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QUINONOID DIHYDROPTERIN REDUCTASE—II

REGIONAL AND SUBCELLULAR DISTRIBUTION OF RAT BRAIN ENZYME*

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Abstract—The regional and subcellular distribution of quinonoid dihydropterin reductase (DHPR, EC 1.6.99.7) was studied in the rat brain. In addition, the subcellular distribution of the enzyme was studied in the rat liver. The activity of rat brain DHPR was found to be lowest in the cerebral cortex (0.14 unit mg^{-1}) and highest in the posterior colliculus (2.35 units mg^{-1}). The distribution of brain DHPR correlated positively with the distribution of aromatic-L-amino acid decarboxylase but not with the distribution of tyrosine hydroxylase or tryptophan decarboxylase. However, this correlation was not observed in the caudate nucleus which contains low DHPR activity and high aromatic-L-amino acid decarboxylase and tyrosine hydroxylase activities. This finding is an indication that the amount of DHPR in the caudate nucleus may be rate limiting. The distribution profiles of brain and liver DHPR were found to be similar to that of lactate dehydrogenase; for both enzymes the greatest specific activity was found in the soluble fraction, indicating that DHPR is a cytoplasmic enzyme.

Quinonoid dihydropterin reductase (DHPR, EC 1.6.99.7) catalyzes the reduction of quinonoid dihydropterins ($q\text{-}H_2P$) to tetrahydropterins (H_4P). It has been suggested that quinonoid dihydropterin reductase may be responsible for the maintenance of the H_4P cofactor for all tetrahydropterin-requiring systems [1, 2]. Whether the enzyme always functions in this general capacity, particularly in brain tissue, is not clear. It is possible that, in brain, DHPR is found mainly in catecholamine and indoleamine neurons and not in all brain cells. Several investigators [2–5] have provided evidence indicating that the availability of H_4P may be an important regulatory factor in the rate of biosynthesis of catecholamine and indoleamine neurotransmitters.

The study of the regional and subcellular distribution of DHPR would provide data for understanding the roles of this enzyme. The regional distribution of DHPR has been previously described in five large brain areas and it was reported to be most concentrated in the brain stem [6]. Since some areas of the brain which contain high concentrations of catecholamine and indoleamine neurons are quite small, we have reinvestigated the distribution of the enzyme with finer dissection techniques. The subcellular localization of the enzyme in brain and liver was also investigated. Although several investigators [1, 2, 5] have provided evidence suggesting that the majority of DHPR occurs in the cytoplasm, the distribution of the enzyme in the various subcellular fractions has never been quantitated. Moreover, Rembold and Buff [7, 8] have proposed that tetrahydrobiopterin, oscillating between tetrahydrobiopterin and quinonoid dihydrobiopterin in a cycle involving DHPR, functions in the control of the mitochondrial respiratory chain and of the cellular concentration of reduced pyridine nucleotides. Their proposed pterin-dependent redox system implies the existence of a cytoplasmic, and possibly a mitochondrial DHPR.

MATERIALS AND METHODS

The following materials were obtained from the indicated companies: sodium dithionate (sodium hydrosulfite), purified, from J. T. Baker Chemical Co., Phillipsburg, NJ; Folin-Ciocalteau phenol reagent from Fisher Scientific Co., Fair Lawn, NJ; fluorescamine from Hoffmann–La Roche, Nutley, NJ; cytochrome *c* (horse heart-lyophilized powder, salt free) and ovalbumin (2× crystallized) from Mann Research Labs, New York, NY; acetylcholine chloride (preweighed vials, 150 mg/vial), albumin from bovine serum (crystallized and lyophilized), nicotinamide adenine dinucleotide, reduced form (dihydrodiphosphopyridine nucleotide, disodium salt from yeast, grade III), catalase from beef liver (2× crystallized), dihydronicotinamide adenine dinucleotide phosphate (triphosphopyridine nucleotide, reduced), peroxidase from horseradish (salt free powder-type II), 2,3,5-triphenyl tetrazolium chloride, and Trizma base—Tris-(hydroxymethyl) aminomethane—from Sigma Chemical Co., St. Louis, MO.

Regional distribution of brain DHPR. Adult, male, Sprague–Dawley rats (200–250 g) were decapitated; their brains were quickly removed and placed in ice-cold saline. Dissection of each brain was performed over ice by combining several procedures [9–13]. Care was taken to exclude any tissue from adjacent areas to insure morphological homogeneity. The dissected regions were homogenized in 3 vol. of 0.03 N acetic acid with a glass homogenizer for 20 sec. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant fraction was assayed for DHPR as

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previously described [14], except that the reaction volume was adjusted to 200 μl.

Liver subcellular fractionation. Subcellular fractions were prepared from fresh rat liver by a modification of the method of Rembold and Buff [7]. All isolation steps were performed at 4° using a buffer composed of 0.01 M Tris, 2 mM EDTA, and 0.3 M sucrose adjusted to pH 7.4 with HCl.

Brain subcellular fractionation. Brain subcellular fractionation was carried out according to the method of De Robertis *et al.* [15] at 4°. Fresh rat brains without the cerebella were homogenized for 60 sec in 4 vol. of 0.32 M sucrose, pH 7.4, in a Potter–Elvehjem homogenizer fitted with a Teflon pestle (clearance = 0.5 to 0.23 mm). The nuclear pellet and crude mitochondria were washed with 0.32 M sucrose. The microsomal pellet was washed with 32 mM NaCl–32 mM sucrose; one half of the resuspended pellet was adjusted to pH 7.4. The washed crude mitochondrial pellet was osmotically shocked by treating it with a volume (equivalent to 90% of the homogenate) of 10 μM $CaCl_2$ prepared in distilled water. Five min after the pellet was resuspended with 1-min rehomogenization, the NaCl concentration of the resuspended crude mitochondria was raised to 32 mM with 3.2 M NaCl, followed by a 1-min rehomogenization. The resuspension was centrifuged at 20,000 *g* (12,930 rev/min) for 30 min in a Sorvall SS34 rotor; the pellet (the mitochondrial fraction) was washed twice by sequential resuspension in 9 vol. of fresh 32 mM NaCl–32 mM sucrose, adjustment of the pH to 7.4, and centrifugation.

Enzyme determinations. Lactate dehydrogenase activity (LDH) was measured according to the method of Kornberg [16] and is expressed in units defined as μmoles NADH oxidized/min. Succinate dehydrogenase activity (SDH) in liver fractions was determined by the method of Bonner [17] and is expressed in units defined as μmoles $K_3Fe(CN)_6$ reduced/min. In brain tissue fractions, SDH was measured by a modification of the method of Kun and Abood [18]. These modifications include the use of 1 ml of incubation mixture, measurement of absorbance at 480 nm, a 20-min incubation time and the use of 1 ml acetone for the formozan extraction. Brain SDH activity is expressed in units defined as μg of 2,3,5-triphenyl tetrazolium chloride (TTC) reduced to formozan/min. Acetylcholinesterase activity was measured by the method of Augustinsson [19] and is expressed in units defined as μmoles acetylcholine hydrolyzed/hr. NADPH cytochrome *c* reductase activity was determined using the method of Williams and Kamin [20] and is expressed in units defined as μmoles cytochrome *c* reduced/min. Protein concentration was measured using the method of Lowry *et al.* [21].

RESULTS

Regional distribution of brain DHPR. The distribution of DHPR activity in fourteen different regions of the rat brain is shown in Table 1. Analysis of variance shows that there are significant differences among the mean DHPR activities per mg of tissue for various regions. However, after arranging the means in increasing order, the single observed difference between consecutive regions is not significant. The means

Table 1. Quinonoid dihydropterin reductase activity in various regions of adult rat brain*

Region	No. of samples	Enzyme activity (units/mg wet weight)	Wet weight (mg/rat)
1. Cerebral cortex	8	0.14 ± 0.01	750 ± 8
2. Hippocampus	7	0.33 ± 0.06	118 ± 3
3. Caudate nucleus	8	0.62 ± 0.11	55 ± 1
4. Thalamus	7	0.93 ± 0.07	64 ± 4
5. Cerebellum	7	1.04 ± 0.12	266 ± 5
6. Substantia nigra	6	1.16 ± 0.06	33 ± 2
7. Hypothalamus	9	1.25 ± 0.12	29 ± 1
8. Olfactory bulb	5	1.30 ± 0.46	43 ± 2
9. Anterior colliculus	8	1.34 ± 0.12	16 ± 1
10. Raphe nuclei	7	1.73 ± 0.22	55 ± 2
11. Spinal cord	5	1.78 ± 0.29	51 ± 1
12. Medulla-midbrain†	7	2.05 ± 0.21	171 ± 10
13. Locus coeruleus	8	2.21 ± 0.42	10 ± 1
14. Posterior colliculus	8	2.35 ± 0.37	24 ± 1

* The number of samples indicates the number of determinations of DHPR activity; a different animal was used for each determination. The reliability of the DHPR assay was established by measuring the DHPR activity for several tissue aliquots of a particular brain region; total DHPR activity was proportional to the amount of tissue extract for any given region. A unit of enzyme activity is defined as μmoles of NADH oxidized/min. All values listed represent the mean ± S.E.M. for each region. Variations in weight for the various regions did not exceed 10 per cent.

† This area is composed of all parts of the brain (excluding the locus coeruleus, substantia nigra, colliculi and raphe nuclei) which come from a block of tissue between: (1) the coronal plane which cuts through the caudal edge of the mammilary bodies and the rostral edge of the anterior colliculus, and (2) the coronal plane which cuts through the rostral edge of the spinal cord.

of the cerebral cortex, hippocampus and caudate nucleus (regions 1–3) do not differ significantly; nor do the means of the posterior colliculus, locus coeruleus, medulla-midbrain and spinal cord (regions 11–14) differ significantly. Regions 1–3, however, do differ significantly from regions 11–14. The maximum difference between two regions (cerebral cortex and posterior colliculus) was found to be 17-fold.

Subcellular localization of rat liver DHPR. Table 2 lists the total activity of DHPR and each marker enzyme found in different rat liver subcellular fractions. Since less than 5 per cent of the total DHPR is in the nuclear pellet, this fraction was discarded. The per cent distribution of each enzyme in the various subcellular fractions is shown in Fig. 1. The 100 per cent activity level is defined as the amount of activity found in the 1000 *g* supernatant fraction. The recoveries of each enzyme were as follows: DHPR, 84 per cent; lactate dehydrogenase, 90 per cent; succinate dehydrogenase, 64 per cent; NADPH cytochrome *c* reductase, 101 per cent; and total protein, 87 per cent.

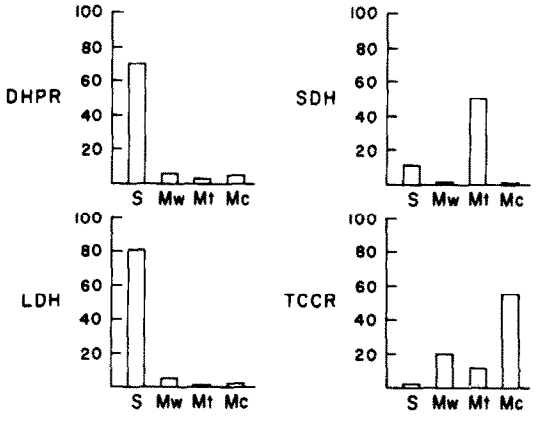

Fig. 1. Per cent distribution of DHPR activity and marker enzyme activity in rat liver subcellular fractions. One hundred per cent activity level is the activity found in the 1000 *g* supernatant fraction. Abbreviations: S = soluble fraction; Mw = mitochondrial wash fraction; Mt = mitochondrial fraction; Mc = microsomal fraction; LDH = lactate dehydrogenase; SDH = succinic dehydrogenase; and TCCR = NADPH cytochrome *c* reductase.

The activity of the enzyme markers in the subcellular fractions corresponds to their known distributions: LDH is localized primarily in the mitochondrial fraction; NADPH cytochrome *c* reductase is localized primarily in the microsomal fraction. The distribution profile of DHPR activity is most similar to the distribution of LDH, the soluble enzyme marker. The results are consistent with a primary, if not exclusive, localization of DHPR in the cytosol. The DHPR and LDH activity found in the mitochondrial and microsomal fractions represents contamination from the soluble fraction.

Subcellular localization of rat brain DHPR. For these studies, LDH was used as the cytoplasmic enzyme marker [22], SDH for the mitochondrial marker [22] and acetylcholinesterase as the marker for "microsomal" particles, which include ruptured external synaptosomal membranes, endoplasmic reticulum and axonal fragments [22, 23].

Shown in Fig. 2 is the per cent distribution of DHPR and the marker enzymes. The amount of enzyme activity present in the total homogenate (H) is taken as the 100 per cent level. The recoveries of each enzyme are as follows: DHPR, 93 per cent; LDH, 94 per cent; SDH, 70 per cent

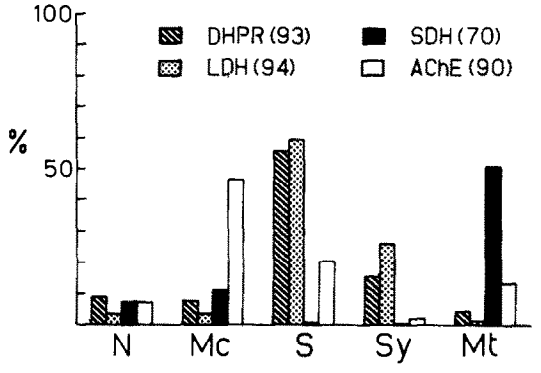

Fig. 2. Histogram of the percentage of DHPR, LDH, SDH and AChE in rat brain subcellular fractions. The numbers in parentheses represent the per cent recovery of each enzyme from the total homogenate. Abbreviations: N = nuclear fraction; Mc = microsomal fraction; S = soluble fraction; Sy = synaptosomal fraction; and Mt = mitochondrial fraction.

Table 2. Subcellular distribution of DHPR activity and marker enzyme activity in rat liver*

	Total activity (μmoles min^{-1} g^{-1}) Fractions				
Enzyme	100 *g* Supernatant	105,000 *g* Supernatant	Mitochondrial washing	Mitochondria	Microsomes
DHPR	79.5	55.9	4.90	2.01	4.10
LDH	296	241	14.4	1.92	5.22
SDH	7.92	0.89	0.10	3.96	0.06
TCCR	6.23	0.28	1.23	0.74	4.04

* Subcellular fractions were prepared as described in Materials and Methods. The units of enzyme activity are: DHPR in μmoles NADH oxidized/min; lactate dehydrogenase (LDH) in μmoles NADH oxidized/min; succinate dehydrogenase (SDH) in μmoles $K_3Fe(CN)_6$ reduced/min and NADPH cytochrome *c* reductase (TCCR) in μmoles cytochrome *c* reduced/min.

acetylcholinesterase, 90 per cent; and total protein, 95 per cent. In all cases, the amount of each enzyme present in the membrane-synaptic vesicle fraction (Mb) is less than 1 per cent and, therefore, was not included in this figure.

The distribution profile of DHPR in the different subcellular fractions is very similar to the distribution profile of LDH. For both enzymes, the greatest amount of activity is in: (1) the soluble fraction (S), 56 and 59 per cent, respectively, and (2) the fraction containing the cytoplasm released from ruptured synaptosomes (Sy) 16 and 26 per cent respectively. The SDH activity is localized primarily in the mitochondrial fraction, 51 per cent. The acetylcholinesterase activity is found primarily in the "microsomal" fraction, 47 per cent. These results indicate that most of the activity of the marker enzymes is contained in the fractions in which they are known to be present.

Table 3 shows the specific activity and relative specific activity of DHPR and the marker enzymes in the different subcellular fractions of rat brain. Relative specific activity (RSA) is defined as the ratio of the percentage of a particular enzyme recovered in a specific fraction to the percentage of protein recovered in that fraction. For example:

$$\frac{\%\ \text{DHPR in fraction S}}{\%\ \text{protein in fraction S}} = \text{RSA of DHPR in S.}$$

The relative specific activity permits an estimation of the degree of concentration or dilution of the enzyme after the isolation procedure.

As indicated by the results in Table 3, DHPR and LDH are diluted relative to the total homogenate in all fractions except the synaptosomal-soluble fraction (Sy) and the soluble fraction (S). In the Sy fraction, DHPR and LDH are concentrated 3.0 and 6.8 times respectively. In the S fraction, they are concentrated to a lesser extent, 2.3 and 2.6 times respectively. SDH, on the other hand, shows a concentration of 2.0 in the mitochondrial fraction, while acetylcholinesterase is concentrated in the microsomal fraction by a factor of 1.6.

Further studies were carried out to measure the effect of ionic concentration and pH on the binding of DHPR by the different organelles or particles characteristic of the various subcellular fractions. Washing the mitochondrial fraction with 32 mM sucrose containing 32 mM NaCl releases up to five times more DHPR and eight times more LDH into the supernatant fraction than a non-ionic 32 mM sucrose washing. The SDH distribution, on the other hand, is the same for either washing solution. Table 4 summarizes the effect of the pH of the washing solution on the binding of DHPR and acetylcholinesterase by the microsomal fraction. The per cent distribution of the DHPR activity and acetylcholinesterase activity between: (1) the microsomal wash-supernatant fraction (105,000 *g* supernatant fraction for the two washings of the microsomal pellet), and (2) the resuspended microsomal pellet, are compared at two different pH levels: 6.6 and 7.4. For each enzyme, activity in both the microsomal wash-supernatant fraction and the microsomal pellet is added together and the total taken as 100 per cent. The total activity for DHPR is the same at either pH, as is the total activity for acetylcholinesterase. When the microsomal pellet is washed at pH 7.4, 74 per cent of the DHPR activity initially present in the unwashed microsomal fraction appears in the microsomal supernatant fraction. When the

Table 4. Effect of pH on DHPR and AChE binding to the microsomal fraction*

	pH 7.4		pH 6.6	
	DHPR (%)	AChE (%)	DHPR (%)	AChE (%)
Mc	26 (1.10)	86 (235)	64 (2.64)	90 (249)
Mc washing	74 (3.13)	14 (38)	36 (1.51)	10 (29)

* The number in parentheses following each percentage is the units/g of tissue of each enzyme found in each fraction under the different conditions of the experiment. Abbreviations: Mc = microsomal fraction, which was the resuspended, washed, microsomal pellet; Mc washing = the 100,000 *g* supernatant fraction resulting from washing the microsomal fraction; and AChE = acetylcholinesterase.

Table 3. Specific activity (units/mg) and relative specific activity (RSA) of DHPR and marker enzymes in subcellular fractions of rat brain*

Fractions	DHPR (units/mg)	RSA	LDH (units/mg)	RSA	SDH (units/mg)	RSA	AChE (units/mg)	RSA
H	0.13	1.00	0.18	1.00	0.46	1.00	4.42	1.00
N	0.08	0.66	0.05	0.27	0.25	0.55	2.39	0.54
Mc	0.03	0.26	0.03	0.14	0.02	0.43	7.11	1.60
S	0.29	2.31	0.47	2.62	0.02	0.04	3.70	0.84
Sy	0.38	3.04	1.23	6.84	0.03	0.05	1.73	0.39
Mt	0.02	0.18	0.01	0.04	0.92	1.99	2.54	0.57
Mb	0.06	0.57	0.06	0.29	0	0	2.41	0.57

* Abbreviations: H = original homogenate fraction; N = nuclear fraction; Mc = microsomal fraction; S = initially soluble fraction; Sy = fraction composed of the cytoplasm released from ruptured synaptosomes; Mt = mitochondrial fraction; and Mb = membrane-synaptic vesicle fraction. The units of activity were defined as follows: DHPR in μmoles NADH oxidized/min; lactate dehydrogenase (LDH) in μmoles NADH oxidized/min; succinate dehydrogenase (SDH) in μg TTC reduced to formozan/min; acetylcholinesterase (AChE) in μmoles acetylcholine hydrolyzed/hr. The respective methods of assay for each enzyme are described in Materials and Methods.

same procedure is carried out at pH 6.6, only 36 per cent of the DHPR activity appears in the microsomal wash-supernatant fraction. On the other hand, the change in pH has little effect on the acetylcholinesterase released into the wash-supernatant fraction; 16 per cent is released at 7.4, whereas 12 per cent is released at pH 6.6. Washing the original microsomal fraction at the two different pH levels causes no significant change in the total protein distribution between the microsomal pellet and the microsomal wash-supernatant fraction.

DISCUSSION

Our studies show that there is a general trend toward higher DHPR concentration in the more caudal brain regions (i.e. hypothalamus, midbrain and medulla). Our results parallel those reported by Turner *et al.* [24]. The locus coeruleus is known to contain a large proportion of noradrenergic neurons [25]. It has one of the highest levels of DHPR, although the difference between it and the area referred to as the midbrain-medulla is not significant. However, since the level of enzymatic activity per unit weight of a dissected region reflects an average for all cells in the region, the relatively higher level of DHPR in individual cells of distinct nuclei, such as the locus coeruleus, is difficult to show by techniques which combine dissection with enzymatic assay. The localization of an enzyme in individual cells is best elucidated by immunohistochemical methods; in preliminary studies [6] we have found that the staining for DHPR in the noradrenergic cell bodies of the nucleus locus coeruleus is much greater than the staining of the surrounding neural tissue.

When we compared the results of the regional distribution investigation of DHPR with the regional distribution of several enzymes involved in catecholamine and/or indoleamine biosynthesis, no correlation to tyrosine hydroxylase, tryptophan hydroxylase or dopamine-β-hydroxylase was found. This lack of correlation indicates that DHPR, as expected, does not seem to be present exclusively in only one of the noradrenergic, dopaminergic or serotonergic neuronal systems.

We also examined the correlation between the distribution of DHPR and aromatic-L-amino acid decarboxylase. Although some evidence indicates that dopa decarboxylase and 5-hydroxytryptophan decarboxylase may be different enzymes, most evidence supports the hypothesis that both types of decarboxylase activity are associated with a single, homogeneous protein: "aromatic-L-amino acid decarboxylase" [26–30]. In addition, the distribution of decarboxylase activity does seem to best represent the regional distribution of both biosynthetic pathways. The distribution of this enzyme should, therefore, correlate with the distribution of DHPR, provided that DHPR is primarily localized in catechol amine and indoleamine containing neurons.

Using the data in the literature [28–30] for the regional distribution of either 5-hydroxytryptophan or dopa decarboxylase, we obtained a correlation coefficient of 0.8 if we exclude the caudate nucleus from our calculation. The reasons why the caudate nucleus has very high aromatic-L-amino acid decarboxylase and tyrosine hydroxylase activities and a very low DHPR activity are not apparent at the present time. It is possible that the amount of DHPR in this region is limiting, as indicated by the experiments of Kettler *et al.* [31]. Measuring the *in vivo* effect of tetrahydrobiopterin on dopamine formation in rat striatum, they showed that an enhanced accumulation of labeled catechols in the striatum paralleled the administration of tetrahydrobiopterin and was probably the consequence of increased synthesis of the amines from labeled tyrosine. They concluded that, *in vivo*, tyrosine hydroxylase apparently does not work to its full capacity due to the limited concentration of tetrahydrobiopterin and that rapid changes in activity of tyrosine hydroxylase may be connected with modifications in the availability of reduced pteridine cofactor at the enzyme site.

In this regard, Kaufman *et al.* [32] and Smith *et al.* [33] have recently found a new variant of phenylketonuria. None of the patients lack phenylalanine hydroxylase. However, the DHPR activity in the only patient examined was less than 1 per cent of control, and a defect in pterin metabolism was postulated to explain the symptoms of the other cases of variant phenylketonuria. A patient deficient in DHPR would be expected to show systemic and nervous system abnormalities related to low levels of catecholamine and indoleamine neurotransmitters. The low or undetectable amounts of dopamine, serotonin, and their metabolites which Kaufman *et al.* [32] observed do, in fact, indicate a deficiency in the production of these two neurotransmitters. However, no clinical evidence of adrenergic system disorders was found, and norepinephrine levels were in normal ranges for those tissues examined.

These studies, along with the results presented in Table 1, indicate that DHPR may not have the same functions of maintenance of H_4P and/or regulation of neurotransmitter biosynthesis in all catecholamine and indoleamine neuronal systems. Since the DHPR activity in the caudate nucleus and the substantia nigra is relatively low, DHPR may be the rate-limiting factor in neurotransmitter biosynthesis in dopaminergic systems. This does not appear to be the case for noradrenergic systems because the enzyme is not only present in relatively large amounts in areas with noradrenergic cells, but also these cells appear to be able to synthesize norepinephrine even in the near absence of measurable DHPR activity, as evidenced by the normal norepinephrine levels in the DHPR-deficient, variant phenylketonuric patients. For serotonergic systems, DHPR seems to be critical for the biosynthesis of 5-hydroxytryptamine, but it may not be involved in the regulation of the rate of neurotransmitter biosynthesis. In addition, because DHPR is also found in relatively high levels in parts of the central nervous system which contain low amounts of catecholamines or indolamines, DHPR probably also functions in the maintenance of the tetrahydrobiopterins for other oxygenase systems.

Our subcellular fractionation studies show that DHPR is primarily, if not exclusively, localized in the cytosol in liver and in brain tissue. The protein and marker enzyme distributions that we found are in good agreement with the distributions reported by previous investigators [15, 34].

We observed small variations from reported results for acetylcholinesterase. Of the total acetylcholinesterase, we found 20 per cent in the soluble fraction compared to the 9 per cent reported by McCaman *et al.* [34]. This disparity could be due to differences in the size of ruptured membrane fragments (which will vary with time of homogenization, sheet force and homogenizer's clearance), centrifugation forces and/or the extent of binding of the enzyme to subcellular particles [35–37].

The solubility of DHPR (i.e. the extent of binding of the enzyme to organelles and particles) is significantly affected by alterations in the ionic strength and pH of the solutions that we used in the subcellular fractionation studies. Similar phenomena for other soluble, small molecular weight proteins (i.e. choline acetyltransferase and lactate dehydrogenase) have been described [15, 38].

Unfortunately, as pointed out by Fonnum [39] and Ichiyama *et al.* [40], subcellular fractionations cannot be carried out at electrolyte concentrations approaching those of extracellular fluid because the sedimentation properties of the particulate material and any enzymes associated with it change due to coacervation. Therefore, we chose a pH of 7.4 and a 32 mM NaCl concentration for the wash solutions of the relevant subcellular fractions (Mt and Mc). We selected these conditions because they resulted in maximum solubility of the bound enzymes.

There are several implications and conclusions which can be drawn from both the primary localization of DHPR to the cytosol and the ability of ionic strength and pH to influence the solubility of this enzyme. First, Rembold and Buff [7, 8] suggested that DHPR regulates the proportion of reduced and oxidized pterins which, in turn, may influence mitochondrial respiration. Since our studies indicate that DHPR is confined to the cytosol, the relationship between DHPR and the mitochondrial electron transport chain can only be indirect. Second, both tyrosine hydroxylase [41, 42] and tryptophan hydroxylase [40, 43–45] are located in the cytoplasm as is DHPR. This implies that the tetrahydropterin coenzyme has immediate access to the hydroxylase and that, in the cell, DHPR and the hydroxylase could be physically associated; both of these factors may be important in the regulation of catecholamine and/or indoleamine biosynthesis. There are several indications that DHPR may have some role in the regulation of catecholamine biosynthesis. Musacchio *et al.* [5] showed that DHPR has *in vitro* the potential to modify the end-product feedback inhibition on tyrosine hydroxylase through changes of the tetrahydropterin concentration. Furthermore, Cote *et al.* [46] reported that the addition of tetrahydrofolic acid or biopterin to chick sympathetic chain cultures significantly increases their catecholamine content. Bjur and Weiner [47] demonstrated that the addition of 6,7-dimethyltetrahydropterin or tetrahydrobiopterin, which naturally depend on the amount of active DHPR, could be an important factor in the regulation of catecholamine biosynthesis in certain regions such as the dopaminergic neurons of the caudate nucleus and substantia nigra as previously indicated.

The fact that the degree of association of DHPR to some subcellular organelles can be changed *in vitro* by varying the ionic strength or pH indicates that similar changes occurring *in vivo* could modify the enzyme activity.

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