the resting neuron part of the enzyme occurs in an inactive state, perhaps bound to the synaptic vesicles or cell membrane; just as cell membrane depolarization will induce release of transmitter from its storage sites, it may cause release also of the tryptophan hydroxylase, but in this case the release will occur into the cytoplasm rather than into the synaptic cleft. A mechanism of this type might, of course, involve a cofactor rather than the enzyme itself.

Another conclusion to be drawn from the present findings is that tryptophan hydroxylation appears to proceed at a fairly high rate even in the absence of an impulse flow. This phenomenon was even more striking in another experimental series involving the ascendant cerebral 5-hydroxytryptamine-carrying fibre system. In fact, axotomy, caused by a transverse cerebral hemisection, induced no detectable decrease in the 5-hydroxytryptophan accumulation on the lesioned side after decarboxylase inhibition, even though some indirect evidence suggested a slight decrease in turnover rate (Bédard *et al.*, 1972). The reason for the different outcome of the spinal and cerebral studies has not yet been elucidated. In any event, both studies emphasize the high rate of tryptophan hydroxylation occurring in the resting 5-hydroxytryptamine neuron. Since very little 5-hydroxytryptamine appears to be released from resting neurons, this would indicate the occurrence of a considerable 5-hydroxytryptamine turnover intraneuronally under these conditions. This invites the speculation that an important control mechanism depends on the degree of saturation of the intraneuronal storage sites. In the resting neuron a high saturation is reached, leading to increased intraneuronal deamination of newly synthesized 5-hydroxytryptamine. When 5-hydroxytryptamine is released by the nerve impulses, the extent of saturation of the storage sites is decreased, leading to increased uptake and diminished intraneuronal deamination.

We have also investigated the possible existence of a feedback regulation of the tryptophan hydroxylase. It was found in mice that treatment with a monoamine oxidase inhibitor (nialamide or pargyline) caused a decrease in the 5-hydroxytryptophan accumulation, induced by a decarboxylase inhibitor. The decrease was not observed when the inhibitors of the two enzymes were given simultaneously but only when the monoamine oxidase inhibitor was given beforehand, leading to an increased 5-hydroxytryptamine concentration. Also, the two hallucinogenic agents LSD-25 and dimethyltryptamine caused a decrease in the 5-hydroxytryptophan accumulation. These agents are believed to activate postsynaptic 5-hydroxytryptamine receptors. The data thus support the existence of a feedback mechanism operating via changes in 5-hydroxytryptamine concentrations and/or in the activity of postsynaptic 5-hydroxy-

tryptamine receptors. Changes in the rate of nerve-impulse flow may be involved in this case, too.

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Metabolic Regulation in the Release and Action of Excitatory and Inhibitory Amino Acids

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A compelling body of evidence supports the postulated role of γ -aminobutyrate as the inhibitory transmitter released at synapses in many different neuronal pathways in the mammalian brain and spinal cord; glycine probably exercises a similar function in the spinal cord (Curtis & Johnson, 1970; Curtis, 1971). Extracellular glutamate and aspartate depolarize central neurons, and numerous electrical and pharmacological comparisons have failed to distinguish between the mechanisms underlying these actions and those whereby unidentified natural excitatory transmitters mediate excitatory postsynaptic potentials (Curtis & Johnston, 1970; Curtis *et al.*, 1972). Concepts of metabolic regulation embracing a transmitter or otherwise 'modulatory' function of these four amino acids must take into account the metabolic correlates of their release, action and uptake.

Little is known of the factors controlling the release of synaptically active amino acids, but these would probably include the movements of ions associated with impulses arriving at axon terminals. Electrical stimulation or a high concentration of K^+ in the medium causes the release of both excitatory and inhibitory amino acids *in vitro* (Machiyama *et al.*, 1970; Hopkin & Neal, 1971; Arnfred & Hertz, 1971; de Belleruche & Bradford, 1972). Factors influencing glucose transport and oxidation, CO_2 fixation, the activity of amino acid-synthesizing enzymes and reuptake of the released transmitters and/or their metabolites must all be considered in relation to the regulation of transmitter synthesis. The claim by Kerpel-Fronius & Hajos (1971) that mitochondria in axon terminals are unable to oxidize α -oxoglutarate and succinate and the proposal that this apparent block in the tricarboxylic acid cycle is related to the synthesis and release of transmitter amino acids are pertinent to these considerations.

The action of excitatory amino acids *in vivo* is associated with an increase in the incorporation of ^{14}C from $[U-^{14}C]$ glucose into amino acids related to the tricarboxylic acid cycle (Watkins, 1971a). The

labelling of endogenous amino acids from radioactive acetate, pyruvate and fructose (Watkins, 1971b, and unpublished work) is decreased in each case by the excitatory amino acids. These results suggest a specific increase in the uptake and oxidation of glucose mediated by the extracellular presence of the amino acid excitants. *In vitro*, both natural and synthetic excitant amino acids cause marked changes in cationic fluxes (Harvey & McIlwain, 1968; Ramsey & McIlwain, 1970) and in the intracellular concentrations of glycolytic intermediates (Takagaki *et al.*, 1959) and phosphorus-containing substances related to energy metabolism (Bradford & McIlwain, 1966). It is possible that the increased glucose uptake mediated by the excitatory amino acids may be directly linked to the influx of Na^+ into neurons, i.e. that the amino acids activate a coupled influx of Na^+ and glucose. Such co-transport of Na^+ and glucose has been observed in other tissues (Schultz & Curran, 1970). Alternatively, the stimulated glucose uptake may be part of a sequence of co-ordinated changes that are secondary to the increases in ionic fluxes initiated by excitant amino acids. The changes in the concentrations of tissue phosphates are probably a consequence of activation of membrane adenosine triphosphatase and can be expected to be involved in the subsequent control of glycolysis and oxidative reactions. The concentrations of reduced and oxidized nicotinamide nucleotides are undoubtedly of fundamental importance in such control. The availability of keto acids for participation in tricarboxylic acid-cycle mechanisms is probably regulated mainly by transaminase activity.

Inhibitory amino acids cause a decrease in the incorporation of radioactivity from $[\text{U-}^{14}\text{C}]$ glucose into brain amino acids, the pattern of labelling being indicative of diminished glucose oxidation and similar to that produced by barbiturate anaesthesia (Watkins, 1971a, and unpublished work). The amino acid depressants have been less well studied than the excitants in systems *in vitro*, and such investigations have led to confusing results (Baxter, 1970), perhaps because of the doubtful electrical integrity of most slice preparations. The metabolic effects *in vivo*, like those of barbiturates, can be attributed to the general damping down of electrical activity, thus diminishing ionic fluxes induced by action potentials and by the release of excitatory transmitters. However, a direct effect of the inhibitory amino acids (and of other neuronal depressants) on glucose transport cannot be ruled out, and, indeed, such a hypothesis has attractive features.

Uptake of L-glutamate, L-aspartate and γ -aminobutyrate from extracellular fluid is associated with glutamine synthesis (Beri & Clarke, 1969). It would appear that the metabolic compartments into which the amino acids are taken up do not contain as large a proportion of the total tissue glutamate (or aspart-

ate) as of the total tissue glutamine. This glutamine-enriched compartment does not seem to be localized within synaptic endings, since isolated synaptosomes do not contain high glutamine synthetase activity (Salganicoff & De Robertis, 1965). Moreover, γ -aminobutyrate formation in this compartment is relatively low (Beri & Clarke, 1969). A paradoxical situation arises from the findings that all the putative amino acid transmitters are taken up into nerve endings by high-affinity transport systems (Weinstein *et al.*, 1964; Iversen & Neal, 1968; Bloom & Iversen, 1970; Logan & Snyder, 1971). However, more than one uptake system has been recognized (Logan & Snyder, 1971), and the resolution of this paradox may be that the lower-affinity systems are associated with glial cells and postsynaptic neuronal somata and dendritic elements wherein the metabolic systems related to glutamine synthesis may be mainly localized. In addition to glutamine synthetase, aspartate aminotransferase, γ -aminobutyrate aminotransferase and glutamate dehydrogenase probably all participate prominently in the post-uptake metabolic processes. The glutamine formed at these sites may function as a precursor for resynthesis of amino acid transmitters after appropriate transport (Balázs, 1971; Van den Berg, 1972).

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The Storage and Release of Acetylcholine

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Preparations of detached presynaptic nerve terminals (synaptosomes) and synaptic vesicles from mammalian brain (Whittaker, 1965, 1969) have proved useful in unravelling the organization of the synapse, identifying the subcellular sites of transmitter synthesis and storage and characterizing the carrier-mediated uptake systems for transmitters and their precursors. For acetylcholine it has been shown that the transmitter is synthesized in the cytoplasm (Fonnum, 1967) from choline taken up by a carrier-mediated transport system in the terminal plasma membrane (Marchbanks, 1968b; Diamond & Kennedy, 1969) and is concentrated and stored in synaptic vesicles. The concentration of acetylcholine in the vesicles is estimated to be at least 200 mM (Whittaker & Sheridan, 1965) and in the cytoplasm to be no more than 3 mM and probably lower (0.4 mM; Marchbanks, 1968a). At these concentrations acetylcholine exerts negligible inhibitory effect on choline acetyltransferase, the enzyme synthesizing it (Glover & Potter, 1971; Morris *et al.*, 1971), and the regulation of acetylcholine synthesis is unlikely to be brought about, under physiological conditions, by acetylcholine inhibition of the synthetic pathway.

One of the limitations of work with synaptosomes and synaptic vesicles isolated from mammalian brain is that the preparations are derived from a mixed population of cholinergic and non-cholinergic nerve terminals in which the latter considerably outnumber the former and cannot be effectively separated from them (Whittaker, 1969). We have therefore been investigating tissues richer in cholinergic terminals than mammalian brain. Two tissues have proved particularly useful: the electric organs of elasmobranch fish belonging to the family Torpedinidae and the head ganglion of the squid (*Loligo pealii* and *Loligo forbesi*).

The electric organ of Torpedinidae has a purely cholinergic innervation, with acetylcholine concentrations of 200 nmol/g or more, i.e. over 15 times that of mammalian cerebral cortex. The tissue contains much collagen and is thus difficult to homogenize in the usual way; however, when crushed to a coarse powder after freezing in Freon 12, the portion of the

presynaptic terminal plasma membrane away from the synaptic cleft is stripped off, leaving an exposed layer of frozen terminal cytoplasm containing intact vesicles attached to morphologically well-preserved fragments of electroplaque cells (D. Soifer & V. P. Whittaker, unpublished work). The frozen cytoplasm and synaptic vesicles can then be extracted into a suitable extraction medium iso-osmotic with elasmobranch plasma (e.g. 0.2 M sucrose–0.3 M NaCl) with relatively little contamination from larger membrane fragments and separated by means of density-gradient centrifuging in a zonal rotor (Essman & Whittaker, 1970; V. P. Whittaker, W. B. Essman & G. H. C. Dowe, unpublished work). In this way milligram quantities of almost pure cholinergic synaptic vesicles may be obtained in a single experiment. Such vesicles contain up to 1300 nmol of acetylcholine/mg of protein and are recovered as a sharp band of density equivalent to that of 0.38 M sucrose–0.21 M NaCl.

Investigations, not yet complete, indicate (M. J. Dowdall, R. M. Facino & V. P. Whittaker, unpublished work) that the vesicles contain not more than four main protein components, three in the membrane and the fourth, accounting for over 50% of the total protein, in the core. After dialysis and freeze-drying, the core protein (vesiculin) is recovered as a protein of molecular weight approx. 10000 rich in acidic and hydroxy amino acids and bound to five or six nucleotide residues (Whittaker, 1971; Whittaker *et al.*, 1971). Freshly prepared vesicles contain considerable amounts of ATP and its breakdown product AMP (M. J. Dowdall, A. F. Boyne & V. P. Whittaker, unpublished work); the molar ratio of acetylcholine to ATP varies in different experiments and in different regions of the vesicle band from about 4:1 to 80:1. It is therefore probable that in the native vesicle the nucleotide bound to vesiculin is largely ATP. Vesiculin, by virtue of its content of nucleotide and acidic amino acid residues, must be strongly negatively charged, and calculations show (a) that the number of negative charges in the vesiculin–nucleotide complex is sufficient to neutralize the positive charges of the amount of acetylcholine cation (up to 24 nmol/mol of vesiculin) associated with it in the vesicle, and (b) that the vesiculin–ATP–acetylcholine complex could largely fill the vesicle core. Vesiculin is thus believed to serve as a non-diffusible polyanion whose presence in the vesicle core ensures the maintenance of the high local concentration of acetylcholine. Spontaneous or enzyme-catalysed hydrolysis of bound ATP would permit loss of vesicular acetylcholine by providing a readily diffusible small anion, i.e. P_i . This and other possible release mechanisms are being examined. The chromogranin–ATP–adrenaline complex in the chromaffin granule (for a review see Winkler, 1971) and the protein–heparin–histamine complex of