Kinetic parameters and statistical tests for L-leucine transport in chicken intestine

Experiment	Preincubation	Incubation	n	$1/V$	V	σ^2	$v\,(1/V)$	$1/K_m$	K_m	σ^2	$v\,(1/K_m)$
1	KHB	KHB	4	0.0107	94	0.00914	4.12×10^{-7}	0.337	2.97	0.855	5.88×10^{-4}
2	ChKHB	KHB	5	0.0249	40	0.0693	2.30×10^{-6}	0.229	4.37	0.621	1.26×10^{-6}
3	ChKHB	ChKHB	8	0.0721	14	0.0301	9.05×10^{-5}	0.227	4.41	0.613	1.05×10^{-3}

	Tests of V			Tests of K_m		
Experiments	t	df	P	t	df	P
1 versus 2	7.5467	5	< 0.001	4.8572	7	< 0.001
1 versus 3	7.0553	8	< 0.001	2.7118	10	< 0.05
2 versus 3	4.1204	9	< 0.01	0.0571	11	N.S.

For details on symbols, see text. The velocity of transport of L-leucine was studied in the chicken ileum and for each substrate concentration was the mean of 8–40 determinations on tissue from 4–20 animals. V is reported in nmol/cm² intestine/min and K_m in mM. Preincubation was for 30 min and incubation with tracer substrate was for 1 min. KHB, Krebs Henseleit buffer; ChKHB, Na^+ -free, choline cation-substituted Krebs Henseleit buffer. For other details, see BURRILL et al.[8]. The t-values were computed on the basis of homogeneous variance factors (see text).

To illustrate the method we evaluated the kinetic parameters for the initial velocity of L-leucine transport in the chicken ileum (Table). The results indicate that the replacement of Na^+ by choline cation in the preincubation solution significantly decreased V and increased K_m. Incubation in the absence of Na^+ led to a further significant decrease in V with no further change in K_m. An objective, properly weighted analysis is essential for all data if the constants are to be compared statistically. This comparison requires an estimate of the variance of the constants; these variances cannot be estimated by simple graphical methods.

[8] P. H. BURRILL, P. A. SATTELMEYER and J. LERNER, Biochim. biophys. Acta *373*, 265 (1974).

The Effects of Various Agents in vitro on Homocarnosine-Carnosine Synthetase from Rat Brain[1]

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Summary. The present paper reports the first evidence that homocarnosine-carnosine synthetase from rat brain requires free sulfhydryl groups for activity. The activity of the synthetase can be stabilized by dithioerythritol and inhibited strongly by Cu^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, and to a lesser extent by Ca^{2+}, Ni^{2+}, Li, chlorpromazine, α-methyl DOPA and norepinephrine at the concentrations tested.

The L-histidine-containing dipeptides carnosine (β-alanyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) are found in excitable tissue. Carnosine was first discovered in muscle in 1900 by GULEWITSCH and AMIRADZIBI[3]. In 1962 ABRAHAM et al.[4] found homocarnosine and small amounts of carnosine in the brains of man and several other mammalian species. Although relatively little is known about the biological roles of these two dipeptides, recent studies[5–7] suggested that they may have unique functions in the central nervous system (CNS). Homocarnosine-carnosine synthetase catalyzes the formations of carnosine from β-alanine and histidine, and of homocarnosine from GABA and histidine. Its isolation and partial purification from rat brain have been reported by SKAPER et al.[8]. However, instability of the enzyme has hindered further purification. The purpose of the present study was to investigate the effects of various agents on the enyzme in order to stabilize its activity. Furthermore, in view of the findings[9,10] that certain drugs lowered levels of homocarnosine and carnosine in rat brain in vivo, several CNS agents were tested in the present study.

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[3] W. GULEWITSCH and S. AMIRADZIBI, Ber. dt. chem. Ges. *33*, 1902 (1900).

[4] D. ABRAHAM, J. J. PISANO and S. UDENFRIEND, Arch. Biochem. Biophys. *99*, 210 (1962).

[5] F. L. MARGOLIS, Science *184*, 909 (1974).

[6] T. L. PERRY, S. HANSEN and M. KLOSTER, New Engl. J. Med. *288*, 337 (1973).

[7] C. O. ENWONWU and B. S. WORTHINGTON, J. Neurochem. *22*, 1045 (1974).

[8] S. D. SKAPER, S. DAS and F. D. MARSHALL, J. Neurochem. *21*, 1429 (1973).

[9] F. D. MARSHALL and W. C. YOCKEY, Biochem. Pharmac. *17*, 640 (1968).

[10] F. D. MARSHALL, Life Sc. *13*, 135 (1973).

Materials and method. L-[ring-2-^{14}C]histidine (specific activity, 55 mCi/mmol) was purchased from Amersham-Searle (Des Plaines, Illinois). Chlorpromazine was obtained from Smith, Klein and French Lab (Philadelphia, Pennsylvania). Other drugs were purchased from Sigma Chemical Company (St. Louis, Missouri). All other chemicals were reagent grade. 5-week-old, male rats of Sprague-Dawley strain were used. Enzyme preparation and assay were carried out essentially as described by SKAPER et al.[8]. Rat brains were homogenized in ice-cold *Tris*-HCl or Na-K phosphate buffer (10 m*M*, pH 7.4) containing 50 m*M* $NaHCO_3$ and 31 m*M* D-glucose. Homogenates were centrifuged at 100,000 *g* for 1 h. The supernatant fluids were immediately shell-frozen and lyophilized. The lyophilized powder was dissolved in distilled water and brought to 30% saturation in ammonium sulfate by addition of solid ammonium sulfate. After centrifuging at 10,000 *g* for 15 min, the pellet was suspended in *Tris*-HCl or Na-K phosphate buffer (10 m*M*, pH 7.4) and served as the source of enzyme to be assayed. In addition to the enzyme solution, the assay mixture contained the assay medium (4 m*M* ATP, 4 m*M* $MgCl_2$, 0.2 m*M* NAD, 32 m*M* sodium bicarbonate and 20 m*M* D-glucose), 10 m*M* β-alanine or GABA, 1 μCi of [^{14}C]histidine and specified concentration of reagent tested in *Tris*-HCl or Na-K phosphate buffer (10 m*M*, pH 7.4). In subsequent studies with inorganic cations and CNS agents, 50 m*M* *Tris*-HCl buffer was used. In addition, 5 m*M* of dithioerythritol (DTE) was included in homogenizing brain tissue and in dissolving lyophilized powder. Also, sodium bicarbonate and D-glucose were omitted in the assay medium.

Under the conditions described above, the enzymatic reaction was allowed to proceed for 52 min. The dipeptide synthesized was separated by paper electrophoresis and its radioactivity was determined by liquid scintillation counting technique.

Results and discussion. The homocarnosine-carnosine synthetase from rat brain is apparently unstable upon storage. As shown in Table I, only 16% of the control activity remained 24 h after the enzyme was prepared. In this investigation, a low concentration (9×10^{-4} *M*) of homocarnosine sulfate did not seem to stimulate or stabilize the enzyme. The addition of the assay medium prior to storage appeared to have slightly greater effect on stabilizing the enzyme. And, this effect was slightly increased by β-alanine. At low concentration (10^{-5} *M*), chlorpromazine (CPZ) failed to stimulate or stabilize the enzyme. However, at a higher concentration (10^{-4} *M*), chlorpromazine significantly stimulated the enzyme activity when assayed immediately after the enzyme was prepared, and appeared to stabilize the enzyme to some extent.

$NaHCO_3$, D-glucose, $MgCl_2$, ATP and NAD are the 5 components in the assay medium. Combinations of chlorpromazine with each one yielded significant stabilization, with the exception of $NaHCO_3$, probably due to the pH difference in the absence of other components normally present in the assay. Storing the brain enzyme in chlorpromazine with the assay medium containing all 5 components resulted in more activity. If β-alanine was added, in addition to the assay medium and chlorpromazine, even more activity was obtained after storing.

The instability of the enzyme activity led us to speculate that free sulfhydryl groups may be involved in the enzyme activity. Therefore, several sulfhydryl reagents were also tested in this study. β-Mercaptoethanol stimulated the enzyme activity but failed to stabilize it during storage. Such inactivation may be due to formation of mixed disulfide bonds between enzyme sulfhydryl groups and β-mercaptoethanol[11]. In view of this finding, dithioerythritol (DTE) and dithiothreitol (DTT) were tested. In the presence of DTE, the enzyme activity was stimulated 2-fold and this level of activity did not decrease after storage of the enzyme for 24 h. DTE was also capable of reactivating the enzyme if added after storage and right before assay. 2-Aminoethylisothiouronium bromide hydrobromide (AET), also a sulfhydryl reagent, had no significant effect on the enzyme when assayed immediately after preparation. The involvement of sulfhydryl groups in the enzyme was further suggested by the finding that both *p*-chloromercuribenzoate (PCMB) and iodoacetamide strongly inhibited the enzyme activity. We also noted that after PCMB inhibition, the enzyme could be reactivated by incubation with DTE. Capitalizing on this observation, it may be possible to facilitate the purification of this enzyme using affinity chromatography with *p*-chloromercurial substituted sepharose.

In order to compare the synthetase with other sulfhydryl enzymes, the effects of several inorganic cations were studied. As seen in Table II, homocarnosine-carnosi-

Table I. Activity of homocarnosine-carnosine synthetase before and after storage

Reagent added	Enzyme activity when assayed	
	Immediately (%)	After 24 h (%)
None (control)	100	16
+ Homocarnosine sulfate (9×10^{-4} *M*)	96	46
+ CPZ (1×10^{-4} *M*) + assay medium + β-alanine (6×10^{-3} *M*)	170	198
+ β-Mercaptoethanol (2×10^{-3} *M*)	215	24
+ DTE (1.5×10^{-3} *M*)	204	239
+ DTT (1.5×10^{-3} *M*)	191	
+ AET (1.5×10^{-3} *M*)	117	
+ PCMB (1.5×10^{-5} *M*)	13	
+ Iodoacetamide (3×10^{-3} *M*)	18	

Carnosine synthesis was determined in duplicate runs with the enzyme solution immediately after preparation, and after storing for 24 h at 0–4 °C with substances indicated. Assay medium minus whatever components present in the storing mixture was added after storage and before assaying.

Table II. Effects of inorganic cations on the activity of homocarnosine-carnosine synthetase

Compound added	Control activity (%)	
	Carnosine synthesis	Homocarnosine synthesis
None (control)	100	100
LiCl	76	46
$NiSO_4$	43	53
$CaCl_2$	31	6
$CuSO_4$	0	4
$CdSO_4$	0	2
$HgCl_2$	1	0
$ZnSO_4$	0	2

Final concentration of the compounds was 0.1 m*M*. Results are means of 2 determinations.

[11] M. ORLOWSKI and A. MEISTER, J. biol. Chem. *246*, 7095 (1971).

ne synthetase from rat brain is highly sensitive to heavy metal ions. Cu^{2+}, Cd^{2+}, Hg^{2+} and Zn^{2+} completely inhibited the enzymatic synthesis of carnosine and homocarnosine. This characteristic property resembles that of sulfhydryl enzymes, e.g. brain L-glutamate decarboxylase[12]. 0.1 mM of Li^{+}, Ni^{2+} and Ca^{2+} also inhibited the enzyme but to a lesser extent.

Among the CNS agents tested at 1 mM, norepinephrine, α-methyl DOPA and chlorpromazine appeared to inhibit the enzyme approximately 50% while hydroxylamine, epinephrine, dopamine and DOPA had no significant effect. In our earlier study[13], the enzyme was prepared in the absence of dithioerythritol and we found that 1.5 mM of hydroxylamine, α-methyl DOPA, dopamine, norepinephrine and epinephrine inhibited the enzyme activity 44% or greater while DOPA had no effect. The inhibition by hydroxylamine, epinephrine and dopamine observed previously may be involved with the availability of free sulfhydryl groups on the enzyme since the enzyme activity is very labile in the absence of a sulfhydryl reagent. Chlorpromazine, although stimulatory at low concentration (10^{-4} M) in earlier study, inhibited the enzyme 45% at 10^{-3} M. These contradictory effects have also been reported in other enzyme systems, e.g., the mitochondrial ATPase is stimulated by chlorpromazine below 1×10^{-4} M but is inhibited by chlorpromazine above 1×10^{-4} M[14]. Our present finding that chlorpromazine is capable of inhibiting the synthetase in vitro may explain the in vivo finding by MARSHALL[10] that chlorpromazine lowered the levels of carnosine and homocarnosine in rat brain but had no effect on the level of histidine.

[12] J.-Y. WU and E. ROBERTS, J. Neurochem. *23*, 759 (1974).
[13] R. H. NG and F. D. MARSHALL, Trans. Am. Soc. Neurochem. *5*, 170 (1974).
[14] H. LÖW, Biochim. biophys. Acta *32*, 11 (1959).

Auxin Dependence and Auxin Oxidase of Cultured Sycamore Cells

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Summary. Quantitative and qualitative differences in auxin-oxidases extracted from auxin-dependent (S) or auxin-independent (MB) sycamore cells were analyzed. MB auxin-oxidases have a higher activity, but the molecular weight of this enzymatic complex is lowered by freeze-drying, without loss of the activity. Correlations with auxin-independence are discussed in this context.

Cultured sycamore tissues are represented by 2 strains, an auxin-independent phenotypic variant (MB)[1] and the original auxin-dependent strain (S)[2]. This auxin-independence might be at least explained by 2 hypotheses: 1. the amount of 3-indolyl-acetic acid (IAA) synthesized via tryptophan is higher in MB than in S; 2. the inactivation of IAA by enzyme systems is lower in MB than in S. The first explanation was in fact not true; as previously demonstrated[3], both strains producing a similar level of IAA after incubation with labelled tryptophan. The aim of the present paper was to test the last hypothesis, analyzing the amount of IAA destroyed in vitro by extracts of tissues from both strains (S and MB).

Preliminary experiments indicated that crude extracts did not destroy IAA, even when cofactors such as $MnCl_2$ and 2,4-dichlorophenol[4,5] were added. The tissues probably contained endogenous inhibitors liberated during

[1] A. M. LESCURE and C. PEAUD-LENOËL, C. r. Acad. Sci., Paris *265*, 1803 (1967).
[2] D. T. A. LAMPORT and D. H. NORTHCOTE, Nature, Lond. *188*, 665 (1960).
[3] A. M. LESCURE, Soc. bot. fr. Mémoires *1970*, 353 (1970).
[4] P. E. PILET, Experientia *13*, 35 (1957).
[5] R. FOX and W. K. PURVES, Plant Physiol. *43*, 454 (1968).

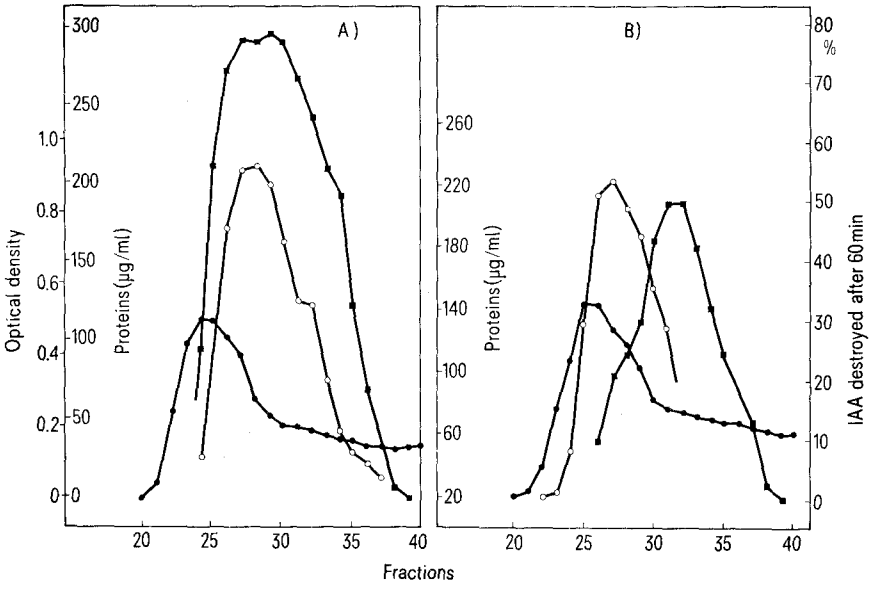

Fig. 1. Elution patterns of G 25 prepurified extracts from 5 g fresh (A) or 500 mg freeze-dried (B) strain MB tissues on Sephadex G 100. Fraction volume: 4.2 ml. Elution speed: 24 ml/h, temp.: 4 °C ± 0.5. Open circles, proteins; plain circles, optical density; squares, IAA destroyed.