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OXIDATIVE STRESS: FREE RADICAL PRODUCTION IN NEURAL DEGENERATION

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Abstract—It is not yet established whether oxidative stress is a major cause of cell death or simply a consequence of an unknown pathogenetic factor. Concerning chronic diseases, as Parkinson's and Alzheimer's disease are assumed to be, it is possible that a gradual impairment of cellular defense mechanisms leads to cell damage because of toxic substances being increasingly formed during normal cellular metabolism. This point of view brings into consideration the possibility that, besides exogenous factors, the pathogenetic process of neurodegeneration is triggered by endogenous mechanisms, either by an endogenous toxin or by inherited metabolic disorders, which become progressively more evident with aging. In the following review, we focus on the oxidative stress theory of neurodegeneration, on excitotoxin-induced cell damage and on impairment of mitochondrial function as three major noxae being the most likely causes of cell death either independently or in connection with each other. First, having discussed clinical, pathophysiological, pathological and biochemical features of movement and cognitive disorders, we discuss the common features of these biochemical theories of neurodegeneration separately. Second, we attempt to evaluate possible biochemical links between them and third, we discuss experimental findings that confirm or rule out the involvement of any of these theories in neurodegeneration. Finally, we report some therapeutic strategies evolved from each of these theories.

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Abbreviations—ACh, acetylcholine; AD, Alzheimer's disease; Al, aluminum; ALC, acetyl-L-carnitine; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propanoic acid; APP, amyloid precursor protein, precursor A4 protein; BDNF, brain-derived neurotrophic factor; Ca^{2+}, calcium ion; $[Ca^{2+}]_i$, intracellular calcium ion concentration; CAT, catalase; ChAT, choline acetyl transferase; DA, dopamine-3,4-dihydroxyphenylethylamine; DNA, deoxyribonucleic acid; EAA, excitatory amino acids; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GABA, γ-amino-butyric acid; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; GSSG-Rd, glutathione disulfide reductase; $HO_2^{\cdot}$, hydrogen dioxide; H_2O_2, hydrogen peroxide; HD, Huntington's disease; IP_3, 1,4,5-inositol triphosphate; LAMMA, laser microprobe mass analysis; LB, Lewy bodies; LC, locus coeruleus; LOOH, lipid hydroperoxides; L-DOPA, L-3,4-dihydroxyphenylalanine; LPO, lipid peroxidation; MAO, monoamine oxidase; MAP, microtubule-associated protein; MBC^+, *N*-methyl-β-carbolinium ion; MDA, malonaldehyde, malondialdehyde; MIQ^+, *N*-methyl-isoquinolinium ion; $MPDP^+$, *N*-methyl-4-phenyl-dihydropyridinium ion; MPP^+, *N*-methyl-4-phenylpyridinium ion; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSA, multisystem atrophy; mtDNA, mitochondrial deoxyribonucleic acid; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NBM, nucleus basalis of Meynert; NFT, neurofibrillary tangles; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; PD, Parkinson's disease; PHF, paired helical filaments; O_2, groundstate triplet dioxygen (if not otherwise specified); $(O_2)^{\cdot-}$, superoxide, dioxide (−1); $(OH)^{\cdot}$, hydroxyl radical; 6-OHDA, 6-hydroxydopamine; Q, coenzyme Q; ROS, reactive oxygen species; SN, substantia nigra; SNC, substantia nigra zona compacta; SNR, substantia nigra zona reticulata; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TH, tyrosine hydroxylase; THBC, 1,2,3,4-tetrahydro-β-carboline; THIQ, 1,2,3,4-tetrahydroisoquinoline; $(TO)^{\cdot}$, α-tocopheroxyl radical; TOH, α-tocopherol.

CONTENTS

1. DEFINITION OF NEURODEGENERATION

The normal functioning of the CNS presupposes a well-balanced interaction between different biochemically and structurally linked neuronal systems. When one member of a neuronal circuit is altered in its structural or biochemical entity, an imbalance in the functional system results and a compensatory mechanism must be activated in order to maintain physiological equilibrium.

Neurodegenerative processes can involve diverse areas of the CNS. The initial etiology of such disturbances is mostly unknown. Nevertheless, we can distinguish between an idiopathic and a symptomatic form of neurodegenerative disease. A close examination of the latter enables one to make valuable conclusions about the nature of the former. Thus, we can amplify our knowledge by examining function, structure and biochemistry of the CNS in symptomatic cases and by looking for comparable alterations in idiopathic forms.

Another way is to mimic the neurodegenerative diseases by means of animal models. Acute symptomatic neurodegeneration can develop during a very short period, a fact from which we deduce the delaying effect of compensatory and regenerative mechanism in slowly developing chronic neurodegeneration. Neurodegeneration appears clinically as a breakdown of functionally connected neuronal circuits, with corresponding alterations in the neurotransmitter system and morphological organization of the affected cell system. In order to understand the pathophysiological consequences of neurodegeneration, it is necessary to know the function of neuronal loops in their normal state (Fig. 1).

Parkinson's disease (PD), Alzheimer's disease (AD), multi-infarct dementia and motoneuron disease (e.g. amyotrophic lateral sclerosis, ALS) represent typical neurodegenerative diseases for which no known etiology has been put forward. Parkinson's disease is characterized by reduced size and velocity of movements. In AD, cognitive impairment is the cardinal clinical symptom. In

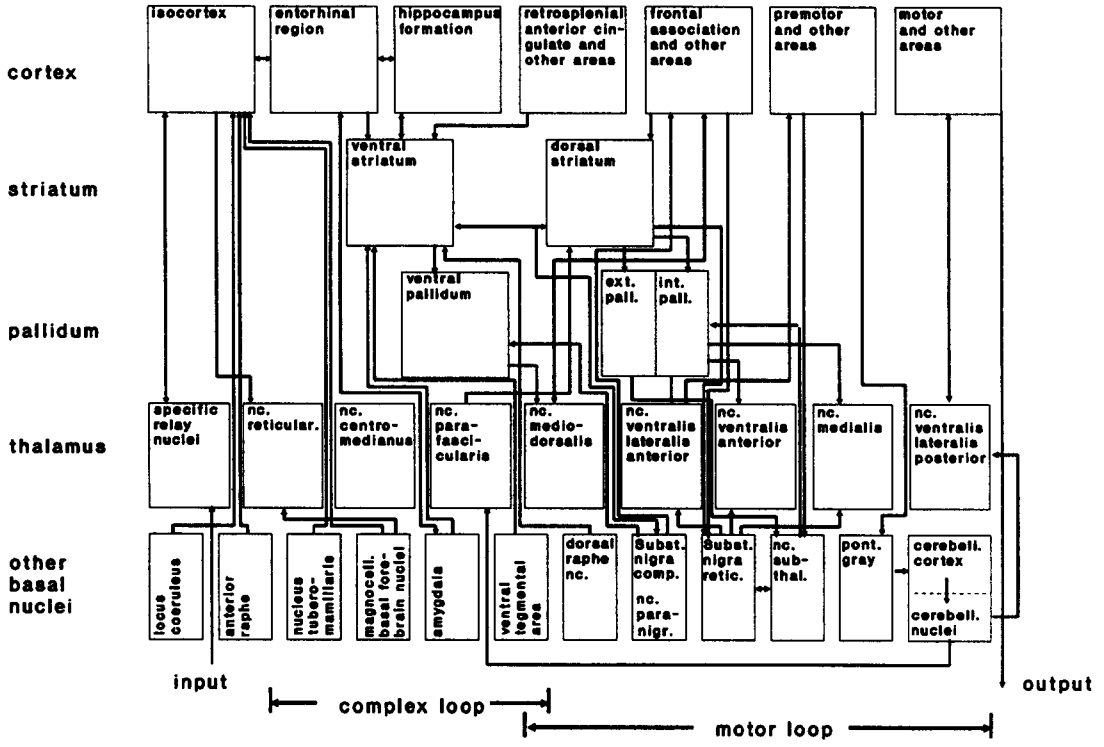

FIG. 1. Integrative aspects of cognitive and motor loops, Disturbances in cognitive loops predominate in AD, and motor loops are predominantly affected in PD. Abbreviations: nc., nucleus; ext. pall., external pallidum; int. pall., internal pallidum; nc. paranigr., nucleus paranigralis; nc. subthal., nucleus subthalamicus; pont. gray, pontine gray. Modified from Alexander *et al.* (1986), Gerlach *et al.* (1991b), Nieuwenhuys *et al.* (1991) and Braak and Braak (1993).

motoneuron disease, a degeneration of the central pyramidal, the peripheral motor system or both is the reason for the clinical picture. A significant overlap exists between these three disorders.

2. CHARACTERIZATION OF NEURODEGENERATIVE DISORDERS

2.1. Movement Disorders

2.1.1. *Physiology of the Motor System*

Several events must occur when a motor action is performed. The acting subject develops the intention to reach a goal by motor action. A motor plan is made. The orientation of the body is adapted to the spatial position of the goal and finally, the motor system must coordinate the activities of the different descending pathways. Before efferent motor actions can be started, sensoric afferent information about body and goal position in space must be established so that the motor system is able to estimate the consequences of actions.

These afferent messages and efferent commands are linked to a functional neuronal network by the following neocortical areas: the motor cortex, the premotor cortex of the frontal lobe, the supplementary motor area and the posterior parietal cortex. The motor cortex covers the precentral gyrus and is organized somatotopically. The premotor cortex, located on the lateral surface of the hemisphere, plays a specific role in the preparation of responsive motor reactions and controls motor responses to tactile stimuli (Fig. 1).

The supplementary motor area plays an indirect preparatory role in movement generation. Both prefrontal cortex and supplementary motor areas receive inputs from posterior parietal areas, but differ in their subcortical projections. The prefrontal cortex receives afferents from the cerebellum, the supplementary motor area from thalamic nuclei.

The posterior parietal cortex processes sensory stimuli before initiating a purposeful movement and projects this information to the premotor area and supplementary motor area. The premotor and supplementary areas represent the highest levels in the hierarchy of motor systems because of their integrating function in programming movements. These areas project to the motor cortex, from where the descending upper motoneurons project to the brain stem nuclei via the corticobulbar system and to the spinal cord through the corticospinal system. In the brain stem, the corticobulbar neurons form synaptic contacts with cranial nerve cells. Besides this, the brain stem is an integrative organ for descending motor commands from higher levels and ascending sensory information from proprioceptive systems and vestibular nuclei. Thus, it provides the postural adjustment of the body. In the spinal cord, the tractus corticospinalis ends on α-motoneurons directly by synaptic contact or indirectly through interneurons that enhance or inhibit the stimulation of motoneurons (Ghez, 1991).

2.1.2. *Clinical Manifestation of Parkinson's Disease*

Idiopathic PD is a movement disorder whose symptomatology is defined by three cardinal symptoms: tremor at rest, rigidity and akinesia (Fahn, 1989).

Since the first description of the syndrome by James Parkinson in 1817, knowledge about this disease has progressed in several directions, but an exact insight into its etiological mechanism has not yet been possible. The mean age of onset of PD is about 60 years. In the age group over 60, the incidence is increased, but an early onset form also exists. The clinical manifestation of PD first appears in most cases as a unilateral subjective feeling of stiffness and clumsiness or of internal tremulousness, later signs of which are the classical symptoms of tremor, rigor and akinesia.

In most cases, clinical diagnosis of PD is correct. Nevertheless, misdiagnoses do occur, especially if additional clinical signs [e.g. gaze paresis and pyramidal signs, which characterize multisystem atrophy (MSA)] are overlooked (Fahn, 1989; Hughles *et al.*, 1992).

From the different expression of the symptoms, the subdivision of the disease into three subtypes can be made. In the first, one can see an equal appearance of rigor, tremor and akinesia. Therefore, it is called equivalence type. In the second form, the clinical picture is dominated by rigor and

akinesia. This is the rigor/akinesia type. In the tremor-dominance type, the tremor at rest is the predominant clinical symptom.

This subdivision can give us important indications about the prognosis of PD. Firstly, there are indications that, in the tremor-dominant form, there is a slower progress of disease (benign form), whereas a positive correlation between rigidity and malign progress is observed. Secondly, a connection between rigidity/akinesia and parkinsonian bradyphrenia and dementia has been described. The risk of developing a cognitive impairment is much lower in the tremor form. A positive correlation between motor symptoms and intellectual impairment in PD patients leads to the conclusion that damage in the same structures, substantia nigra zona compacta (SNC), may be responsible for both parkinsonian symptoms and the associated dementia (Mortimer *et al.*, 1982). This assumption is supported neuropathologically by semiquantitative estimation of neuronal loss in locus coeruleus (LC) and substantia nigra (SN) (Fig. 1) and cognitive impairment (Gaspar and Gray, 1984). Nevertheless, the course of the disease is a progressive one. The beneficial effect of L-3,4-dihydroxyphenylalanine (L-DOPA) therapy has increased patients' life expectancy to a significant degree. The advanced stage of the disease is dominated by the complications of L-DOPA therapy and lack of L-DOPA responsiveness. Indeed, little can be said about the natural history of the disease.

2.1.3. *Pathophysiology of Parkinson's Disease*

Lesions in different anatomical sites of the structures that make up the motor loop can be used to create models of movement disorders (Fig. 1). When the subthalamic nucleus is lesioned, a disruption of the indirect pathway results: the decline of glutamatergic stimulation of internal globus pallidus/substantia nigra zona reticulata (SNR) neurons via subthalamico-pallidonigral pathways reveals diminished inhibition of the excitatory thalamocortical pathways. The clinical consequence of this lesion is a proximal ballistic movement of the limbs.

Degeneration of striatal neurons, as seen in Huntington's disease (HD), increases the inhibitory activity of the indirect pathway and causes an increase in activity of the direct pathway. This leads to an enhanced activation of the thalamocortical input, evidenced by clinical correlates distal to irregular choreiformic movement of limbs.

In hypokinetic disorders, the discharge of efferent striatal neurons is diminished in the direct and increased in the indirect way, so that an inhibition of thalamocortical connections originates from a dual alteration (Alexander and Crutcher, 1990). The purpose of internal globus pallidus/SNR, as part of the motor loop, is to make a copy of the corticospinal instruction, sending this copy back to the cortex via thalamocortical connections (Fig. 1). This copying system is markedly important in the automatic execution of learned motor plans. A motor plan consists of a consecutive series of single motor programs that are integrated by neuronal interaction between sensoric perception and motoric action.

Whereas single programs are unimpaired in PD, motor planning, an action performed by the basal ganglia, is disrupted. Although the patient is able to perform several single motor acts slowly, he cannot perform the same actions successively as part of a smoothly executed motor plan. In single motor actions, we distinguish fast ballistic and slow movements. The ballistic movements depend mostly on an intact fast interaction of perceptive judgement and motor command. This kind of movement cannot be performed by parkinsonian patients because of the lack of sensorimotor interaction as irreplaceable presupposition in motor planning. Slowly performed movements in PD indicate that motor programming and sensory perception, *per se*, are still intact (Marsden, 1982).

2.1.4. *The Pathology of Parkinson's Disease*

The morphologic changes in PD are characterized by neuronal cell death in the pigmented cell nuclei of the brain stem, which frequently appear symmetrically (Jellinger, 1986). One can also find changes in the nucleus dorsalis nervi vagi and nucleus basalis of Meynert (NBM). The most important degenerative process is in the SNC. These neurons project to the striatum, which plays a role in programming of motor actions (Marsden, 1982). As an important functional member of

the motor loop (Riederer *et al.*, 1989b), the dopaminergic nigrostriatal connections are primarily involved in the frictionless performance of planned movements, and their disruption results in a heavy imbalance of the motor system. The basis of the neurodegeneration in the SNC is an active pathological process (McGeer *et al.*, 1988). There is a natural age-related cell loss in the SNC (Fearnley and Lees, 1991). Comparing the number of pigmented cells in young (20 years) and aged (90 years) non-parkinsonian subjects, a decline of 47% can be observed. This corresponds to a cell loss of 5% per decade. In parkinsonian patients there is an age-dependent correlation between duration of disease and decline of cell number. The cell loss occurs in an exponential manner, being 10 times greater in the first decade (45%) than in age-matched control persons. Whereas the first clinical symptoms appear at a time when dopamine (DA) content is reduced by 80%, at the same time point, the cell number in the SNC is reduced by only 50%. This indicates a partial biochemical dysfunction of the remaining neurons.

Comparing pathologically two different subtypes of PD, the rigor/akinesia-dominance type and the tremor-dominance type, more pronounced neuronal cell loss in the medial and lateral part of the SNC, more severe gliosis, dystrophic axons and nigral extraneuronal melanin deposits have been found in the rigor/akinesia-dominance type (Paulus and Jellinger, 1991).

The loss of neurons in 80–100% of PD is associated with morphological changes of cytoplasmic structure: these consist of the appearance of Lewy bodies (LB) (Jellinger, 1986; Forno, 1986), hyalinic cytoplasmic inclusion bodies in several brain regions, such as SNC, LC, NBM, spinal cord, sympathetic ganglia and cerebral cortex (Jellinger, 1986, 1989). The ultrastructure of LB is described as follows: inclusion bodies with an electron-dense, amorphous or circulate dense core from which filaments radiate to the neuromelanin particles. LB can appear singly or multiply in the pigmented cell cytoplasm (Forno, 1986). LB are not pathognomonic for PD.

2.1.5. *Biochemical Alterations in Parkinson's Disease*

Biochemical changes in PD are strongly correlated with the morphological degeneration of neuronal systems. The pathomorphological hallmark of this disease is the decline of dopaminergic nigrostriatal neurons, which histologically appears as loss of melanin-containing neurons in the SNC. Our knowledge of the physiological function of these neurons in the motor loop explains the clinical signs of reduced size and velocity of volitional movements. Indeed, the chemoarchitectural profile of the motor loop would enable several diverse neurochemical alterations to provoke a picture of hypokinesia (Gerlach *et al.*, 1991a; Braak and Braak, 1993):

(1) reduction of dopaminergic nigrostriatal transmission enhances the excitotoxic effect of the corticostriatal afferents;
(2) a shift in glutamatergic activity can explain the phenomenon of hypokinesia by inhibiting the direct pathway or exciting the indirect pathway via increased excitatory action of the subthalamic nucleus; and
(3) an increased action of striatal cholinergic interneurons could also result in an imbalance of motor regulation and, therefore, in parkinson-like hypokinesia.

The most prominent biochemical finding in PD is the DA decline in the striatum, the main DA containing region (80%) in human brain (Ehringer and Hornykiewicz, 1960; Hornykiewicz and Kish, 1986; Agid *et al.*, 1987; Montastruc, 1991; Calne, 1992). Nigrostriatal degeneration in patients with postencephalitic parkinsonism and idiopathic PD differ with respect to the progression of nigrostriatal cell loss (Agid, 1991). Postencephalitic parkinsonism is characterized by an initial rapid cell decline at the onset of the disease, which is followed by a slow, age-related degeneration of nigrostriatal neurons (0.5% cell loss per year). By contrast, in PD, there is a continuous fast progression of nigrostriatal cell death, exceeding that observed in normal aging (Scherman *et al.*, 1989; Agid, 1991; Kish *et al.*, 1992). However, aging can play an additional role in degeneration of dopaminergic nigrostriatal neurons because of the early continuing natural cell loss of 0.5% per year in healthy persons.

Tyrosine hydroxylase (TH) is the key enzyme in DA synthesis, by means of which transmitter production is regulated in relation to the physiological need. The discharge frequency of dopaminergic neurons is correlated positively to the DA synthesis from L-DOPA. A high DA

concentration in the synaptic cleft suppresses the firing rate of the neuron, the activity of TH and DA release via presynaptic autoreceptors (Riederer *et al.*, 1989b; Melamed, 1992). By means of TH-staining, using immunohistochemical methods, the dopaminergic neurons and their synaptic boutons can be labelled in a quantitative manner (Pasik *et al.*, 1986), and a decrease of TH-positive cells in the ventral tegmental area and perforant path has also been observed in PD (Torack and Morris, 1990, 1992). The loss of dopaminergic cells in the ventral tegmental area, together with the decrease of DA content in neocortical and limbic structures, demonstrates that mesolimbico-cortical tracts degenerate in PD (Dubois *et al.*, 1992), although the DA decline in the mesolimbic system does not reach the degree of cell loss in the nigrostriatal system (Agid, 1991).

It is not quite clear how much these alterations contribute to the cognitive slowing and affective alterations in PD patients because, in contrast to the undoubted improvement of motor actions after L-DOPA treatment, the results of this therapy on cognitive and affective functions are very contradictory (Riklan *et al.*, 1976; Mortimer *et al.*, 1982; Delis *et al.*, 1982).

In the nigrostriatal system of PD patients, the activity of remaining TH increases following cell loss (Nagatsu, 1990). The elevated homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid)/DA ratio in the striatum, which begins at a DA reduction of 50–60%, is proof of increased metabolism in the remaining neurons.

Another way to compensate for the cell decline in PD is the D_2 receptor up-regulation seen in untreated patients, whereas the L-DOPA substitution, depending on the dose, provokes either normalization or down-regulation (Rinne *et al.*, 1981; Guttman and Seeman, 1986; Riederer *et al.*, 1989b; Montastruc, 1991; Playford and Brooks, 1992).

The denervation-receptor hypersensitivity develops after a nigrostriatal cell reduction of 90% and more (Riederer *et al.*, 1989b; Agid, 1991; Montastruc, 1991). A positive correlation between decline of D_2 receptor density and duration of disease, age and duration of L-DOPA therapy cannot be found (Guttman and Seeman, 1986).

Besides the cardinal symptoms in PD, many autonomic disturbances, such as hypotension, hypersalivation and constipation, which are a part of a parkinson-plus syndrome, are present and also can be attenuated by DOPA-application (Guttman and Seeman, 1986). Therefore, it can be suspected that these symptoms also result from a central DA deficiency, perhaps related to the observed biochemical effects on DA-containing centers in the hypothalamus and area postrema.

In PD brain, there is a significant decrease of serotonin and its metabolite 5-hydroxyindoleacetic acid in fiber tracts from the nuclei raphe to the forebrain, in forebrain areas, basal ganglia, hippocampus and cerebral cortex (Mayeux, 1990) (Fig. 1). A strict attribution of defective transmitter systems to symptoms is not really possible. Nevertheless, it has been proposed that depression in PD is related to a decrease in the serotonergic system (Agid *et al.*, 1987), while the known decline in noradrenergic coerulocortical connections is thought to contribute to depression in PD (Mayeux 1982, 1990) and, furthermore, to provoke a cognitive decline.

A very interesting field with regard to the connection between several neurodegenerative diseases is the appearance of dementia in PD patients. As reviewed by Boller (1985), clinical dementia is a frequent finding, of which the etiologic explanation is far from clear. Pathological investigations have not been able to show a higher incidence of Alzheimer-specific findings in PD than in controls. Nevertheless, a significant percentage (17% younger than 70, 35% aged over 70) of PD brains shows typical alterations as observed in AD (Jellinger, 1986). Because of the possibility of PD-associated dementia without corresponding AD pathology, the conclusion has been drawn that, analogous to the DA decline of 80–90% necessary for clinical manifestation of motor disturbance, a comparable reduction of acetylcholine (ACh) in several areas must have occurred before the appearance of clinical signs of dementia (Whitehouse *et al.*, 1983a; Jellinger, 1989; Ruberg *et al.*, 1989; Agid, 1991). In the striatum and SNC of PD patients, there is, however, no alteration of the cholinergic system. In contrast, in the striatum, a functional hyperactivity occurs. Some degeneration of cholinergic systems, as measured by choline acetyl-transferase (ChAT) immunohistochemistry, occurs in the NBM-cortical and the septohippocampal pathway. Concerning the ACh receptors, a reduction only of the hippocampal nicotinic receptors has been detected in either AD or PD (Perry, E. K. *et al.*, 1987), whereas the muscarinic receptor density in PD frontal cortex can even be elevated (Ruberg *et al.*, 1989). Signs of ChAT, ACh and nicotine receptor alterations are absent from thalamic nuclei and subthalamic nucleus in both AD and PD.

The contradictory role of DA in cognitive disturbances has been discussed recently (Mohr *et al.*, 1989; Dubois *et al.*, 1992), while the reduction of norepinephrine in coerulocortical neurons, which has also been found in demented patients (Cash *et al.*, 1984; Hardy *et al.*, 1985), could also contribute to the dementia in PD. Further parallel biochemical findings between PD and AD are:

(1) diminution of substance P in cortical regions of demented parkinsonian patients (Clevens and Beal, 1989) with Alzheimer-specific pathological findings in SNC, SNR, NBM and internal globus pallidus;
(2) reduction of cortical corticotropin releasing factor (Whitehouse *et al.*, 1987); and
(3) low somatostatin content in cerebrospinal fluid, correlating more with the degree of motor disadvantage than with that of dementia (Strittmatter and Cramer, 1992).

Other investigations have also elaborated a decline of somatostatin in frontal cortex and hippocampus of demented parkinsonian patients, which corresponds to the decline of ChAT (Eppelbaum *et al.*, 1983).

2.1.6. *Pathophysiology of Motoneuron Disease*

Lesions of the corticospinal tract produce both positive and negative signs. The positive signs originate from normally suppressed, inadequate motor responses to sensoric stimuli. Negative signs correspond to the affected ability to perform volitional movements forcefully and rapidly.

Lesion of the upper motoneuron results in weakness of groups of muscles and a spastic muscle tone, which is produced by a brain stem-mediated increased excitation of α-motoneurons. Lesions of the lower motoneuron affect single muscles. Weakness, atrophy and a slack muscle tone are the clinical symptoms of this damage. A further symptom occurs after degeneration of a motoneuron: the muscle fibers innervated by it discharge sporadically and not in coordination with other muscle fibers that are still controlled by intact motoneurons. These visible single discharges are called fasciculations (Ghez, 1991).

2.1.7. *The Clinical Manifestation of Amyotrophic Lateral Sclerosis*

In the typical form of ALS, the upper and the lower motoneurons of the spinal cord and the bulbar system are affected. The oculomotor nuclei and the sphincter muscles of the anus and bladder are spared.

When the upper motoneuron is predominantly affected, the clinical term 'primary lateral sclerosis' or 'progressive pseudobulbar palsy' is used, while isolated affection of the lower motoneuron is called 'progressive muscle atrophy' or 'progressive bulbar palsy' (Tandan and Bradley, 1985a). In the Scottish motoneuron disease register, ALS, progressive bulbar palsy and progressive muscle atrophy are characterized as clinical subtypes of a single disease, whereas primary lateral sclerosis is considered to be a separate disease (The Scottish Motor Neuron Research Group, 1992). The overall term for all these disturbances is motoneuron disease.

ALS is a chronic progressive degenerative disorder, which, in its classical form, appears sporadically. It is noteworthy that there exists a familial form of ALS in 5–10% of the cases (Roe, 1964; Finlayson *et al.*, 1973; Rosen, 1978). This form has been described as being of early onset, with a longer course, as compared with the predominant late onset form, with a shorter course.

A study exists that was able to detect a familial ALS form caused by a gene on chromosome 21 (Siddique *et al.*, 1991). The combination of ALS with PD or dementia is not so rare and appears in a familial and sporadic form (Hudson, 1981). Encephalitis lethargica, usually seen in connection with PD, has also sometimes caused a combination of ALS and PD (Hufschmidt *et al.*, 1960).

In the Western Pacific form, ALS appears as one member of a complex disease in association with PD and dementia.

ALS may occur as a consequence of a remote poliomyelitis infection, as reported in cases of ALS in patients who suffered from a polio infection years ago. Proof of a connection between these two diseases is still lacking.

There also sometimes seems to be metal intoxication in ALS patients. The best investigated toxic metal is lead, which has been described as a trigger of ALS symptoms in many cases, although

there is no knowledge of a potential pathophysiological mechanism (Rowland, 1984; Tandan and Bradley, 1985b).

The average annual incidence rate of ALS is about 1:100 000: a male predominance of 2:1 is described, the mean age of onset is 56, the duration of disease 3 years (Hudson, 1981; Tandan and Bradley, 1985a; Li *et al.*, 1990). In the majority of cases (43–37%), the disease starts in the upper or lower limbs; in a minor percentage (20%), in the bulbar muscles (Li *et al.*, 1990). The first symptoms are mostly difficulty in walking or diminution of manual dexterity due to increasing muscle weakness. The brain stem symptoms are difficulty in speaking or swallowing. Finally, the patient becomes unable to speak or swallow food or saliva. The esophageal dysmotility results in dysphagia and predisposes to aspiration. An additional respiratory failure makes respiratory support necessary to keep the patient alive. In the classical form of ALS, the patient's cognition and consciousness is unimpaired until death (Tandan and Bradley, 1985a; Rowland, 1984).

2.1.8. *Neuropathology of Amyotrophic Lateral Sclerosis*

A number of pathological changes are seen in the CNS of ALS patients, with an expected regional distribution. The most prominent of these is a loss of large motoneurons in the motor cortex, brain stem and spinal cord. It has been debated whether this degeneration represents a dying back mechanism, spreading from the axon back to the perikaryon (Appel, 1981; Rowland, 1984). The remaining neurons show atrophy, lipofuscin accumulation or LB-like or eosinophilic (Bunian-bodies) intracytoplasmic inclusions (Appel, 1981; Tandan and Bradley, 1985a).

A non-obligatory, but frequently seen, change is a cerebral atrophy and abnormal gliosis in cerebral cortex, striatum, pallidum, subthalamic nucleus and SN (Hudson, 1981). The Guamanian ALS is characterized pathologically by many neurofibrillary tangles (NFT) throughout the brain (Kurland, 1988). In familial ALS combined with dementia, there is a symmetrical involvement of amygdala, insula and frontotemporal cortex, whereas the cell loss in spinal cord anterior horn is identical to the sporadic changes seen in uncomplicated ALS.

2.1.9. *Biochemistry in Amyotrophic Lateral Sclerosis*

The biochemical changes in ALS-affected patients have been widely and inconsistently construed. L-glutamate is the neurotransmitter of the primate tractus corticospinalis; from the degeneration of this system a derangement of glutamatergic systems results (Eisen and Calne, 1992). Since the ALS of Guam is highly suspected to be caused by an excitatory amino acid (EAA) affecting glutamate receptors, a damaging role of glutamate has been proposed in sporadic ALS also (Meldrum and Garthwaite, 1990, 1991; Farooqui and Horrocks, 1991). However, other authors have found a glutamate deficiency in sporadic ALS brain which is absent in ALS of Guam (Plaitakis *et al.*, 1988; Plaitakis, 1990; Perry *et al.*, 1991).

A reduction of muscarinic receptor sites in the ventral horn has also been found to be related to this neurodegenerative disorder (Whitehouse *et al.*, 1983b), while the observation of decreased citrate synthase activity of isolated anterior horn cells has led to the proposal that neuronal ACh production may be depressed (Hayashi and Tsubaki, 1982).

Other biochemical abnormalities reported for ALS patients have included increased anterior horn levels of ornithine and ammonia (Patten *et al.*, 1982), reduced glycine receptor binding in anterior horn (Hayashi and Tsubaki, 1982) and reduced levels of γ-amino-butyric acid (GABA) and norepinephrine in the cerebrospinal fluid. A lack of some specific motoneuron growth factor has also been postulated (Appel, 1981).

2.2. Alzheimer's Disease, a Cognitive Disorder

2.2.1. *Physiology of Cognition and Emotion*

The hippocampal formation as a relay station in the origin of emotions was proposed by Papez in 1937 in the circuit constructed by himself (Gray, 1987). The gyrus cinguli receives afferents from

a mamillothalamic tract and sends inputs to the hippocampal formation, from where neurons project back to the gyrus cinguli. Neocortical areas send their information to the gyrus cinguli via a hippocampo-mamillo-thalamic pathway. In a new version of the classic Papez circuit, the nucleus accumbens has been added as a basal ganglia structure with emotional function that receives afferents from thalamic nuclei and from the gyrus cinguli directly or via a cingulo-hippocampal loop (Fig. 1).

While involvement in emotion is one function of the hippocampal formation, contribution to cognition and memory is another. For instance, kindling experiments involving hippocampal structures provoke disturbances of memory (Gaffan, 1987). The pathological and biochemical alterations in the hippocampal formation of Alzheimer patients discussed in Sections 2.2.3 and 2.2.4 show the importance of this anatomical substrate in learning and cognition.

As reviewed by Rolls (1990), the hippocampus proper consists of a system of high plasticity, which is modified in its specificity by long-term potentiation. In this process, randomly arriving inputs are filtered and, thus, specified by partly increasing and partly decreasing strength of synapses between the pre- and postsynaptic neuron. This neuronal plasticity means that neurons discharging at high frequency develop closer synaptic linkages with the postsynaptic neuron, whereas subthreshold firing is not able to alter synaptic properties.

Because of this process, partly mediated by *N*-methyl-D-aspartate (NMDA)-responsive receptor activation (Monaghan *et al.*, 1989), the hippocampus becomes a memory and recognition organ for individual experiences. Neocortical inputs entering the hippocampal formation induce synaptic plasticity, and back projections to the neocortex prepare cortical areas for further perceptional stimuli by storing the hippocampus-mediated information. As a consequence of this learning system, the neocortex is enabled to react faster and more specifically upon a known stimulus (Rolls, 1990).

2.2.2. *The Clinical Manifestation of Alzheimer's Disease*

The first case of AD was reported in 1907 by Alois Alzheimer, who investigated a woman who displayed symptoms that today are characteristic of a progressed stage of the disease. After death, he found histologically cortical NFT and plaques, a finding that represents a milestone in the history of this disease (Brun *et al.*, 1990).

According to the definition of dementia given by the World Health Organization, this term means "an acquired global impairment of higher cognitive functions including: memory, the ability to resolve problems of daily living, the performance of sensorimotoric and social functions, language communication and control of emotional reactions without marked reduction of consciousness. This process is mostly progressive but not absolutely irreversible".

The diagnostic criteria for dementia (American Psychiatric Association, 1987) represent a more detailed description of this definition, confirming that the term 'dementia' is adequate only when the above-mentioned disturbances of personality, memory, language, abstract thinking, judgement and social behavior that interfere with work and social activities occur in the absence of delirium, functional or organic impairment, such as major depression (Thompson, 1987),

The clinical diagnosis of AD is made in the absence of histopathological findings. However, comparing the percentage of diagnostic pathological and clinical agreement, we see that there has been an increase of correct clinical diagnoses over the last 10 years.

Having diagnosed AD in an aged person, other dementias, such as multi-infarct dementia, Pick disease and HD, must be excluded. It is also important to distinguish between dementia and pseudodementia in depressive patients. It is a helpful hint that true dementia worsens at night and becomes more prominent when antidepressant, anxiolytic or hypnotic drugs are prescribed (Thompson, 1987).

The age of onset of the disease can be about 40 years, but more usually, it begins at ages over 60 (Khachaturian, 1985). Two to five percent of individuals over 65 suffer from dementia, and half of these have AD (Thompson, 1987). It is probable that the rate doubles every 4–5 years from 60 to 90 years of age. Further, a higher incidence in women is apparent.

Investigations have led to the conclusion that some AD cases have a positive familial history

of the disease. In some cases, there is an upward shift in maternal age, and in some, an increased frequency of Down's syndrome is found in relatives (Henderson, 1990).

The course of dementia is progressive and can be subdivided into three phases: in the early phase, there is no cognitive impairment, but the patient complains of other diffuse symptoms. He suffers from anxiety, mild depression, feeling frustrated, insomnia or multiple somatic symptoms. The cognitive deficits can still be compensated. A marked alteration of personality develops, and patients show conspicuous accentuation of certain character qualities.

In the middle phase the cognitive decline can no longer be compensated. There is a memory impairment firstly for recent events, later for long-distance events. Arithmetical properties, judgement and social living are disturbed.

In the late phase, patients often develop paranoid ideas, are no longer able to recognize their family members, lose completely the capacity to participate in conversation and cannot perform the simplest activities, such as feeding themselves and caring for their bodies. The consequences are reduction of body weight and incontinence (Thompson, 1987). Some patients die in bed, a consequence of decubitus or pneumonia; others die from accidents that occur due to the spatial disorientation.

The oldest group of demented patients differs clearly from the younger. In this group, head trauma and cerebrovascular diseases often complicate a mild subclinical Alzheimer dementia. The patients mostly die of non-dementia-related organic diseases (Brun *et al.*, 1990).

2.2.3. *Pathology of Alzheimer's Disease*

Cognitive decline is the essential criterion of AD during life, but only after death can the diagnosis be confirmed pathologically.

The pathology of normal, aged persons and mildly demented patients is broadly overlapping, so that only after crossing the defined neuropathological threshold can the diagnosis be made (Roth, 1986).

The autopsy criteria for AD described by Alois Alzheimer in 1907 were NFT and neuritic plaques. The presence of NFT is the presupposition for the diagnosis of AD. Whereas in brains of normal, aged persons, NFT in greater extent are never found, numerous amyloid and neuritic plaques can frequently be observed (Perry, E. K., 1986; Perry, R. H., 1986).

In order to diagnose AD, at least three neocortical regions, amygdala, hippocampal formation, basal ganglia, SN, cerebellar cortex and spinal cord should be examined (Khachaturian, 1985). The distribution of neuritic plaques is irregular, whereas the NFT show a characteristic pattern with minimal interindividual variations.

The pathologic process develops in a clear temporal order. The region first affected is the transentorhinal region, followed by the regio entorhinalis, hippocampal formation, isocortical regions and extrapyramidal system (Braak and Braak, 1991) (Fig. 1).

The senile plaques represent extracellular round or ovoid structures, their diameters ranging between 1.5 and 20 nm. Typically, these plaques consist of three components: abnormal nerve processes, glial processes and a central or amyloid core. There are different stages in plaque development: primitive plaques consisting of neuritic components only, the classic plaque with central amyloid core surrounded by dystrophic neurites and, in the final stage, the burned-out plaque with a great amyloid core and no or only a small surrounding neuritic border. Biochemically, catecholaminergic and cholinergic components have been detected (Perry, E. K., 1986; Perry, R. H., 1986). Besides amyloid-containing neuritic plaques, pure amyloid deposits, without pathologic neuritic components, also exist. Such deposits can appear without accompanying neurofibrillary changes, whereas severe neurofibrillary changes characteristically accompany amyloid plaques (Braak and Braak, 1991).

The NFT are an abnormal intracytoplasmatic accumulation of neurofilaments. They develop within the nerve cell soma, from where they can extend into the dendrites. The parent cell may disappear and the NFT persist as ghost tangles (Braak and Braak, 1991). Ultrastructural investigations of the NFT show that they are composed of altered neurofilament peptides that form paired helical filaments (PHF). The PHF are composed of two identical filaments twisted around each other with a periodicity of 80 nm (Perl and Pendlebury, 1987). The PHF are insoluble,

covalent-bound cytoskeletal elements of high stability that, therefore, could disrupt intraneuronal axonal transport and cytoskeletal metabolism.

By biochemical and immunochemical techniques it is possible to stain three PHF components: microtubule-associated protein (MAP), tau and ubiquitin. The question is whether the PHF represent intracellular amyloid deposits (Masters and Beyreuther, 1990). Both the PHF and extracellular amyloid plaques are formed from globular subunits that consist of aggregates of A4 protein. Experimentally, the amyloid protein precursor fragments are transformed to their insoluble aggregating form by meta-catalysin oxidation systems. This transformation can be prevented by radical scavengers (Dyrks *et al.*, 1992).

The gene of amyloid precursor protein (APP) is located on chromosome 21. It is speculated that the precursor A4 protein (APP) forms intracellular NFT and amyloid plaques in the extracellular space (Masters and Beyreuther, 1990; Beyreuther *et al.*, 1991). On the question of whether NFT consist of A4 protein or not, opinions are still divided, but intracellular NFT are stained by both APP and NFT antibodies (Murphy *et al.*, 1992). As well, in hippocampal and cells of NBM in AD patients, an overexpression of APP was detected by *in situ* hybridization (Goldgaber and Schmechtel, 1990).

The gene location of APP on chromosome 21 is interesting because patients suffering from trisomia 21 develop NFT by age 35–40 years. In cases of sporadic AD, a duplication of chromosome 21 around the A4 protein locus has been reported. This allows the logical conclusion that the pathological changes of both AD and Down's syndrome are due to an increased A4 protein production (Wisniewski *et al.*, 1988).

2.2.4. *Biochemical Alterations in Alzheimer's Disease*

In conventional terminology, AD, as cortical dementia, is distinguished from the subcortical forms that accompany diseases of subcortical structures, such as PD, HD, morbus Wilson and progressive supranuclear palsy. This classification neglects the fact that, in both forms, both cortical and subcortical systems are affected: NBM and LC in AD and cortical areas in the subcortical dementias. Therefore, a clear distinction in biochemical findings of the two forms cannot be expected. Rather, a reasonable course of study might be to examine common biochemical characteristics of both forms (Rossor, 1985).

A further difficulty is that the typical pathological changes in neocortex and hippocampus represent valuable diagnostic criteria in young AD patients, but can be numerous in brains of non-demented aged persons (Khachaturian, 1985). Thus, where to draw the limits between pathology and physiology of aging becomes very problematic. Clearly, biochemical and pathological data can only be interpreted usefully in combination with clinical observations.

The neurotransmitter profiles in normal aging and AD must be interpreted with caution, keeping in mind the influence of terminal conditions (medical treatment, hypoxic states, cachexia) on *post mortem* measured transmitter content (Hardy *et al.*, 1985). In normal aged brain, a decline of TH in putamen and nucleus caudatus, of ChAT in nucleus caudatus and of glutamic acid decarboxylase and DA in putamen and nucleus caudatus has been described, although the ChAT decline was not consistent. Norepinephrine decline was found in hippocampus, 5-hydroxytryptamine decline was measured in gyrus cinguli. A prominent finding in all investigated regions is an increase of monoamine oxidase (MAO)-B in comparison with that in young control brains; this could be interpreted as one marker for neurodegeneration in the aging brain.

Thinking is a complex process that includes several levels and activities. A certain attentional state is necessary to absorb new information. Information must then be transferred from a short-term memory depot to long-term memory storage. In order to apply stored information usefully, new contents must be compared with that of semantic storages in an associative manner. In order to resolve problems further, we make use of logical convergent and creative divergent thinking. The former depends on high attention and tends to try known solution concepts, the latter appears at a low arousal state and enables one to find new simple solutions.

These aspects of thinking can be connected to neurotransmitter systems. Numerous experiments have been able to show that anticholinergic drugs decrease focused attention and inhibit transformation of information from short- to long-term memory storage, whereas cholinergic

agonists have the opposite effect. Concerning norepinephrine systems, we know that they facilitate associative thinking (Holttum and Gershon, 1992).

Starting from this point, the goal of investigating these neurotransmitter systems in demented persons is a logical consequence. The synthesis of ACh from mitochondrial coenzyme A and extracellular choline is mediated by ChAT (Tucek *et al.*, 1990). By electrical stimulation of cholinergic neurons, a large production of ACh has been seen, despite a complete lack of extracellular choline. Therefore, the suggestion has been made that neurodegeneration develops by an autocannibal mechanism taking the choline of membrane phosphatidylcholine for ACh synthesis, which is followed by membrane destruction (Wurtman *et al.*, 1990).

The impairment of the cholinergic system in AD is an undoubted fact.

ACh histochemistry and ChAT immunohistochemistry in NBM of AD brains show marked reductions, corresponding to cell loss of about 75%. In cortical target areas, there is also a cholinergic pathology, which may provoke the cholinergic deficit in NBM by retrograde degeneration (Perry, E. K., 1986; Perry, R. H., 1986).

The hippocampal formation, the anatomic structure most closely related to the cognitive decline in AD, is innervated intrinsically by glutamatergic and GABA-ergic neurons, and extrinsically by cholinergic projections from frontal forebrain. These topographically organized cholinergic projections originate from the medial septal nucleus and the vertical limb of the diagonal band of Broca. The projections end in CA2/3, hilus and gyrus dentatus of the hippocampus (Emson and Lindvall, 1986).

Measuring the ChAT activity in neocortical areas, 70% is estimated to belong to extrinsic projections from basal forebrain and 30% to intrinsic cholinergic neurons (Emson and Lindvall, 1986).

Reduced ChAT activity in temporal, frontal and parietal neocortex, hippocampus and NBM from both autopsy and biopsy specimens of AD patients further demonstrates the degeneration of cholinergic cells. Since ChAT is a marker for presynaptic ACh synthesis, the diminution is interpreted as presynaptic axon degeneration. Therefore, the degenerating axons should belong to NBM neurons that project strongly to the frontal neocortex (Adolfsson *et al.*, 1979; Gottfries, 1985a,b; Hardy *et al.*, 1985; Ichimiya *et al.*, 1986; Perry, 1987; Sparks *et al.*, 1988). On the other hand, cases have also been described that show no cell degeneration in NBM, despite cortical ChAT diminution (Quirion *et al.*, 1986; Etienne *et al.*, 1986). Nevertheless, overall there is a relationship between quantity of specific neuropathological findings (NFT, plaques), and ChAT-deficiency and choline uptake is also reduced.

A neurotransmitter reduction is also seen in the monoaminergic system of AD brains. Whereas norepinephrine and serotonin are shown to be reduced by most investigators (Mann, D. M. A. *et al.*, 1980; Ichimiya *et al.*, 1986; Palmer *et al.*, 1987; D'Amato *et al.*, 1987, Blennow *et al.*, 1991), the statements about DA are quite different (Adolfsson *et al.*, 1979). Sometimes a diminution of DA in the striatum and nucleus caudatus exists reproducibly (Rossor, 1985; Bowen and Davison, 1986).

Norepinephrine reduction can be due to a severe cell loss in LC of AD brains. Serotonin, its metabolite 5-hydroxyindole-3-acetic acid and norepinephrine are measured to be decreased in hippocampal formation. The substantia innominata may be unaffected (Baker and Reynolds, 1989). The loss of serotonin correlates to many neuropathological findings in serotonergic cells of the nuclei raphe (Quirion *et al.*, 1986).

Somatostatin is the only consistently reduced neuropeptide in AD. It is directly involved in histopathological changes, as evidenced by the fact that it has been found in NFT-containing cells and plaques. In frontal and temporal cortex, a decline in content of somatostatin and substance P is possible (Quirion *et al.*, 1986; Hardy *et al.*, 1985).

There is no alteration of GABA content in AD *post mortem* brain, despite occasional detecting of glutamate decarboxylase diminution. Whereas GABA is not affected by perimortal circumstances, reduction of glutamic acid decarboxylase activity is often due to premortal hypoxia (Bowen *et al.*, 1990).

The role of EAA in cognition and learning is well established (Monaghan *et al.*, 1989). The high level of EAA receptors in neocortical and hippocampal regions is also undoubted (Young and Egg, 1991).

Therefore, in AD, there seems to be a logical connection between cognitive impairment, neuropathologic changes in neocortical and hippocampal areas and alterations of EAA. The neurodegeneration caused by EAA has been shown experimentally, just as has the vulnerability of limbic and cortical neurons to this neurotoxic effect. Instead, corresponding studies of AD brains showed a reduction of EAA-binding sites in hippocampal areas (Jansen *et al.*, 1990; Geddes *et al.*, 1992). As further proof of this connection, we can take investigations that explain that EAA alter the polymerization of cytoskeletal elements in cultured cells, a fact that may elucidate the origin of NFT in AD (Kato *et al.*, 1992).

2.3. Pathological Overlapping of Neurodegenerative Diseases

In approximately 10% of persons over 60 years and also in 15% of Alzheimer patients, LB can be found (Forno, 1986). Another term is the 'diffuse LB disease' which is clearly distinguished from PD: here the LB infiltrate the limbic system and cortical areas; the clinical correlate is dementia with or without PD (Gibb, 1989). Tests exist to distinguish between parkinsonian LB and diffuse LB disease: using antineurofilament antibodies, the LB of the latter disease were stained by τ-protein antibodies only (Galloway *et al.*, 1989). The immunoreactivity against other cytoskeletal elements, such as ubiquitin, was the same in PD and diffuse LB disease (Galloway *et al.*, 1989; Dale *et al.*, 1992). Many cases of unclear parkinsonian-like movement disturbances are misdiagnosed as PD (Fearnley and Lees, 1991). The striatonigral degeneration, Steel–Richardson–Olszewsky syndrome and corticobasal degeneration can be distinguished from PD by the lack of LB in SNC and the presence of structural pathology outside the SNC (Gibb, 1989).

The LB can be taken as a good example for a pathological, pathophysiological correlation between diverse degenerative disorders. Other examples are the NFT and the neuritic plaques.

The NFT consist of intracytoplasmatic filaments with antigen determinants of abnormal neurofilament and non-neurofilament protein and of microtubules.

The senile plaques are spherical dense structures composed of pre- and postsynaptic degenerating and regenerating neuritic terminals, abnormal synapses, glia, filamentous protein and extracellular amyloid (Jellinger, 1989).

NFT and senile plaques are typical structural pathological findings in AD, where they are found in the limbic system, cortical areas and NBM. The number of NFT corresponds to the stage of the disease and the several brain regions are invaded in a specific order. At the beginning of the disease, NFT appear in the regio entorhinalis, then progress to the hippocampus and later involve neocortical areas (Braak and Braak, 1991) (Fig. 1).

The NFT are an irreplaceable index proof of AD, whereas senile plaques are not necessarily linked with pathological diagnosis (Braak and Braak, 1991).

The finding of single NFT and senile plaques in the brain of non-demented elderly persons is not unusual (Katzman and Saitoh, 1991). Comparing pathological findings in early and late onset PD, a similar Parkinson-specific pathology can be detected, whereas changes typically found in Alzheimer's brains are more frequent in the later onset of PD, a fact that shows a probable decompensation of subtle damages by an additional influence of aging processes (Jellinger, 1986). Age-dependent number of NFT and senile plaques correspond to the clinical signs of dementia in PD. Clinically, senile parkinsonism presents dementia, mild parkinsonian signs and a lack of L-DOPA responsiveness. Pathologically, there is a mild cell loss in SNC, LB in 50% and NFT in 60% of the cases (Jellinger, 1989).

The prevalence of dementia and pathologically diagnosed Alzheimer's changes in PD patients is six-fold higher than in the age-matched, healthy population (Boller, 1985). Also described are cases of L-DOPA responsive, clinically non-demented Pd, which on pathological examination, show a severe decline of neurons in SNC, without LB or other pathological findings outside this region, which could support the diagnosis of a PD-like syndrome. At the same time, many NFT and senile plaques were detected in various brain regions.

The severity of dementia in PD patients correlates to the severity of Alzheimer's pathology in parkinsonian brains whereby a mild degree of dementia can be ascribed to degeneration of SNC, and a marked degree of cognitive impairment results from additional cortical Alzheimer lesions

(Paulus and Jellinger, 1991). The neuropathological distinction between PD/dementia and PD plus dementia is very difficult.

One test is to compare the degree of brain atrophy in several regions by photographic analysis of coronare slices. The result shows a global cerebral atrophy in PD plus AD with moderate atrophy of white matter and a more partial cerebral atrophy without impairment of white matter in PD dementia (De la Monte *et al.*, 1989).

As we can see, pathological findings in different neurodegenerative disorders overlap to a great extent. As a probably useful model for a common etiopathologic cause in clinically diverse neurodegenerative diseases, the Guam disease can be considered to be a combined syndrome of PD, AD and ALS.

This disease shows neuropathological findings of PD and AD: loss of neurons and appearance of LB and NFT in cortical and subcortical structures. In the spinal cord of ALS and PD patients of Guam, NFT can be observed. Distribution and number are similar in both diseases. In immunohistochemistry, a reactivity against anti-τ-protein antibodies has been seen, a result that could not be confirmed by examination of the cytoplasm of sporadic ALS and controls (Kato *et al.*, 1992).

A hypothesis of abiotrophic interaction between aging and environment in PD, ALS and AD exists (Calne *et al.*, 1986). Another way to reach a unifying hypothesis for several neurodegenerative disorders is to analyse inclusion bodies linked with it. The result is that the inclusion bodies in all these cases are composed of cytoskeletal components, so that neurodegeneration of several functional systems can be subordinated to a common term of cytoskeletal disorders (Calne and Eisen, 1989).

3. POSSIBLE BIOCHEMICAL CAUSES OF CELL DEGENERATION

3.1. Oxidative Stress

3.1.1. *Reactive Oxygen Species*

Metabolically, the brain is one of the most active organs in the body. This is reflected by cerebral O_2 consumption in normal, conscious, young men which amounts to 3.5 mL O_2 per 100 g brain per min (Sokoloff, 1960). Thus, 2% of total body weight accounts for 20% of the resting total body O_2 consumption. Nearly all O_2 is utilized for the oxidation of carbohydrates (Sokoloff, 1960) and results in an estimated steady-state turnover of approximately 4×10^{21} molecules of ATP per min in the entire human brain. Oxygen maintains brain function and is crucial for life. However, O_2 supplied at concentrations greater than those in normal air is highly toxic. High pressure O_2 can lead to convulsions, which are attributed to an inhibition of the enzyme glutamate decarboxylase by O_2 or reactive oxygen species (ROS) (Halliwell and Gutteridge, 1989). Even normal O_2 consumption could lead to toxic cellular reactions mediated by oxidative stress.

'Oxidative stress' is an expression used for a process that implicates reactions with biomolecules of O_2 or derived substances, such as hydrogen peroxide (H_2O_2), superoxide [$(O_2)^{\cdot-}$], hydroxyl radicals $(OH)^{\cdot}$ or singlet $O_2^{\cdot}$. If a reaction is thermodynamically feasibile, its reaction rate depends primarily on the concentrations of the reacting partners. Thus, to evaluate effects of ROS on biomolecules, their concentrations and sites of production have to be considered. In the following Section, some general comments on chemistry will be made before discussing the biochemistry of radicals in relation to neurodegeneration.

Groundstate O_2 is in the triplet or diradical electronic configuration, having two unpaired electrons, each located in a different π^* antibonding orbital. These two electrons have the same spin quantum number ('parallel spins') in contrast to singlet O_2, which has antiparallel spins. A description of molecular orbital chemistry in the biomedical context has been made by Halliwell and Gutteridge (1984). A prerequisite for exergonic reactions is the rule that reacting electrons in an energetic groundstate have to have antiparallel spins. Thus, in order to achieve spin conversion, groundstate O_2 must react in a two-step, energy-dependent process. This is the reason for the slow reactivity of groundstate O_2 when this energy is not provided by enzymes or light (McMurry and

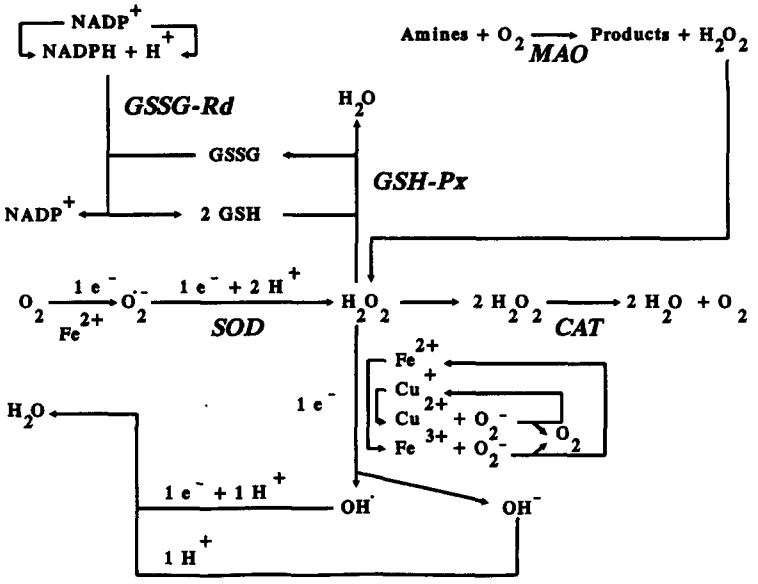

FIG. 2. Possible redox reactions leading to ROS (H_2O_2; $(O_2)^{\cdot-}$; $(OH)^{\cdot}$) and pathways degradating H_2O_2 directly via CAT or via NADPH-dependent mechanisms utilizing GSH. Adapted from Benzi *et al.* (1988).

Groves, 1986). Among all oxygen species, dioxide(1−) [$(O_2)^{\cdot-}$] (which is a new term for the old, but still allowed, name 'superoxide'), $(HO)^{\cdot}$ and H_2O_2 are supposed to be the most abundant ROS in biological systems. Although O_2 is a diradical and may be represented as $(O_2)^{\cdot\cdot}$, we prefer to omit the radical dots in the cases of groundstate O_2 and of transition metal ions. For a discussion of new nomenclature for oxygen species, see Koppenol (1990).

3.1.1.1. *Dioxide* (1−), *superoxide* $(O_2)^{\cdot-}$. $(O_2)^{\cdot-}$ is mainly produced in biological systems through one-electron reduction of triplet O_2 mediated by enzymes (Fig. 2). In brain, xanthine oxidase (E.C. 1.2.3.2.) and aldehyde oxidase (E.C. 1.2.3.1.), located in nuclear membranes and cytoplasm, seem to be the only enzymes responsible for $(O_2)^{\cdot-}$ production (eqns 1, 2).

$$\mathrm{RCHO} + \mathrm{H_2O} + 2\mathrm{O_2} \rightarrow \mathrm{RCOO^-} + 2(\mathrm{O_2})^{\cdot-} + 3\mathrm{H^+} \quad (1)$$

$$\mathrm{Xanthine} + \mathrm{H_2O} + \mathrm{O_2} \rightarrow \mathrm{Urate} + (\mathrm{O_2})^{\cdot-} + \mathrm{H^+} \quad (2)$$

As well, these two enzymes generate H_2O_2 and are dependent on pO_2 and pH. Xanthine oxidase can oxidize a variety of substrates, including aldehydes, pteridines, purines and hypoxanthine (Halliwell and Gutteridge, 1989). Xanthine oxidase can be converted from a dehydrogenase (non-superoxide-producing) to the oxidase form during tissue hypoxia (Granger *et al.*, 1981). The relative rates of $(O_2)^{\cdot-}$ production by these enzymes may vary with the concentrations of the enzymes in various cell types and the availability of substrates and cofactors (for $(O_2)^{\cdot-}$ production by mitochondria and microsomes see Section 3.2.2).

$(O_2)^{\cdot-}$ can behave as a free radical, a weak nucleophile, a one-electron oxidant or a one-electron reductant (for a review, see Fridovich, 1986a). Free radicals usually are very likely to abstract hydrogen or to add to double bonds; however, as for superoxide, these reactions are slow (Bors *et al.*, 1979). Only if protons (3) are present will the reactive free radical hydrogen dioxide ($HO_2^{\cdot}$) be formed.

$$(\mathrm{O_2})^{\cdot-} + \mathrm{H^+} \leftrightarrow \mathrm{HO_2^{\cdot}} \qquad pK_a = 4.88. \quad (3)$$

Thermodynamically $(O_2)^{\cdot-}$ tends to dismutate to H_2O_2 and O_2 (eqns 4, 5).

$$(\mathrm{O_2})^{\cdot-} + \mathrm{H^+} \leftrightarrow \mathrm{HO_2^{\cdot}} \quad (4)$$

$$\mathrm{HO_2^{\cdot}} + (\mathrm{O_2})^{\cdot-} + \mathrm{H^+} \leftrightarrow \mathrm{H_2O_2} + \mathrm{O_2} \quad (5)$$

In aprotic media, $(O_2)^{\cdot-}$ behaves like a strong nucleophile but in protic media, it is only weakly nucleophilic, due to the presence of protons. Thus, reactions of $(O_2)^{\cdot-}$ in protic media are determined by kinetic rather than by thermodynamic parameters (concentrations, pH, ionic strength). The most important reaction in terms of biological effects is the dismutation of $(O_2)^{\cdot-}$ (eqns 4, 5), which proceeds rather slowly in physiological conditions ($K_{dis} \sim 5 \times 10^5\ M^{-1}\ sec^{-1}$ at pH 7.4; Halliwell and Gutteridge, 1989), but can be considerably favoured by superoxide dismutases (SOD) (E.C. 1.15.1.1.) (Chance *et al.*, 1979; $K_{dis} \sim 1.6 \times 10^9\ M^{-1}\ sec^{-1}$).

$(O_2)^{\cdot-}$ can act as a reductant (eqn 6) of peroxides only if transition metals (Mn^+) are present (Sawyer and Nanni, 1981; Wood, 1988) (eqn 7) or of quinones (eqn 8) (Sawada *et al.*, 1975).

$$O_2 + e^- \leftrightarrow (O_2)^{\cdot-} \qquad E^\circ = -0.33\ V \text{ in protic media} \tag{6}$$

$$(O_2)^{\cdot-} + H_2O_2 \overset{Mn+}{\leftrightarrow} HO^- + (HO)^{\cdot} + O_2 \tag{7}$$

$$\text{Quinone} + (O_2)^{\cdot-} \leftrightarrow \text{Semiquinone radical} + O_2 \tag{8}$$

On the other hand, $(O_2)^{\cdot-}$ can act as a one-electron oxidant, oxidizing e.g. hydroquinones to semiquinone radicals, or oxidizing ascorbate or epinephrine with concomitant production of H_2O_2. Thus, if dismutation reactions and scavenging of $(O_2)^{\cdot-}$ are impaired, degradation of alkylperoxides (ROOH) to alkoxylradicals $(RO)^{\cdot}$ via eqn (9) could lead to potent cytotoxic substances.

$$HO_2^{\cdot} + ROOH \leftrightarrow O_2 + RO^{\cdot} + HO^- + H^+ \tag{9}$$

$(O_2)^{\cdot-}$ biochemistry is strongly influenced by transition metals. Tyler (1975) reasoned that lipid peroxidation (LPO) in membranes occurs only in the presence of iron (for iron and oxidative stress see Section 4.1.4).

3.1.1.2. *Hydrogen peroxide, iron, copper and hydroxyl radical.* The metabolism of H_2O_2 in mammalian organs was reviewed by Chance *et al.* (1979). Enzymes known in the liver to generate H_2O_2 are assumed to be also present in the human brain. These oxidases bear flavins [flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD)] or pyridoxale phosphate (P_i) and metal ions as prosthetic groups. Pyridoxamine phosphate oxidase (E.C. 1.4.3.5.) triggers H_2O_2 production (eqn 10).

$$\text{Pyridoxamine-}P_i + H_2O + O_2 \rightarrow \text{Pyridoxale-}P_i + NH_3 + H_2O_2 \tag{10}$$

D-amino acid oxidase (E.C. 1.4.3.3.) (eqn 11), converting glycine to glyoxylate, is present in the CNS (Gaunt and De Duve, 1976), but glycine is a very poor substrate for the enzyme (DeMarchi and Johnston, 1969).

$$d\text{-}RCHNH_2COOH + H_2O + O_2 \rightarrow RCOCOOH + H_2O_2 + NH_3 \tag{11}$$

For R=H the reaction product of eqn (11), glycolate, is converted by glycolate oxidase (E.C. 1.1.3.1.) to glyoxylate and H_2O_2.

The most prominent oxidase in brain tissue, however, is the flavin-containing MAO (E.C. 1.4.3.4.), which preferentially deaminates primary, secondary and tertiary monoamines (Fig. 2) according to eqn (12) and is located at the outer mitochondrial membrane (see Section 4.1.1; Tipton, 1967).

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + H_2O_2 + NH_3 \tag{12}$$

Moreover, a great source of H_2O_2 production in the intact cell appears to be generated by the auto-oxidation of chemically reactive compounds during reductive processes associated with the mitochondrial and microsomal electron transport systems and during action of SOD (Chance *et al.*, 1979; Forman and Boveris, 1982).

In rat liver, microsomal compartments make the greatest contribution (about 5% of liver uptake of O_2) to the intracellular oxidant load, followed by peroxisomes, mitochondria and cytosol (Boveris *et al.*, 1972). If not enhanced by transition metals or enzymes, such as catalase (CAT) (eqn 13) in peroxisomes (E.C. 1.11.1.6.), or by various peroxidases, such as glutathione peroxidase (GSH-Px) (E.C. 1.11.1.9.) in cytoplasm (eqn 14), reactivity of H_2O_2 is rather low.

$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O \quad (13)$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad (14)$$

Thus, H_2O_2 or $(O_2)^{\cdot-}$ may diffuse some distance from their sites of production. Consequently, radical generation by subcellular compartments may be a threat for the whole cell. H_2O_2 diffusion to reach nuclear DNA may be even enhanced by histidine (his) (Schubert and Wilmer, 1991). Oya and Yamamoto (1988) found that L-his enhanced the induction by H_2O_2 of chromosomal aberrations eight-fold in human embryonic fibroblasts and suggested involvement of a his-H_2O_2 adduct for transport of H_2O_2. It has been estimated that the steady-state concentrations in normal aerobic liver cells are 10^{-12} M to 10^{-11} M $(O_2)^{\cdot-}$ and 10^{-9} M to 10^{-7} M H_2O_2, respectively (Chance *et al.*, 1979). Due to its high need for O_2, the brain appears to produce $(O_2)^{\cdot-}$ and H_2O_2 in similar amounts.

If H_2O_2 is not detoxified by CAT or peroxidases, one-electron reduction results in the formation of the $(HO)^{\cdot}$. This species is assumed to be the most toxic reactive oxygen radical, with an approximate intracellular half-life of 10^{-9} sec (Pryor, 1986) so that reactions with biomolecules become diffusion-controlled. In a variety of biological phenomena, for example aging (Sohal and Allen, 1990; Masoro, 1991; Pacifici and Davies, 1991; Le Bel and Bondy, 1992), cancer (Sahu, 1991; Cerutti, 1991), diabetes (Wolff *et al.*, 1991), phagocytosis and cataractogenesis (Halliwell and Gutteridge, 1989), ischemia-reperfusion injury (Braughler and Hall, 1989; Sussman and Bulkley, 1990; Agardh *et al.*, 1991), quinone toxicity (Powis, 1989; Sinha and Mimnaugh, 1990; O'Brien, 1991), 6-hydroxydopamine (6-OHDA) toxicity (Kostrzewa, 1989) and radiation injury (Girotti, 1990), the hydroxy radical is assumed to contribute to or to cause toxic processes.

Irradiation of water leads to formation of $(HO)^{\cdot}$. By contrast, in the brain, strong water-soluble electron donors (DH) such as nicotinamide adenine dinucleotide phosphate (NADPH), catechin, hydroquinone, ascorbic acid or glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) can promote formation of $(HO)^{\cdot}$ from H_2O_2 in the presence of Cu^+ or some iron complexes (e.g. Fe^{2+}–adenosine diphosphate complexes) according to eqns (15) and (16) (Florence, 1984; Kadiiska *et al.*, 1992).

$$\begin{aligned} DH + Fe^{3+}(\text{chelate}) &\rightarrow Fe^{2+}(\text{chelate}) + D^{\cdot} + H^+ \\ DH + Cu^{2+}(\text{chelate}) &\rightarrow Cu^{+}(\text{chelate}) + D^{\cdot} + H^+ \end{aligned} \quad (15)$$

$$\begin{aligned} H_2O_2 + Fe^{2+}(\text{chelate}) &\rightarrow HO^- + (HO)^{\cdot} + Fe^{3+}(\text{chelate}) \\ H_2O_2 + Cu^{+}(\text{chelate}) &\rightarrow HO^-(HO)^{\cdot} + Cu^{2+}(\text{chelate}) \end{aligned} \quad (16)$$

Reaction (16) is a Fenton-type reaction (Fenton, 1894; Croft *et al.*, 1992). There is still ccntroversy as to whether the reaction of Cu^+ and H_2O_2 leads to the formation of $(HO)^{\cdot}$ or even a Cu^{3+} species (Bielski and Cabelli, 1991). As for iron-catalysed reactions, Fenton chemistry probably involves (oxygen)iron(2+) $[FeO]^{2+}$ intermediates, which are strong oxidants as well. The reaction of Fe^{3+}(chelate) + H_2O_2 could yield $(O_2)^{\cdot-}$, but is reported to be rather slow (Halliwell and Gutteridge, 1989, 1992; Chiueh *et al.*, 1992). *In vivo* formation of $(HO)^{\cdot}$ is determined by measurement of hydroxylated salicylic acid (Van Steveninck *et al.*, 1985) or nitrophenol (Florence, 1984). Involvement of oxygen radicals will often be detectable by means of electron spin resonance spectroscopy using spin traps, such as 5,5-dimethyl-1-pyrroline-*N*-oxide (Buettner, 1987), if care is taken of possible pitfalls (Buettner, 1987; Makino *et al.*, 1990; Davies *et al.*, 1992). This method has been successfully used to determine that mainly low molecular weight complexes of iron catalyse formation of substantial amounts of $(HO)^{\cdot}$ (Ozaki *et al.*, 1988) in contrast to complexes of desferrioxamine, an iron chelator often used therapeutically (Desferal®) in experimental hepatic iron overload (Bacon and Britton, 1989). Thus, it is believed that iron–oxygen complexes, rather than free $(HO)^{\cdot}$, are involved in initiating cytotoxic mechanisms, at least those involving stimulation of LPO by decomposing preexisting lipid peroxides or oxidation of proteins and nucleic acids (for effects of ROS on biomolecules, see Section 3.1.3).

3.1.1.3. *Singlet dioxygen.* The singlet O_2 can be generated by an input of energy (e.g. irradiation with light) to normal groundstate triplet O_2 or can arise from H_2O_2 reacting with hypochlorite (ClO^-), which can be formed by myeloperoxidase (E.C. 1.11.1.7.) during phagocytosis (Khan *et al.*, 1983). Exposure of cells in culture to high-intensity visible light causes damage especially to

mitochondria, which are rich in haem proteins known to be potent sensitizers of singlet O_2 formation (Srivastava *et al.*, 1986). Since formation of singlet O_2 is predominantly dependent on light or presence of ozone, and its relevance to oxygen-mediated tissue injury in the CNS is not yet elucidated, we shall focus on cell damage that possibly involves reactions of $(O_2)^{\cdot-}$, H_2O_2 and $(HO)^{\cdot}$-iron species with biomolecules.

3.1.2. *Factors Scavenging Reactive Oxygen Species*

3.1.2.1. *Enzymes.* Two types of enzymes exist to remove H_2O_2 within cells (Fig. 2): the haem protein CAT, present in most aerobic cells, which catalyses the degradation of H_2O_2 to triplet O_2 and water (eqn 13), and the haem- or selenocysteine-bearing peroxidases utilizing electron donors to reduce H_2O_2 to water (eqn 17).

$$DH_2 + H_2O_2 \rightarrow D + 2H_2O \tag{17}$$

As mentioned in Section 3.1.1.2, H_2O_2 detoxification prevents formation of reactive oxidants like $(HO)^{\cdot}$. However, in contrast to liver and erythrocytes, which contain high levels of CAT ($\sim$1300 U/mg protein), the brain contains less than 20 U/mg protein (Marklund *et al.*, 1982). CAT is predominantly located in small peroxisomes (microperoxisomes). In brain mitochondria, there is very little CAT activity (Sorgato and Sartorelli, 1974). Histochemical techniques have revealed that, in the CNS, at least two classes of microperoxisomes exist, mainly in astrocytes, and that D-amino acid oxidase (which contributes to generation of H_2O_2) and CAT are not in the same peroxisome. Studies of the regional distribution of CAT (Brannan *et al.*, 1981) revealed highest activity in hypothalamus and SN and lowest activity in striatum and frontal cortex. The distribution was reported to correspond to the localization of CAT to catecholaminergic nerve cell bodies (McKenna *et al.*, 1976). The observation that ethanol can be metabolized to acetic aldehyde by H_2O_2-activated CAT ('compound I') points towards a role for peroxidatic activity of CAT in brain (Cohen, 1983b). It was further suggested that CAT may play a role in the metabolism of lipids (Masters and Holmes, 1977) and is very dominant in the liver. However, current evidence supports the view that, in certain cell types, GSH-Px is probably more important than CAT for protecting cells from H_2O_2 (Cohen and Hochstein, 1963; Nathan *et al.*, 1980). The relative importance of GSH-Px in brain, compared with that of CAT, remains to be elucidated.

Meister (1991) has reviewed the enzymology, metabolism and transport of GSH. GSH is an essential tripeptide present in virtually all animal cells. It is synthesized by the consecutive actions of γ-glutamyl-cysteine synthetase and GSH synthase. The rate of the former enzyme is regulated through feedback inhibition by GSH. During action of GSH-transdehydrogenases and GSH-Px, glutathione disulfide (GSSG) is formed. GSH is regenerated via glutathione disulfide reductase (GSSG-Rd) utilizing NADPH resulting from nicotinamide adenine dinucleotide (NADH) by transdehydrogenation and, mainly, from glucose-6-phosphate dehydrogenase, which is very specific for $NADP^+$ and is regulated by intracellular contents of ATP, NADPH and ribose-5-phosphate. Due to its high nucleophilicity, GSH forms conjugates with endogenous compounds, such as estrogens and leukotriene A, or with xenobiotics and products of LPO (Spitz *et al.*, 1991). These reactions are often catalysed by GSH transferases.

GSH-Px (E.C. 1.11.1.9.) catalyses the reductive destruction of H_2O_2 (eqn 17) and organic hydroperoxides (ROOH), using GSH as an electron donor (eqn 18) (for a review on structure and function of peroxidases, see Spallholz and Boylan, 1991).

$$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O \tag{18}$$

Oshino and Chance (1977) pointed out that in contrast to liver peroxisomes, H_2O_2 is destroyed in mitochondria and cytoplasm mainly by GSH-Px and not by CAT. GSH-Px activity of human brain amounts to approximately 70 U/mg protein (Marklund *et al.*, 1982). GSH-Px is a selenium-dependent enzyme (Rotruck *et al.*, 1973; Flohe *et al.*, 1973) and accounts for about one-fifth of total brain selenium (Prohaska and Ganther, 1976). In perfused rat brain, activities of GSH-Px and GSSG-Rd are highest in the striatum. GSH-Px, but not GSSG-Rd, activity was high in SN (Brannan *et al.*, 1980a,b). This points towards a high need for peroxide-detoxifying enzymes in

dopaminergic neurons. However, GSH-Px activity is more pronounced in glial cells than in neurons. Comparing primary cultures of murine astrocytes and neurons with respect to their content of total glutathione (GSH + GSSG), differentiated astrocytes contained about 16-fold higher levels (~16 nmol/mg protein) than neurons (Raps *et al.*, 1989). The overall concentration of GSH in rat brain is about 2 mM. The ratio GSH/GSSG is roughly 10:1 or higher in favor of the reduced form (Cooper *et al.*, 1980; Rehncrona *et al.*, 1980).

Since MAO is also predominantly localized in glial cells (see Section 4.1.1), deamination of catecholamines appears to be linked to GSH-Px content (Maker *et al.*, 1981; Spina and Cohen, 1989).

In addition to reaction with H_2O_2, the selenium-dependent GSH-Px is postulated to act together with phospholipase A_2 in converting potentially harmful phospholipid hydroperoxides (LOOH) to free fatty acid alcohols (ROH) via production of lysophospholipids and free fatty acid hydroperoxides (ROOH; 19) (van Kuijk *et al.*, 1987).

$$\text{LOOH} \xrightarrow{\text{PLA}_2} \text{lysophospholipid} + \text{ROOH} \tag{19}$$

$$\text{ROOH} + 2\text{GSH} \xrightarrow{\text{GSH-Px}} \text{ROH} + \text{GSSG} + \text{H}_2\text{O}$$

Moreover, a specific phospholipid hydroperoxide GSH-Px has been described (Ursini *et al.*, 1982), which reduces directly the hydroperoxide moiety of the still esterified fatty acid to phosphatidylglycerol without the necessity of phospholipase A_2 activity. This prevents successive formation of prostanoids and lysolipids, which otherwise would affect cellular metabolism and destabilize membranes. GSH is located in a key position of cellular defense against free radical-mediated injury (Benzi *et al.*, 1990, 1991). Protective potency against membrane protein oxidation (Section 3.1.3.2; Reglinski *et al.*, 1988), lipid oxidation (Section 3.1.3.1; Thomas *et al.*, 1990) and chelation of free haem (Shviro and Shaklai, 1987) has been ascribed to GSH. In addition, maintenance of the proper GSH/GSSG ratio (Miller *et al.*, 1990) may be of significance in the metabolic regulation of the cell. Gilbert (1982) speculated that modulation of the thiol/disulfide ratio *in vivo* may serve as a 'third messenger' in response to cyclic adenosine monophosphate levels, and that the activity of key enzymes of glycolysis/gluconeogenesis may be regulated in response to changing thiol/disulfide ratios. However, since regeneration of GSH is dependent on NADPH, activity of glucose-6-phosphate dehydrogenase could be rate limiting for the activity of GSSG-Rd (Scott *et al.*, 1991). Since GSH synthase is dependent on ATP, the overall pool of GSH is linked to oxidative phosphorylation, implying that impairment of mitochondrial respiration could lead to decreased synthesis of GSH (Section 3.2.1; Meister, 1991). Thus, a proper balance of antioxidant enzyme activities and reducing equivalents (NADH, NADPH, GSH, ascorbate) is crucial for optimal cell function and resistance to oxidative stress.

SOD (E.C. 1.15.1.1.) are metalloenzymes that are widely distributed among oxygen-consuming organisms (yeasts, plants, animals). McCord and Fridovich (1969) discovered $(O_2)^{\cdot-}$ to be a substrate for a copper- and zinc-containing protein (Fig. 2), formerly known as 'haemocuprein', in which copper is associated with enzymatic activity, while zinc serves as a stabilizer of protein structure. Interestingly, a manganese-dependent (MnSOD; Keele *et al.*, 1970) and an iron-dependent SOD were first characterized in *Escherichia coli* (FeSOD; Yost and Fridovich, 1973). Localization of these enzymes is very different, indicating functional changes during the evolutionary history of SOD. MnSOD in eucaryotic cells is strictly a mitochondrial enzyme in the inner membrane and is synthesized by nuclear genes (Autor, 1982; Wisp *et al.*, 1989). It resembles the FeSOD found in procaryotes, while cytosolic and peroxisomal CuZnSOD (Keller *et al.*, 1991) are different from MnSOD with respect to amino acid sequence and secondary structure (Harris *et al.*, 1980), supporting the idea for an endosymbiotic origin for mitochondria (Steinman and Hill, 1973; Beyer *et al.*, 1991). In rat brain, SOD is homogeneously distributed (Thomas *et al.*, 1976) with respect to brain region. In human grey matter, CuZnSOD amounts to 3.7 μg/mg protein, almost equalling the amount of CuZnSOD in liver (Hartz *et al.*, 1973). In brains of mice, total SOD activity was reported to be 408 U/mg protein (Grankvist *et al.*, 1981), while in rat brain only 3 U/mg protein were measured (Peeters-Joris *et al.*, 1975).

The subcellular localization of brain SOD is highest in cytoplasm.

Mitochondria and microsomes showed only 13–15% of cytoplasmic levels. Glial cells from rat cortex contain higher specific activity of SOD than neurons. Several studies (Fridovich, 1986a) have demonstrated a direct toxicity of $(O_2)^{\cdot-}$ without invoking $(HO)^{\cdot}$ produced by the metal catalyzed Haber–Weiss reaction (eqn 20). The direct reaction of $(O_2)^{\cdot-}$ with H_2O_2 is very unlikely to proceed because of a reaction rate constant of $10^{-4}\,M^{-1}\,sec^{-1}$ (Rigo *et al.*, 1977).

$$Fe(chelate)^{3+} + (O_2)^{\cdot-} \leftrightarrow Fe(chelate)^{2+} + O_2 \tag{20}$$

$$Fe(chelate)^{2+} + H_2O_2 \rightarrow Fe(chelate)^{3+} + HO^- + HO^{\cdot}$$

Evidence for toxicity of superoxide is exemplified by the following observations: bovine liver CAT is inactivated by $(O_2)^{\cdot-}$ but protected by SOD (Kono and Fridovich, 1982); exposure of a purified GSH-Px to an enzymatic source of $(O_2)^{\cdot-}$ and H_2O_2 causes inactivation of the enzyme, which is preventable by SOD, but not by CAT, suggesting, in each case, that superoxide-mediated cytotoxicity is not dependent on dismutation to H_2O_2 (Blum and Fridovich, 1985). SOD provide *in vivo* cellular protection by virtue of their ability to catalytically dismute $(O_2)^{\cdot-}$ (Fridovich, 1975, 1986b). Failure of SOD could result in increased production of $(O_2)^{\cdot-}$ during respiratory bursts of phagocytic leukocytes (Babior, 1982) and could aggravate inflammatory processes and reperfusion injury (McCord, 1987). Thus, biosynthesis of CAT, peroxidases and SOD have to be rigorously controlled to ensure protection.

Mechanisms of regulation of enzyme synthesis in eucaryotes depend on many diverse factors, including age, organ, developmental stage, prevailing hormone profile and the availability of active site cofactors. However, just as our understanding of genetic regulation has been helped by studies of bacteria, studies of the bacterial response to H_2O_2 have given general insight into how cells defend themselves against deleterious oxidants. *E. coli* and *Salmonella typhimurium* respond to H_2O_2 with the induction of synthesis of over 30 proteins, including CAT, GSSG-Rd and alkylhydro-peroxidase. Sensing of the H_2O_2 is achieved by a DNA-binding protein, the OxyR protein. It is encoded by a genetic locus, the OxyR regulon, and assumed to be a redox-sensing protein capable of reversibly altering its conformation in response to the prevailing redox environment of the cell. For eucaryotes, the regulatory mechanisms of antioxidant enzymes await elucidation. Since spontaneous or catalytic dismutation of $(O_2)^{\cdot-}$ by SOD provides cells with H_2O_2, cellular response must not only elevate SOD activity to counteract $(O_2)^{\cdot-}$ toxicity, but those of CAT and of GSH-Px as well. If SOD activity is increased selectively in brain of transgenic mice by introduction of the CuZnSOD gene, a significant increase of LPO (measured as malondialdehyde (MDA); Section 3.1.3.4) in the pyramidal cells of Ammon's horn and the granule cells of gyrus dentate can be detected (Ceballos-Picot *et al.*, 1991). In contrast to GSH-Px, levels of CuZnSOD mRNA and protein, as well as susceptibility to LPO increase with age in mice (De Haan *et al.*, 1992), suggesting involvement of ROS in aging, trisomy 21 (Down's syndrome) and possibly neurodegenerative diseases.

3.1.2.2. *Antioxidants.* Excessive concentrations of ROS can have serious effects on membranes, nucleic acid bases and proteins (Section 3.1.3). If uncontrolled, mutations and membrane damage could lead to cell death. To minimize damage, defensive control systems exist. Besides enzymes, there are hydrophilic- and lipophilic-soluble molecules called 'antioxidants', scavenging free radicals to prevent destruction of cellular biomolecules crucial for cell viability. Non-enzymatic biological antioxidants include tocopherols, carotenoids, quinones, bilirubin, steroids, ascorbate, uric acid, GSH, cysteine and metal-binding proteins, such as ferritin (Krinsky, 1992).

Iron-binding proteins (transferrin, haemosideroin and ferritin) (Halliwell and Gutteridge, 1990) remove iron from the cytosol so that it is no longer able to catalyse oxidation of biomolecules (eqn 21) through formation of alkoxylradicals $(RO)^{\cdot}$ or $(HO)^{\cdot}$.

$$ROOH + Fe^{2+} \rightarrow (RO)^{\cdot} + HO^- + Fe^{3+} \tag{21}$$

The most important of these seems to be ferritin, which will be discussed later (Section 4.1).

Due to their long, conjugated double-bond systems, carotenoids are excellent substrates for radical attack, thus scavenging singlet O_2 or alkoxylradicals in membranes. Some of the reaction products have been described recently (Kennedy and Liebler, 1991; Handelman *et al.*, 1991; Mordit

et al., 1991), and the role of carotenoids in the risk of lung cancer, coronary heart disease and cataract has been discussed (Canfield *et al.*, 1992; Rousseau *et al.*, 1992). However, to date, there is no study on the role of carotenoids in neurodegeneration. The same is true for bilirubin and uric acid, the end-products of haem metabolism and of purine metabolism, respectively. In addition to the features of GSH discussed in Section 3.1.2.1, either GSH or cysteine can react directly with $(HO)^{\cdot}$, generating thiyl radicals ($RS^{\cdot}$; reaction rate constant $> 10^9\ M^{-1}\ sec^{-1}$), which can also be formed when GSH is oxidized by peroxidases or by O_2 in the presence of copper or iron (Rowley and Halliwell, 1982). Although less reactive than $(HO)^{\cdot}$, thiyl radicals also react with biomolecules, suggesting that thiol compounds are not ideal antioxidants.

Coenzyme Q (Q) in its reduced form (ubiquinol) is known to inhibit LPO in subcellular membranes (Mellors and Tappel, 1966; Forsmak *et al.*, 1991), either by reducing the α-tocopheroxyl radical $(TO)^{\cdot}$ back to α-tocopherol (TOH) (Kagan *et al.*, 1990) or by reacting directly with radicals. However, by far the most information exists, to date, for vitamin E and vitamin C (ascorbate).

Vitamin E is the term used for eight naturally occurring fat-soluble nutrients (Fritsma, 1983). Four compounds bear a saturated phytyl side chain and differ only with respect to number and position of methyl groups at the chromanol ring (α, β-, γ-, and δ-tocopherols). Four other compounds contain phytyl side chains with three double bonds (α-, β-, γ-, and δ-tocotrienols). However, TOH predominates in many species. The phytyl side chain in the 2-position facilitates incorporation and retention of TOH in biomembranes, while the active site of radical scavenging is the 6-hydroxyl group of the chromanol ring (Lucy, 1972; Burton and Ingold, 1981). Since eight stereoisomers exist, the name tocopherol should not be used without clarification of stereochemistry (Horwitt, 1991). However, *R,R,R*-tocopherols are the only stereoisomers to occur in nature (Slover and Thompson, 1981; Cohen *et al.*, 1981; Vecchi *et al.*, 1990).

The most widely accepted physiological function of TOH is its role as a scavenger of free radicals. Thus, it prevents oxidant injury to polyunsaturated fatty acids and thiol-rich proteins in cellular membranes and cytoskeleton. It is thought to preserve the structure and functional integrity of subcellular organelles (Chow, 1991). Each TOH molecule can react with two peroxyl radicals (eqns 22, 23).

$$TOH + R^{\cdot} \rightarrow (TO)^{\cdot} + RH \tag{22}$$

$$(TO)^{\cdot} + ROO^{\cdot} \rightarrow \text{ROO-TO adduct} \tag{23}$$

or

$$(TO)^{\cdot} + DH \rightarrow TOH + D^{\cdot} \tag{24}$$

The first product is the $(TO)^{\cdot}$, which is a resonance-stabilized, oxygen-centered radical. It can react with other peroxyl radicals to form stable adducts (eqn 23), some of which have already been isolated (Matsumoto *et al.*, 1986), or can react with electron donors (e.g. ascorbate) to become re-reduced to TOH (eqn 24; Bendich *et al.*, 1984, 1986).

The absorption, transport and metabolism of TOH in animals has been reviewed on several occasions (Bjorneboe *et al.*, 1989; Drevon, 1991). TOH is transferred from circulating lipoproteins to the brain, spinal cord and peripheral nerves and muscle by unknown mechanisms (Sokol, 1989). There is no uniform distribution of TOH in the central and peripheral nervous system (Vatassery *et al.*, 1984a).

In contrast to other brain regions, the cerebellum is particularly active in the metabolism or utilization of TOH (Vatassery, 1987). During experimental TOH deficiency, nerve tissue retains a greater percentage of TOH than do serum, liver and adipose tissue (Goss-Sampson *et al.*, 1988). Morphological and functional studies performed on experimental TOH-deficient rats have revealed axonal dystrophy and degeneration of peripheral nerve. This can be aggravated by increasing dietary polyunsaturated fatty acids providing increased quantities of peroxidizable substrate and reduced by feeding a synthetic antioxidant (ethoxyquin) (Southam *et al.*, 1991). These experiments provide evidence in favour of an antioxidant role for TOH in the nervous system (Nelson, 1987). In brain, TOH is predominantly localized in the mitochondrial, microsomal and synaptosomal fractions (Vatassery *et al.*, 1984b), suggesting that protection by TOH from peroxidative damage

to subcellular membranes may be important for mitochondrial energy production or microsomal enzyme activity (Chow and Gairola, 1984).

Ascorbic acid is an extremely water-soluble antioxidant essential for humans, primates and guinea pigs, but not for rodents, which can synthesize it from glucose. Ascorbic acid serves as a cofactor in several iron-dependent hydroxylases (Padh, 1991) important for collagen synthesis, (prolyl- and lysyl-hydroxylases), for carnitine biosynthesis (6-*N*-trimethyl-L-lysine-hydroxylase) and for catabolism of tyrosine (4-hydroxyphenyl-pyruvate-hydroxylase). Two major functions of ascorbate are support of the synthesis of norepinephrine and α-amidation of neurohormones, explaining in part its higher concentrations in brain and endocrine tissues (adrenal gland). The copper-containing DA-β-hydroxylase (E.C. 1.14.17.1.) catalyses the final step in the synthesis of norepinephrine (eqn 25), the hydroxylation of DA.

$$\text{DA} + \text{ascorbate} + \text{O}_2 \rightarrow \text{norepinephrine} + \text{semidehydroascorbate}^{\cdot} \quad (25)$$

Ascorbate is most likely required by hydroxylases to maintain iron or copper at the active enzyme site in the reduced form, since it is necessary for hydroxylation. The semidehydroascorbate radical is not very reactive (Bielski and Richter, 1975; Rose, 1989). It decays by disproportionation to ascorbate and dehydroascorbate (the latter subsequently degrades to oxalic acid and L-threonic acid), rather than acting as a reactive free radical. Reaction of ascorbic acid with $(OH)^{\cdot}$ is rapid and diffusion-dependent ($K \sim 7.2 \times 10^9$–$1.3 \times 10^{10}\ \text{M}^{-1}\,\text{sec}^{-1}$) (Cabelli and Bielski, 1983). $(O_2)^{\cdot-}$ oxidizes ascorbic acid with a rate constant of 10^4–$10^5\ \text{M}^{-1}\,\text{sec}^{-1}$ (Bielski *et al.*, 1985). Besides direct scavenging of radicals, ascorbic acid is known to have a number of physiological effects (Padh, 1991), with a role in leukotriene biosynthesis (Schmidt *et al.*, 1988), tetrahydrofolate reduction (Stone and Townsley, 1973), immunity (Anderson, 1984) and cancer (Wittes, 1985). Many membrane proteins are sensitive to tissue redox state (Levine, 1983), such as the NMDA receptor, thought to be involved in neuronal degeneration in seizure and ischemia (Choi, 1988a). It is inhibited by ascorbate, whereas reductants, such as dithiothreitol and penicillamine, which break protein disulfide bonds, potentiate receptor function (Majewska *et al.*, 1990). The mechanism of this effect is not fully understood, but it must be important for survival of cells in cerebral ischemia.

Furthermore, an important protective action of ascorbic acid is its ability to act synergistically with TOH in the inhibition of various oxidation reactions (McCay, 1985; Craw and Depew, 1985; Bendich *et al.*, 1986; Burton and Ingold, 1986; Niki, 1987a,b). Packer *et al.* (1979) have shown in pulse radiolysis studies that in solution $(TO)^{\cdot}$ reacts rapidly with ascorbic acid ($K \sim 1.55 \times 10^6\ \text{M}^{-1}\,\text{sec}^{-1}$) to yield TOH again. This synergism seems to function in liposomal membranes as well (Scarpa *et al.*, 1984; Niki *et al.*, 1985; Doba *et al.*, 1985). Some studies indicate that ascorbic acid helps to maintain tissue levels of TOH *in vivo* (Hruba *et al.*, 1982; Bendich *et al.*, 1984). However, by contrast, other *in vivo* studies found no evidence for an interaction between TOH and ascorbic acid (Yen *et al.*, 1985; Burton *et al.*, 1990). Thus, the interaction of TOH and ascorbate remains an open question for discussion.

GSH was observed to protect against LPO *in vitro* (Reddy *et al.*, 1982; Wefers and Sies, 1988; Graham *et al.*, 1989), probably involving a GSH-dependent heat labile factor(s) capable of reducing $(TO)^{\cdot}$. In addition, GSH is needed to reduce dehydroascorbate to ascorbate (eqn 26) by a dehydroascorbate reductase, or even non-enzymatically (Winkler, 1992).

$$\text{dehydroascorbate} + 2\text{GSH} \rightarrow \text{GSSG} + \text{ascorbate} \quad (26)$$

Moreover, an NADH-dependent semidehydroascorbate reductase is thought to be involved in the regeneration or restoration of ascorbate (Diliberto *et al.*, 1982; Chow, 1988). Under certain circumstances, ascorbic acid functions as a pro-oxidant rather than an antioxidant. Similarly to superoxide, ascorbate is able to reduce Fe^{3+} to Fe^{2+}, and in the presence of H_2O_2, it can promote $(HO)^{\cdot}$ production. *In vitro* concentrations of ascorbate up to 0.2 mM can induce LPO in rat liver microsomes (Samuni *et al.*, 1983; Shinar *et al.*, 1983). By contrast, at concentrations above 0.2 mM, it protects against LPO. Normal cytosolic concentrations would favour GSH over ascorbic acid as a cytosolic antioxidant in most tissues (McCay, 1985). However, if GSH is compromised *in vivo* by administration of its antimetabolite, L-buthionine-(*S*,*R*)-sulfoximine, ascorbate is utilized to protect against cell damage due to GSH deficiency (Martensson and Meister, 1991). If enzymatic or non-enzymatic antioxidants are inactivated or depleted, ROS can trigger various deleterious

events, including oxidation of lipids, proteins and nucleic acid bases, as described in the next section.

3.1.3. *Consequences of Excess of Reactive Oxygen Species*

3.1.3.1. *Lipid peroxidation.* One hypothesis to explain mechanisms of cellular aging and chronic progressive cell degeneration suggests the impairment of enzymatic and/or non-enzymatic antioxidant defence (Section 3.1.2), resulting in uncontrolled damage of biomolecules by ROS. In addition, presence of endogenous or exogenous toxins could affect cellular antioxidant defence systems. Thus, a common, but not necessarily primary, cause of oxidation of lipids, proteins and DNA could be an overflow and/or decreased detoxification of ROS. However, primary targets of ROS depend on sites of formation. Since compartmentalization is crucial for cell viability, severe damage to membrane structure could be an irreversible step towards cell death. Impairment of membrane function can be triggered either directly, by oxidation of polyunsaturated fatty acids of lipids (called LPO), or indirectly, by mechanisms leading to decreased lipid synthesis, decreased fatty acid desaturation, impaired redox equilibrium or increased activities of lipases.

LPO involves the direct or metal-catalyzed reaction of oxygen and unsaturated fatty acids associated with polar lipids, generating free radical intermediates and semistable peroxides (Tappel, 1973). Since subcellular membranes in brain cells contain high amounts of polyunsaturated fatty acids, formation of a single carbon-centered radical within a membrane can lead to peroxidation of many fatty acids. This can occur when O_2 is present. The complex process of LPO is commonly described by three stages:

(1) Initiation: the generation of a radical with sufficient reactivity to extract hydrogen atoms from methylene groups of fatty acids [$(HO)^{\cdot}$; $(HO_2)^{\cdot}$];

(2) Propagation: reaction of these radicals to yield another radical, which likewise is capable of generating more radicals (radical chain reaction);

(3) Termination: recombination of two radicals or reactions yielding stabilized radicals no longer capable of propagating chain reactions.

$(HO_2)^{\cdot}$ and $(HO)^{\cdot}$, but not $(O_2)^{\cdot-}$, are able to extract hydrogen from allylic or *bis*-allylic positions of polyunsaturated fatty acids (Girotti, 1985; Kappus, 1985). The carbon radicals tend to be stabilized by molecular rearrangements to form conjugated dienes. In the presence of sufficient amounts of O_2, peroxyl radicals are formed ($K = 10^9$–$10^{10}\,M^{-1}\,sec^{-1}$). In media of low hydrogen-donating capacity, the peroxyl radical is free to react further by competitive pathways, resulting in cyclic peroxides, double-bond isomerization or formation of dimers and oligomers (Gardner, 1989). Thus, random peroxidation of, for example, arachidonic acid could give a complex mixture of isomers of cyclic peroxides and hydroperoxides. If peroxidation of free fatty acids is driven enzymatically by cyclooxygenases or lipoxygenases, stereospecific hydroperoxides and endoperoxides are produced, which are precursors of eicosanoids (prostaglandins, thromboxanes, leukotrienes). If the peroxyl radical extracts a hydrogen atom from an adjacent fatty acid to yield another lipid radical ($L^{\cdot}$), which subsequently reacts with O_2, a hydroperoxide (LOOH) chain reaction is propagated. Other peroxyl radical reactions are the β-scission, intermolecular addition and self-combination. These reactions and those of phenols (e.g. TOH), aromatic amines and conjugated polyenes (e.g. β-carotene) with various radicals (carbon- and oxygen-centered) can terminate radical chain reactions. If LOOHs are not removed by GSH-dependent peroxidases (see Section 3.1.2.1) transition metal ions, especially iron and copper, can catalyse the decomposition of peroxides to form either alkoxyl ($LO^{\cdot}$) alkyl ($L^{\cdot}$) or $(OH)^{\cdot}$ – radicals (eqns 27, 28; $K = 1.5 \times 10^3\,M^{-1}\,sec^{-1}$; Garnier-Suillerot *et al.*, 1984).

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + HO^- + LO^{\cdot} \quad (27)$$

$$LOOH + Fe^{3+} \rightarrow Fe^{2+} + H^+ + LOO^{\cdot} \quad (28)$$

These radicals could initiate a secondary propagation of radical chain reactions called LOOH-dependent LPO (Bast and Haenen, 1984). Consequently, iron, or complexes of iron, with low molecular iron chelators stimulate LPO by lipid decomposition reactions (Gutteridge *et al.*, 1984). Moreover, ferritin, an iron-storage protein holding 4500 mol of Fe^{3+} per mol of protein, is able

to stimulate LPO by releasing Fe^{2+} (Wills, 1966), and ascorbate enhances the rate of ferritin-stimulated LPO (Gutteridge *et al.*, 1983). In contrast to popular belief, alkoxyl radicals of polyunsaturated fatty acids do not significantly abstract hydrogens, but rather, are channeled into epoxide formation through intramolecular rearrangement (Gardner, 1989). Moreover, besides homolytic reactions of polyunsaturated fatty acids, one has to keep in mind the susceptibility of hydroperoxides to heterolytic transformations, such as nucleophilic displacement and acid-catalysed rearrangement (Gardner, 1989).

In 1990, Babbs and Steiner published a computational model of kinetics of LPO in a two-compartment model system (membrane and cytosol), assuming an iron-catalyzed, $(O_2)^{\cdot-}$ driven Fenton reaction as the initiator of LPO (eqn 20). Kinetic interactions of up to 109 simultaneous enzymatic and free radical reactions thought to be involved in the initiation, propagation and termination of LPO were calculated using rate constants from the literature. From these model studies it was concluded that:

"1. Segregation and concentration of lipids within membrane compartments promote chain propagation;
2. In the absence of antioxidants, computed concentrations of LOOH increase linearly at a rate of 40 μM/min during oxidative stress;
3. LPO is critically dependent on O_2 concentration and the modeled dependence is similar to the experimental function;
4. LPO is rapidly quenched by the presence of TOH-like antioxidants, SOD and CAT;
5. Only small (1 to 50 μM) amounts of 'free' iron are required for initiation of LPO;
6. Substantial LPO occurs only when cellular defense mechanisms have been weakened or overcome by prolonged oxidative stress. Hence understanding of the balance between free radical generation and antioxidant defense systems is critical to the understanding and control of free radical reactions in biology and medicine".

Dependent on the fatty acid hydroperoxide (primary product of oxidation of unsaturated fatty acids with O_2), and on catalytic degradation by either iron complexes or by NADPH cytochrome P450 reductase, a huge range of secondary products of LPO is formed. These include conjugated dienes (Corongiu *et al.*, 1989), hydrocarbon gases (e.g. ethane, ethene from linoleic acid; Burk and Ludden, 1989) and carbonyl compounds (e.g. MDA, alkenals, alkadienals and α-β-unsaturated aldehydes; Kaneko *et al.*, 1987; Yoshino *et al.*, 1991; Esterbauer *et al.*, 1991). Carbonyl compounds are formed by β-scission of alkoxyl radicals or thermic- or metal-catalysed degradation of cyclic endoperoxides. The latter process produces MDA. In addition, it is suggested that MDA can also be formed *in vivo* as a byproduct of eicosanoid biosynthesis (Hecker and Ullrich, 1989). Various techniques exist to evaluate products of LPO in tissues (Gutteridge and Halliwell, 1990; Hageman *et al.*, 1992), but all are limited either with respect to sensitivity, specificity or practicability, since the most accurate assays for measuring lipid peroxides are the most chemically sophisticated, requiring sample preparation under inert gas to ensure no further peroxidation during handling of lipid material (e.g. gas–liquid chromatography/mass spectrometry; Hughes *et al.*, 1986). Measurement of MDA has been employed to detect and quantify LPO in a variety of chemical and biological matrices (Valenzuela, 1991), but there is increasing doubt of the specificity of MDA as a quantitative indicator of *in vivo* preformed lipid peroxides (Choi and Yu, 1990; Janero, 1990). In order to release MDA from cyclic endoperoxides, elevated temperature is often applied. The MDA released is trapped by thiobarbituric acid to yield a pink pigment (Nair and Turner, 1984). This step is seldom done under inert gas conditions. The levels of pigments resulting from reaction of MDA and various other aldehydes with thiobarbituric acid (called thiobarbituric acid reactive substances, TBARS) are indicative of levels of TBARS originating from both preformed lipid peroxides *in vivo* and newly formed peroxides *in vitro* during incubation (Götz *et al.*, 1993). Since assay of TBARS is influenced by many experimental conditions (e.g. pH, temperature, O_2, antioxidants, buffers, transition metals; for a review, see Janero, 1990), measurement of TBARS is not sufficient to give precise evaluations of LPO in pathophysiological states. At best, it can be an empirical indicator of the potential occurrence of peroxidative lipid injury *in vivo* and of the susceptibility of tissues to oxidative stress *in vitro*. Thus, whenever possible, a combination of methods measuring primary and secondary, as well as tertiary, products of LPO (amino acid

adducts, nucleotide adducts and glutathionyl conjugates) is advisable. Excellent overviews concerning analytical aspects of monitoring oxidative stress *in vivo* are provided by Saran and Bors (1991), Hageman *et al.* (1992) and Pryor and Godber (1992).

3.1.3.2. *Oxidation of proteins.* ROS can directly oxidize free or protein-bound amino acids, leading to deactivation of enzymes (Stadtman, 1990; Stadtman and Oliver, 1991; Stadtman and Berlett, 1991). Cysteine, methionine, histidine and tryptophan are preferentially oxidized, resulting in sulfenic, sulfinic or sulfonic acids from thio-containing amino acids and in histidine- and tryptophan-endoperoxides, which subsequently degrade (Sies, 1986). Oxidation of thiols in proteins is often involved in regulation of enzyme activity, such as glucose-6-phosphate dehydrogenase, pyruvate kinase, brain adenylate cyclase, γ-glutamyl-synthetase and others (Elstner, 1990). Carbonyl compounds can be attacked by amino groups. Increase of MDA *in vivo* could result in both intra- and intermolecular cross links of proteins, giving fluorescent products (conjugated imines, R—N=CH—CH=CH—NH—R′, fluorescence maximum at 470 nm with excitation maximum at 395 nm; Tappel, 1973). Interestingly, accumulating lipofuscin pigments in the aging brain and heart (Brizzee and Ordy, 1979; Brunk and Ericsson, 1972; Masoro, 1981; Mann *et al.*, 1978) show characteristic fluorescence spectra similar to those of MDA cross-linked proteins. This may possibly result from interactions between ROS and autophagocytosis (Brunk *et al.*, 1992). Histological and biochemical studies of lipofuscin have provided evidence that they contain lipid–protein adducts, which are extractable by mixtures of chloroform plus methanol (Davies, 1988). Besides lipids and proteins, lipofuscin contains a high concentration of metal ions, such as zinc, copper and iron. Lipofuscin-like fluorophores can result from reactions between oxidized ascorbic acid and glutamine (Yin and Brunk, 1991; Yin, 1992). Histological and ultrastructural evidence in hippocampal pyramidal and Purkinje neurons of rat brain indicates that lipofuscin probably originates from lysosomes (Masoro, 1981; Schlote and Boellaard, 1983) or mitochondria (Glees and Hasan, 1976; Brizzee and Ordy, 1979; Heinsen, 1979). Since lipofuscin deposition is promoted by very different factors, including inherited abnormalities of fat metabolism (e.g. in patients suffering from abetalipoproteinemia), administration of inhibitors of lysosomal proteases or feeding diets deficient in TOH or abnormally rich in polyunsaturated fatty acids (Halliwell and Gutteridge, 1989), it seems more likely that increase in pigments with age results from impairment of lysosomal functions (degradation of lipids and proteins) rather than oxidative stress outside the lysosomes (Youngman *et al.*, 1992; Stadtman, 1992). Interestingly, degeneration of striatal tissue in HD is accompanied by a massive accumulation of the fluorescent pigment lipofuscin in the brain. However, the role of lipofuscin pigment in cellular aging is still unknown (Amenta *et al.*, 1988).

Proteins that have been oxidatively modified become excellent substrates for degradation by proteases (Davies and Goldberg, 1987), probably because of concomitant denaturation and subsequent increase in their hydrophobicity (Pacifici *et al.*, 1989). High molecular weight proteolytic complexes, called ingensin, macropain, macrosin, proteasome, multicatalytic protease or macroxyproteinase (Rivett, 1985; Pacifici *et al.*, 1989), are assumed to be responsible for the degradation of oxidatively modified proteins, providing amino acids for *de novo* synthesis. Such modifications mark enzymes for degradation by proteases (Stadtman, 1992).

α-β-Unsaturated hydroxy-alkenals are far more toxic than is MDA (for a review, see Esterbauer *et al.*, 1991). *Trans*-4-hydroxy-2-nonenal is the most prominent of these (Van Kuijk *et al.*, 1986). It probably results from peroxidation of arachidonic acid (Pryor and Porter, 1990). It has been shown to inhibit protein synthesis and to interfere with growth of bacterial and animal cells in culture. Hydroxyalkenals are mainly detoxified by alcohol and aldehyde dehydrogenases, or by forming adducts with cysteine or GSH, the latter process being catalysed by GSH transferases (Witz, 1989; Spitz *et al.*, 1991). In addition, adducts of *trans*-4-hydroxy-2-nonenal with nucleosides have been identified (Hageman *et al.*, 1992). This makes it clear that oxidative damage to lipids can affect proteins and DNA by secondary products of LPO. In addition, it is likely that α-β-unsaturated aldehydes are potentially able to serve as cellular messengers interfering with signal transduction pathways. This hypothesis is supported by observations that hydroxyalkenals can stimulate oriented migration of neutrophils (chemotaxis; Curzio, 1988; Curzio *et al.*, 1990) and phospholipase C activity (Rossi *et al.*, 1990).

3.1.3.3. *Nucleic acid damage caused by reactive oxygen species.* There is increasing interest in the potential role of ROS as mediators of metal-catalysed carcinogenesis (Klein *et al.*, 1991; Kasprzak, 1991) and in genetic changes occurring as a consequence of ionizing radiation, chemical carcinogens and various other tumor promoters (e.g. phorbolesters; Frenkel, 1992). Besides ribonucleic acids, DNA is the most important factor damaged by ROS *in vivo* (Kasai *et al.*, 1986; Adelman *et al.*, 1988; Richter *et al.*, 1988; Simic *et al.*, 1989), resulting in the disruption of transcription, translation and DNA replication. The amount of oxidative damage, even under normal physiological conditions, may be quite extensive, with estimates as high as one base modification per 130 000 bases in nuclear DNA (Richter *et al.*, 1988). Damage to mitochondrial DNA is estimated to be as much as one per 8000 bases (Richter, 1988, 1992). DNA–DNA and DNA–protein cross links, sister chromatid exchange, single- or double-strand breaks and base modifications are reported to occur due to reactions of ROS with DNA (Teebor *et al.*, 1988; Simic *et al.*, 1989). In principle, all four DNA bases can be oxidatively modified, thymidine being most susceptible to ROS. As for the reaction mechanisms, it is thought that H_2O_2 interacts with metal ions (Fe, Cu) on DNA bases and the sugar backbone, causing site-specific $(HO)^{\cdot}$-mediated DNA damage. For example, a strong correlation exists between the concentration of H_2O_2 in culture medium and the degree of strand breaks in human peripheral lymphocytes (Cochrane *et al.*, 1987). In the presence of Fe^{2+} (micromolar range), H_2O_2 concentrations of even lower than 100 μM can induce strand breaks in cultured cells, an effect that can be inhibited by iron chelators (phenanthroline) or CAT, but not by SOD, suggesting involvement of $(HO)^{\cdot}$ or reactive iron–oxygen species. The nucleosides thymidine glycol and 8-hydroxy-2′-deoxyguanosine are considered to be biomarkers of DNA damage by ROS (Simic, 1991; Hageman *et al.*, 1992). These are specific since, in contrast to the free bases, they are not absorbed through the digestive system (Cathcart *et al.*, 1984) and can be measured by HPLC in urine using electrochemical detection (Kasai and Nishimura, 1986; Floyd *et al.*, 1986; Shigenaga and Ames, 1991; Halliwell and Dizdaroglu, 1992). In eucaryotes, several glycosylases, which act on DNA oxidation products, have been characterized, including a 3′ repair diesterase in yeast (Johnson and Demple, 1988), a mammalian endonuclease specific for oxidatively modified DNA (Doetsch *et al.*, 1986, 1987) and GSH transferases and peroxidases recognizing thymidine hydroperoxide as a substrate (Johnson and Demple, 1988). Simic (1991) has pointed out that not only do exogenous factors, such as ionizing radiation or chemicals (bleomycin, adriamycin, benzo(a)pyrene), increase urinary levels of hydroxylated nucleosides, but high dietary caloric intake and high metabolic rates correlate with urinary thymidine glycol and 8-hydroxy-2′-deoxyguanosine excretion (Cathcart *et al.*, 1984; Simic and Bergtold, 1991). It has been further documented that DNA repair is less efficient in older organisms (reviewed by Rao and Loeb, 1992). In contrast to the known inherited metabolic disorders, there is little evidence of DNA damage in relation to the pathophysiology of PD or AD (see Section 5.3). Of course, mutations in nuclear or mitochondrial DNA could be the ultimate cause of disturbed cellular metabolism leading to nerve cell death. On the other hand, chronic exposure of cells to ROS as a consequence of normal aging can be aggravated and accelerated by exogenously or endogenously produced toxins. This could be a cause of damage to biomolecules (Holmes *et al.*, 1992; Harman, 1992). Since the steady-state level of oxidized biomolecules ultimately will depend on the efficiency with which they are removed, much effort is being made to quantify markers of oxidative damage to proteins, lipids and nucleic acids. The topics dealt with in this Section are briefly summarized in Fig. 3.

3.2. Impairment of Energy Metabolism

3.2.1. *Defects in Energy Metabolism*

Defects in energy metabolism cause profound disturbances in the function of muscle or the brain. Such defects may be represented by myopathy, encephalopathy or encephalomyopathy, the latter concomitantly affecting both tissue types. In the post-absorptive state, the brain utilizes glucose predominantly, with regional variations in the metabolic rate, depending on the mental or motor task being performed (Sokoloff *et al.*, 1977; Kennedy *et al.*, 1978). Brain concentrations of glycogen are low (0.1 g/100 g fresh weight) (Sokoloff, 1989), and the role of fatty acids as oxidizable fuels

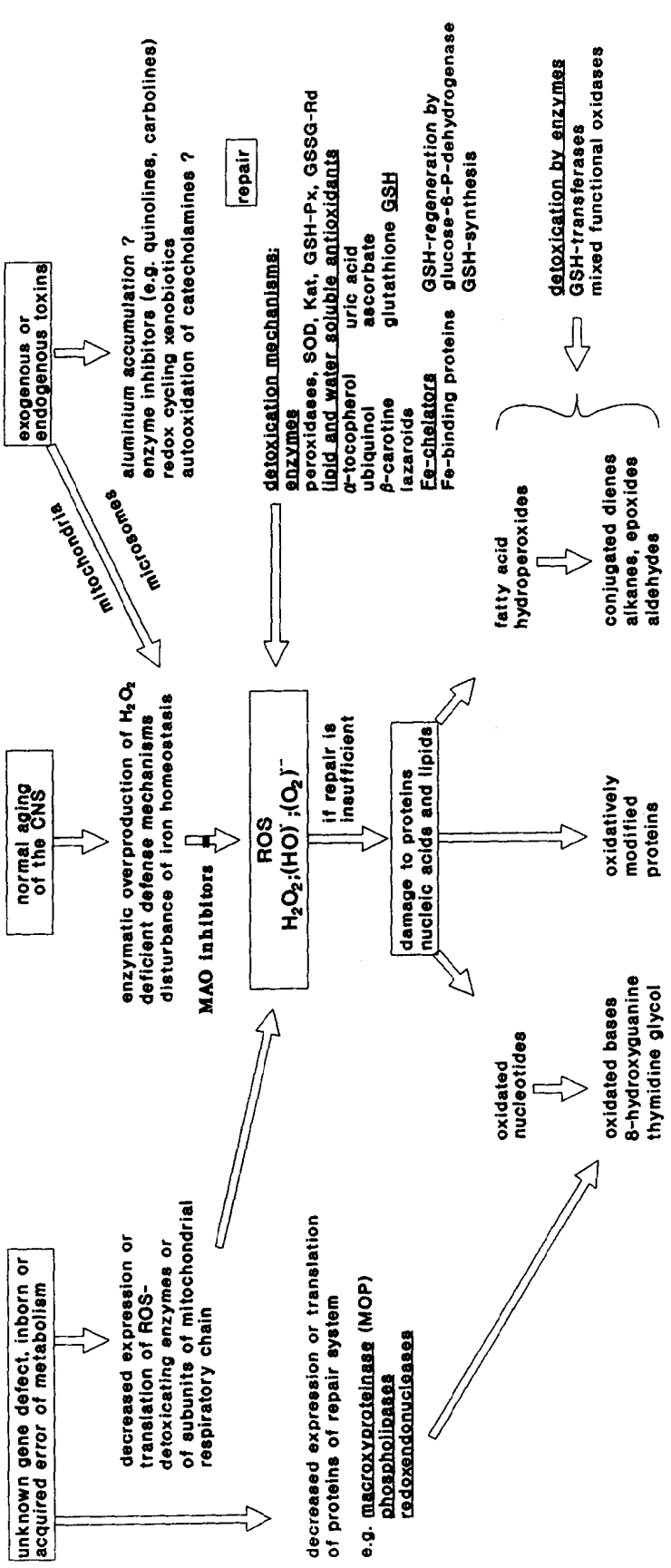

FIG. 3. Putative pathogenetic causes assumed to contribute to ROS toxicity, consequently leading to damage to biomolecules, if repair mechanisms become insufficient. Putative sites of therapeutic intervention are indicated by double lines crossing arrows.

for brain metabolism is considered to be negligible. Thus, brain tissue is extremely sensitive to fluctuations in the blood glucose concentration, since no satisfactory endogenous substitute exists. Only in prolonged fasting are ketone bodies formed in liver (D-β-hydroxybutyrate and acetoacetate), passively taken up by the brain from the bloodstream and utilized to produce acetyl-coenzyme A.

Most diseases with impairment of enzymes of glycolysis and mitochondrial metabolism are inherited and result in serious malfunction of the nervous system. They can be classified into five major groups, resulting in defects of:

(1) mitochondrial transport
(2) substrate utilization
(3) the Krebs cycle
(4) oxidation–phosphorylation coupling
(5) the mitochondrial respiratory chain.

The clinical picture of deficiencies in glucose utilization can be subdivided into groups in which myopathy is the predominant manifestation or in which brain dysfunction predominates (cerebellar ataxia, pyramidal signs and dementia). In this review, however, we focus on the role of putative defects in energy metabolism as a possible factor in neurodegenerative diseases.

In the majority of patients suffering from AD or PD, there seems to be no major genetic factor in its etiology (see, however, Section 5.3). Nevertheless, defects in mitochondrial and microsomal function could be involved causatively in the pathogenetic process, possibly by their ability to produce $(O_2)^{\cdot-}$ *in vivo*.

3.2.2. *Microsomal Production of Superoxide*

The body is continually threatened by toxic substances, which are inhaled, absorbed or ingested. Thus, enzymatic detoxication systems have been developed for elimination of toxic compounds (Minn *et al.*, 1991). For these purposes, and for the catalysis of oxidations of fatty acids and steroids, microsomal membranes (including membranes from lysosomes, peroxisomes and endoplasmic reticulum) possess a large number of enzymes (Jakoby and Ziegler, 1990). Microsomes contain two electron transport systems, one being dependent on NADH and consisting of NADH cytochrome b_5 reductase and cytochrome b_5 (needed for fatty acid acyl coenzyme A desaturase system), and the other involving NADPH-dependent cytochrome P450 reductase (also referred to as NADPH cytochrome c reductase) and many isoenzymes (Nebert *et al.*, 1989). Cytochrome P450 is involved in the oxidation of a wide range of substrates at the expense of O_2 (known as mono-oxygenation or mixed-function oxidation) and requiring a reducing agent (normally NADPH). The reaction stoichiometry is given in eqn (29).

$$2NADPH + O_2 + RH \rightarrow 2NADP^+ + H_2O + R—OH \tag{29}$$

However, normally more NADPH is oxidized and more O_2 is consumed than needed. The excess of O_2 can lead to production of $(O_2)^{\cdot-}$ (Gorsky *et al.*, 1984; Zhukov and Archakov, 1982). $(O_2)^{\cdot-}$ can be generated both from dissociation of the oxygenated complex of reduced cytochrome P450 (Sligar *et al.*, 1974) and from the auto-oxidation of cytochrome P450 reductase containing FAD and FMN (Nakamura and Yamazaki, 1969; Aust *et al.*, 1972). Like other flavoproteins, reaction of flavinsemiquinone with O_2 generates $(O_2)^{\cdot-}$. Although liver is the major organ involved in the P450-mediated metabolism, this process has also been detected in the brain (Mesnil *et al.*, 1984) of mice (Ravindranath and Anandatheerthavarada, 1989), rats (Warner *et al.*, 1988) and humans (Bahmre *et al.*, 1992). Immunocytochemical study of the rat brain P450 reductase using an antibody to the rat liver enzyme had revealed the presence of the enzyme in catecholaminergic neurons in SN, LC and the ventrolateral medullary region (Haglund *et al.*, 1984). Spectral quantification has revealed that the level of cytochrome P450 in brain microsomes is approximately 40 pmol/g tissue, which is roughly 0.25% of that found in the liver microsomes of control rats (Warner *et al.*, 1988), and 30–120 pmol/mg protein in human brain regions obtained at autopsy (Bahmre *et al.*, 1992). Regional variation in NADPH cytochrome c reductase activity was immunohistochemically observed in human brain stem neuronal cell bodies (Ravindranath *et al.*, 1990). Roles of P450 in

the CNS may include metabolism of xenobiotics (Das *et al.*, 1981), aromatization of androgens and estrogens by 2- and 4-hydroxylations (Reddy *et al.*, 1974) and formation of catecholestrogens (Paul *et al.*, 1977; Fishman and Norton, 1975). Their action in the CNS is linked to inhibition of catechol-*O*-methyltransferase (Ball *et al.*, 1972) and inhibition of TH (Lloyd and Weisz, 1978). In addition, it has been proposed (Sasame *et al.*, 1977) that NADPH cytochrome P450 reductase could be involved in the formation of the catecholamine neurotoxin 6-OHDA (Section 4.1.3) or in potentiation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced (MPTP) toxicity in mouse brain slices, *in vitro* (Pai and Ravindranath, 1991; Section 5.1). These results suggest that MAO is not the only enzyme producing neurotoxic metabolites of MPTP in brain. Moreover, a role for the cytochrome P450 enzymes in formation of carbolines (possible ligands for benzodiazepine receptors; Section 5.1) and of hallucinogenic indoleamine derivatives (*N*,*N*-dimethyltryptamine) has been suggested (Seth *et al.*, 1990).

It is important to learn more about the cytochrome P450 enzyme family in the CNS. This is because they have a putative role in the synthesis and metabolism of endogenous compounds and of xenobiotics, resulting in acute or chronic generation of toxic agents. Interestingly, Armstrong and colleagues (1992) reported that the risk of PD is more than twice as great for individuals with a P450 genetic polymorphism associated with deficient debrisoquine metabolism than in those without, implicating a genetic factor in pathogenesis of PD.

3.2.3. *Superoxide Production in Mitochondria*

In brain, the oxidation of NADH and $FADH_2$ produced in the Krebs cycle from various substrates in mitochondria is mediated by an electron transport chain consisting of flavoproteins [E-FMN], non-haem iron–sulfur proteins [Fe_m-S_n], iron- and copper-containing cytochromes (b_{562}, b_{566}, c_1, c, a, a_3) and coenzyme Q (ubiquinone, oxidized form of Q; ubiquinol, reduced form of Q) located in the inner mitochondrial membrane of mitochondria (Jung and Brierley, 1983). The electron transfer chain can be resolved into four catalytically active complexes by fractionation with detergents and salt (Fleischer and Packer, 1978; De Pierre and Ernster, 1977). These are complex I, the NADH ubiquinone reductase containing [E-FMN] and [Fe_m-S_n]; complex II, succinate-ubiquinone reductase; complex III, ubiquinone cytochrome c reductase containing cytochromes b_{562}, b_{566}, c_1 and iron–sulfur proteins; and complex IV, cytochrome c oxidase containing cytochromes a, a_3 and copper (Fig. 4). Of the redox centers that have been implicated in electron transport, only Q and cytochrome c are not firmly associated with one of these complexes, and the four complexes, together with these two so-called mobile components, can be reconstituted to yield electron transport activity corresponding to that in the native membrane. Free radicals are formed during activity of the mitochondrial electron transfer chain (Boveris and Chance, 1973; Paraidathathu *et al.*, 1992) and the rate of $(O_2)^{\cdot-}$ formation is proportional to mitochondrial O_2 utilization. Considerable amounts of $(O_2)^{\cdot-}$ are produced when the electron flow is inhibited (antimycin or rotenone). There are two separate sites of $(O_2)^{\cdot-}$ production: the flavoprotein NADH dehydrogenase (located in complex I) and the ubiquinone cytochrome b segment (Boveris *et al.*, 1976; Turrens and Boveris, 1980). Whether $(O_2)^{\cdot-}$ formation is coupled with auto-oxidation of ubisemiquinone or with auto-oxidation of cytochrome b_{566} is still unclear, but the latter hypothesis is favoured (Nohl and Jordan, 1986; Beyer, 1990; Glinn *et al.*, 1991; Nohl and Stolze, 1992). In contrast, ubiquinone has been shown to act as a potent protectant against free radical damage to subcellular membranes *in vitro* (Ernster *et al.*, 1992). It is assumed that under 'normal' conditions little of the $(O_2)^{\cdot-}$ formed escapes the mitochondria due to the high levels of MnSOD within the matrix (Section 3.1.2.1). However, during aging, decreased levels of GSH and cytochrome aa_3 were measured in brains from old rats (Benzi *et al.*, 1992), supporting the theory of increased oxidative stress due to $(O_2)^{\cdot-}$ production of the respiratory chain as one of several causes of cell aging (Sohal and Sohal, 1991). Endogenous or exogenous inhibitors of the mitochondrial electron transfer chain could cause a continuous chronic oxidative stress to mitochondria, finally leading to cell death. Thus, it seems reasonable to assume that a decrease in enzymic activity in the electron transfer chain, due to a decreased formation of enzymes (Sections 5.2 and 5.3) or due to inhibitors, probably results in a chronic decrease in ATP levels and an increase in $(O_2)^{\cdot-}$ formation.

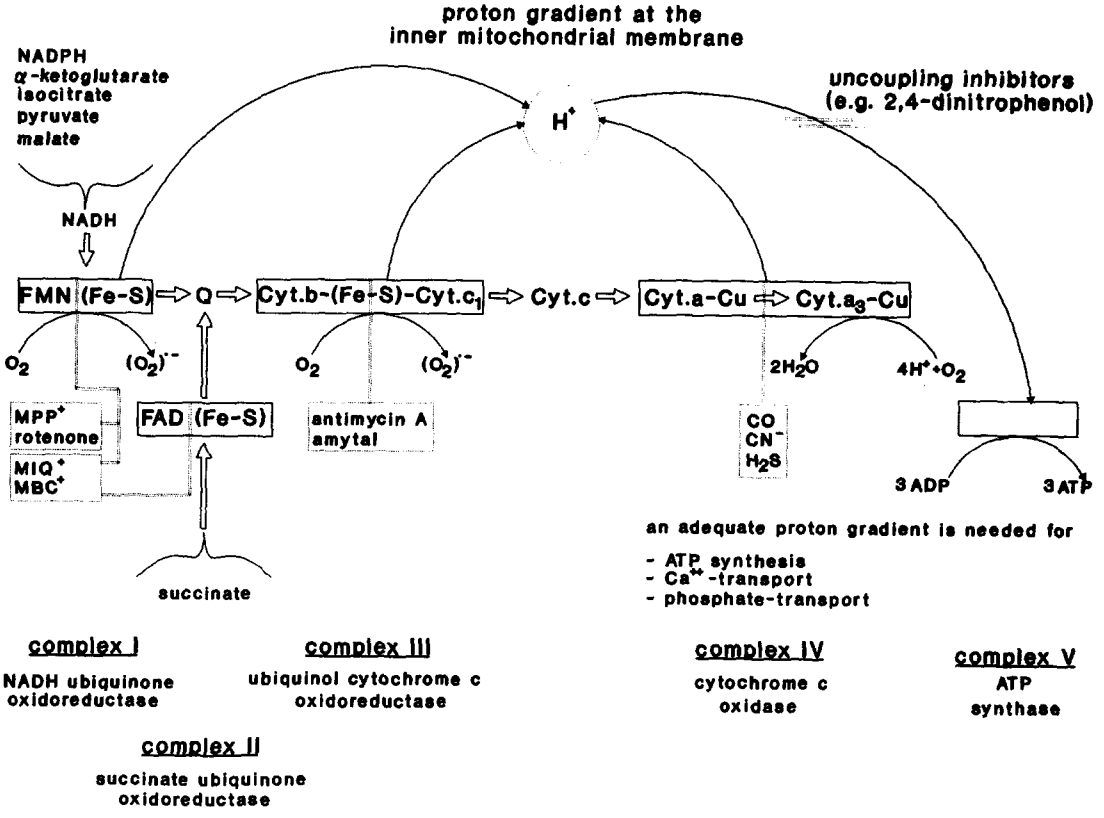

FIG. 4. The pathway of electron transfer from various substrates of the Krebs cycle to water in the inner mitochondrial membrane, including the most relevant flavin- and cytochrome-containing enzyme complexes (I–V) with known and putative inhibitors. Potential sites of $(O_2)^{\cdot-}$ production are indicated. NADH-DH, NADH-dehydrogenase.

3.3. EXCITOTOXIN-INDUCED CELL DEATH

In addition to the acute effects of generalized forms of CNS trauma (e.g. hypoglycemia, hypoxia or ischemia), topologically restricted and cell-selective damage occurs within hours or days following brain injury, mainly affecting pyramidal neurons of the hippocampus, neocortical and striatal neurons. To explain this regional pattern of neuropathology, the existence of selective vulnerable structures in brain has to be postulated. The affected targets are those neurons expressing postsynaptic receptors sensitive to EAA (NMDA-, quisqualate-, kainic acid-sensitive and metabotropic receptors; Farooqui and Horrocks, 1991) and, within the last decade, EAA (glutamate, aspartate) have been implicated as mediating damage to neurons and glial cells (Rothman, 1984; Collins, 1987; Choi, 1988a,b; Olney, 1990; Bridges *et al.*, 1992). For example, pretreatment with EAA-receptor antagonists (Rothman, 1984; Simon *et al.*, 1984) prevents the regional damage (Section 8).

3.3.1. *Excitotoxicity*

Excessive and prolonged release of glutamate and/or aspartate from nerve terminals, their insufficient glial clearance from the extracellular space and decreased GABA-ergic postsynaptic input are prerequisites to regarding EAA as excitotoxic substances (Olney, 1990). Vulnerability of postsynaptic neurons to excitotoxin-mediated damage depends on the nature of the receptors, which can be stimulated by NMDA, kainic acid, quisqualate, ibotenate and quinolinic acid directly. *In vitro* studies indicate that excitotoxin-induced neuronal injury may involve acute swelling of cells due to the depolarization-mediated influx of sodium chloride, water and calcium (Ca^{2+}) (Choi, 1988a,b; Rothman and Olney, 1986). Ca^{2+} is regarded as the triggering agent of many biological

reactions and has attained the status of a second messenger (Berridge, 1975; Rasmussen and Goodman, 1977). It alters membrane stability and permeability (Seeman, 1972) and is involved in nerve impulse propagation by coupling the electrical signal to neurotransmitter release (Llinás and Nicholson, 1975). Ca^{2+} regulates an enormous number of enzyme activities (Carvalho, 1982), including protein kinases, endonucleases, proteases and lipases. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has to be maintained at a low level of about 0.1 μM (in contrast to extracellular levels of *ca.* 1 mM; Orrenius *et al.*, 1989) by rigorously controlling Ca^{2+} entry, intracellular sequestration of Ca^{2+} (endoplasmic reticulum, mitochondria) and binding to high affinity binding proteins, such as calmodulin, calretinin and calbindin-D28K (Billingsley *et al.*, 1985; Baimbridge *et al.*, 1992; Heizmann and Braun, 1992). When the energy state of a cell is normal, Ca^{2+} is transported out of the cell via the Na^+/Ca^{2+} antiporter and Ca^{2+}-activated ATPase. However, in the case of an energy deficit (impairment of mitochondrial respiration, hypoxia, hypoglycemia), intracellular $[Ca^{2+}]_i$ may rise many-fold due to channel-mediated influx or mobilization of Ca^{2+} from internal stores by 1,4,5-inositol triphosphate (IP_3). IP_3 and diacylglycerol are formed during receptor- and G-protein-coupled activation of phospholipase C. Diacylglycerol, in concert with Ca^{2+}, activates protein kinase C, which catalyses the activation of many proteins (Barnard, 1992). In addition, phospholipase A_2 is dependent on Ca^{2+} and calmodulin and is suggested to participate in the detoxication of LOOH (Orrenius *et al.*, 1989). However, by its releasing fatty acids (predominantly arachidonic acid) and membrane destabilizers, such as lysophospholipids (Zaleska and Wilson, 1989), the prostaglandins, leukotrienes and thromboxanes are formed, all of which are known to be mediators of inflammatory and allergic reactions. In addition, a high level of $[Ca^{2+}]_i$ leads to activation of non-lysosomal proteases (e.g. calpains), which induce the conversion of xanthine dehydrogenase to xanthine oxidase and may help in the production of $(O_2)^{\cdot-}$ (Dykens *et al.*, 1987; Section 3.1). Cellular targets for these enzymes include cytoskeletal elements and integral membrane proteins (Mirabelli *et al.*, 1989; Kosower *et al.*, 1983; Melloni and Pontremoli, 1989) leading to dissociation of actin microfilaments from anchoring proteins in the plasma membrane with subsequent membrane blebbing and increasing membrane permeability (Fig 5). Finally, Ca^{2+} may even activate endonucleases, catalysing nuclear DNA fragmentation, a process that could be involved in apoptosis, or programmed cell death (Nicotera *et al.*, 1989; Fawthrop *et al.*, 1991).

3.3.2. *Calcium and Mitochondrial Function*

ATP is essential for maintaining the normal voltage gradient across the cell membrane. Thus, reduced ATP levels, following impairment of energy metabolism, depolarize cell membranes, thereby permitting intracellular accumulation of sodium. This can relieve the voltage-dependent magnesium block of NMDA channels and cause opening of voltage-dependent Ca^{2+} channels. Impairment of energy metabolism also prevents ATP-dependent extrusion of Ca^{2+} and the storage of excess $[Ca^{2+}]_i$ in endoplasmic reticulum and mitochondria by ATP-dependent mechanisms (Siesjö and Bengtsson, 1989; Blaustein, 1988; Choi, 1988a). In cultured neurons, inhibitors of oxidative phosphorylation or of the sodium–potassium pump allow NMDA or glutamate to become neurotoxic (Novelli *et al.*, 1988). Chemically induced hypoglycemia results in excitotoxic lesions, which can be prevented with NMDA antagonists (Zeevalk and Nicklas, 1991), but which are not accompanied by increased glutamate release. This suggests that ambient glutamate is sufficient to induce excitotoxic damage if intracellular energy metabolism is compromised (Sah *et al.*, 1989; LoTurco *et al.*, 1990; Beal, 1992). Depletion of ATP from rat hepatocytes by treatment with potassium cyanide and iodoacetate leads to a sustained elevation of cytosolic free Ca^{2+} preceded by depletion of GSH and loss of ATP.

3.3.3. *Calcium and Oxidative Stress*

It is generally accepted that Ca^{2+} mobilization is crucial for the activation of phospholipases. However, Sevanian and coworkers (Sevanian *et al.*, 1981; Sevanian and Kim, 1985) demonstrated that phospholipase A_2 can also be activated in the absence of elevated Ca^{2+} by the presence of peroxidized fatty acids in phospholipids. The degree of phospholipase activation was correlated

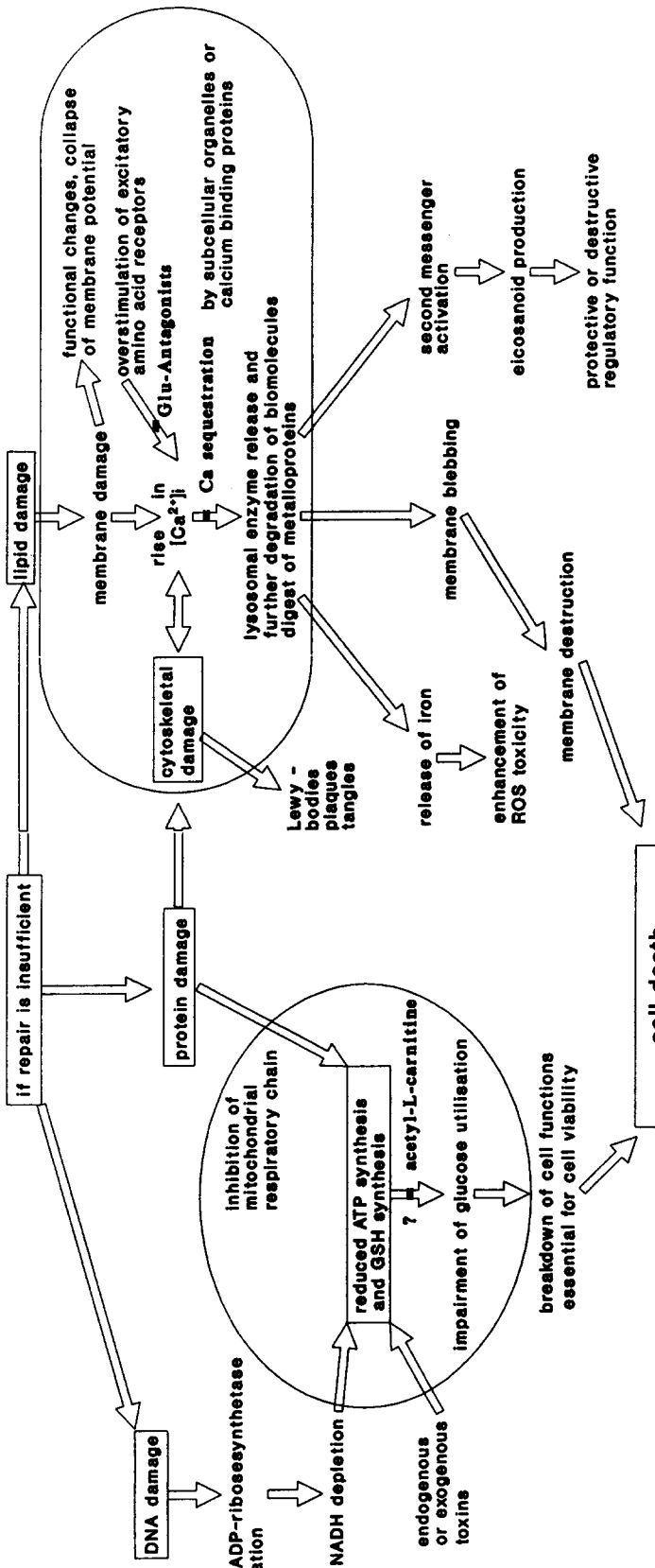

FIG. 5. Putative consequences of excess of ROS, of excess intracellular Ca^{2+} and impairment of mitochondrial oxidative phosphorylation to membrane structure and function. The putative sites of therapeutic intervention are indicated by double lines crossing the arrows.

with the extent of TBARS. Thus, both peroxidized fatty acids and Ca^{2+} can independently trigger degradation of membrane lipids, but may also act synergistically. Certainly, whenever ROS are involved in membrane damage, Ca^{2+} must be suspected as a participant.

In Fig. 5, the consequences of excess of EAA and Ca^{2+} as responses of neurons to various types of CNS trauma (Choi, 1988a,b; Baumgarten and Zimmermann, 1992) are briefly summarized.

3.4. Relationships Between Oxidative Stress, Impairment of Energy Metabolism and Calcium Cytotoxicity

Impairment of mitochondrial ATP regeneration, as a consequence of electron transfer chain inhibition or of a decrease in activities of enzymes of energy metabolism, could enhance $(O_2)^{\cdot-}$ production in mitochondria. This can also impair clearance of ROS due to less effective detoxification systems (loss of GSH), leading to increased influx of Ca^{2+} and peroxidation of lipids and proteins (Fig. 5).

Cells overstimulated by excitotoxic inputs or suffering from decreased levels of ATP react by taking up sodium and water, resulting in swelling. Subsequently, the cells are exposed to an increase of cytoplasmic free Ca^{2+} via channel-mediated influx, mobilization of Ca^{2+} from internal stores resulting from activation of second messengers and alterations in Ca^{2+} clearance due to depletion of energy reserves or of ATP resynthesis. Increased Ca^{2+} levels activate proteases, lipases and endonucleases, with subsequent degradation of phospholipids and production of prostaglandins known to involve production of ROS. In the final phase, cytoskeletal components and membranes are degraded. Prolonged oxidative stress episodes associated with depletion of the energy reserve may contribute, therefore, to neurodegeneration in a wide variety of pathological conditions (Fig. 5).

Although many of the biochemical mechanisms of cell damage are well established, there is little information concerning the primary causes that trigger these mechanisms and induce the selective neurodegeneration in distinct brain regions. The clinical and neuropathological features of neurodegenerative diseases and the possible common mechanisms of neuronal cell death have been discussed. Next, we wish to focus on animal models of neurodegeneration and on *post mortem* data from brains of patients who suffered from neurodegenerative disorders.

4. RELEVANCE OF OXIDATIVE STRESS TO NEURODEGENERATION

4.1. Factors Favouring Damage by Reactive Oxygen Species

4.1.1. *Hydrogen Peroxide Production by Monoamine Oxidase*

The enzyme MAO exists in two forms, termed A and B, in the mammalian brain. Both enzymes are flavoproteins localized in the outer mitochondrial membrane (Youdim *et al.*, 1988). They play a key role in the metabolism of monoamine neurotransmitters and xenobiotic amines (Blaschko *et al.*, 1937; Youdim *et al.*, 1988). The A-form is mainly responsible for the deamination of serotonin and norepinephrine and is pharmacologically defined by its sensitivity to inhibition by clorgyline (Johnston, 1968; Fowler *et al.*, 1980a). MAO-B, on the other hand, is known to deaminate predominantly non-polar amines, phenethylamine and methylhistamine (Glover *et al.*, 1977). The B-form is characterized by its high sensitivity to inhibition by L-deprenyl (Knoll and Magyar, 1972; Riederer *et al.*, 1978; Oreland *et al.*, 1983; Riederer and Jellinger, 1983; for reviews see Denney and Denney, 1985; Dostert *et al.*, 1989; Youdim and Finberg, 1990; Gerlach *et al.*, 1992). Both enzymes metabolize DA and tyramine.

Equation 12 (Section 3.1.1) depicts oxidation of primary amines by MAO, leading to the production of H_2O_2, aldehydes and ammonia. As for ammonia and aldehydes, detoxification enzymes, such as glutamine synthetase, aldehyde reductase and aldehyde dehydrogenase, seem to be up-regulated if MAO activity increases (Strolin-Benedetti *et al.*, 1986), as could be observed in the whole brains of aging rats. However, activities of CAT, GSH-Px and GSSG-Rd responsible for detoxification of H_2O_2 did not change significantly in brains of old rats compared with young

controls. Thus, a greater sensitivity to oxidative damage arising from amine oxidation might be expected to accompany aging or various pathological stages in which turnover by MAO is increased, in particular in combination with drugs known to elevate amine concentrations, such as L-DOPA or catecholaminergic uptake inhibitors (Strolin-Benedetti and Dostert, 1989). In human brain, MAO-B increases with increasing age (Robinson *et al.*, 1971; Fowler *et al.*, 1980b; Jossan *et al.*, 1991a) due to an increase in MAO-B concentration. Moreover, this increase in MAO-B activity in brain is further accelerated in neurodegenerative disorders, such as senile dementia of Alzheimer type (Adolfsson *et al.*, 1980; Jossan *et al.*, 1991b), HD (Mann, J. J. *et al.*, 1980b, 1986), ALS (Ekblom *et al.*, 1992) and maybe PD (Schneider *et al.*, 1981; Riederer and Jellinger, 1983). However, increase in MAO-B activity in PD could not be confirmed in a further study (Jellinger and Riederer, 1984), possibly because of treatment of patients with L-DOPA, which is now known to alter MAO-B activity (McIntyre *et al.*, 1985; LeWitt *et al.*, 1985). The results from animal studies utilizing surgical (Oreland *et al.*, 1983) and neurotoxic lesions (Francis *et al.*, 1985; Jossan *et al.*, 1989) and from investigation of ALS brains have supported the idea that the increase in MAO-B activity is a consequence of gliosis (Oreland *et al.*, 1989). Astrocytes have been shown to be rich in MAO-B activity (Levitt *et al.*, 1982) and astrocytosis has been demonstrated in senile brains (Schechter *et al.*, 1981), and, interestingly, MAO-B activity was detected in astrocytes of senile plaques (Nakamura *et al.*, 1990). The less consistent changes in MAO-A activities may reflect neuronal loss, but this is still uncertain.

The human isoforms of MAO have been purified and cloned (Bach *et al.*, 1988; Grimsby *et al.*, 1991). MAO-A and -B are derived from different genes closely linked to each other and located on the short arm of the X-chromosome (Shih *et al.*, 1990). Tissue specificity of MAO-A and -B activity and gene expression has been demonstrated (Stenström *et al.*, 1987; Shih *et al.*, 1990). In addition, within the same brain, there is a great difference in localization in different regions of the isoenzymes (Saura *et al.*, 1992). Thus, the relative extents of MAO-A and MAO-B breakdown of DA varies markedly in different brain regions (Glover *et al.*, 1980). The different promoter organization of MAO-A and -B genes provides the basis for their different tissue- and cell-specific expression (Zhu *et al.*, 1992). It is tempting to assume that the knowledge about regional differences in MAO activity could be a clue to the understanding of region-specific brain damage in neurodegeneration. However, immunohistochemical investigations (Glenner *et al.*, 1957; Graham and Karnovsky, 1965; Levitt *et al.*, 1982; Westlund *et al.*, 1985, 1988; Arai *et al.*, 1986; Thorpe *et al.*, 1987; Konradi *et al.*, 1988, 1989) and quantitative enzyme autoradiography (Saura *et al.*, 1992) revealed that the cellular localization of the isoenzymes of MAO in both rat and human brain differs markedly and does not reflect the distribution of the presumed natural substrates (e.g. absence of MAO-B in melanin-containing neurons of the SN, Konradi *et al.*, 1989; Moll *et al.*, 1990; absence of MAO-A in serotonergic neurons, Saura *et al.*, 1992). In contrast, glial cells and melanin-free neurons contain both MAO-A and MAO-B in SN (Konradi *et al.*, 1989; Moll *et al.*, 1990). Thus, degeneration of melanin-containing neurons of SNC in PD cannot be directly attributed to increased levels of MAO-B. Perhaps MAO-A found in dopaminergic neurons metabolizing DA and producing H_2O_2 contributes to degeneration (Spina and Cohen, 1989) and/or increased MAO-B activity in glial cells leads to elevated production of ROS (secondary to increased metabolism of increased activity of surviving neurons). This could be followed by depletion of GSH stores subsequent to H_2O_2 production (Spina and Cohen, 1988; Sandri *et al.*, 1990; Werner and Cohen, 1991) and by oxidation of catecholamines, leading to neuromelanin generation. However, there is no certain answer to this question, and it seems likely that, in addition to H_2O_2 production by MAO in glial cells, other factors have to be considered to be pathogenetic in the degradative process of catecholaminergic neurons.

4.1.2. *Catecholaminergic Toxicity and Neuromelanin*

An increased DA turnover in PD (relative rise of acidic metabolites) as a consequence of an 80–90% loss of nerve cells in the SNC and amplified DA-liberation and reuptake in remaining axons of the striatum is a process that may contribute to the progressive loss of DA neurons in PD (Cohen, 1983a). An elevation in DA turnover (Mogi *et al.*, 1988) may be a compensating mechanism in PD to overcome the effects of the loss of dopaminergic neurons (Hornykiewicz and

Kisch, 1986). Although it has been shown that long-term administration of L-DOPA does not damage dopaminergic neurons in the mouse (Hefti *et al.*, 1981), studies on two groups of patients with PD, matched for age and with one group which was treated with L-DOPA, provide some evidence for an increase of the striatal DA loss in the advanced decompensatory phase of the disease (Riederer and Wuketich, 1976). In C57B16 mice, DA synthesis is increased during aging to compensate for loss of dopaminergic neurons (Tatton *et al.*, 1991). This could indicate that, in aging or pathological states, surviving neurons contain higher concentrations of catecholamines. It is known that DA is unstable in solution at neutral pH and easily undergoes auto-oxidation (Graham *et al.*, 1978; Rodgers and Curzon, 1975). In the presence of transition metal complexes, catechols enhance the formation of $(HO)^{\cdot}$ from H_2O_2 (Iwahashi *et al.*, 1989), as examined by spin-trapping techniques. Thus, the presence of catechols in cells could provide a threat to cell viability, especially if low molecular weight iron complexes are present (Section 4.1.3; Bindoli *et al.*, 1992). Interestingly, Mann and Yates (1983) showed that the more heavily pigmented neurons of the SN appear to be preferentially lost in PD and during the course of aging, when both iron and melanin are known to increase. When comparing the SN of control and parkinsonian brains, Hirsch *et al.* (1988) demonstrated the greater vulnerability of the population of DA neurons containing neuromelanin to the neurodegenerative process of PD. Their studies also showed a direct relationship between the distribution of pigmented neurons normally present and the distribution of cell loss in the SN of individuals dying with the disease.

Recently, an inverse relationship was observed between the percentage of surviving neurons in PD compared with controls and the amount of neuromelanin they contain. Moreover, the largest pigmented neurons in SN are lost preferentially in PD (Moller, 1992), suggesting that the vulnerability of the dopaminergic neurons is related to their neuromelanin content (Kastner *et al.*, 1992). However, this hypothesis was challenged recently by Gibb (1992). Neuromelanin is not found in the cerebral cortex, thalamus, strio-pallidal complex, cerebellum or spinal cord (Marsden, 1983). It accumulates during life in pigmented brain-stem nuclei, appearing first in cells of the LC around the time of birth and then in SN around the age of 18 months (Foley and Baxter, 1958; Mann and Yates, 1974).

In normal subjects, the intracellular content of neuromelanin has been shown to increase with aging, up to 60 years. Then it begins to decrease, presumably due to destruction of melanin-containing cells.

Many investigators have suggested that DA is a precursor of neuromelanin, a dark brown pigment, mainly located in the cell bodies of SN and LC (Van Woert *et al.*, 1967; Nordgren *et al.*, 1971; Das *et al.*, 1978). In the skin, the formation of melanin is catalysed by tyrosinase, a bifunctional enzyme, oxygenating tyrosine to DOPA and oxidizing DOPA to DOPA-quinone (Lerner *et al.*, 1949). However, this enzyme does not seem to be present in the SN (Barden, 1969). Thus, auto-oxidative mechanisms may play a primary role in neuromelanin formation. The exact chemical composition of this pigment is still unknown and debated. Since there seem to be structural similarities of lipofuscin and neuromelanin (Lillie and Yamada, 1960a,b; Van Woert *et al.*, 1967), it is likely that neuromelanin results from the deposition on lysosomes of a melanin derived from the catecholamines DA or norepinephrine, or from deposition on lipofuscin of a melanin derived from lysosomes (Marsden, 1983). Incubation of DA with tissue homogenates results in the formation of cysteinyl-DA (via glutathionyl-DA; Ito *et al.*, 1986), and cysteinyl-catechols, such as cysteinyl-DA, cysteinyl-3,4-dihydroxyphenylacetic acid and cysteinyl-DOPA, are detected in brains of several mammalian species; the ratio of cysteinyl-DA/DA amounts to about 1:40 in human SN (Rosengren *et al.*, 1985; Fornstedt *et al.*, 1989). Most melanins of the skin are co-polymers of indole and cysteinyl DOPA-derived eumelanin and pheomelanins, respectively (Prota *et al.*, 1976). However, the presence of cysteinyl-DA in neuromelanin is still debated (Wakamatsu *et al.*, 1991; Zecca *et al.*, 1992). In contrast, it is believed that cysteinyl-DA or glutathionyl-DA is actually formed in the brain as a protective mechanism in neurons (Fornstedt *et al.*, 1989; Fornstedt and Carlsson, 1991a) to absorb and excrete an overflow of catecholamines (Fornstedt and Carlsson, 1991b). The presence of indoles in neuromelanin has led to the assumption that, once thiols are depleted, neuromelanin formation is favoured (Carstam *et al.*, 1991). Jellinger *et al.* (1992) and Good *et al.* (1992a) provided evidence for an iron–melanin complex in SNC neurons in parkinsonian brains, but not in LB or in non-melaninized cytoplasm of SNC

neurons, and concluded that an iron–melanin interaction could significantly contribute to dopaminergic neurodegeneration in PD. Depending on conditions, melanin can significantly increase or decrease the yield of reactive products of iron-catalyzed decomposition of H_2O_2 *in vitro*, as determined by spin trapping of the products (Pilas *et al.*, 1988). They found that for low concentrations of ferrous ions, melanin decreased the yield of $(OH)^{\cdot}$ due to binding of ferrous ions by melanin. In their experiments, ferrous ions bound to melanin did not decompose H_2O_2 efficiently. Melanin increased the rate of $(OH)^{\cdot}$ production if the predominant form of iron was Fe^{3+}, presumably due to the ability of melanin to reduce Fe^{3+} to Fe^{2+}. Thus, it is possibly not the presence of melanin *per se*, but the interaction between catechols, iron and H_2O_2 that determines the vulnerability of melaninized DA neurons to neurodegeneration in PD (Ben-Shachar and Youdim, 1990; Youdim *et al.*, 1990; Ben-Shachar *et al.*, 1991b; Section 4.1.3).

The participation of catecholamines in neurotoxicity seems very likely (Sandyk and Willis, 1992). However, the commonly accepted cellular marker of parkinsonian pathology, the LB (consisting of pathologically phosphorylated proteins, ubiquitin, phospholipids and sphingomyelin), is not confined to neurons containing neuromelanin and is not correlated with a selected neurotransmitter system. There are areas of the brain, of which the substantia innominata is an example, that contain virtually no neuromelanin or catecholaminergic neurons, but whose cells in PD patients are packed with LB and die (Marsden, 1983). Because there is no neuroanatomical evidence that regions like substantia innominata receive dense catecholaminergic innervation, it is hard to imagine that their degeneration in PD invokes a transsynaptic degeneration (Marsden, 1983).

Unfortunately, to our knowledge, LB are not well characterized with respect to their chemical nature and origin. Perhaps there is a relationship to lipofuscin pigments and lysosomal disorders or breakdown of cytoskeleton, possibly due to excess Ca^{2+}, to yield inclusions similar to the NFT or amyloid plaques of AD. Indeed, it was shown recently by immunohistochemistry that LB in cortex and SN contain epitopes similar to the APP found in Alzheimer brains (Arai *et al.*, 1992).

So, is there a common cause of neurodegeneration despite the presence of morphologically and biochemically different neuropathological hallmarks? To give an answer, it seems crucial to define the exact compositions, possible modes of formation and features in common of LB, tangles, plaques, lipofuscin and neuromelanin.

4.1.3. 6-*Hydroxydopamine Model of Neurodegeneration*

This review is concerned with endogenous factors putatively involved in the pathogenesis of various neurodegenerative diseases. However, research is inspired to a great extent by animal models. Functional relationships between experimental parameters are clearly more easily evaluated in animal models or cell culture than with post-mortem tissue. Thus, we at least want to mention some interesting topics dealing with ROS and a neurotoxin derived from catecholamines, 6-OHDA (for reviews concerning neurotoxins see Kostrzewa, 1989; Calne, 1991; Herken and Hucho, 1992).

6-OHDA accumulates in catecholamine-containing neurons and exhibits selective toxicity towards them. 6-OHDA is often administered intrastriatally to rats to induce degeneration of nigrostriatal neurons (Berger *et al.*, 1991; Ichitani *et al.*, 1991). However, to be selective for the dopaminergic system, norepinephrine uptake has to be inhibited by desmethylimipramine (Kostrzewa, 1989). Two mechanisms for the toxicity of 6-OHDA have been proposed. First, auto-oxidation could generate ROS and subsequently oxidize unsaturated fatty acids of lipids or thiol groups of proteins. Second, 6-OHDA uncouples mitochondrial oxidative phosphorylation (Wagner and Trendelenburg, 1971). Whether the neurotoxicity of 6-OHDA can be attributed to the production of ROS or dihydroxyindoles (for a review, see Thoenen and Tranzer, 1973) is not yet defined. Degeneration of nigrostriatal neurons after intracerebral injections of 6-OHDA to rats is potentiated by administration of iron (Ben-Shachar and Youdim, 1991) or after depletion of brain GSH by intracerebral administration of L-buthionine sulfoximine (Pileblad *et al.*, 1989). In contrast, long-term oral administration of TOH (Cadet *et al.*, 1989) or intraventricular injection of desferrioxamine (Ben-Shachar *et al.*, 1991b) attenuated the 6-OHDA-induced depletion of striatal dopamine. Although these experimental conditions are very harsh, the participation of ROS in the toxicity of 6-OHDA seems likely. These experiments could be a rational basis for the use

of antioxidants or iron chelators in treatment of diseases suspected to involve ROS, if adverse effects can be minimized.

4.1.4. *Iron Distribution in Brain and its Role for Oxidative Stress*

As described in Section 3, iron can promote peroxidation of biological macromolecules due to its reactions with ROS and, thus, is of high toxic potential for cells, if it is not kept in a toxicologically inactivated form bound to specific proteins. Only when iron is tightly bound to a chelator is its capacity for promoting LPO minimal. Amongst synthetic chelators of iron, *bis*-(2-aminoethyl)-amine-*N*,*N*,*N'*,*N'*-penta-acetic acid, desferrioxamine, *o*-phenanthroline and bathophenanthroline are able to complex Fe^{3+} and, thus, slow down reduction of Fe^{3+} to Fe^{2+} by reductants like ascorbic acid or $(O_2)^{\cdot-}$ *in vitro*, but EDTA is ineffective. Desferrioxamine was originally developed for the treatment of iron overload disease because it binds Fe^{3+} rather selectively, but there are current efforts to create more specific iron chelators that pass the blood–brain barrier (Section 8).

The role of ferritin in iron-promoted LPO is ambiguous. Ferritin stimulates LPO proportionately to the amount of iron it contains, provided that mechanisms exist that release iron from ferritin. In liposomes, LPO by ferritin or haemosiderin, presumably a product of proteolytic attack on ferritin in lysosomes, is almost completely inhibited by desferrioxamine, suggesting that it is mediated by released iron ions (Halliwell and Gutteridge, 1989). Recently, it was argued that ferritin serves as a source of iron for oxidative damage (Reif, 1992) in the presence of redox-cycling xenobiotics, such as paraquat, adriamycin or alloxan, which cause $(O_2)^{\cdot-}$ production (Winterbourn *et al.*, 1991; Minotti *et al.*, 1991). On the other hand, as long as iron is correctly bound to ferritin, it seems that it does not initiate peroxidation of biomolecules.

Iron is an essential participant in many metabolic processes, including DNA, RNA and protein synthesis, the formation of myelin and the development of the neuronal dendritic tree, and as a cofactor of many haem and non-haem enzymes. A deficiency in iron metabolism, therefore, would be expected to alter some or all of these processes (Youdim, 1985; Youdim *et al.*, 1991), and excessive accumulation of tissue iron may lead to oxidative stress via the formation of ROS (Halliwell, 1989a,b). Cytotoxicity of iron was confirmed by *in vitro* studies to cultured neurons (Tanaka *et al.*, 1991; Michel *et al.*, 1992) or by intranigral injection of iron into rats (Ben-Shachar and Youdim, 1991; Sengstock *et al.*, 1992). Moreover, iron causes a time- and concentration-dependent opening of dihydropyridine sensitive Ca^{2+} channels in rat cortical synaptosomes, resulting in parallel increased uptake of $^{45}Ca^{2+}$ and stimulation of LPO, as measured by formation of TBARS *in vitro* from intact rat cortical synaptosomes. Both the Ca^{2+} uptake and LPO can be inhibited by dihydropyridines (nifedipine) and iron-chelating agents (desferrioxamine). The ability of iron salts to induce opening of Ca^{2+} channels, resulting in alteration of intracellular Ca^{2+}, would support the recent hypothesis that iron could be the agent that induces neurotoxic events in iron-rich regions of the brain. Therefore, in order to be toxic to cells, iron has to be present in the brain in a more or less loosely bound form. This could mean that minimizing the amount of non-haem iron without depleting enzyme bound iron in biological systems is an important part of antioxidant defense.

4.1.5. *Uptake and Distribution of Iron and Copper in Normal and Pathological Brain*

There are two major problems associated with the biological use of iron, namely the poor solubility of Fe^{3+} at physiological pH and the involvement of iron in potentially harmful redox reactions. These problems have led to the evolution of a variety of elegant biochemical processes, high affinity binding and cellular uptake of iron (Fatemi *et al.*, 1991). Recent investigations (Morris *et al.*, 1992a,b; Crowe and Morgan, 1992; Roberts *et al.*, 1992) suggest that iron uptake into the brain does not involve the transcytotic pathway of transferrin-bound iron via transferrin receptors into endothelial cells, but deposition of transferrin-bound iron within endothelial cells, followed by recycling of apotransferrin to the circulation. The deposited iron is then delivered to brain-derived transferrin for extracellular transport within the brain and, subsequently, taken up as Fe^{3+} via transferrin receptors on neurons and glial cells. Iron is used in several enzymes

(Wrigglesworth and Baum, 1988), such as mitochondrial complexes of the respiratory chain, MAO, cytochrome P450, CAT, TH and others, or for storage in ferritin.

Reaction of Cu^+ ions with H_2O_2 appears to generate $(HO)^{\cdot}$ and reactive Cu^{3+} species *in vitro*. However, there is doubt whether copper is available to promote production of ROS *in vivo*. Copper transport seems not to involve transferrin (Thorstensen and Romslo, 1990) but Cu^{2+}complexes with histidine-imidazoles, α-amino groups of amino acids or nitrogen of peptide bonds of proteins, such as albumin or ceruloplasmin. Following binding, the Cu^{2+} is reduced to Cu^+, possibly by a reductase or perhaps by ascorbate, and then carried across the membrane into the cell (McArdle, 1992). It has been suggested that ceruloplasmin may be able to donate copper within cells for incorporation into copper proteins, such as CuZnSOD (Dameron and Harris, 1987a,b). Ceruloplasmin obviously functions as an important scavenger of excess copper since low ceruloplasmin concentrations in the blood, as observed in Wilson's disease, lead to excess copper in various organs, including the brain, and concomitantly to lack of coordination, to tremors and to progressive mental retardation. If iron or copper were causally involved in neurodegenerative diseases, transition metal distribution in brain should ideally reflect neuropathological changes and perhaps explain the region-specific cell loss. Thus, many investigators were and are still concerned with the question of metal distribution in normal and pathological brain. In principle, it would be necessary to quantitate cellular and subcellular iron levels in pathologically affected brain regions. However, until recently, iron determinations were based on analytical approaches utilizing iron chelators for histochemical staining, spectrophotometric analysis or magnetic resonance brain imaging, which provide data only on regional bulk iron concentrations. Now, more sensitive methods are available, such as laser microprobe mass analysis (LAMMA; Jellinger *et al.*, 1990) and energy-dispersive radiographic microanalysis (Perl and Good, 1992), which allow identification and localization of cellular structures in histological sections and provide sensitive trace-elemental detection and characterization. Using the LAMMA technology, Good and colleagues (1992a,b) measured increased concentrations of aluminum (Al) and iron in the NFT within tangle-bearing and adjacent neurons in patients with AD. However, another microprobe analytical technique (nuclear microscopy) failed to demonstrate the presence of Al in plaque cores of chemically untreated tissue (Landsberg *et al.*, 1992).

It is obvious that each method has advantages and drawbacks, thus making it sometimes difficult to compare reported findings. Using histochemical techniques, the presence of iron in the brain was first detected at the end of the last century and, subsequently, was a subject of intense investigation (for a review, see Hill, 1988). Recently, a detailed study of the anatomical distribution of bulk iron in non-pathologic human *post mortem* brain was published; using Perl's and Turnbull's methods with the diaminobenzidine intensification procedure for the demonstration of non-haem Fe^{3+} and Fe^{2+}, respectively (Morris *et al.*, 1992a). This confirmed and extended the findings of earlier studies, showing highest levels of stainable iron in the extrapyramidal system (globus pallidus, SNR, red nucleus and myelinated fibres of the putamen). Moderate staining with Perl's technique was found in the thalamus, cerebellar cortex and SNC. Microscopically, the non-haem iron appears to be predominantly in glial cells as fine cytoplasmic granules. Neurons, in general, show low reactivity for iron, and this is difficult to discern, often because of the higher reactivity of the surrounding neuropile. In the globus pallidus and SNR, however, neurons with highly stainable iron content are found with granular cytoplasmic iron reactivity similar to that seen in the local glial cells. Although there seem to be no apparent correlations of iron staining with known transmitter systems, the extrapyramidal system is favored in iron uptake and storage. This could point towards involvement of iron in the pathogenesis of disorders involving striatonigral degenerations (Uitti *et al.*, 1989), such as Hallervorden–Spatz disease (Dooling *et al.*, 1974), MSA, progressive supranuclear palsy, ALS, HD and PD. Here, we want to focus on PD as the most frequently occurring disease.

4.1.6. *Iron and Neurodegeneration in Parkinson's Disease*

Sofić and colleagues' (1988, 1991) biochemical studies of total iron, Fe^{2+} and Fe^{3+}, using spectrophotometry in different brain regions of patients with PD with and without AD, showed an increase of total iron in the SN of patients with PD vs patients with AD and control subjects.

Fe^{3+} in the SN of patients with PD was nearly twice as high as in patients with AD and control subjects; in both the SNC and SNR it was increased by approximately one-third, whereas Fe^{2+} levels showed no differences. In the cortex, hippocampus, putamen and globus pallidus, there were no differences in the levels of total iron or Fe^{3+}. The findings of increased total iron in the SN of patients with PD were confirmed by Dexter and colleagues (1989b, 1991, 1992a). Subcellular regions in SN of patients with PD and control subjects were investigated for iron by Jellinger *et al.* (1992) using transmission electron microscopy and energy dispersive radiographic microanalysis. Only the analysis of neuromelanin in SN neurons of patients with PD showed iron levels that were significantly greater than baseline control levels. No significant demonstration of iron accumulation was observed in the central core or the periphery of LB or in the cytoplasm and neuromelanin of SN neurons of control subjects. These results agree with previous histochemical findings that LB are consistently negative for Fe^{3+} (Jellinger *et al.*, 1990). However, they are at variance with the radiographic microanalysis data reported by Hirsch and colleagues (1991), who found higher iron concentrations in LB in SN neurons of patients with PD than in control subjects. An increase in total iron content in the SN seems not to be specific to PD, but is detected in other neurodegenerative diseases affecting the striato-nigral system, namely MSA and progressive supranuclear palsy. Total iron levels were also increased in striatal areas affected by the pathology of those diseases and of HD (Dexter *et al.*, 1991, 1992a). Copper levels were reduced in the SN in PD and were elevated in the putamen of HD. The same authors found no consistent alterations in manganese levels in the basal ganglia in any of these diseases, but increased levels of zinc in SN, caudate nucleus and putamen in PD. Other studies have demonstrated increased iron levels in multiple sclerosis brain (Valberg *et al.*, 1989) and accumulation of iron in the striatum of patients with ALS and AD (Olanow *et al.*, 1989). The potential toxicity of the increased iron load in these disorders would be determined by the extent to which iron is deactivated by binding to ferritin and other moieties. Whether ferritin levels increase or decrease in PD is difficult to judge at this point, since both an increase and a decrease of SN ferritin in PD have been reported, using virtually the same methodology but different polyclonal antibodies to quantitate brain ferritin (Riederer *et al.*, 1989c; Dexter *et al.*, 1990, 1992a).

Taken all together, these findings suggest that increased iron levels are likely to be involved in neurodegenerative diseases affecting basal ganglia. However, it is still questionable whether iron acts as primary initiator of nerve cell death in PD or represents a secondary response to another yet unknown pathological cause. Nevertheless, iron in the SN may exist in a form capable of contributing to the toxic processes occurring in PD by stimulating formation of ROS. To date, changes in other metals are difficult to explain. Zinc and Mn are essential in the human diet and needed, for example, by SOD. Perhaps increased levels of Zn could be an expression of an answer by cells to increased levels of $(O_2)^{\cdot-}$.

4.1.7. *Aluminum Neurotoxicity*

It was the studies of Gutteridge *et al.* (1985) and Quinlan *et al.* (1988) that shed light on the possibility that Al could be involved in ROS-mediated cell damage. However, Al^{3+} salts do not themselves stimulate LPO. In the presence of Fe^{2+} salts (100 μM), Quinlan and colleagues (1988) observed a 3-fold increase of TBARS induced by Al^{3+} salts (300 μM) in rat liver microsomes. Accumulation of Al and iron not efficiently bound to storage proteins, therefore, could provide a risk factor for brain cells. In contrast to a study by Fleming and Joshi (1987), who found a 5-fold increase of Al content in brain ferritin in AD vs controls, Dedman and colleagues (1992) observed no difference in ferritin isolated from the cerebral cortex of AD patients vs controls. In a further study, Fleming and Joshi (1991) demonstrated a concentration-dependent decrease in the initial rate of iron loading into human brain ferritin in the presence of Al, suggesting that both Al and iron can be stored in ferritin. In contrast, Dedman *et al.* (1992) showed that brain ferritin from chronic renal-dialysis patients had less than nine atoms of Al per ferritin molecule, despite markedly increased concentrations of Al in the cerebral cortex in these patients. These authors suggested that Al does not accumulate in ferritin *in vivo*. Some authors have reported that Al is associated with various lesions of Alzheimer's brain—lipofuscin granules in the abnormal processes of some senile plaques and in the cytoplasm of neurons (Duckett and Galle, 1976; Duckett *et al.*, 1985), nuclei

of tangle-bearing neurons (Perl and Brody, 1980; Crapper *et al.*, 1980) and amyloid cores of senile plaques and NFT (Candy *et al.*, 1986). On the contrary, McDermott *et al.* (1979), Markesberry *et al.* (1981), Stern *et al.*(1986), Chafi *et al.* (1991) and Landsberg *et al.* (1992) did not find prominent concentrations of Al in the brain of Alzheimer patients. Likewise, Dedman *et al.* (1992) found no increase in bulk Al in the parietal cortex in AD, but an increase in ferritin (38%) and the non-haem iron content (45%) predominantly located in microglial cells (Kaneko *et al.*, 1989) associated with senile plaques (Grundke-Iqbal *et al.*, 1990). Moreover, Connor *et al.* (1992a,b) demonstrated intense ferritin immunoreactivity in senile plaques and blood vessels of brains from AD patients, suggesting a disruption of brain iron homeostasis. Since Al has also been found in the senile plaques and tangles of the functionally normal elderly (Perl and Brody, 1980; Candy *et al.*, 1986), there are doubts whether Al may be involved in neuronal degeneration and dementia. To date, there is only sparse experimental evidence for a role of Al in iron-induced oxidant stress *in vivo*.

Nevertheless, there is evidence from animal experiments and from cell culture systems that Al affects many biochemical and neurochemical metabolic events (for reviews, see Crapper McLachlan *et al.*, 1991; Van der Voet *et al.*, 1991; Mera, 1991). The experimental administration of Al or its salts by intracerebral or subcutaneous injection results in encephalopathies and in the production of Al-containing NFT (Wisniewski *et al.*, 1980; Trancoso *et al.*, 1982), due to accumulation of neurofilaments in the cell body and processes (axon, dendrites) of large neurons. However, the individual fibrils making up NFT in AD appear as PHF and are ultrastructurally different from the normal neurofilaments and those induced by Al (Munoz-Garcia *et al.*, 1986). On the other hand, AD-type tangles share determinants with normal and Al-induced neurofilaments and also appear to contain MAPs, tau and MAP-2 (Langui *et al.*, 1988). Although Shigematsu and McGeer (1992a) reported an accumulation of APP in damaged neuronal processes and microglia following intracerebral administration of Al salts, the authors state that this would not be specific to Al-induced pathology, but is rather a general response to disturbance of axoplasmic flow, regardless of the causative factors. They conclude that the value of models, such as the Al model of neuroskeletal toxicity, would be in revealing that APP accumulation can be secondary to interruption of axoplasmic transport from any cause, for example toxins, such as colchicine (Shigematsu and McGeer, 1992b), or impairment of mitochondrial energy production. Thus, there has to be a primary, maybe long-lasting, event that directly or indirectly precedes neuroskeletal degenerative changes. Epidemiologic studies linking AD to Al concentrations in water supply remain a matter of controversy (Reynolds *et al.*, 1992; Whalley *et al.*, 1992). There are far more important sources of Al in the diet (Davenport and Goodall, 1992). Thus, there is much to do in the future to elicit the real risk of Al for neurodegenerative diseases. Although Al may not be causative for the development of neurofibrillary pathology in AD, its contribution cannot be so far discounted (Shea *et al.*, 1992).

4.2. Factors Protecting Cells from Oxidative Stress

4.2.1. *Detoxifying Enzymes*

In order to prevent oxidative damage to DNA, proteins and lipids, cells are equipped with different antioxidative enzymes (Section 3.1.2.1). There is some evidence that these enzymes are altered in PD, whereas for AD very little information exists. In 1975, Ambani and colleagues found in PD patients that the non-GSH-dependent peroxidase was decreased in homogenates from SN, caudate and putamen (reduction $\sim 50\%$ of controls), but not in other brain regions. Kish *et al.* (1985) observed a lower, but significant, decrease of GSH-Px in frontal cortex, putamen, globus pallidus externus and SN (reduction $\sim 20\%$ of controls). However, this could not be verified by Marttila *et al.* (1988). Recently, GSH-Px was reported to be reduced in erythrocytes in PD. This reduction was correlated with the duration of the disease, but not with the age of patients (Johannsen *et al.*, 1991). In contrast, serum levels of GSH-Px and SOD seem to be increased in PD (Kalra *et al.*, 1992). Moreover, GSH-Px activity and levels of the vitamins E, C and A are reported to be decreased in erythrocytes of patients with AD (Jeandel *et al.*, 1989).

Since GSH-transport seems not to be affected in PD (GSH-transferase activity is not altered in SN in PD; Perry and Yong, 1986), decreases in peroxidase and GSH-Px activity would imply a possible increase in susceptibility to oxidative stress of some brain regions in PD.

The other important antioxidative enzyme, CAT, is in rat brain, predominantly located in microperoxisomes of catecholaminergic neurons and oligodendrocytes (McKenna *et al.*, 1976). Again, Ambani *et al.* (1975) detected significant reductions in CAT activity in PD in SN and putamen, but Marttila *et al.* (1988) could not confirm this.

It is evident that the extent of damage would be increased if H_2O_2-degrading enzyme activities are decreased or if production of H_2O_2 is enhanced. As outlined in Section 3.1, SOD generates H_2O_2. Interestingly, within the SNC, CuZnSOD gene is preferentially expressed in the neuromelanin-pigmented neurons (Ceballos *et al.*, 1990). Analyses of SOD activity in homogenates of PD patients have provided conflicting results. Whereas Marttila *et al.* (1988) reported increased CuZnSOD in temporal cortex, nucleus ruber, thalamus, SN and NBM, but not the caudate nucleus or putamen, Saggu *et al.* (1989) found an increase only in mitochondrial MnSOD in SN, but not in cerebellum. The altered activity of MnSOD may be an adaptive increase due to excess formation of $(O_2)^{\cdot-}$ from the mitochondrial respiratory chain or various enzymes, such as xanthine oxidase (Jenner *et al.*, 1992).

In AD patients, Ceballos *et al.* (1991), using immunohistochemical methods, detected a high level of CuZnSOD protein in large pyramidal neurons of the hippocampus, which are known to be susceptible to degenerative processes in AD. They argued that biochemical pathways leading to $(O_2)^{\cdot-}$ generation were specially active in these neurons, requiring an active transcription of the CuZnSOD gene. Alternatively, a high cellular CuZnSOD activity might also, by promoting H_2O_2 production, contribute to the vulnerability of these neurons, in particular within compartments low in GSH-Px or CAT activity (Hirsch, 1992).

4.2.2. *Antioxidants*

GSH-Px needs GSH as a substrate, and many attempts have been made to evaluate the levels of GSH and GSSG in PD. *Post mortem* changes can dramatically affect measurements of GSH (Reed *et al.*, 1980), with loss of GSH without concomitant increase of GSSG, indicating that peptidase activity could play a role in brain GSH levels. However, careful attention to selection of samples with respect to *post mortem* delay, sex and age should overcome these problems. In SN, there is a 40–50% reduction of GSH (Riederer *et al.*, 1989c; Jenner *et al.*, 1992; Sofić *et al.*, 1992). By contrast, no differences in GSH levels were observed in other brain regions in the same studies. There were no changes in the levels of GSSG, in agreement with the normal levels of GSSG-Rd found in PD (Marttila *et al.*, 1988). In addition, GSH-transferase was reported to be unchanged in *post-mortem* tissue (Perry and Yong, 1986). Thus, the decrease of GSH levels can only be explained by impairment of GSH synthesis (depletion of ATP, enzyme inhibition, decreased transcription or translation rates) or by oxidative degradation, maybe via thiyl radicals. To date, no data are available concerning activity of the rate-limiting synthetic enzyme for GSH formation in SN (γ-glutamyl cysteine synthetase). A decrease in levels of GSH and of GSH-Px activity could provide a source for H_2O_2 accumulating in cells. Thus, GSH concentration could be a key factor determining the fate of a cell at the threshold between life and death. It has been argued (Uhlig and Wendel, 1992) that, in order to be really a cause of cell death, GSH levels would have to be decreased to about 10% of that value existing in healthy cells (1 μmol/g GSH, 10 nmol/g GSSG; Slivka *et al.*, 1987b). GSH depletion to a lesser extent, however, renders cells more susceptible to impairments of cellular metabolism. Interestingly, the levels of GSH are decreased to the same extent in incidental LB disease, considered to reflect early presxymptomatic stages of PD (Gibb and Lees, 1988), as in advanced PD, despite a far less intensive neuronal loss in the SN. This could be an indication that impairment of GSH/GSSG equilibrium is an early event in neuronal degeneration. The importance of GSH for mental function is underlined by the observation that patients with GSH synthetase deficiency showed a gradual neurological deterioration of motor functions, retardation of movement, tremor and rigidity and psychomotor retardation beginning in childhood (Jellum *et al.*, 1983).

In addition to its antioxidant role, GSH recently was proposed to function as a neuroactive

peptide in the CNS (Guo and Shaw, 1992). Binding sites for GSH, possibly coupled to inositol phosphate production, were identified in cell membranes of astrocytes and oligodendrocytes. Since GSH is predominantly localized in non-neuronal cells (Slivka *et al.*, 1987a; Raps *et al.*, 1989; Philbert *et al.*, 1991), the severe loss of GSH in SN in PD has to be, at least in part, attributed to impairment of glial functions or to extensive neuronal loss. The latter seems unlikely because, in LB disease, cell loss was very moderate, but GSH depletion was nearly 40% (Jenner *et al.*, 1992).

Interestingly, patients with AD and AD plus PD exhibited increased levels of GSH in the hippocampus compared with controls (Adams *et al.*, 1991), and TOH content was doubled in the midbrain of both groups of patients. This raises the question whether these increases result from reactive gliosis or nerve terminal proliferation in response to neuronal loss. Whether this is specific for AD is not known but, as in PD, no elevated GSH levels were reported, despite gliosis.

In PD, there were no alterations in levels of TOH in serum (Férnandez-Calle *et al.*, 1992) nor in various brain regions, including SN, when compared with control subjects (Dexter *et al.*, 1992b). In addition, brain levels of ascorbate are not altered in PD (Riederer *et al.*, 1989c).

In conclusion, decreased activities of GSH-Px and CAT, as well as decreased GSH levels, increased activity of SOD and elevated levels of non-ferritin-bound iron concomitant with a high turnover of catecholamines, may participate in production of ROS. All these factors may render SN cells in PD more susceptible to hereto undefined toxic noxae and may provoke LPO and/or, as a consequence, lead to increased levels of intracellular Ca^{2+}, with activation of proteases, lipases and endonucleases.

As for AD, such a story seems less conclusive since, to date, investigations into the free radical theory of AD are scarce. Nevertheless, possible roles of Al or EAA in the pathogenesis of AD are currently under debate, and an impairment of Ca^{2+} homeostasis in AD brains, affecting membrane integrity and cytoskeleton, has been postulated.

4.3. Possible Consequences of Oxidative Stress in the Central Nervous System

If there is a real increase in H_2O_2 and $(O_2)^{\cdot-}$ production in PD or AD markers of LPO, hydroxylated nucleotides or signs of protein oxidation should be found in *post mortem* tissue, at least in those brain regions that undergo the pathogenetic process of degeneration. To date, there is very little information available on markers of oxidative stress in neurodegeneration. For PD, only two parameters were studied, namely the substrate for LPO, polyunsaturated fatty acids, and the content of TBARS. Levels of polyunsaturated fatty acids of parkinsonian SN were decreased compared with controls (Dexter *et al.*, 1989a). Thus it appeared that perhaps increased degradation of polyunsaturated fatty acids did occur in parkinsonian SN. This view was confirmed by the finding of a selectively increased level of TBARS in SN of parkinsonian patients. To our knowledge, there is currently no information available concerning oxidatively damaged proteins or nucleotides in parkinsonian SN. There is, however, an indication of increased protein oxidation in aging (Stadtman, 1992) and increased vulnerability of frontal cortex of AD brains to age-related protein oxidation (Smith *et al.*, 1991). In addition, Zemlan *et al.* (1989) suggested that hydroxyproline residues found in amyloid deposits of Alzheimer brain arose from free radical-induced oxidation of proline. Furthermore, a selective increase in susceptibility to iron/oxygen- or oxygen-induced formation of TBARS in frontal cortex of AD brains could be detected by Subbarao *et al.* (1990) and Götz *et al.* (1992), respectively. This could be indicative of defective defense systems against ROS.

Fatty acid peroxides become rapidly deacylated by phospholipase A_2. The resulting lysophospholipid leads to labilization of the lipid membrane, if it is not removed or reacylated. Levels of glycerophospholipids, plasmalogens and polyphosphoinositides are markedly decreased in patients with AD compared with age-matched control subjects (Suzuki *et al.*, 1965; Stokes and Hawthorne, 1987; Farooqui *et al.*, 1988a; Gottfries, 1990; Nitsch *et al.*, 1992; Söderberg *et al.*, 1992; Jellinger *et al.*, 1993). This decrease in glycerophospholipids is correlated with elevations of phospholipid degradation metabolites, such as glycerophosphocholine, phosphocholine and phosphoethanolamine, in autopsy samples of AD patients (Barany *et al.*, 1985; Miatto *et al.*, 1986; Pettegrew *et al.*, 1988; Blusztajn *et al.*, 1990). These changes may be associated with elevated activities of lipolytic enzymes in AD (Farooqui *et al.*, 1988b, 1990). As a result of deacylation of

fatty acids from lipids, increases in levels of prostaglandins may occur in AD (Iwamoto *et al.*, 1989). Fatty acid composition seems to be altered only in selected phospholipids of frontal grey matter and in hippocampus, with a substantial increase in the relative amounts of the saturated components 14:0, 16:0 and 18:0 paralleled by a decrease in polyunsaturated fatty acids 20:4, 22:4 and 22:6 (Söderberg *et al.*, 1991). In contrast, analysis of fatty acid composition of total lipid fraction from grey or white matter of the frontal cortex of AD brains and occipital lobes (Antuono *et al.*, 1991) showed no differences from controls. This could mean that the free fatty acid pool increases with an increase in eicosanoid production. Besides elevated formation of lipid peroxides, impairment of phospholipid synthesis, of the microsomal desaturase system (Strittmatter *et al.*, 1974; Jeffcoat, 1979) or of deficiency in uptake or reacylation of fatty acids could account for the above-mentioned changes in lipids (Dhopeshwarkar and Mead, 1973). Thus, the changes in fatty acid composition of selected lipids (phosphatidylcholine and phosphatidylethanolamine, predominantly located in subcellular membranes; Söderberg *et al.*, 1991) are very unspecific in nature, albeit consistent with oxidative stress. There may be also a link between EAA and abnormal phospholipid metabolism in AD (Section 3.3). Since no correlations have been found between levels of phosphatidylcholine and choline acetyltransferase activity in AD frontal cortex, the changes in membrane phospholipids may not be confined to cholinergic terminals. This supports the hypothesis of a defective biosynthesis or stimulated degradation of phospholipids in AD, pointing towards a generalized defect preceding the formation of neuritic plaques. Since amyloid formation in AD brains requires abnormal processing of the APP (Müller-Hill and Beyreuther, 1989; Sisodia *et al.*, 1990; Esch *et al.*, 1990; Katzman and Saitoh, 1991; Selkoe, 1991), defective membrane metabolism could expose the APP transmembrane domain to proteolytic cleavage, enhanced by increased Ca^{2+} influx; alternatively, amyloidogenic APP fragments may be poorly anchored to defective membranes and thus, released into the neuropile (Nitsch *et al.*, 1992).

Therefore, taking into account the existing knowledge concerning the etiology of AD and PD, further effort is needed to define the physiological importance of the accumulation of iron or Al in specific brain regions, of antioxidant systems and of alterations in membrane composition, in order to confirm or to rule out a role for oxidative stress as a pathogenetic factor in neurodegeneration.

5. IMPAIRMENT OF MITOCHONDRIAL FUNCTION

5.1. TETRAHYDROPYRIDINES, TETRAHYDROISOQUINOLINES AND TETRAHYDRO-β-CARBOLINES, NEUROTOXINS PRODUCING A PARKINSON-LIKE SYNDROME IN ANIMALS AND HUMANS

The neurotoxic substance MPTP produces a clinical syndrome strikingly similar to idiopathic PD in humans (Langston *et al.*, 1983), and induces nigrostriatal cell loss with concomitant decrease in DA and its metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid), in monkeys (Burns *et al.*, 1983; Jenner *et al.*, 1984; Langston *et al.*, 1984a), mice (Heikkila *et al.*, 1984a; for reviews, see Heikkila *et al.*, 1989a,b; Gerlach *et al.*, 1991b), cats (Schneider *et al.*, 1986), dogs (Johannessen *et al.*, 1985) and even goldfish (Pollard *et al.*, 1992). Although the chronic progressive nature of the symptomatology of parkinsonism could never be elicited in this model (Birkmayer and Riederer, 1985; Gerlach *et al.*, 1991a), administration of MPTP provides, at present, the most thoroughly investigated animal model for PD.

The mechanism of toxicity of MPTP clearly involves bioactivation to *N*-methyl-4-phenyl-dihydropyridinium ion ($MPDP^+$) by MAO-B in glia (Heikkila *et al.*, 1984b; Langston *et al.*, 1984b) and serotonin-containing neurons (Westlund *et al.*, 1985). The *N*-methyl-4-phenyl-pyridinium ion (MPP^+), the degradation product of $MPDP^+$, is an effective substrate for the catecholaminergic synaptosomal uptake system (Javitch *et al.*, 1985; Chiba *et al.*, 1985). Active uptake of MPP^+ was also observed in synaptosomes prepared from extrastriatal brain regions (e.g. hypothalamus, hippocampus, cerebellum; Chiba *et al.* 1985). At least two possible pathways exist by which MPP^+ has been postulated to be toxic:

(1) oxidative stress could ensue (Johannessen *et al.*, 1986); or

(2) mitochondrial respiration could be inhibited in vulnerable neurons (Nicklas *et al.*, 1985; Ramsay *et al.*, 1986a,b).

While some preliminary reports indicated that administration of ascorbic acid (Sershen *et al.*, 1985; Wagner *et al.*, 1985) or TOH (Perry *et al.*, 1985) to mice is partially protective against MPTP toxicity, other reports dispute protection (Smith *et al.*, 1987; Perry, T. L. *et al.*, 1987; Baldessarini *et al.*, 1986; Martinovits *et al.*, 1986; Mihatsch *et al.*, 1991; Gong *et al.*, 1991). However, the toxicity of MPTP has been found to be potentiated by TOH deficiency (Odunze *et al.*, 1990) or after depletion of brain GSH by intracerebroventricular injections of diethylmaleate (Adams *et al.*, 1989). Young *et al.* (1986), as well as Riederer *et al.* (1987), found that MPTP slightly lowers mouse brain stem GSH levels, an effect that could be explained by reduced NADPH-dependent GSSG-Rd or by higher need of GSH by GSH-peroxidase because of detoxification of putatively increased amounts of hydroperoxides. Therefore, it seems crucial to protect cells from loss of antioxidants and potent reducing substances, such as NADPH or NADH. In turn, depletion of cells from ATP by inhibition of oxidative phosphorylation could result in decreased activity of 5-oxoprolinase, glutamyl-cysteine-synthetase and GSH synthetase, all known to be dependent on ATP (Meister, 1991), resulting in impaired detoxification mechanisms and subsequent cell damage because of oxidative stress.

On the other hand, ATP depletion caused by MPP^+, potassium cyanide or antimycin A (the latter two substances are known as definite inhibitors of mitochondrial respiratory chain) could lead to decreased GSH concentrations independent of oxidative stress, because substrates for glycolytic production of ATP counteract the GSH depletion caused by mitochondrial respiratory chain inhibitors (Mithöfer *et al.* 1992).

Thus, we have to consider a second hypothesis of MPTP neurotoxicity. Metabolism, transport and storage of MPTP within cells is functionally closely connected with mitochondria. Once created by the MAO in glial cells, MPP^+ is suspected to be actively concentrated in mitochondria of astrocytes and of dopaminergic neurons (Ramsay and Singer, 1986). Reports of MPP^+ interference with mitochondrial function and ATP formation provide an alternative possible mechanism to explain toxicity of MPP^+ (for a review, see Gerlach *et al.*, 1991b).

MPP^+ is a potent inhibitor of oxidation of the NAD^+-linked substrates pyruvate/malate and glutamate/malate in isolated rat liver and brain mitochondria, while leaving the oxidation of succinate unaffected (Nicklas *et al.*, 1985). The locus of inhibition of the mitochondrial respiration is assumed to be between the highest potential Fe-S cluster in NADH dehydrogenase and the coenzyme Q located probably at the rotenone-binding site (Ramsay *et al.*, 1991). As a consequence of inhibition of respiration, cellular energy supplies in the form of ATP would rapidly be consumed, followed by depolarization of membranes, probable Ca^{2+} influx and overstimulation of Ca^{2+}-dependent lysosomal enzymes.

Whichever mechanism of toxicity is exerted by MPP^+, the result could be impairment of 'GSH homeostasis' in mitochondria. Adopting this hypothesis, some investigators have sought to identify possible endogenous compounds similar in structure to MPTP and MPP^+ in parkinsonian patients and in animal models of parkinsonism. Amongst these substances, tetrahydroisoquinolines (THIQ) and tetrahydro-β-carbolines (THBC) gained considerable interest recently, because they produce parkinsonism in animals after long-term treatment (Nagatsu and Yoshida, 1988; Yoshida *et al.*, 1990) and are found in foods, such as cheese, milk, chocolate powder and wine and even endogenously in brain (Makino *et al.*, 1988; Adachi *et al.*, 1991a,b; Rommelspacher *et al.*, 1991).

The mechanisms of toxicity of THIQ and THBC are supposed to be similar to that of MPTP. Subsequent to uptake by catecholaminergic transporters and MAO-catalyzed oxidation, the resulting isoquinolinium ions or carbolinium ions and/or *N*-methylated forms could accumulate within mitochondria and disturb electron flow in the respiratory chain (Suzuki *et al.*, 1988, 1992; Albores *et al.*, 1990; Collins *et al.*, 1992; Fields *et al.*, 1992). Naoi *et al.* (1989a,b) and Matsubara *et al.* (1992a,b) have provided evidence that it is predominantly the *N*-methylated THIQ and THBC, which are oxidized by MAO. The resulting *N*-methyl-isoquinolinium ions (MIQ^+) or *N*-methyl-β-carbolinium ions (MBC^+) are effective enzyme inhibitors *in vitro* (Naoi *et al.*, 1989c; Sayre *et al.*, 1991). Selectivity of TH-like immunoreactive cells for MIQ^+ at 100 μM was demonstrated (Niijima *et al.*, 1991), suggesting its uptake and accumulation selectively by

catecholaminergic neurons. Despite less severe toxicity of MIQ^+ compared with that of MPP^+ *in vivo* (to induce a similar decrease of DA in striatum by intranigral administration of MIQ^+ or MPP^+, a 20-fold higher concentration of MIQ^+ than of MPP^+ is needed; Sayre *et al.*, 1991), prolonged accumulation of such compounds over a lifetime from foods or because of severe ethanol uptake (Collins *et al.*, 1990) could putatively be a risk factor for catecholaminergic neurons. This is especially true in subjects with a poor metabolizing capacity involving cytochrome P450 reductase, where brain accumulation of THIQ is enhanced (Ohta *et al.*, 1990). Interestingly, it has been found that, among patients with PD, many are poor debrisoquine metabolizers (Poirier *et al.*, 1987). This implies that PD may be associated with low levels of the specific form of cytochrome P450, which oxidizes debrisoquine and potentially MPTP-like environmental toxins. Recently, however, using immunoassays sensitive to a broad range of compounds structurally related to MPTP and MPP^+, Ikeda *et al.* (1992) could not demonstrate altered immunoactivity in striatum of parkinsonian versus control brains, suggesting that compounds chemically related to MPTP are not likely to exist (at a nanogram range) in PD brain at the time of death. Nevertheless, it can be imagined that such compounds could be initiators of toxic events (genetic defects, impairment of mitochondrial function), leading to loss of a certain amount of cells, but not enough to produce parkinson-like symptoms early in life. However, due to the age-related decline of dopaminergic cells, PD would become manifest at a later stage of life.

If isoquinolines or carbolines played a major role in PD, consumers of large amounts of cheese, chocolate powder and wine would be expected to show at least a greater prevalence of PD than others, since, for example, THIQ may be formed by ring cyclization of 2-phenylethylamine in foods containing formaldehyde or in combination with ethanol in beverages. However, this seems not to be the case (Andrade, 1991).

The discovery of MPTP has rekindled the environmental toxin theory of PD that started with PD in manganese miners (Barbeau, 1984). Nevertheless, despite a number of epidemiological studies that suggest an unisotrope occurrence of PD in some populations, perhaps related to exposure to environmental toxins (Barbeau *et al.*, 1986; Rajput *et al.*, 1987; Tanner, 1989; Ho *et al.*, 1989; Goldsmith *et al.*, 1990) and the considerable efforts made to elucidate the mechanisms of toxicity of various exogenous and even endogenous compounds, to date it still is not possible to define the ultimate 'parkinsonism-inducing toxin'.

5.2. Activities of Enzymes of the Respiratory Chain in Parkinson's and Alzheimer's Diseases

Mitochondria are one of the main generators of ROS (Section 3.2.2.2). Consequently, at the site of cellular free radical generation, the enzymes of the respiratory chain and the mitochondrial DNA (mtDNA) are particularly susceptible to damage by ROS. The rate of mitochondrial $(O_2)^{\cdot-}$ and H_2O_2 generation increases with age in houseflies and in the brain, heart and liver of the rat (Sohal and Sohal, 1991; Sohal *et al.*, 1990; Sohal, 1991; Sohal and Brunk, 1992). In addition, mitochondrial respiratory chain functions in human muscle (Trounce *et al.*, 1989) or human liver (Yen *et al.*, 1989) have been reported to decline with increasing age. Therefore, we have to ask whether these accelerated age-related events are important in the pathology of neurodegenerative diseases with adult onset, such as PD and AD. In PD, several groups of investigators have reported mitochondrial respiratory dysfunctions in brain, muscle and platelets. Using immunoblotting techniques with specific antisera against enzyme complexes I, III and IV, Mizuno *et al.* (1989) found a decrease in four out of five patients with PD in the 30-, 25- and 24-kDa subunits of complex I. In contrast, Schapira *et al.* (1989, 1990a,c) and Mann *et al.* (1992a) detected a decrease in complex I activity in the SN of PD, but not in cerebral cortex, cerebellum, globus pallidum, caudate nucleus and tegmentum. Due to the high amount of glial cells in the brain, it has to be assumed that, in addition to that of neuronal cells, glial complex I is also defective. The absence of changes in activities of mitochondrial enzyme complexes I–IV in MSA suggests that complex I deficiency in PD is not due to cell death and, perhaps, may be specific to PD (Schapira *et al.*, 1990a). Immunohistochemical studies in PD showed a fair proportion of the nigral neurons with reduced staining against complex I and, in three patients, against complex II antibodies, whereas staining for complexes III and IV appeared normal (Hattori *et al.*, 1991).

Two studies using platelets failed to detect abnormalities in the enzyme activities of respiratory

chain complexes (Mann *et al.*, 1992a; Bravi *et al.*, 1992), in contrast to Parker *et al.* (1989), who reported a 55% decrease in the mean platelet mitochondrial complex I activity, and Yoshino *et al.* (1992), who published small but significant decreases in platelet complex I and II activities.

Studies on skeletal muscle from PD patients have produced conflicting results, also. As summarized by Schapira and Cooper (1992), three studies have shown multiple respiratory chain deficiencies in some patients, pure complex I deficiency in others and normal activities in one patient with advanced disease (Bindoff *et al.*, 1991; Shoffner *et al.*, 1991; Nakagawa-Hattori *et al.*, 1992).

Because the reasons for these inconsistent findings are still unknown, it seems impossible, to date, to develop a diagnostic test for PD using blood cells or biopsies from peripheral tissues, such as muscle.

A similar inconsistency exists concerning oxidative phosphorylation in AD. Although activities of enzymes of the mitochondrial electron transfer chain are reported to be normal in AD brain, partial uncoupling of oxidative phosphorylation (electron transfer and phosphorylation of adenosine diphosphate are normally functionally linked; Sims *et al.*, 1987) and overexpression of cytochrome oxidase subunit-3 gene in cerebral temporal cortices (Alberts *et al.*, 1992) have been reported. In addition, substantial decreases of complex IV activity were detected in platelets from five patients with AD (Parker *et al.*, 1990).

Although not entirely convincing, these results could point towards a more generalized defect of oxidative phosphorylation in neurodegeneration, with a possible genetic determination.

5.3. Alterations in Mitochondrial Deoxyribonucleic Acid

The 16.6 kb human mtDNA codes for two ribosomal ribonucleic acids, 22 transfer ribonucleic acids and 13 peptides, which are part of enzyme complexes of the respiratory chain in the inner mitochondrial membrane (Capaldi, 1988). Mitochondria are largely, but not entirely, maternally inherited (Gyllensten *et al.*, 1991). They proliferate independently of the cell cycle. In mammals, mtDNA mutates much faster than nuclear DNA, possibly because mtDNA is not covered by histones, and is at least transiently attached to the inner mitochondrial membrane, where large amounts of ROS are produced. Therefore, mtDNA is particularly susceptible to oxidative damage. The steady-state level of oxidized bases in mtDNA is about 16 times higher than in nuclear DNA (Richter *et al.*, 1988; Hruszkewycz and Bergtold, 1990). Numerous base modifications are detectable when ROS react with DNA (von Sonntag, 1987). The most studied oxidized base is 8-hydroxydeoxyguanosine, which can be measured in the femtomolar range (Halliwell and Aruoma, 1991). ROS generate strand breaks in mtDNA (reviewed by Richter, 1988, 1992), and DNA repair in mitochondria is much less efficient than in the nucleus. These mammalian organelles do not have significant recombinational repair, but may excise damaged bases. Mutations of mtDNA are the cause of some oxidative phosphorylation diseases with prominent basal ganglia pathology, such as Leigh disease (Montpetit *et al.*, 1971; Pincus, 1972) and Leber's disease with dystonia (Novotny *et al.*, 1986). Kearns–Sayre syndrome and mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes may show basal ganglia calcifications, neuronal loss and spongiform histopathological changes (Horwitz and Roessmann, 1978; Driscoll *et al.*, 1987; Ichiki *et al.*, 1988). These mitochondrial diseases are predominantly maternally transmitted (Harding, 1991). It is now evident that large deletions of mtDNA, duplication of mtDNA or point mutations of mtDNA account for the metabolic changes observed in these diseases (Harding, 1991; Wallace, 1989, 1992).

However, as for PD, pedigree analysis has identified only a few families with familial PD, suggesting autosomal dominant inheritance with variable penetrance (Golbe *et al.*, 1988, 1990), but providing no evidence of maternal inheritance (Maraganore *et al.*, 1991). Twin studies in PD have not provided very conclusive data on inheritance susceptibility, although there are promising activities in progress (Johnson *et al.*, 1990; Duvoisin and Johnson, 1992). Initial reports of an increase in mtDNA deletion in SN of parkinsonian patients (Ozawa *et al.*, 1990; Ikebe *et al.*, 1990) could not be confirmed by others (Schapira *et al.*, 1990b; Lestienne *et al.*, 1990, 1991), but was attributed to an age-related phenomenon (Yen *et al.*, 1991; Mann *et al.*, 1992b). The role of genetic factors in the etiology of sporadic cases of PD remains to be determined. There is no evidence that

major deletions of mtDNA occur in SN in PD, indicating that a further biochemical insult (toxins, ROS) could act directly on mitochondrial enzymes or that the nuclear genome is damaged. Clearly, further effort is needed in this field of research.

6. EXCITATORY AMINO ACIDS AND NEURODEGENERATION

The importance of EAA in physiological function of neural transmission is well known. Glutamate was the first of them to be recognized. In the meantime, a further subdivision of the glutamate receptors has taken place. At the moment, we know of four glutamate receptor subtypes (Watkins *et al.*, 1991): (1) the NMDA, (2) the α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA), (3) the kainate and (4) the metabotropic receptor.

The EAA are involved as neurotransmitters in the sensory input of spinal and supraspinal systems. Another important function of EAA is to contribute to the programming and execution of movements in the motor loop (Riederer *et al.*, 1989b). A further EAA-mediated neuronal function is the process of learning and memory, where the NMDA receptor, in particular, is thought to be important because of its electrophysiological action in long-term potentiation, which introduces synaptic plasticity (Monaghan *et al.*, 1989; Collingridge and Singer, 1991).

Besides the role of EAA in physiological actions, an excitotoxic etiology of neurodegenerative diseases has been proposed in the last few years. A lot of investigations show a noxious effect of EAA, which is mediated by Ca^{2+} influx into the cytoplasm (Monaghan *et al.*, 1989; Mayer and Miller, 1991). Endogenous and environmental EAA receptor agonists can cause acute and chronic neurodegenerative diseases, resulting in dysfunction of motion and memory. Environmental diseases, such as Guam disease, neurolathyrism and mussel poisoning, can be used for studying the putative role of excitotoxic substances in PD, ALS and dementia.

In Guam disease, a still unidentified ingredient of the cycas seed, perhaps the sago palm toxin 2-amino-3-(methylamino)-propanoic acid (Kurland, 1988; Meldrum and Garthwaite, 1990, 1991; Duncan *et al.*, 1990), causes a syndrome characterized by dementia and by aspects of ALS, PD or both, in people living on the islands of Guam and Rota (Zang *et al.*, 1990). The noxious agent acts by binding on the NMDA-receptor ion channel (Meldrum and Garthwaite, 1990, 1991). Although there are valid doubts concerning the participation of the sago palm toxin in the etiology of Guam disease (Spencer *et al.*, 1990), the improvement of MPTP-provoked hypokinesia by treatment with EAA antagonists (Löschmann *et al.*, 1991; Klockgether *et al.*, 1991) supports the general hypothesis of a glutamate dysbalance in PD.

Investigation of glutamate receptor density in PD SNC has shown a loss of glutamate-receptor subtypes (Difazio *et al.*, 1992). The heterogeneous distribution of glutamate-receptor subtypes in the striatum and the participation of these receptors in movement function has lead to the hypothesis of glutamate imbalance in motor system disturbances that show opposite clinical pictures (Albin *et al.*, 1991, 1992).

One implication for the excitotoxic cell damage in PD and HD is the binding of 6-hydroxylated L-DOPA on non-NMDA receptors. Thus, the hypothesis arises that an abnormal derivative of the natural DA precursor may be responsible for destroying nigral and striatal cells in these diseases (Hanson *et al.*, 1985; Olney *et al.*, 1990; Cha *et al.*, 1991).

Application of kainic acid, and the more specific quinolinic acid, into the striatum is followed by Huntington-like structural and biochemical changes; excitotoxin injection into the striatum produces locomotor hyperactivity and deficits in learning tests (DiFiglia, 1990).

Quinolinic acid is an endogenous NMDA receptor agonist formed from L-tryptophan, thought to be elevated in HD. Although there is still disagreement that neurodegeneration in HD is mediated by altered tryptophan/quinolinic acid ratios, the excitotoxic model of HD is a solid approach to the elucidation of the role of glutamate in movement disorders (Schwarcz *et al.*, 1987; Reynolds *et al.*, 1988; Connick *et al.*, 1988; Brogn and Stoof, 1990; Bakker and Foster, 1991).

In AD, impairment of memory and cognition could reflect disturbances in NMDA-mediated long-term potentiation, and a decline of NMDA receptors in hippocampal and cortical regions has been found (Jansen *et al.*, 1990). Under hypercalcemic conditions, stimulation of NMDA receptors

can produce formation of cytoskeletal NFT-like structures. Therefore, NFT could be seen to be a consequence of the degenerative process.

One of the best investigated environmental diseases is neurolathyrism, a spastic paresis, resulting from damage of the corticospinal tract. A reliable connection between the lathyrus toxin β-*N*-oxalylamino-L-alanine and the degenerative process of the upper motoneuron has been shown (Mertens, 1947; Spencer *et al.*, 1990). In this case, the neurotoxic process is initiated by binding on non-NMDA receptors (Ross *et al.*, 1989). Implication of EAA in cognitive processes is documented in a further exotoxic environmental disease: in domoic acid poisoning, many people have shown a severe memory impairment after eating mussels (Sutherland *et al.*, 1990; Teitelbaum *et al.*, 1990; Strain and Tasker, 1991). Domoic acid is well known to bind on the kainate receptor.

Excitotoxicity also plays a role in epileptic seizures and cerebral ischemia. During ischemic injury, a rapid accumulation of glutamate takes place. This induces a noxious Ca^{2+} influx into the cytoplasm. The Ca^{2+} conductance of NMDA receptors explains the efficacy of NMDA antagonists against hypoxic damage. In addition, AMPA antagonists have also a protective function in hypoxia (Siesjö *et al.*, 1991)

In kindling experiments systemic administration of kainic acid in rats induces epileptic seizures (De Vera *et al.*, 1991).

The activation of non-NMDA receptors is sufficient for epileptogenesis, but the latency before the onset of convulsions, their duration and the resulting brain damage depends critically on NMDA participation (Hwa and Avoli, 1991).

The action of glutamate in epilepsy is assumed to be the consequence of NMDA-receptor binding and of AMPA-receptor activation as well. *In vivo* kindling studies provoke epileptic activity. *In vitro* application of glutamate on hippocampal cell cultures burst firing. These phenomena can be reduced by NMDA antagonists.

7. CURRENT THERAPY OF PARKINSON'S DISEASE

PD is the only neurodegenerative disorder we can symptomatically treat in a very specific and effective manner. Since the first description of the disease by James Parkinson in 1817, a number of procedures for handling this disease have been developed.

For a long time, anticholinergic drugs were the only effective treatment of parkinsonian symptoms. The therapeutic mechanism of these drugs is suppression of the relative cholinergic overactivity in the striatum of parkinsonian patients.

Since the detection of DA deficiency in PD, neurotransmitter replacement has been the therapy of choice (Coleman, 1992). The transmitter has been applied in form of its precursor L-DOPA, combined with a peripheral decarboxylase inhibitor (e.g. benserazide), so that the drug's efficacy develops only in the CNS. L-DOPA is stored in the remaining nigrostriatal dopaminergic neurons, decarboxylated to DA and then released. It acts postsynaptically on D_1 and D_2 receptors, so that the natural function of the transmitter is imitated and the clinical signs improve. The patients reach a non-fluctuating, constant degree of mobility.

Over the years the L-DOPA-storing nigrostriatal neurons continue to diminish, so that DA acts on its receptors in an uncontrolled and pulsatile manner. The clinical correlate of this is an immediate switch of the patients' mobility from bad to good, and *vice versa*, depending on L-DOPA application. The receptors become supersensitive and dyskinetic phases develop (Coleman, 1992). These are also thought to be triggered by DA acting on the D_1 receptors of remaining descending striatonigral neurons and, in this way, increasing the GABA release in SNR (Robertson, 1992). This undesired L-DOPA effect should be prevented by application of low and frequent L-DOPA doses, not really a successful strategy (Rajput *et al.*, 1984; Poewe *et al.*, 1986a), or by controlled L-DOPA release over the day, a useful but difficult technique (Poewe *et al.*, 1986b; Ceballos-Baumann *et al.*, 1990; Gauthier and Amyot, 1992).

L-DOPA therapy may have some disadvantages: the glial MAO-B metabolizes dopamine, decreasing the neurotransmitter content and increasing H_2O_2 (Fig. 2) (Riederer *et al.*, 1989a; Olanow, 1992). With MAO-B inhibitor treatment, the DA content increases and oxidative damage is diminished (Riederer *et al.*, 1989a; Koller, 1992). This is an important implication of combining

L-DOPA with L-deprenyl (Coleman, 1992). Protection from the complications of L-DOPA therapy is possible by giving direct DA-receptor agonists, such as the ergot derivates bromocriptine, lisurid, pergolid and apomorphine (Lataste, 1984).

The most important effect of combining a D_1/D_2 agonist, such as bromocriptine, with L-DOPA is a decrease in duration of dyskinetic periods (Montastruc, 1991; Rabey *et al.*, 1991). The motor fluctuations in advanced DOPA-treated patients can be well controlled by subcutaneous apomorphine or lisurid (Poewe *et al.*, 1986b), while an early combination of lisurid with low L-DOPA doses can prevent the development of fluctuations and dyskinesias (Madeja, 1992). A limiting factor, especially in lisurid therapy, is the psychotic potency of antiparkinsonian drugs. One possible method of suppressing this side effect is to add the atypical neuroleptic drug clozapine, which does not bind on D_2 receptors and, therefore, cannot reverse the effect of dopaminergic drugs (Wolters *et al.*, 1989).

In view of the interaction of glutamate and DA of the motor loop in physiological and pathological conditions, glutamate-receptor blockade may offer another avenue for PD treatment (Riederer *et al.*, 1992). Amantadine, which is thought to increase DA release (Coleman, 1992), has been reported to act by blocking the NMDA ion channel (Kornhuber *et al.*, 1991). This well-noted antiparkinsonian drug has also a potent psychotic activity (Danielczyk, 1980).

Antagonists at AMPA-receptors, in combination with low-dose L-DOPA, may open new perspectives in PD treatment because of their antiakinetic effect (Löschmann *et al.*, 1991).

Another antiparkinsonian drug is piribedil, a direct DA agonist acting in the nigrostriatal and mesolimbic systems; its properties are reduction of tremor and decrease of age-related cognitive decline (Jenner, 1992; Ollat, 1992; Randot and Ziegler, 1992).

8. PROTECTIVE VS SYMPTOMATIC THERAPY

8.1. Antioxidative Strategy for Neuroprotection

If ROS are involved in the initiation or progression of neurodegeneration, then scavengers of ROS should ideally stop the pathogenetic process and preserve the function and integrity of vulnerable neurons. Because iron may promote formation of ROS, its abnormal presence may contribute to oxidative stress. Thus, strategies have been designed to reduce entry of iron into the brain, to increase non-toxic brain storage of iron and to remove iron through chelation. Desferrioxamine, the most widely used chelating agent, however, does not enter the brain after systemic administration, thus diminishing only the peripheral iron (Halliwell, 1989a,b). D-penicillamine was shown to reduce brain iron content when it was given intraperitoneally to rats. It was shown to be an inhibitor of iron-induced LPO *in vitro* and *in vivo* (Ciuffi *et al.*, 1992) and to chelate copper efficiently. For this reason, penicillamine aroused reasonable interest for the treatment of Wilson's disease. In this autosomal recessive disorder, agents that deplete brain copper, such as penicillamine, are effective in slowing basal ganglia damage, supporting restoration of neurological function and preventing the onset of illness in presymptomatic homozygotes (Scheinberg and Sternlieb, 1984). Because iron is involved in many biologically important processes, long-term treatment with iron chelators could provoke many adverse effects and, therefore, seems not to be feasible. Thus experimental neuroprotective strategies may be focused on preventing deleterious biochemical consequences of increased brain iron. A variety of agents are purported to inhibit iron-dependent oxidative stress in brain, including ascorbic acid, aminosteroids, TOH and L-deprenyl (a selective MAO-B inhibitor). The latter two substances are currently being subjected to clinical trials for the treatment of PD (Fig. 6).

Novel steroids (21-aminosteroids, lazaroids and 2-methylaminochromans) were developed for acute treatment of traumatic or ischemic CNS injury (for reviews, see Jacobsen *et al.*, 1990; Hall, 1992). They were shown to be potent inhibitors of iron-dependent LPO in liposomes or of peroxidation of linoleic acid in the presence of the methanol-soluble free radical generator [2,2′-azobis(2,4-dimethyl valeronitril) (Braughler *et al.*, 1987; Braughler and Pregenzer, 1989)], presumably by scavenging lipid peroxyl radicals and thus, blocking lipid–radical chain reactions in a manner similar to TOH. Indeed, administration of the lazaroid U74006F (tirilazad mesylate)

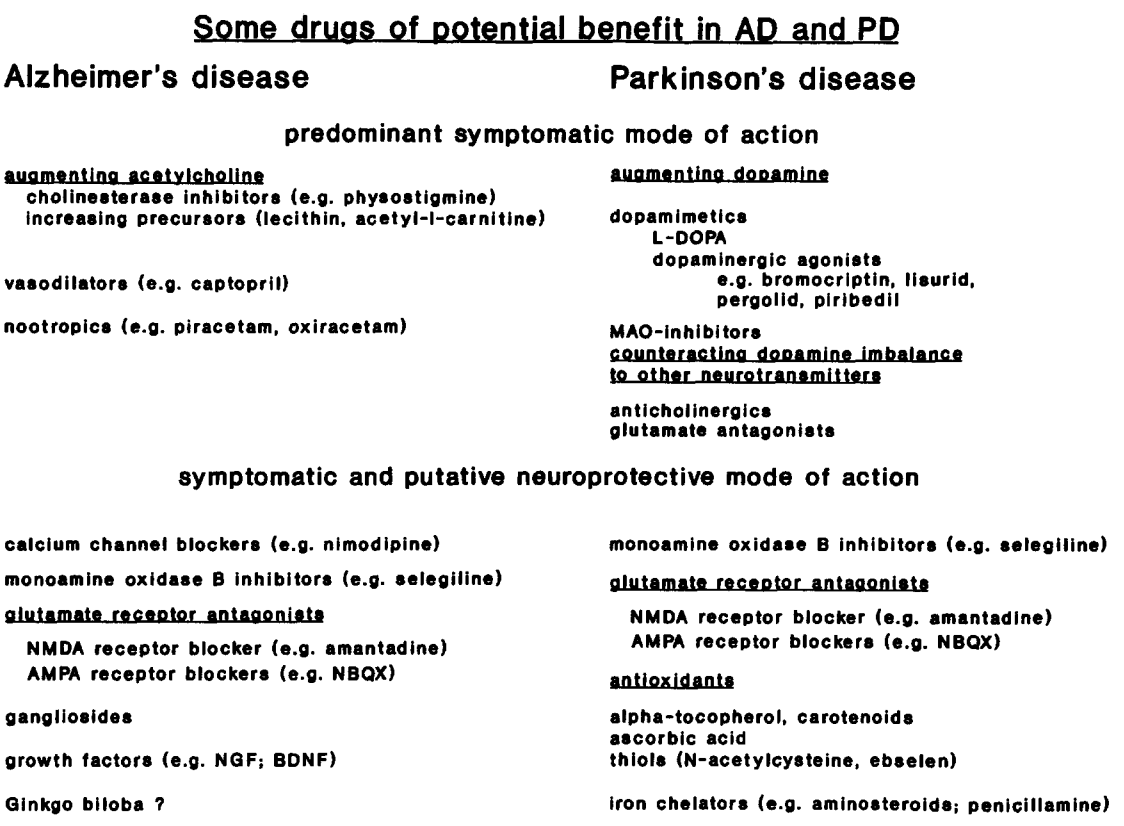

FIG. 6. Drugs with known and putative effects in treatment of neurodegenerative diseases or in treatment of animal models of these diseases. NBQX, α-amino-3-hydroxy-5-methyl-4-isoxazole-propanoic acid.

prevented decreased brain levels of ascorbate and TOH during brain ischemia reperfusion injury in gerbils (Sato and Hall, 1992).

The importance of TOH for human brain function becomes dramatically evident in patients with intestinal fat malabsorption syndromes, who suffer from a severe and prolonged deprivation of TOH (Muller and Goss-Sampson, 1990). *In vitro*, TOH supports the survival and neurite extension of neurons of fetal rat brain (Nakajima *et al.*, 1991) and thus, could be putatively beneficial for viability of neurons *in vivo*. This hypothesis has prompted a pilot study of high dose TOH and ascorbate in early PD (Fahn, 1992). Twenty-one patients were treated with antimuscarinics and amantadine, but not with L-DOPA, and received TOH and ascorbic acid up to 3200 U/day and 3000 mg/day, respectively. The end-point for analysis was the time at which L-DOPA or DA agonist had been required (i.e. when symptoms were severe enough to become a threat to employment or to social or physical capacity). Compared with an independent control group, the need for L-DOPA was delayed by 2–3 years in patients receiving vitamins. TOH is part of the large North American multicenter, controlled trial known as DATATOP (Deprenyl And Tocopherol Antioxidant Therapy Of Parkinsonism), which is evaluating 2000 U/day TOH and 10 mg/day L-deprenyl (Section 8.2) in 800 patients with early PD. However, there was no beneficial effect of TOH alone or any synergistic interaction between TOH and L-deprenyl (The Parkinson Study Group, 1989a,b, 1993). By contrast, a slight beneficial effect on tardive dyskinesia ratings following TOH could be observed in patients who had had this disease for 5 years or less (Egan *et al.*, 1992).

8.2. L-DEPRENYL® (SELEGILINE®), A SELECTIVE MONOAMINE OXIDASE-B INHIBITOR, IN THE TREATMENT OF EARLY PARKINSON'S DISEASE

If oxidative products of catecholamine metabolism indeed do provide free radical production and provoke progressive deterioration of the DA nigrostriatal system, then blockage of formation of

H_2O_2 by MAO and blockage of accumulation and high turnover of DA without depriving the postsynaptic DA receptors of ligand (e.g. by using agonists selectively acting on presynaptic D_2-receptors, such as pergolid) should result in a halt of the neuronal and clinical decline in PD (Felten *et al.*, 1992). The selective inhibitor of MAO-B, L-deprenyl, has gained wide acceptance as a useful form of adjunct therapeutic drug in the treatment of PD (Knoll *et al.*, 1978; Knoll, 1987; Gerlach *et al.*, 1992) and has been reported to be effective in improving the life expectancy of patients with PD (Birkmayer *et al.*, 1985). The pharmacological basis of the therapeutic effect of L-deprenyl was fully reviewed recently (Chrisp *et al.*, 1991; Knoll, 1992a,b; Gerlach *et al.*, 1992) and has been an important topic in some recent international symposia (published in: Riederer and Przuntek, 1987; Rinne and Heinonen, 1991; Lieberman, 1992). As mentioned in Section 8.1, L-deprenyl is currently under investigation in the DATATOP study, where no other drugs, except TOH, are allowed. An unplanned interim analysis of this trial indicated that L-deprenyl reduced the risk of disability requiring L-DOPA therapy by approximately 50% and similarly reduced the loss of full-time employment (The Parkinson Study Group, 1989a,b). Although encouraging, these interim results, and that of the final report (The Parkinson Study Group, 1993), do not necessarily support a neuroprotective effect of L-deprenyl in PD because the observed functional benefits were also accompanied by slight, but statistically significant, improvements in the clinical measures of PD. Thus, the findings may reflect the symptomatic antiparkinsonian effects of L-deprenyl (Shoulson, 1992) that could be expected to occur also during treatment with anticholinergics, amantadine or DA agonists. These drugs would provide temporary therapeutic benefits and, thus, might delay the need for introduction of a more potent antiparkinsonian drug, such as L-DOPA (Olanow and Calne, 1992). Whether L-deprenyl enhances DA and/or phenylethylamine availability is still a matter of discussion (Paterson *et al.*, 1990). Furthermore, L-deprenyl metabolites might contribute to clinical effects. Virtually all administered L-deprenyl is metabolized to (−)methamphetamine and to a lesser extent to amphetamine (Reynolds *et al.*, 1978), which enhance the release of DA and block its reuptake. However, they are present in low concentrations, rapidly cleared and are far less potent than the (+)-enantiomers, which cannot be generated from L-deprenyl. It, therefore, appears unlikely that an amphetamine-like action is the only cause of symptomatic effects of L-deprenyl in PD (Heinonen and Lammintausta, 1991). However, a comparison between a selective MAO-B inhibitor, which does not metabolize into amphetamine metabolites, and L-deprenyl will probably give new insight to this question.

The findings of Spina and Cohen (1988, 1989; Cohen and Spina, 1989) have demonstrated that increased extracellular DA turnover induces increased concentrations of GSSG. This rise in GSSG can be suppressed by coadministration of L-deprenyl, indicating that it is caused by the oxidative metabolism of DA. Moreover, inhibition of MAO protects rat brain from hyperbaric oxygen toxicity (Zhang and Piantadosi, 1991), suggesting that H_2O_2 production by MAO is a biologically significant risk factor for brain degeneration if cellular defense mechanisms are impaired, as is obviously the case in SN in parkinsonian patients. Interestingly, depending on the sex and age of experimental animals, L-deprenyl increases the activities of CuZnSOD, MnSOD in striatum and SN, and to a lesser extent, in cerebral cortices, as well as CAT activity in striatum, but not in the hippocampus of rats (Knoll, 1988; Carrillo *et al.*, 1992a,b). Despite the fact that the mechanisms of action of L-deprenyl on activities of SOD and CAT remain obscure, there could be a hypothetical link to the free radical theory of aging. Knoll and coworkers (Knoll, 1988; Knoll *et al.*, 1989) observed an increased life-span of male rats after long-term administration of L-deprenyl.

Nevertheless, because MAO-B is unlikely to be present in dopaminergic neurons in SNC, the importance of MAO-induced production of H_2O_2 for catecholaminergic cell death must be seriously questioned. Tatton and Greenwood (1991) noted that following administration of MPTP, neuronal degeneration in the SNC of mice could be dramatically attenuated by L-deprenyl, even when administered 72 hr after the last dose of MPTP. Thus, a trophic effect similar to that reported with brain-derived neurotrophic factor (BDNF; Hyman *et al.*, 1991) was attributed to the drug. This was further substantiated by the findings of Salo and Tatton (1992), who reported the number of surviving motoneurons 21 days after axotomy to be 2.2 times higher than in mice not given L-deprenyl. Thus, L-deprenyl can rescue neurons other than those in the SN and can compensate

in part for the loss of target-derived trophic support caused by axotomy. These findings are also encouraging for use of L-deprenyl in treatment of neurodegenerative diseases other than PD. In AD patients, L-deprenyl has been shown to improve verbal memory (Finali *et al.*, 1991) and a wide variety of other cognitive functions, without frequent or severe adverse effects (Mangoni *et al.*, 1991).

8.3. Maintaining Neuronal Plasticity

The major symptoms of AD result from massive destruction of cholinergic synaptic terminals in cerebral cortex and subcortical structures (Bartus *et al.*, 1982), although other non-cholinergic synaptic terminals are also affected. Autopsy studies have shown that age-related loss of cholinergic cells in the NBM has a dropout rate very close to that observed in the LC and the zona compacta of the SN, two non-cholinergic nuclei (McGeer *et al.*, 1977, 1984; Mann *et al.*, 1984a,b). In AD, the level of ChAT, the synthesizing enzyme for ACh, is reduced in the neocortical projection fields of basal forebrain cholinergic neurons (Etienne *et al.*, 1986). Perry *et al.* (1978) found a positive correlation between these cholinergic markers and clinical severity on dementia scales. Thus, present therapeutic strategies are directed towards enhancing cholinergic function by:

(1) increasing various precursor molecules utilized for ACh synthesis (choline, phosphatidylcholines, acetyl-L-carnitine (ALC), L-α-glycerylphosphorylcholine)
(2) improving ACh function (through administration of aminopyridines to increase reuptake of free choline from synapses, or cholinesterase inhibitors, or muscarinic agonists) and
(3) maintaining membrane function and neuronal viability (growth factors, gangliosides, phosphatidylserine; for reviews, see Cooper, 1991; Holttum and Gershon, 1992).

By antagonizing progressive destruction of membrane phosphocholines by a putative mechanism of 'autocannibalism' (Wurtman, 1992), choline precursors should slow the degenerative process. However, results of treatment with choline (Ferris *et al.*, 1982) and with phosphatidylcholines (Bartus *et al.*, 1982) have been reported to be negative or at least equivocal. Although not yet subject to clinical trials for treatment of AD, cytidine-5′-diphosphocholine, a major precursor in the synthesis of phosphatidylcholines, phosphatidylserines and phosphatidylethanolamines in cell membranes, affects the synthesis and levels of cell membrane phospholipids in PC-12 cells when simultaneously incubated with choline (López G.-Coviella and Wurtman, 1992) and in brain of mice after long-term treatment for 27 months (López G.-Coviella *et al.*, 1992).

In contrast, a large multicenter study from Italy reported improvements in logical intelligence, verbal critical abilities, long-term verbal memory and selective attention in AD patients receiving oral ALC for 1 year (Spagnoli *et al.*, 1991; critically reviewed by Bowman, 1992). Although progression of the disease was not halted, it was slowed markedly.

Carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid) is a naturally occurring important metabolite in higher organisms that plays a key role in the transport of fatty acids from the cytosol into the mitochondrial matrix for β-oxidation (Bahl and Bressler, 1987; Bieber, 1988). Carnitine is synthesized from protein-bound lysine, mainly in the liver, kidney and brain (Shug *et al.*, 1982) and is additionally present in plasma and muscle in its free form and as acylcarnitine esters (Bieber and Lewin, 1981).

Acylcarnitines can be exchanged across subcellular membranes and ALC serves as a pool of acetyl groups from which to regenerate acetyl-coenzyme A (Dolezal and Tucek, 1981). ALC is structurally similar to ACh and has been shown to increase ACh synthesis, to promote ACh release (Imperato *et al.*, 1989) and to increase ChAT activity. These modes of action are possibly responsible for the symptomatic effects of ALC.

In addition, a putative protective effect of ALC should be considered in neurodegeneration. ALC appears to be effective in reversing certain aging processes in the brain (Sershen *et al.*, 1991), such as reducing some of the morphological changes in lipofuscin of aged rat Purkinje neurons (Dowson *et al.*, 1992) and decreasing lipofuscin accumulation in hippocampal and prefrontal brain areas of aged rats (Badiali De Giorgi *et al.*, 1987; Ramacci *et al.*, 1988). Moreover, ALC as well as carnitine increases the metabolic rate of mitochondria, thereby improving mitochondrial oxygen utilization

in experimental brain ischemia (Matsuoka and Igisu, 1992; Rosenthal *et al.*, 1992). ALC mechanisms of action in potentiating brain energy metabolism remain to be elucidated (for a review, see Calvani and Carta, 1991). ALC partially protects nonhuman primates against MPTP toxicity (Bodis-Wollner *et al.*, 1991), which could be explained, in part, by an ALC-induced increase in cytochrome c oxidase activity (Villa and Gorini, 1991; Petruzzella *et al.*, 1992).

Extended ALC administration causes an increase in nerve growth factor (NGF) receptors in the striatum of developing rats (De Simone *et al.*, 1991) and in cultured PC-12 cells (Taglialatela *et al.*, 1991, 1992). In cultures of aged dorsal root ganglia, ALC did not affect axonal regeneration, as was seen with NGF, but substantially attenuated the rate of neuronal mortality (Manfridi *et al.*, 1992).

Regardless of the ultimate cause of neurodegeneration, therefore, ALC appears to attenuate impairment of mitochondrial energy metabolism *in vitro* and in neuronal cell cultures, and may support regenerative processes in neurons via growth factor biochemistry.

Although AD is not usually considered a metabolic disorder, evidence supporting this hypothesis exists. Abnormalities in a number of brain enzymes involved in glucose metabolism (Friedland *et al.*, 1989; Kalaria and Harik, 1989) and in Ca^{2+} homeostasis in AD have been detected (Martin *et al.*, 1989). Thus, ALC may be useful to attenuate cellular energy deficiency in aging and AD.

A causal relationship of NGF depletion and AD seems unlikely because levels of NGF messenger ribonucleic acid in AD are not decreased (Goedert *et al.*, 1986) and because there is a consistent degenerative effect on neural populations in AD, which are not dependent upon or responsive to NGF (Hefti and Weiner, 1986). Nevertheless, intraventricular administration of NGF produces trophic actions on cholinergic neurons and prevents age-related neuronal atrophy (Hefti and Schneider, 1991), justifying the evaluation of NGF as a therapeutic tool for the treatment of AD. The protective function of NGF, even in the presence of ROS, is underlined by experiments in cultured rat astrocytes expressing NGF and basic fibroblast growth factor, following 0.2–1 mM H_2O_2 (Pechan *et al.*, 1992). As well, NGF restores normal CAT activity and increases CuZnSOD and selenium-dependent GSH-Px activity in several brain areas of aged rats (Nisticò *et al.*, 1992) and cultured PC-12 cells (Jackson *et al.*, 1990a,b), suggesting that the effects of different toxic events (impairment of mitochondrial respiratory chain induced by MPP^+ or accumulation of ROS) can be attenuated by NGF.

Similarly, BDNF (Hyman *et al.*, 1991) selectively protects DA neurons against 6-OHDA and MPP^+ toxicity (Spina *et al.*, 1992) by increasing the activity of GSSG-Rd, but not of CAT, and thereby preventing loss of GSH.

Although growth factors are obviously not able to prevent onset of neurodegeneration, they could enforce regenerative processes of already damaged but still viable neurons. They, therefore, have been seriously considered for treatment of degenerative diseases. However, growth factors have to be given intracerebrally because, being peptides, they do not cross the blood–brain barrier, rendering their usage in clinical practice more difficult. Thus, will there perhaps be strategies in the future to stimulate growth factor synthesis at specific brain regions in the adult brain?

Another therapeutic approach could be the use of membrane-stabilizing agents, such as gangliosides. Gangliosides are sialic acid-containing glycosphingolipids, which are highly concentrated in neuronal membranes (Leeden, 1984; Mahadik *et al.*, 1992). Morphological, developmental, biochemical and behavioral studies have demonstrated that gangliosides participate in the maturation and repair of neural tissue (Thomas and Brewer, 1990; Bull Zeller and Marchase, 1992). They enhance recovery from MPTP-induced parkinsonism in rodents and primates (Schneider *et al.*, 1992; Hadjiconstantinou *et al.*, 1989, 1990; Fazzini *et al.*, 1990; Gupta *et al.*, 1990) and have been used for the treatment of stroke and AD (Bassi *et al.*, 1984; Porsche-Wiebking, 1989; Ala *et al.*, 1990). During aging, and in AD, some gangliosides (GM1, GD1a) were found to be decreased in NBM, frontal cortex and temporal cortex, but the simple gangliosides GM2 and GM3 were elevated (Kracun *et al.*, 1992a,b). These authors correlated their results with accelerated lysosomal degradation of gangliosides and/or astrogliosis occurring during neuronal death. Despite these intriguing data, gangliosides are unlikely to reverse the disease process. Clearly, further effort is needed to find new compounds, or at least efficient combinations of all these drugs mentioned, in order to slow down the destructive processes in brain.

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