasma of normal subjects at any time.*

(ii) Plasma concentrations of cysteine-homocysteine disulphide. Concentrations of this amino acid were significantly higher in heterozygotes than normals during the post-load period. Clear distinction of the two groups was observed at 6, 9, and 12 hours post load.

(iii) Urine concentration of homocystine and homocystine:cystine ratios. These parameters gave a clear distinction of the two groups in the 16- to 24-hour and 0- to 24-hour post-load periods.

(iv) Urine cysteine-homocysteine disulphide:cystine ratios. There was no overlap of the normal and heterozygote ranges of this parameter in the 8- to 16-hour and 0- to 24-hour post-load periods.

(v) Homocysteic acid:cysteic acid ratios in oxidized urine. Differences in this parameter were highly significant and there was no overlap of the two groups at 16 to 24 hours post load.

Although any single parameter considered in isolation may not provide an unequivocal distinction, the summation of the evidence affords a conclusive distinction between those heterozygotes for homocystinuria and the normal subjects investigated and underlines the value of this method when heterozygote detection is required for genetic counselling in affected families. It is of particular value in laboratories where enzyme assay using liver or cells from fibroblast culture (Uhlendorf, Conerly, and Mudd, 1973) cannot be carried out.

In this investigation 5 urine and 8 blood samples

were collected from each subject for sulphur-containing amino acid analysis. It would be desirable to reduce this number of samples for a practical test of the carrier state. Differences between the two groups are maximal in blood at 6, 9, and 12 hours post load and in urine in the 16- to 24-hour or 0- to 24-hour post-load period. A simplified test could be based on L-methionine loading with determination of cysteine-homocysteine disulphide and homocysteine concentrations in plasma at 6, 9, and 12 hours and collection of urine at 16 to 24 hours or 0 to 24 hours post load with determination of cysteine-homocysteine disulphide/cystine, homocysteine/cystine, and homocysteic acid/cysteic acid ratios.

The reliability of the test will be increased by the use of standardized meals as used in the latter part of this investigation. A consideration of the methionine intake of individuals who received as much food as they wished in the first half of this study suggests that a diet containing a total of 27 mg methionine/kg over the 0- to 12-hour post-load period is satisfactory. The methionine intake could be split thus: 6 mg at breakfast, 9 mg at lunch, and 12 mg at the evening meal.

Of the 12 parents investigated, 2 were parents of a child responsive to pyridoxine. There were no conclusive differences in the response to the methionine load in these 2 parents compared to the other 10 parents and it seems likely that the methionine load is equally suitable for detection of parents of pyridoxine-responsive and pyridoxine-resistant homocystinurics.

In conclusion, it is clear that the methionine loading test is of value in detection of heterozygotes for homocystinuria. It should be emphasized that sulphur-containing amino acids should be identified in urine by a method other than ninhydrin detection, and the iodoplatinate system used in this investigation is ideally suited. Also, methionine intake should be controlled during the post-load period.

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