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REDUNDANT MECHANISMS REGULATING BRAIN TYROSINE AND TRYPTOPHAN HYDROXYLASES

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The following is a review of current and seminal work in six areas of research relevant to the biochemical regulation of the brain's tyrosine and tryptophan hydroxylases, critical enzymes in the synthesis of the neurotransmitters norepinephrine, dopamine, and serotonin. In addition to presenting a view of some of today's issues, this paper emphasizes the *simultaneity and redundancy* of the mechanisms being discussed. The brain must maintain both its sensitivity and inertia, and it appears to do so through a group of control mechanisms that are layered over each other along the dimension of time, with varying degrees of specificity and potency. The breadth attempted in this review looks toward the day when regulatory influences will no longer be discussed on an either/or basis, because investigators in different laboratories focus their attention on separate mechanisms, and the issues will be seen as requiring the simultaneous measurement of multiple parameters and data reduction through pattern analyses using multivariate statistics. The areas to be covered in this review include regulation by (*a*) the kinetic interactions of reactants and products, (*b*) conformational alterations in enzyme, (*c*) supply of amino acid substrate, (*d*) supply of reduced pterin cofactor, (*e*) amount of enzyme protein, and (*f*) neurophysiological organization.

REGULATION BY THE KINETIC INTERACTIONS OF REACTANTS AND PRODUCTS

In vitro studies of the kinetics of interactions of tyrosine and tryptophan hydroxylases with their reactants, products, and other effectors have revealed four groups

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of regulatory phenomena which are used frequently to explain the results of research conducted in intact preparations (1–7). These are classical competitive interactions of aromatic amino acids, catechols, pterin analogues, and other compounds with tyrosine, tryptophan, and reduced pterin (8); binding and desorbtion sequence restraints in multisubstrate systems, with the regulatory influences of out-of-sequence binding and "noncompetitive" inhibition (9); the influence of substrates and other molecules as ligands altering the affinity constants of the enzymes for substrates, called *allosteric regulation* (10, 11); and factors introduced into the assay procedure which relate to the maintenance of a reduced pterin cofactor and/or enzyme form, such as ascorbic acid, ferrous iron, catalase, 2-mercaptoethanol, and glutathione, and which may or may not have relevance to circumstances in vivo (12–15). Generalizing about the kinetic characteristics of tyrosine hydroxylase is made difficult by the different molecular properties of native enzyme from heterogeneous sources used in various studies (12, 16–18); varying techniques used in enzyme preparation that have the potential for altering kinetic properties, such as proteolytic digestion and the use of detergents (12, 16, 17, 19); and the common use of the 6,7-dimethyl pterin and more recently the 6-monomethyl pterin analogue (pterin is 2-amino-4-hydroxypteridine), which appear not to be the natural cofactor (tetrahydrobiopterin; see below) and not to reproduce its effects with adequate fidelity, either kinetically or stoichiometrically (14, 20, 21). For example, the reaction using the dimethyl pterin analogue is loosely coupled such that in assays for adrenal tyrosine hydroxylase activity the pterin:dopa product ratio is 1.75, compared to 1.15 for tetrahydrobiopterin:dopa, and the former reaction runs at less than half the latter reaction's relative velocity (20). This uncoupling is responsible for the adventitious generation of peroxides which in turn alters the reduced state of the cofactor and may alter the reduced form of the enzyme which binds cosubstrates (13). This phenomenon has been thought to account for the apparent stimulation and/or protection of enzyme activity afforded by ferrous iron, catalase, ascorbate, and other reducing substances (13–15, 22, 23), although it should be mentioned that ferrous iron manifests saturation kinetics (24, 25). The K_m for tyrosine in the presence of tetrahydrobiopterin is one twentieth of that when the 6,7-dimethyl congener is used (26); the K_m for oxygen is 1%, versus 6% in a comparison of these pterin cofactors using the adrenal enzyme and less than 1% versus 1% using the brain enzyme (21, 27, 28). The K_m of the soluble brain (caudate) enzyme for 6,7-dimethyl pterin is about an order of magnitude above that for tetrahydrobiopterin (25, 26). Another characteristic dependent on the substituents at the 6 and 7 positions of the pterin is substrate specificity. Adrenal tyrosine hydroxylase was initially reported as absolutely specific for L-tyrosine and inhibited competitively by phenylalanine with respect to tyrosine (24). Later the enzyme was shown to hydroxylate phenylalanine with 6,7-dimethyl pterin as the cofactor, but with low yields (29). When tetrahydrobiopterin was used as the cofactor, however, phenylalanine was hydroxylated at a rate equal to or greater than that for tyrosine by the adrenal tyrosine hydroxylase (13), and the same seems to be true for the brain enzyme (30).

Studies of adrenal and brain tyrosine hydroxylase have suggested that some L-aromatic amino acids (24, 31–33); catechols (24, 27, 29, 32, 34); pterin analogues

(13, 20); phenylcarbonyl compounds with various ring hydroxylations (34, 35); ferrous iron-binding compounds (20); apparently nonspecific compounds related to the maintenance of the redox environment of the enzyme (13, 15, 22, 23); the substrates tyrosine and oxygen (13, 26, 28, 36, 37); and product dopa, with different mechanisms dependent on the type of pterin cofactor (13, 24, 26, 27), may be inhibitory and reveal different regulatory properties of the enzyme. Competition with respect to tyrosine by the α-alkyl, ring-halogenated, and combination-substituted aromatic amino acids is straightforward, with the L-isomers, as expected, more effective (32). The competitive inhibition of tyrosine hydroxylase by various catechols with respect to the pterin cofactor (16, 24, 32, 37), in which dopamine appears to be the most potent, manifesting sigmoidal kinetics when the brain enzyme is in the soluble but not the bound form (25), can be seen with all three commonly used pterins, but with varying K_i's (13). This interaction, called *simple product–feedback inhibition,* has in the past been the most prominent mechanism invoked to explain the increase in synthesis of catecholamines associated with neural activation (38, 39).

Previous attempts to establish the sequence of binding of substrates and release of products to elucidate the reaction mechanism have resulted in differing conclusions although all three used the 6,7-dimethyl pterin as the cofactor and adrenal tyrosine hydroxylase as the enzyme, albeit variously prepared. In these studies, one substrate is varied at several fixed levels of the second, and the third is held constant to ascertain the interactional dependence of the reaction velocity with respect to each on the varying presence of the other (9). The first group, assuming oxygen to be saturating at ambience (20%)—an assumption unproved in their studies—reported parallel lines in their reciprocal plots, indicating no interactional effect on the enzyme between the reduced pterin and tyrosine which, when considered with their demonstration of dopa's noncompetitive interaction with tyrosine and competitive interaction with 6,7-dimethyl pterin, led the group to decide that the mechanism was ping-pong [what Cleland (9) would call a uni-uni-bi-uni ping-pong]. That is, after the enzyme is reduced by the reduced pterin, the oxidized pterin (quinoid dihydropterin) leaves before the binding of tyrosine and oxygen to the reduced enzyme and the aerobic oxidation of tyrosine (27). Using a range of pterin cofactor an order of magnitude below that used by the first group, a second group reported convergent lines in their reciprocal plots, suggesting a sequential mechanism in which all three cosubstrates (oxygen, reduced pterin, and tyrosine) add to the enzyme, forming a quaternary complex, before any product is released (36). A third group, using cofactor concentrations comparable to those used by the first, but a lower tyrosine substrate range, also reported intersecting lines on reciprocal plots, similarly suggesting a sequential rather than a ping-pong mechanism (37). We have been doing similar studies of soluble brain tyrosine hydroxylase (from striate cortex) using 6-methyltetrahydropterin and a new pterin analogue, 5-deaza-6-methyltetrahydropterin (W. P. Bullard and A. J. Mandell, unpublished observations). Our work has suggested that the binding of oxygen, tyrosine, and reduced pterin to tyrosine hydroxylase occurs in a semiordered sequence of reversible steps, while release of the products dopa and quinoid dihydropterin, since dopa was not competi-

tive with respect to either tyrosine or reduced pterin, follows an obligatory sequence. The sequential binding of reactants was evident from the family of double-reciprocal plots in which the slope for any reactant depended on the concentrations of both co-reactants. Linear noncompetitive inhibition by dopa with respect to both tyrosine and tetrahydropterin and competitive inhibition by 5-deaza-6-methyltetrahydropterin with respect to tyrosine and pterin established the random addition of the amino acid and reduced pterin (although oxygen may be an ordered addition) and mandated the release of dopa prior to oxidized pterin. The similarity between kinetic constants seen in the assay dependent on the hydroxylation of 3,5-ditritiotyrosine and those in the assay using 1-[^{14}C]-L-tyrosine as substrate, coupled to dopa decarboxylase (40), indicates the absence of an apparent tritium isotope effect, which would be significant even with 1% of the substrate radioactive, and suggests that the interconversion of the quaternary central complex may *not* be rate-limiting, as suggested by other groups (36, 37); rather it may be thermodynamically obscured by a slower step involving perhaps oxygen activation by the reduced pterin (41) or desorbtion of dopa or quinonoid dihydropterin. When these findings are combined with the above-noted critical role of the 6 and 7 substituents of the reduced pterin in relation to the apparent affinities and sequence of tyrosine hydroxylase binding of oxygen, tyrosine, dopa, and alternate substrates like phenylalanine, and considered with the absence of a significant ^{3}H-isotope effect, the implication is that the dynamics of binding and desorbtion of the reduced and oxidized pterin may dominate the functional characteristics of tyrosine hydroxylase. Substrate inhibition by tyrosine requiring high levels of tyrosine with 6,7-dimethyl pterin as the cofactor, but only twice tissue concentrations (36) with tetrahydrobiopterin (13, 30), would thus be explained as a result of out-of-sequence binding of tyrosine to the dopa product site. Phenylalanine, hydroxylated at rates equal to or greater than those of tyrosine hydroxylation when tetrahydrobiopterin is the cofactor (30), does not manifest substrate inhibition, perhaps because of its greater dissimilarity to dopa. Inhibition of the brain enzyme by oxygen (28) spans the range of current models in the multiplicity of its possible explanations. While it may be that oxygen in high concentrations alters the enzyme by oxidation of SH groups necessary for its function, it is unlikely because, as noted above, the oxygen effect is dependent upon the 6,7 substituents of the pterin cofactor (28). Another potential explanation is suggested by the findings of one group that when oxygen was the variable substrate across different levels of tyrosine and reduced pterin there were downward curved plots suggestive of an allosteric influence of oxygen, with negative cooperativity (42). A recent study demonstrated a change in the oxygen dependence of brain tyrosine but not tryptophan hydroxylation following electroshock and immobilization stress consistent with such allosteric properties (43). Another explanation for inhibition by oxygen lies in the model of a semi-ordered sequential ter-bi reaction mechanism developed above, suggesting that an out-of sequence binding may be responsible. Further systematic kinetic studies will be required to resolve these questions.

As is seen in all the following sections, with the exception of research on factors influencing amino acid substrate availability, studies of the regulation of tryptophan hydroxylase lag considerably behind those on tyrosine hydroxylase, and this has

certainly been true of kinetic studies. Hampered by lack of extracerebral sources of enzyme except from the pineal, mast cells, and an occasional carcinoid tumor—there may be another source in the recently elucidated serotonergic nerves of the gastrointestinal plexes (44, 45)—and with more enzyme instability manifested during enrichment procedures than was the case for tyrosine hydroxylase, only two- to tenfold purifications over that obtainable from a supernatant fraction derived from high speed centrifugation have been used in kinetic characterizations of tryptophan hydroxylase (14, 46–48). The problem of multiplicity of forms presenting kinetic diversity characteristic of brain and adrenal tyrosine hydroxylase studies has also been operative in tryptophan hydroxylase studies (47, 49–51).

An early confusion about tryptophan hydroxylation not requiring tetrahydrobiopterin (52) was probably due to the partial maintenance of "synaptosomal" integrity during preparation, with the pterin and the reductase regenerating system entrapped, allowing assays that were linear with time without added cofactor or reducing system for as long as 30 min (53, 54). When the enzyme was first studied systematically, it was found to require pterin as well as 2-mercaptoethanol. Ferrous iron was thought by one group to play a direct role in the reaction (55), although in light of later work (14) it appears that it probably stabilizes the cofactor and enzyme by protecting them from the peroxides generated by the use of the 6,7-dimethyl pterin in these assays (13, 46, 55), particularly since when catalase was used there was no stimulation by iron (14). Although a comparative stoichiometry for the three commonly used pterins is not available in the literature, it has been shown that when tetrahydrobiopterin is used as the cofactor there is a mol-for-mol ratio of tetrahydrobiopterin consumed to 5-hydroxytryptophan formed (14).

Another problem that has made work in this area difficult to interpret from the standpoint of kinetic mechanisms is that with the commonly used assay coupling the hydroxylase and decarboxylase, which exploits the two orders of magnitude between the K_m's of aromatic amino acid decarboxylase for tryptophan and 5-hydroxytryptophan (46, 47, 55), a full range of substrate curves, either alone or in relation to the other reactants, is not obtainable. A tritium-release assay analogous to the one often used in studies of tyrosine hydroxylase has been developed (56), but because of expense and general unavailability, it has not been used. Studies exploiting the discriminable fluorescence of 5-hydroxytryptophan, being less sensitive, require much more enzyme protein, and this requirement combined with the enzyme's rapid loss of activity during preparation also contributes to the relative slowness of advance in this research.

With rabbit brain stem enzyme, a dihydropteridine reductase–reduced pyridine nucleotide system was found to stimulate tryptophan hydroxylase and eliminate the protective function of 2-mercaptoethanol. The K_m for tryptophan with tetrahydrobiopterin as cofactor was 50 μM [close to brain concentrations (57)]; for oxygen, 2.5%; and for the cofactor itself, 31 μM, compared to the values when the dimethyl pterin was used, which were 290 μM, 20%, and 130 μM respectively. The V_{max} was not significantly increased by changing the cofactor analogue used (14).

The enzyme, as might be expected, is inhibited competitively by halogenated and nonhalogenated aromatic amino acids (48, 58), but problems involved with the

definition of "particulate" tryptophan hydroxylase (synaptosomal?) confound interactions at the neuronal membrane transport site (see below) with those at the amino acid substrate site on the enzyme (59). Studies of *p*-chlorophenylalanine demonstrated the multiplicity of these factors: *p*-chlorophenylalanine appears to inhibit competitively the uptake of tryptophan into the nerve; exhibits competitive reversible inhibition of the enzyme with respect to tryptophan; and also produces a nondialyzable inhibitory effect thought to be due to either covalent binding with the enzyme or incorporation of the halogenated amino acid in the enzyme protein near the active site (49, 60). Serotonin in concentrations higher than 0.1 mM does not inhibit rat brain stem tryptophan hydroxylase with either the dimethyl pterin or tetrahydrobiopterin (14, 48). Many catechols, however, are competitive with respect to the pterin cofactor—dopamine manifests a K_i of 0.05 mM (61)—suggesting that the "product" part of product-feedback inhibition for brain catecholamines may be adventitious, the inhibition arising from the relationship of the stereochemistry of catechols and tropolones to that of the pterins. This absence of enzyme inhibition by serotonin in vitro was originally a source of confusion in light of studies in intact brain or brain slices suggesting that an increase in brain serotonin reduced its rate of synthesis (62, 63). This issue has recently been clarified somewhat by the demonstration that a serotonin uptake blocker, chlorimipramine, did not eliminate the reduction in serotonin synthesis by striatal slices incubated in a high serotonin environment. This has suggested the possible role of autoreceptors in the control of synthesis (64). Other catechols and tropolones inhibit synthesis, but the mechanism remains to be elucidated (65).

Substrate inhibition was manifested at tryptophan concentrations above 0.2 mM when tetrahydrobiopterin was used as the cofactor, but over the full range of oxygen concentrations there was no inhibition by this cosubstrate (14). If this property is not altered by further purification of tryptophan hydroxylase, it may suggest a reaction mechanism different from that for the other two pterin-dependent aromatic amino acid hydroxylases (28). It has been shown that the immediate product of tryptophan hydroxylase, 5-hydroxytryptophan, in the presence of tetrahydrobiopterin inhibits the enzyme at concentrations of 0.1 mM (66), and rough calculations based on those data (66) suggest that the interaction is noncompetitive with respect to tetrahydrobiopterin. If this is confirmed and is also true with respect to tryptophan, it may be that both tryptophan and 5-hydroxytryptophan inhibit by interfering with the sequential desorbtion of 5-hydroxytryptophan and quinoid dihydropteridine in a way analogous to the mechanism speculated to account for the effects of tyrosine and dopa on tyrosine hydroxylase. These issues require further work, but a recent report of the acute modulation of tryptophan hydroxylase activity in both directions by treatments that alter tryptophan substrate supply (67) suggests an out-of-sequence binding of substrate as one possible explanation. The other is the evidence of sigmoidal kinetics evoked by changing concentrations of tryptophan, demonstrated for hepatic tryptophan hydroxylase (68) and the brain enzyme (69), which suggests the potential for allosteric regulation of tryptophan hydroxylase by tryptophan concentration, a potential in addition to those factors influencing substrate supply that is discussed below. Phenylalanine hydroxylase, which may have been the enzyme under study in the liver since it can hydroxylate tryptophan with

reasonable kinetic parameters (70), has been shown to manifest substrate control, and the property was dependent on the type of pterin cofactor used (71).

REGULATION BY ALTERATIONS IN ENZYME CONFORMATION

Following the seminal observation by Carlsson & Lindqvist in 1963 that the neuroleptics chlorpromazine and haloperidol, but not phenoxybenzamine, enhanced the accumulation of methylated catecholamines 3 hr after monoamine oxidase inhibition in the mouse brain and their speculation that it was reasonable "to assume that this receptor blockade results in a compensatory activation of monoaminergic neurons" it has been difficult to establish the mechanism(s) of this acute activation of synthesis (72). Unlike the drug-induced compensatory changes in catecholamine synthesis associated with increases in soluble brain tyrosine hydroxylase activity usually requiring several hours or days for their appearance and disappearance (73, 74), after acute administration of biogenic amine agonists or antagonists changes in indices of synthesis in vivo may appear without evidence in vitro of either increased enzyme activity—assayed in the presence of saturating concentrations of cosubstrates (75–77)—or an increase in immunoprecipitable protein (78). A similar dissociation of alterations in synthesis in vivo and enzyme activity in vitro has been reported for serotonin and tryptophan hydroxylase in response to reserpine by one laboratory (79) but attributed to a change in the amount of enzyme by another (80). Neuroleptic drugs, their clinical potency correlated with their affinity for dopamine receptors (81), induce an increment in the spontaneous firing rate of dopamine-containing cells (82), and on the basis of studies conducted with preparations of peripheral adrenergic tissue, some of the neural activation-induced increase in synthesis has been attributed to discharge-related loss of amine transmitter and the subsequent cofactor-sensitive release of tyrosine hydroxylase from product-feedback inhibition (27, 83, 84). As noted above, competitive kinetic interaction has not been demonstrable for tryptophan hydroxylase with respect to the reduced pterin cofactor and serotonin (66); rather the interaction is "uncompetitive" (14, 48, 85) with a K_i of more than 100 μM (86). As noted above, what appeared to be product-feedback inhibition of serotonin synthesis, when the turnover of tryptophan to serotonin was studied in brain slices after monoamine oxidase inhibition (62), has recently been reinterpreted as a response to a presynaptic receptor signal (87). Consistent with this speculation is the finding that serotonin inhibits striatal synaptosomal serotonin synthesis at concentrations of 1.0 μM (the effect is not reversed by methysergide) and without increasing intrasynaptosomal serotonin or interfering with the uptake of substrate (S. Knapp and A. J. Mandell, unpublished observations). Thus, to account for some aspects of the short-latency changes induced by drugs in catecholamine synthesis and perhaps all aspects of such changes in serotonin synthesis, regulatory mechanisms other than the kinetic interaction of amine product with cofactor at catalytic sites must be sought.

Nagatsu et al (24) observed that tyrosine hydroxylase in adrenal extracts exists in both soluble and particulate forms. In the late 1960s there was considerable debate about whether the existence of the "particulate" form in the adrenal medulla

and brain reflected the enzyme's natural state or was a function of its highly polar character and tendency to adhere to membranes and to itself, especially when prepared in nonpolar solvents such as sucrose (12, 87a). However, recent studies of the ultrastructural location of tyrosine hydroxylase in noradrenergic neurons in the nucleus locus coeruleus, using the peroxidase-antiperoxidase immunohistochemical method, have shown diffuse staining in the perikarya and orderly, plasma membrane-related staining in axonal and dendritic processes, suggesting that two forms of the enzyme may in fact exist in vivo (88). Multiple forms of tryptophan hydroxylase have also been suggested (49), and similar diversity in histofluorescent appearance has been reported (89).

The finding that different physical forms have varying kinetic properties and can be interconverted by psychotropic drugs was first suggested to explain the dissociation of the effect of amphetamine (90) on catecholamine synthesis in relatively intact systems (brain slice or synaptosome) and on tyrosine hydroxylase activity measured as soluble enzyme (75, 77). More recently, studies have shown that the acute administration of neuroleptics could predictably alter the activity state of striatal tyrosine hydroxylase, as manifested by increased affinity for the reduced pterin cofactor (91, 92). That this finding has physiological significance is suggested by the successful manipulation of the K_m of tyrosine hydroxylase for its cofactor with drugs acting farther from the dopamine synapse, in the multitransmitter nigrostriatal circuit (93, 94). A reduction in the affinity of striatal tyrosine hydroxylase for the monomethyl pterin cofactor, i.e. "deactivation" of the enzyme, has been demonstrated recently after the repeated administration of a neuroleptic (92). The increment observed in striatal tyrosine hydroxylase activity immediately after axotomy (95) or neurotoxin administration (96), as well as studies of tyrosine and tryptophan hydroxylases in vivo which have shown loss of oxygen dependence (alteration in the enzyme's affinity for oxygen) after immobilization, footshock, or electroconvulsive shock (43, 97), may be other examples of acute conformational adaptation by amine-synthesizing enzymes. Activation of brain stem tryptophan hydroxylase, with respect to the affinity for tryptophan substrate, follows methiothepin administration, although the kinetics of the effect also reflect the abolition of what appears in controls to be negative cooperativity with respect to tryptophan concentration (10, 69). Active and inactive forms of dopamine-β-hydroxylase have also been reported (98).

Efforts to understand the mechanism(s) of the kinetic activation of brain tyrosine and tryptophan hydroxylases manifested by decreased K_m for substrate and/or cofactor have been directed toward the explanation of the effects of a number of potential ligands in vitro. For tyrosine hydroxylase these have included binding to neural membranes (25, 90), the sulfate ion and sulfated mucopolysaccharides (99; W. Lovenberg, unpublished observations), limited tryptic proteolysis (19), treatment with detergents (17), alterations in microenvironmental pH (100), phospholipids (101–103), phosphorylating conditions without demonstration of P^{32} incorporation when the possibility of phosphatases removing the label had not been ruled out (104–106), and phosphorylating mixtures to which a protein kinase had been added (105, 107, 108). Ethyleneglycol-bis (β-aminoethyl ether) N,N'-tetra-

acetic acid (EGTA) in striatal preparations can activate dopamine-related tyrosine hydroxylase (109), and calcium has been reported both to inhibit it (110) and to have no effect (111, 112). Findings of calcium-invoked kinetic activation of tyrosine hydroxylase from the noradrenergic locus coeruleus-hippocampal system (113), in cell suspensions from pheochromocytoma tumor (114) and in the snail (115) have not been invalidated, although failure to replicate the effect in preparations of noradrenergic tyrosine hydroxylase from hypothalamus (112) and in peripheral adrenergic preparations (116) should be noted. It is not beyond reason that brain tyrosine hydroxylase from dopaminergic systems would have different regulatory properties than the enzyme from noradrenergic systems because differing molecular forms of tyrosine hydroxylase have been reported for central dopaminergic and noradrenergic neurons [the former characterized by low (≃65,000) and the latter by high (≃200,000) molecular weight and an adherent RNA moiety], although their antigenic properties were similar (18). The forms were also differentially responsive to reserpine as a stimulus for enzyme induction (118). Studies of physical influences on tryptophan hydroxylase activity are not as advanced as those for tyrosine hydroxylase. Kinetic activation by partial proteolytic digestion (69, 87), calcium (111, 119, 120), and a detergent (120) has been reported. We have been unable to predictably activate tryptophan hydroxylase with polyanions, phospholipids, or phosphorylating conditions (S. Knapp and A. J. Mandell, unpublished observations), nor have such phenomena been established by other laboratories.

The mechanisms for conformational regulatory influences have not been established. It appears that activation by partial proteolysis and detergent of tyrosine hydroxylase (17) and tryptophan hydroxylase (120) may involve the removal or an alteration in the relationship of a residue to the active site of a regulatory subunit, as has been shown for a comparable mixed-function oxydase, hepatic phenylalanine hydroxylase (121). The allosteric properties of tyrosine hydroxylase are consistent with this possibility (25). Sulfate and sulfated mucopolysaccharides (25, 99), the phospholipids (101–103), protonation (100), and phosphorylating conditions in an enriched but impure enzyme preparation may activate the enzyme via the creation of favorable electrostatic interactions for tyrosine hydroxylase, as in a nonspecific polyanionic environment (122). A recent study in conflict with previous ones (104, 105) has reported incorporation of P^{32} in a gel electrophoretic isolation of tyrosine hydroxylase after phosphorylating conditions, so conformational alteration of the enzyme via phosphorylation has not been ruled out (123). The findings vis-à-vis calcium have not been consistent for tyrosine hydroxylase (which may be partially explained by whether the enzyme comes from a dopaminergic or a noradrenergic system), and myriad potential mechanisms for its action make the specific circumstances of each study crucial. Since calcium has been demonstrated to promote membrane binding in preparations of striatal tyrosine hydroxylase (25), and membrane binding can lead to the occlusion of active sites (90) as well as enzyme activation (25, 90), it may be that the EGTA effect on striatal tyrosine hydroxylase involves alterations in the physical state of the enzyme in relation to subcellular organelles when studied in slices or with the characteristic impure enzyme preparations. Histofluorescence evidence for "bound" and "free" tyrosine and tryptophan

hydroxylases has been noted (88, 89). Calcium has been shown to activate a brain proteolytic effect, inhibitable by benzethonium and phenylmethylsulphonyl chloride, and this has been used to explain calcium's activation of tryptophan hydroxylase via partial proteolytic digestion (120). These serine residue-directed inhibitors may not be the proper ones for brain proteases. There appears to be a calcium-dependent regulator in brain for adenylate cyclase (124, 125) as well as a calcium-dependent phosphodiesterase (126) which may play roles in the phosphorylation of the hydroxylases (if it indeed occurs) or perhaps in changing the ionic environment of the enzymes via the phosphorylation of another proximate synaptic protein (127, 128). One research group has reported as additive the activating effects of phosphorylating conditions and phospholipids (102), but another did not find them so (103). There is a calcium-dependent exchange reaction between phosphatidylserine and phosphatidylethanolamine (129), and an interaction of the activating effect of ethanolamine with that of EGTA on hippocampal tyrosine hydroxylase has been reported (103). Dependence on calcium by an ATP-Mg^{2+} activation of brain tryptophan hydroxylase has also been noted (64). Mention should also be made of the calcium dependence of the exocytotic process and the relationship between changes in intracellular catecholamine concentrations and rate regulation via product inhibition (130). Thus calcium, if it has a modulatory role in the regulation of tyrosine and/or tryptophan hydroxylase, may act through its influence on the enzyme's affinity for co-reactants, or exert effects on membrane binding, on partial proteolysis, on the process of phosphorylation of the enzyme or an adherent molecule, on phospholipid interactions with the enzyme, on an alteration in the amine-release process, or on some other as yet unexplored mechanism(s).

REGULATION BY AMINO ACID SUBSTRATE SUPPLY

As is the case with many aspects of central nervous system regulation, the parameter of amino acid substrate supply to biosynthetic enzymes in the catecholamine and indoleamine systems involves interdependent relationships with factors operating beyond the limits of the brain, in this case, perhaps the quality and quantity of the protein in the food supply. It has been shown that the protein hunger of an animal can be influenced by the amount and ratio of tryptophan to the other amino acids (aromatic and branch-chain) in the preconditioning diet (131). This ratio, when combined with a measure of the pH-dependent albumin binding of tryptophan (132), constitutes what has been called the effective tryptophan concentration and determines the rate of uptake of tryptophan into the brain (133, 134) and, within a specifiable range, the serotonin concentration in some but not all brain regions (67, 135). The limits of such influence appear to be established by the modulatory influence of tryptophan loads on raphe cell firing rate (136); tryptophan inhibition of tryptophan hydroxylase (substrate inhibition) as demonstrated in vitro with tetrahydrobiopterin as cofactor (14); and other mechanisms (demonstrable in brain slices or synaptosomes) that appear unrelated to nutritional supply of substrate or

intraneuronal tryptophan hydroxylase activity. These might include neuronal membrane uptake of tryptophan; intraneuronal regulatory influences such as cofactor level and the effects of ligands (85, 87); and the activity of hepatic tryptophan pyrrolase (137) which oxidatively cleaves the indole ring. A high protein diet, by creating a relative increase in the amino acids that compete with tryptophan for overlapping systems transporting neutral amino acids into the brain (138), reduces brain tryptophan and brain serotonin, thus invoking environmental supply and such behavioral factors as appetite to close what has been called the open loop regulation of serotonin biosynthesis in the brain (139). This work has led to speculation that control of protein hunger via plasma and brain levels of the body's amino acid in lowest supply, tryptophan, and its substrate-dependent rate of conversion to serotonin have adaptive significance. A recent study using the serotonergic neurotoxin 5,7-dihydroxytryptamine and protection of catecholamine neurons with desmethylimipramine has shown a relation between serotonin depletion and hyperphagia (140), which may indicate that the appetitional influence of tryptophan-dependent serotonin levels is not limited to protein hunger.

It has been known for some time that tryptophan loads increase brain serotonin concentration (141, 142). This is in marked contrast to the effect of tyrosine on brain catecholamine concentration. Substrate influences on brain catecholamine biosynthesis can be demonstrated only under contrived conditions. For example, the inhibition of aromatic amino acid decarboxylase eliminates "feedback" influences via pre- or postsynaptic receptors or competition with cofactor binding to tyrosine hydroxylase (143); drug-induced neural activation, as in the case of pretreatment with the dopamine receptor blocker haloperidol, creates a circumstance in which substrate can become limiting (144).

There has been considerable debate concerning the relative influence of peripheral factors on the tryptophan supply to the brain. Studies using psychotropic drugs and dietary manipulations have shown that total plasma tryptophan can be meaningfully related to brain tryptophan and serotonin levels (67, 87, 145, 146); other work suggests that the degree of binding to serum albumin is a drug-sensitive regulatory influence of importance (147–149); in other studies, the ratio of free or total tryptophan to other neutral amino acids in the plasma appears determinant (150–153). Two recent studies have monitored these variables in concert, and, as might be expected, it appears that they all contribute to the variance in the relationship between plasma and brain tryptophan, with the relative contributions of each depending on the circumstances of the experiment (133, 134).

A peripheral regulatory influence of importance, especially when tryptophan loads are to be given on a regular basis, is the activity of hepatic tryptophan pyrrolase, which is sensitive to both induction by tryptophan (substrate) and induction by glucocorticoids (hormonal) (154). The activity of this pathway, shunting tryptophan away from the indoleamines to nicotinic acid and energy metabolism, has been shown to be increased by these influences in intact animals (155) and man (156). Its quantitative significance has been challenged in the past (157), but a recent study using an intact organ preparation has demonstrated that the liver can turn

over its entire tryptophan store via this pathway in less than 7.5 min (137). In light of the rather predictable elevation of plasma glucocorticoids in states of severe clinical depression but not mania (158) and the speculated role of serotonin in mood disorders (159), we can wonder whether this, like the regulation of appetite, is an example of the brain in functionally significant metabolic interaction with peripheral influences which themselves are influenced by the brain.

In addition to transport across the blood-brain barrier, the mechanism for which has not been established, there is another regulatory transport system for amino acids, one across the neuronal membrane to the enzyme site, which studies in brain slices or synaptosomes have shown to manifest the same overlapping stereospecificity for aromatic and branch-chain amino acids as does the transport system across the blood-brain barrier (160–162). This process is stimulated by progesterone (87), lithium (163), phenelzine (164), and preloading with tryptophan (165), and it is inhibited by cocaine (166). The uptake was reported originally to manifest double hyperbolic kinetics with respect to substrate concentration (a "low" affinity system manifesting a K_m of 6 mM and a "high" affinity system with K_m in the 50 μM range) (53,167). The "high" affinity system has been shown to be differentially responsive to metabolic and pharmacological manipulations (161,162). This has not been as clear for the "low" affinity system, which appears to have the characteristics of a mass-action controlled process when statistically resolved into component functions and fitted with models of carrier-mediated movement exhibiting Michaelis-Menten kinetics versus diffusion (168). Since in studies of both brain slices and synaptosomes, tryptophan uptake has been indicated by the retention of radioactivity after short incubations with radioactive tryptophan, and it has been shown that at least with respect to aromatic amino acids there is preferential and active exchange of "newly taken up" amino acids for neutral amino acids in the incubation medium (161), there has been speculation that some uptake studies may reflect not increase in intraneuronal tryptophan but labeling of the intracellular pool. In the actions of lithium and cocaine respectively, however, a net increase and decrease in intrasynaptosomal tryptophan concentration have been clearly demonstrated (162).

A change in intracellular tryptophan concentration in brain following drug or dietary manipulation is a complex function of plasma level in relation to other neutral amino acids and albumin (and substances competing for the binding such as free fatty acids) (146), their relative rates of conveyance into the brain via the blood-brain transport system, and the influence of the neuronal membrane transport system. Learning how the kinetic constants of these processes relate to those of brain tryptophan hydroxylase (itself sensitive to inhibition by substrate), and therefore modulate the rate of serotonin biosynthesis by the degree of saturation of this initial-step biosynthetic enzyme, will require study of several indices simultaneously and multivariate statistical determinations. The K_m for tryptophan transport across the blood-brain barrier in the presence of normal levels of the other neutral amino acids has been calculated to be 723 μM (134); that across the neural membrane, 50 to 100 μM (69, 169); that of tryptophan hydroxylase with tetrahydrobiopterin as cofactor, 50 μM (14, 85). In simultaneous comparison of three tryptophan pools (i.e. levels in plasma, extrasynaptosomal brain, and intrasynaptosomal brain) after

the administration of small amounts of tryptophan (10 to 20 mg/kg) to rats, it was shown that the intrasynaptosomal fraction was increased preferentially (162). The congruence in K_m's for tryptophan of the neuronal membrane transport mechanism and tryptophan hydroxylase suggest that under some circumstances the neuronal membrane transport system may be limiting for serotonin biosynthesis. This has been demonstrated following lithium and cocaine, which alter the relative velocity of neuronal membrane transport (163, 166) as well as synaptosomal serotonin biosynthesis. A recent study using methiothepin, thought to be a serotonin receptor blocker, has demonstrated, in addition to an alteration in the kinetic properties of tryptophan hydroxylase, a facilitated tryptophan uptake into brain slices (69), which may indicate that alterations in the rate of substrate uptake can be produced by neural activation.

The concept of the prepotency of tryptophan substrate supply to the brain over all other regulatory factors influencing serotonin biosynthesis [suggested by such findings as the failure of pretreatment with a monoamine oxidase inhibitor to alter the increase in brain tryptophan and associated increase in serotonin after a high carbohydrate meal (169)] must be tempered by the awareness of differences among brain regions in this regard. For example, an area with high concentration of serotonin endings like the striate cortex responds little or not at all to increased "effective" plasma tryptophan concentrations (67, 135). Moreover, it may be that factors regulating substrate supply are redundant and coordinated. For instance, the well-established diurnal rhythm in brain serotonin (170) is led by alterations in both plasma and brain tryptophan levels (171) *and* associated with a cyclic variation in the rate of neuronal membrane transport (172). It may be that brain tryptophan levels regulate neuronal membrane transport (165).

REGULATION BY REDUCED PTERIN COFACTOR SUPPLY

Following Kaufman's demonstration that a reduced, unconjugated pteridine could function as a cofactor in the enzymatic conversion of phenylalanine to tyrosine (173) and that the phenylalanine hydroxylating cofactor isolated from rat liver was dihydrobiopterin (174), work elucidating the role of the reduced pterins in the regulation of the activity of biogenic amine biosynthetic mixed-function oxidases in brain has seemed increasingly important to an understanding of the regulation of biogenic amine synthesis in brain. Since the reduced pterins, probably tetrahydrobiopterin (175–176a) were shown to be necessary to optimal activity of brain tyrosine hydroxylase (24) and tryptophan hydroxylase (46), they have been invoked prominently in models of regulation of biogenic amine synthesis, although the old debates about the role of ascorbate, iron, and the nucleotides NADH and NADPH continue (15). The potential rate-limiting properties of brain reduced pterin levels were an unstated factor in most of the older in vitro and in vivo turnover studies that made use of a multiple-pool concept and the interaction of biogenic amine product with the cofactor site–product-feedback inhibition (1,38) and more recent work demonstrating the conformational regulation of biogenic amine hydroxylase activity expressed through changes in the affinity of the enzyme for the cofactor (25, 80, 104,

177). It had been demonstrated (178) that the activity of tyrosine hydroxylase in vitro could be stimulated by the addition of quinonoid dihydropteridine reductase, the enzyme that reductively regenerates the paraquinonoid (179) but *not* the stable tautomer of the dihydro form of the pterin cofactor (180) and suggested that the activity of this enzyme could be regulating biogenic amine synthesis in brain. More recent studies of the regional and subcellular distribution of this reductase suggest that it is as much as 1000-fold in excess when the stoichiometry of the combined rates of catecholamine and indoleamine biosynthesis are estimated in a region like the striate cortex, and, in addition, its regional distribution appears poorly related to that of the biogenic amine systems in the brain (180–182). However, it should be noted that about 25% of the brain reductase is associated with a synaptosomally enriched fraction (182).

Recent findings have reemphasized the potential regulatory role of reduced pterins. Catecholamine biosynthesis has been stimulated in brain by the intraventricular administration of tetrahydrobiopterin (183, 184) and by its addition to intact preparations of peripheral adrenergic structures (185), cultures of sympathetic neurons (186), and striatal synaptosomal preparations (187). These demonstrations are interesting from a qualitative point of view, but should not be seen as suggesting that the reduced pterins are supplied to the brain from a distant organ such as the liver because little is picked up by the brain after peripheral administration (188), and much is required for an effect to be observed even with intraventricular administration (183). This circumstance is unlike that of conjugated pterins (folates), which appear to have a specific transport system into the brain (189). Calculations have indicated that tetrahydrobiopterin levels as measured in the brain are limiting for the activity of tyrosine and tryptophan hydroxylases (176, 182, 188), and recent studies have demonstrated a coupling of changes in the rate of conversion of tyrosine to dopamine by striate synaptosomes with reduced pterin levels in experiments involving electrolytic and neurotoxic lesions as well as psychoactive drugs (182, 190). Since it appears that the level of quinonoid dihydropteridine reductase is not likely to be the controlling factor, and the peripheral sources can play only a minor role in maintaining brain reduced pterin levels, current attention is focused on the potential for regulation along the biosynthetic pathway from the purine precursor guanosine triphosphate to 7,8-dihydrobiopterin, and its subsequent reduction. There is emerging evidence that the same enzymatic sequence is involved in the brain as has been demonstrated in bacterial and mammalian cell lines (191–193). However, a major area of concern currently involves a missing step that would convert 7,8-dihydrobiopterin to its quinonoid form, making it a suitable substrate for quinonoid dihydropteridine reductase. As the research now stands, it appears that once the quinonoid form spontaneously rearranges, which happens facilely in solution (194) since the nonquinonoid form is thermodynamically favored, or guanosine triphosphate is converted to 7,8-dihydrobiopterin by a two or three enzyme biosynthetic sequence (191–193), there is no established way in brain to get this pterin into the cycle involving its oxidation and regenerative reduction coupled to the activity of the biogenic amine hydroxylases. Liver dihydrofolate reductase is able to convert the nonquinonoid form of 7,8-dihydrobiopterin to the tetrahydro active form, but

with relatively poor yield and a K_m 430 times higher than that for its normal dihydrofolate substrate (195). Dihydrofolate reductase activity has been found in brain (196), although it has also been reported to be missing (197). Its kinetic constants and yield with respect to 7,8-dihydrobiopterin present the same interpretive problems in brain as in liver in light of the wide difference in affinities for the pterin versus the folate substrate (R. Spector, unpublished observations) and the likelihood that the much higher concentration of reduced folates in brain (198) in combination with their higher affinity for the reductase would swamp the enzyme, making it a poor candidate to close the gap between the biosynthesis of 7,8-dihydrobiopterin and its isomerization and reduction to the active tetrahydro form. This step, the elucidation of which remains a research frontier, probably shares with the initial enzyme in the biosynthesis of reduced pterins, guanosine triphosphate cyclohydrolase, the greatest potential for metabolic regulatory influence. Since 7,8-dihydrobiopterin is the only intermediate present in brain to any significant extent, 0.8 nmol/g (188, 192), it is possible that it is bound in a storage form and its isomerization and/or reduction may be rate limiting.

REGULATION BY CHANGES IN ENZYME PROTEIN

Since the first observations that reserpine could increase soluble tyrosine hydroxylase activity under assay conditions of its saturation with respect to tyrosine and pterin cofactor from rabbit and rat brain stem (73, 74), the possibility of the induction of biosynthetic enzymes as an important regulatory mechanism for the rate of biogenic amine synthesis in brain has been a prominent consideration. When amine storage granules are depleted, the brain appears to respond by an increase in biosynthetic enzyme activity. The time courses of the depletion and repletion curves for dopamine and norepinephrine and those for tyrosine hydroxylase and dopamine-β-hydroxylase protein in the locus coeruleus after reserpine administration are mirror images (118). The observation itself has been confirmed with more anatomical specificity: the major change occurs in the noradrenergic nucleus locus coeruleus (199, 200); the increase in enzyme activity is associated with an increase in immunoprecipitable protein of both tyrosine hydroxylase and dopamine-β-hydroxylase (118); a delayed appearance in nerve-ending regions depends upon axonal distance and slow-to-intermediate axoplasmic flow rates (200–202); the effect is absent in the nigrostriatal system (118, 199, 200); and the effect is selective for tyrosine hydroxylase and dopamine-β-hydroxylase, the level of dopa decarboxylase not being altered (118, 203). Desmethylimipramine, a potentiator of noradrenergic transmission, has been demonstrated to have an inverse effect on tyrosine hydroxylase activity in the locus coeruleus and its hippocampal projections (204), as has amphetamine on the nigrostriatal enzyme, where the effect can be blocked by pretreatment with haloperidol (205). Tyrosine hydroxylase activity has been increased in rat brain stem by cold exposure (199, 206); in the locus coeruleus following contralateral lesioning (207); in the locus coeruleus after electrolytic coagulation of the dorsal and median raphe nuclei sources of inhibitory serotonin input (208, 209); in the arcuate nucleus and hypothalamus following immobilization (210–211); in the hypothalamus of sponta-

neously hypertensive rats (212); and in the median eminence after gonadectomy and thyroidectomy (213). Tyrosine hydroxylase activity has been reduced in brain stem by treatment with thyroid hormone (213, 214).

Genetic control of protein complement and the apparent relationship between behavior and the catecholamine products of brain tyrosine hydroxylase have led to studies relating genetic strain and developmental factors to behavior and the enzyme. Five strains of inbred mice manifested genetic variation in adrenal and brain catecholamine biosynthetic enzymes (215) which related to their fighting behavior (216) and, via backcross, suggested control by a single gene locus for tyrosine hydroxylase, dopamine-β-hydroxylase, and phenylethanolamine N-methyltransferase (217). In exploitation of the accessibility of the chick embryo, a critical period for the development of brain tyrosine hydroxylase was elucidated between 10 and 20 days of development, and prenatal administration of reserpine or α-methyl-p-tyrosine produced an elevation of brain tyrosine hydroxylase that persisted for at least 29 days postnatally (218). In another approach, the question was asked whether the brain tyrosine hydroxylase level was the primary genetic manifestation or an adjustment to the activity of another genetically determined protein. A series of studies in rat was begun with the observation that there was an inverse relationship between midbrain and striatal tyrosine hydroxylase activity and spontaneous exploratory behavior previously shown to be dose-related to intraventricular norepinephrine infusion (219, 220). Four of these strains were subsequently found to manifest a direct correlation between this behavior and the responses of a norepinephrine-sensitive adenosine 3',5',-monophosphate (cyclic AMP)-generating system studied in combined midbrain-striatal slices (221), and two of the strains responded to intraventricular infusion of norepinephrine in the direction consistent with the genetic difference being one of "receptor sensitivity," with the inverse relationship between tyrosine hydroxylase activity and behavior as evidence of enzyme adaptation (222).

The issue of the primacy of drug-induced changes in receptor sensitivity versus biosynthetic enzyme activity in neurochemical genetics has also been reflected in adaptive changes to drugs. Tricyclics, as noted (204), reduce tyrosine hydroxylase activity in locus coeruleus and to a lesser degree in substantia nigra, but also reduce the reactivity to norepinephrine of a noradrenergic cyclic AMP-generative system in limbic forebrain (223, 224). Dopa loads and amphetamine reduce behavioral and neurochemical manifestations of receptor sensitivity while appearing to decrease tyrosine hydroxylase (205, 225, 226). A model suggesting simultaneous receptor and enzymatic adaptation to drugs and environmental stimuli has been presented (165, 204, 227). With regard to both receptor sensitivity via response of norepinephrine-sensitive cyclic AMP-generative systems (228) and adrenal catecholamine biosynthetic enzyme activities (216), it appears that the expression rather than the potential range of expression of the genetic determinants of these variables is operative because extreme control values are difficult to move further with experimental manipulations. In addition, since the denominator in these genetic studies is "per milligram of protein," part, and part weight, the possibility that the genetic expression is not enzyme or receptor function per neurone (some measure of DNA would be preferred) but number of cells in a particular neurotransmitter system cannot be

ruled out. A recent study of the inbred mouse strains discussed above (215–217) has revealed that the *number of dopaminergic neurons* in the substantia nigra (A-10) correlates well with the difference in tyrosine hydroxylase activity reported (229).

With the discovery that the glucocorticoids played a prominent role in the regulation of hepatic tryptophan metabolism, it was not surprising that the first agent reported to change tryptophan hydroxylase activity in rat brain was corticosterone (230). Confusion introduced into the literature by that account remains; the enzyme preparation was made in isomolar sucrose, thus leaving the synaptosomes intact and confounding the effects of neuronal membrane uptake of tryptophan to the enzyme's active site (161, 162) with enzyme activity (231). The monitoring of brain serotonin before and after progressive tryptophan loads has indicated that in vivo the enzyme is less than half saturated with respect to substrate (with many mechanisms participating in maintaining the steady-state rate of serotonin synthesis still not elucidated), so brain serotonin concentration can be more than doubled in whole brain and regionally (57, 232) in synaptosomes following drug manipulation of the tryptophan uptake process (233), and in synaptosomes in which the tryptophan uptake process is stimulated by glucocorticoids or progesterone administered before sacrifice (167, 231). In a sequence of studies acutely manipulating brain tryptophan levels with chlorimipramine, neutral amino acids loads, and a one-day tryptophan-deficient diet, using discrete "punch" tissue collection and high pressure liquid chromatographic measurement of product, it was shown that tryptophan hydroxylase activity (V_{max}) moved within 15 to 30 min in a direction opposite to that of brain tryptophan when assayed under conditions of saturation with its cosubstrates (67). These studies suggest that substrate may be controlling enzyme activity through substrate stabilization against proteolysis (if there is a fraction of the tryptophan hydroxylase pool that turns over quickly); allosteric regulation as has been demonstrated for liver phenylalanine hydroxylase by phenylalanine (71); or some other mechanism. Substrate control would certainly explain the quick changes in tryptophan hydroxylase activity in nerve-ending regions induced by drugs that alter tryptophan uptake into synaptosomes (163, 233). When the enzyme was prepared without associated neural membranes, the glucocorticoid influence on tryptophan hydroxylase activity was not confirmed (234), and, as a matter of fact, a thorough exploration of endocrine influences (adrenelectomy, thyroidectomy, castration, and treatment with dexamethasone, testosterone, or thyroxine) has evidenced no changes in tryptophan hydroxylase in the relevant hypothalamic nuclei or in a number of limbic sites or the raphe nuclei (235).

A few hours after the administration of reserpine there is an increase in striatal synaptosomal synthesis of serotonin from tryptophan without an increase in intrasynaptosomal enzyme activity freed by lysis (79), and a few hours later and continuing for three or four days or more there is an elevation in soluble enzyme activity (80, 236). Other manipulations have been reported to alter soluble brain tryptophan hydroxylase activity, including chlorimipramine (67, 85, 232), lysergic acid diethylamide (236), monoamine oxidase inhibitors (164, 236), fenfluramine (237), cocaine (233), lithium (163), the amphetamines (79), tryptophan loads (67, 85), desmethylimipramine (85), circadian rhythms (238), and stressors such as formalin injection and forced immobilization (239). The increased activity of trypto-

phan hydroxylase seen after administration of methiothepin, reputed to be a serotonin receptor blocker, is instructive in its complexity. One group has attributed the effect to increased brain tryptophan content (240), which, as noted, has been considered controlling for enzyme activity (67), and another group, working with less-than-saturated conditions in their assay, has reported a drug-induced increase in enzyme affinity for the tryptophan substrate, although the drug appears to alter a curvilinear control substrate curve manifesting evidence of allosteric regulation by substrate (64, 69). Definitive work in relation to drug and environmental alteration of brain tryptophan hydroxylase activity awaits more nearly complete enzyme purification and antibodies of the sort available for tyrosine hydroxylase because of the cross-reactivity with ample adrenal source for that enzyme. The brain being the only source of tryptophan hydroxylase antigen has afforded less specific and available antibodies for titration of enzyme protein molecules (50).

The weakness inherent in using kinetic parameters to characterize changes in enzyme activity without the methodology to determine whether changes in the number of enzyme molecules have occurred has been exemplified in a recent study demonstrating an increase in tyrosine hydroxylase activity in the locus coeruleus peaking in 24 hr and lasting two weeks after the administration of oxytremorine, physostigmine, pilocarpine, or nicotine which, though characterized by no change in the affinity constants and a change in V_{max}, was nonetheless *not* accompanied by an increase in immunoprecipitable tyrosine hydroxylase protein (241). It has been characteristic of workers in this area to think of increases in brain biogenic amine biosynthetic enzyme activity that were of long latency, long duration, appeared in cell body regions before nerve ending regions, and demonstrated changes in relative velocity without changes in affinity constants, as indicative of induction of new enzyme protein (or a decrease in the rate of the disaggregation and proteolysis of enzyme protein molecules). Now it appears that more definite evidence is required. Even inhibition of enzyme changes by protein synthesis inhibitors such as cycloheximide (236) is not definitive because cycloheximide inhibits the uptake of tryptophan into synaptosomes (S. Knapp and A. J. Mandell, unpublished observations), and it has been seen that rather than changing protein synthesis, substrate can alter the activity state of tryptophan hydroxylase (67). It is of interest that the cholinergic drug-induced prolonged activation of tyrosine hydroxylase in the locus coeruleus does not affect dopamine-β-hydroxylase, which, as we have seen, is also consistent with the genome for tyrosine hydroxylase being uninvolved (216, 217). What is a change in activity state and what is a change in enzyme protein in studies of drug influences on brain tyrosine and tryptophan hydroxylases remain open questions.

Until a transsynaptic sequence is established as a mechanism for producing changes in central nervous system tyrosine hydroxylase and dopamine-β-hydroxylase, we will not know whether the elegant work detailing the events involving cholinergic receptors, a cyclic AMP-mediated dissociation of a regulatory subunit of protein kinase with its resultant activation, translocation to the nucleus, and the resulting nuclear mechanisms leading to new protein synthesis of catecholamine biosynthetic enzymes in peripheral adrenergic tissue is relevant to the brain (242-246). A similar limitation exists for generalizing to the brain the work demonstrating

the regulation of dopamine-β-hydroxylase by transsynaptic induction of synthesis and hormonal regulation of steady state levels by regulation of enzyme degradation (247). Recent work using developmental phase (248, 249), tissue damage (250), nerve growth factor (251), and other messages involving the establishment and maintenance of a normal morphological relationship between adrenergic neurons and their end organs (252, 253) suggests the possibility that the germinal finding—the induction of tyrosine hydroxylase in brain by the administration of reserpine (73, 74)—did not result from the transsynaptic influence of an increase in acetylcholine resulting from a drug-induced increase in choline acetyltransferase (254, 255), which is high in concentration in the locus coeruleus (256), but may have followed upon the destruction of vesicular integrity by reserpine, signaling through retrograde axonal transport (257) events relevant to the triggering of the protein-synthesizing processes of neuronal repair. The discrimination between enzyme activation and inhibition versus enzyme synthesis and degradation in response to transsynaptic functional rather than growth and tissue-reparative processes (258, 259) is of major theoretical importance to an understanding of the regulation of biogenic amine synthesis in brain and awaits further technological advance.

SOME NEUROPHYSIOLOGICAL ASPECTS OF THE REGULATION OF TYROSINE AND TRYPTOPHAN HYDROXYLASES

Before leaving the subject of the regulation of tyrosine and tryptophan hydroxylases, an increasingly important area should be mentioned, here briefly due to limitations of space: the changes in amount and/or activity of the tyrosine and tryptophan hydroxylases consequent to neurophysiological events, i.e. regulatory influences expressed from outside the neuron, from other neurons, or by the neuron upon itself. Studies of the functional organization of neural systems using dependent variables reflective of changes in the biosynthetic machinery rather than electrical activity are a currently burgeoning research area revealing an au courant fusion of neurophysiology and neurochemistry. The relationship between electrical discharge rates of nerve cells and their biosynthetic activity, however, looms extraordinarily complex. To trace a relatively simple example: whereas central nervous system modulation of adrenal tyrosine hydroxylase through cerebral catecholaminergic and serotonergic systems has been demonstrated (260–263), and studies have indicated a pathway from the preganglionic cholinergic message to the induction of tyrosine hydroxylase (242–246), it has also been shown that depolarization by high concentrations of potassium or veratridine did *not* initiate induction in sympathetic ganglia in culture and that the selective blockage of sodium channels and therefore the action potentials by tetrodotoxin did not prevent the initiation of induction of tyrosine hydroxylase by the nicotinic receptor agonist carbamylcholine (264). This is in the face of reports of an increase in tyrosine hydroxylase activity in peripheral adrenergic structures after electrical stimulation of the preganglionic nerve (265). A more complex example of the problems facing those integrating neurophysiological and biosynthetic events is the interesting group of studies signaled by the report of the

triggering of an allosteric activation of striatal tyrosine hydroxylase by a dopamine antagonist, haloperidol (91, 92), and the elucidation of a nigrostriatal dopamine circuit regulating dopamine biosynthesis (perhaps through the activity state of tyrosine hydroxylase) involving multiple neurotransmitters (dopamine, acetylcholine, GABA, and substance P) as well as postsynaptic receptors and autoreceptors, but without as yet established relationships to electrical events (93, 266–272). Activation of brain noradrenergic tyrosine and tryptophan hydroxylases by electrical stimulation has been reported (273–275), suggesting the possibility of a relationship between artificially induced changes in firing rate and adaptive changes in the mechanisms of biosynthesis (274, 275). Even in simple systems such approaches have proved to be difficult. The germinal series of studies relating electrical stimulation to biosynthetic mechanisms using peripheral adrenergic preparation began with the observation that there was an electrical stimulation-linked increase in norepinephrine synthesis which manifested a critical relationship to frequency, "off" time, and now the theorizing invokes not only "product-feedback" inhibition but changes in the activity state of tyrosine hydroxylase, the role of ions, and that of nucleotides as mechanistic explanations; so, even this relatively simple system continues to present myriad unresolved questions (84).

An explanation of the relationship between the spontaneous firing rate of central biogenic amine neurons and their biosynthetic state has been another approach to this problem. One group studying the spontaneous discharge rate of cells of the mesostriatal (276) serotonergic system and regional serotonin turnover has reported that chlorimipramine, an inhibitor of serotonin uptake into neurons, lysergic acid diethylamide, and tryptophan loads all reduce the firing rate of dorsal raphe neurons, but with varying influences on serotonin turnover, suggesting a state in which the cell senses its own amount of biosynthetic activity (277, 278). However, these studies again underline the difficulty in relating electrophysiological events to enzyme regulation. Whereas all three treatments reduced dorsal raphe cell firing rate, chlorimipramine was associated with an increase, tryptophan loads with a decrease, and lysergic acid diethylamide with no change in the activity of tryptophan hydroxylase (85). It may be that messages to neurons changing biosynthetic mechanisms such as enzyme amount and/or activity are related in a different way from those reflected in action potentials. Another conceptual framework may come from a consideration of the time after the perturbation that the biosynthetic events occur. The release of stored transmitter and kinetic alteration would be the fastest, changes in enzyme conformation next, and a change in the amount of enzyme protein still later. Each could be influenced by a different set of signals.

Another area relating neurophysiological organization to the regulation of biosynthesis, one receiving much attention currently, involves the relative influences of hetero- and auto-receptor function. It may be that many results of previous turnover studies conducted in the intact brain which were explained in terms of product-feedback inhibition controlling enzymatic activity were in fact secondary to a receptor-mediated effect of the biogenic amine on its cell of origin (64, 269, 270, 279). The experimental model with which this possibility has been most thoroughly explored in the central nervous system has involved the administration of an aromatic amino decarboxylase inhibitor followed by axotomy and determination of the

levels of hydroxylated tyrosine and tryptophan over time—they appear to accumulate in a linear manner for 30 min or longer (280, 281). Deprived of neural feedback input from other cells by a transverse cerebral hemisection, apomorphine-haloperidol agonist-antagonist interactional effects on the rate of tyrosine hydroxylation in vivo were maintained (270, 281; A. Carlsson, personal communication). The best cases for autoreceptors regulating the activity of an aromatic amino acid hydroxylase appear to exist in the nigrostriatal dopaminergic system and, to a lesser extent, the limbic forebrain dopaminergic system (269, 270, 281–283). It has been shown that indoleamine agonists can slow down the synthesis of 5-hydroxyindoles (284–286), and "self" regulation of neurophysiological parameters by serotonin cell bodies via an indoleamine message has been rather convincingly demonstrated (136) and may be mediated by axon collaterals (287).

Although it is too early in this exciting new field to generalize, it appears now that biogenic amine synthesis may be modulated by receptor-mediated events of both a hetero- and autosynaptic nature. A recent exemplifying study, using a wide range of apomorphine doses, demonstrated an autoreceptor-mediated alteration in hydroxylation at very low levels of the drug and a heteroreceptor effect at larger doses (270). The dynamics of change in "sensitivity" of receptors involving both alterations in number of binding sites (288–290) and affinities for the ligand manifesting allosteric characteristics (291–294) are reminiscent of the two major groups of regulatory properties of the biosynthetic enzymes themselves. When taken in combination with the literature portraying receptor-mediated influences on biosynthesis, they add another redundant layer of control for brain tyrosine and tryptophan hydroxylases.

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