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Trans-sulfuration in rat brain—Effects of 3,4-dihydroxyphenylalanine (L-dopa)

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IN LARGE daily doses, L-3,4-dihydroxyphenylalanine (L-dopa) is the most effective therapeutic agent presently available for the treatment of Parkinson's disease.[1,2] The rationale for its use is based on the dopamine replacement theory,[3] and much evidence in support of this theory has been published.[4–7] When experimental animals receive intraperitoneal injections of large amounts of L-dopa, comparable to the quantities used in therapy, a major portion of the drug is converted to 3-*O*-methyl-dopa and to homovanillic acid. The metabolism of large amounts of exogenous L-dopa causes a marked decrease in brain and kidney levels of *S*-adenosylmethionine (SAM), the methyl donor for the *O*-methylation.[8,9] Since the conversion of SAM to *S*-adenosylhomocysteine, i.e. demethylation, is a major step in trans-sulfuration reactions in tissues, we have investigated the problem of whether large amounts of L-dopa affect the levels of some sulfur containing compounds, particularly cystathionine, in the brain, liver and kidney of rats. The synthesis and degradation of cystathionine are mediated in the tissues by pyridoxal phosphate-requiring enzymes. Since pyridoxine will reverse the therapeutic effects of L-dopa,[10,11] the effect of this vitamin was also investigated.

Adult male rats (Sprague–Dawley derived) were housed in pairs and were matched on the basis of similar body weight. The two animals were treated identically, except that one of each pair received an intraperitoneal injection of L-dopa (100 mg/kg) dissolved in 0·05 M HCl (10 mg/ml) every 24 hr for 7 days;[8] the other member of the pair served as control and was injected with the diluent only. Some animals received a single injection. Forty-five min after the final injection, the rats were decapitated and the brains, livers and kidneys were removed and rinsed in cold water. A cystathionine-containing fraction was then isolated from each tissue by ion-exchange chromatography as previously described.[12] In some experiments, both rats received pyridoxine-HCl *ad lib.* in their drinking water (0·05 mg/ml) and were injected daily for 7 days with pyridoxine (5 mg/kg given in saline).

In previous studies,[12] we have shown that cystathionine concentrations in normal rat brain and liver are low and difficult to evaluate quantitatively in unpooled samples. The following procedure was devised specifically for measuring this amino acid in an extract from a single rat brain. The cystathionine-containing fraction from ion-exchange columns was evaporated to dryness under reduced pressure and redissolved in 1 ml of aqueous solution containing sodium pyrophosphate (200 mM) and EDTA (50 mM) at pH 8·0. An aliquot (usually 0·8 ml for brain and 0·2 ml for other tissues) was transferred to a cuvette containing 50 μmoles pyridoxal phosphate and the total volume was adjusted to 0·9 ml with sodium pyrophosphate buffer. Absorbance was measured at 412 nm in a Gilford model 200 spectro-photometer against a blank, identical to the sample except that the tissue was replaced with an equal volume of buffer. The cuvettes were allowed to equilibrate at 37°, then 0·83 μmole of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added and the absorbance measurements were repeated. γ-Cystathionase (25 μl), purified from rat liver,[13] was added (total volume, 1 ml) and the increase in absorbance at 412 nm with time was recorded automatically. The reaction was essentially completed in from 3 to 5 min, so the absorbance value at the latter time period was chosen for all calculations.

In this procedure, each sample serves as its own blank. An absorbance increase with DTNB over the original blank reading is a measure of total free sulfhydryl groups present.[14] This value subtracted from the final reading gave a value which was proportional to the amount of cystathionine present.

The amount of enzyme required for each analysis depends upon the specific activity of the preparation. However, we found variation in response from batch to batch. For that reason, standard curves were run for each enzyme preparation used in the studies. The data in Fig. 1 show that increasing enzyme concentrations do not give linear increases in response. They also show that the sensitivity of the method can be increased by increasing the amount of enzyme used in the assay. Data analogous to those shown in Fig. 1 were compiled for each preparation, and a concentration of enzyme to be used was chosen. We arbitrarily selected a concentration to give a slope of about 3·0. In Fig. 1, the standard error for each experimentally determined point, using the preferred amount of enzyme (100 units) is $\pm$ 0·012, 0·002 and 0·002, respective to increases in cystathionine concentrations. Cystathionase, by the way, is stable for at least 6 weeks at −20°. With brain and kidney extract, recovery of internal standard of L-cystathionine ranged from 92 to 100 per cent. With liver extract, recoveries were consistently in the 50 per cent range (52 $\pm$ 2), so the data for this tissue were corrected for the difference.

Concentrations of cystathionine in the control rat tissues, expressed in μmoles/g of tissue, are: for brain, 0·036 $\pm$ 0·003; liver, 0·048 $\pm$ 0·007; and kidney 0·614 $\pm$ 0·068. These values are the baseline, or controls, from which the data in Table 1 were derived. Except for kidney, which contains considerably more of this amino acid than was observed previously by us[12] and others,[15] control levels are consistent with

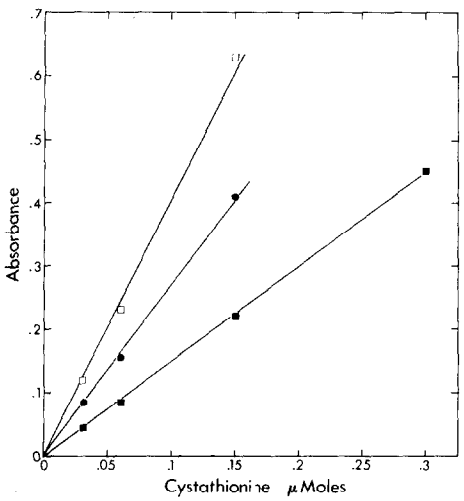

FIG. 1. Standard curves for the enzymatic determination of cystathionine. Expermimental conditions are described in the text. Each flask contained cystathionase in the following quantities: □—□—□, 200 units; ●—●—●, 100 units; ■—■—■, 40 units. Units of activity are defined in terms of the α-ketobutrrate formed in the reaction and equal 1 μmole of the latter hr mg of protein.[13] In a linear regression analysis of these data, the slopes are given by the beta coefficient (b) with standard error as follows: □—□—□ (b) 4·3 ± 0·014; ●—●—● (b) 3·01 ± 0·003; ■—■—■ (b) 1·51 ± 0·003. Correlation coefficient, (r) = 0·99.

amounts reported for normal rats. Kidney extracts, prepared from control and dopa-injected rats, had little or no effect on cystathionase when assayed in the presence of saturation levels of substrate.

Increased amounts of cystathionine were extracted from the brains of rats which had been injected with L-dopa as compared to controls. This effect was increased by almost 2-fold in rats which had received pyridoxine, but only after chronic administration of L-dopa. A single injection of the latter caused increased levels of cystathionine, but no further increase was observed in those animals which received pyridoxine-HCl. These conclusions are based on the data shown in Table 1.

Cystathionine levels in kidney, were decreased by about 25 per cent. A single injection of L-dopa also caused a decrease which was not affected by pyridoxine administration. The decrease did not occur in animals which received pyridoxine concomitantly with daily injection of L-dopa. These data are also shown in Table 1. L-Dopa administration had no effect on cystathionine levels in liver, in either single or chronic doses.

These data are consistent with the hypotheses that increased utilization of SAM, and the resulting decreased steady-state levels of this compound, would cause changes in the concentrations of obligatory pre-

TABLE 1. CHANGES IN CYSTATHIONINE CONCENTRATIONS IN RAT TISSUES AFTER TREATMENT WITH L-DOPA.*

Treatment	Brain (% change)	Kidney (% change)
(A) L-Dopa	+33 (N = 16)	−22 (N = 10)†
L-Dopa + PYDR‡	+64 (N = 10)	+ 9 (N = 5)§
(B) PYDR	0 (N = 6)§	− 3 (N = 10)§
PYDR + L-dopa	+20 (N = 6)	−25 (N = 10)

* Values represent per cent difference in means as compared to controls and are significantly different from the latter ($P < 0·01$) unless otherwise noted. Procedural details are given in the text. (A). Animals were injected daily for 7 days; (B). animals received pyridoxine-HCl for 7 days prior to a single injection of L-dopa.

† $P < 0·05$.

‡ Pyridoxine-HCl.

§ Not significant.

cursors and derivatives.[16] However, brain methionine levels are not reduced and the enzymes responsible for the *de novo* syntheses of this amino acid are not affected by L-dopa treatment.[17] This disparity had led to the suggestion that methionine may not be an essential amino acid for brain.[17] The amounts of sulfhydryl-containing material, as measured by the procedure above, were not altered in either brain or kidney after L-dopa injections. Disulfide levels in brain, as determined on pooled extracts by the nitroprusside procedure, also were not altered; disulfide levels were not determined in kidney. The increases in cystathionine with L-dopa could reflect, therefore, a diversion of the increased levels of *S*-adenosylhomocysteine, through the trans-sulfuration pathway. Rat brain, like human brain, is relatively rich in cystathionine synthase as compared to cystathionase, and tissue pyridoxine levels influence the activity of both enzymes.[18-20] In pyridoxine deficiency, cystathionine concentrations are increased in most rat tissues, including brain.[12,15,18] The increased levels found with L-dopa are probably not due to a dopa-induced pyridoxine deficiency, since the increase is restricted to brain and repleting the vitamin enhances rather than reverses the dopa effect. The possibility of a localized deficiency cannot be ruled out however.

The effects of L-dopa on kidney cystathionine levels parallel the changes observed in kidney SAM concentrations,[16] in direction, if not in magnitude. In kidney, pyridoxine appears to be effective, as it is in brain, only after chronic administration of L-dopa. In each case, that effect is to increase the amount of cystathionine. Originally this study was designed to determine L-dopa effects on brain. For that reason and because L-dopa had been extensively evaluated prior to its therapeutic use in humans, some of the interesting observations on kidney have not been, as yet, further investigated by us. Among these is the fact that the kidney extracts are deeply pigmented. Patients who receive L-dopa may excrete urine which becomes reddish brown, particularly at alkaline pH. The pigment is due to large amounts of dopa metabolites, probably unknown, which are considered to be harmless.[2] Also, there is no good explanation for the greater amounts of kidney cystathionine found in these rats, unless kidney function is altered by the sizable amounts of diluent (0·5 M HCl) injected into these animals.

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