The importance of brain banking in Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a progressively disabling neurological disorder caused by the degeneration of nerve cells within the brain that produce dopamine, a neurochemical substance necessary for normal movement, walking and balance. The primary reason for the destruction of the brain's dopamine-producing cells remains obscure. The way to unravel the causes underlying this disease as well as the mechanisms causing the neurodegenerative changes in the brain is by looking into the brain itself. The development of sophisticated neurobiological techniques which can be applied on human brain causes an increased demand for post-mortem tissue for research. Brain banks for neurological diseases form an important link between the clinician, neupathologist and basic scientist by supplying clinically and neuropathologically well-documented specimens for neurobiological research. The various techniques which are currently applied on human brain tissue have one drawback in common: many patient-related data and factors introduce a huge variation and systemic errors, which have to be corrected or carefully matched for. Neurochemical measures are affected by the various pre- and post-mortem factors and the interpretation of the results of post-mortem studies therefore requires adequate consideration of these factors in order to separate changes that are due the disease from those which occur as a result of epiphenomena. In the current routine of brain banking it is of crucial importance to match samples for various factors. Ante-mortem factors include age, sex, circadian and seasonal variation, lateralization, medication and agonal state of the patient. Postmortem factors include the postmortem delay, freezing procedures, fixation duration and storage time.

INTRODUCTION

Parkinson's disease (PD) is a progressively disabling neurological disorder caused by the degeneration of nerve cells within the brain that produce dopamine, a neurochemical necessary for normal movement, walking and balance. The primary reason for the destruction of the brain's dopamine-producing cells remains obscure. Various factors, including toxins, autoimmunity, infections and genetic predisposition have been suggested as basis for what may be a disease with multiple causes. Except for some optimistic indications of a protective effect of MAO-B inhibitors, no therapy is known to actually slow or stop the progressive loss of nerve cells in PD
ANTEMORTEM FACTORS

Age

Age related changes occur in many structures in the brain both in normal aging and in various neurodegenerative diseases. At about 50 years of age the human brain starts to lose weight but the atrophy is not uniform, being more pronounced in the temporal, frontal and parietal lobes than in brainstem. This difference is also apparent in cell counts; the neurons of the pars compacta of the substantia nigra and of the locus coerules show significant losses with increasing age, in contrast to brainstem neurons in general. The integrity of the nigrostriatal tract declines with age. A similar picture appears when different neurotransmitters are examined. Age must therefore be taken into consideration when studying monoamines, their metabolites and enzyme activities in the human brain (2).

One of the biochemical constituents of the brain which has been found to significantly decrease with age is the enzyme glutamate decarboxylase (GAD) which is localized in γ-aminobutyric acid (GABA)-containing nerve terminals (3,4).

For striatal dopamine (DA) a significant age related reduction has been found. MAO-B activity, however, is reported to increase with age (for review see Kuiper et al., ref. 5). Noradrenalin (NA) and serotonin (5-HT), in certain brain regions, and muscarinic and nicotinic receptors in the hippocampus, have been reported to be significantly negatively correlated with age (6) whereas in the thalamus, an increase in muscarinic binding sites has been observed. An increase in muscarinic receptors has also been reported in the frontal cortex of demented PD patients (7).

The age related decrease of monoamine levels is probably at least partly due to the loss of monoaminergic neurons observed morphologically. One of the possible explanations for the neurochemical changes in PD is that increased turnover of dopamine (due for instance to increased MAO-B activity) and 5-HT may partly account for the discrepancy between amines and metabolite levels (8). Using quantitative autoradiography, 5-HT$_2$ receptors were found to be heterogeneously distributed in human brain and to respond to aging in a non-specific manner (9). This finding emphasizes the need for sampling a large variety of neuroanatomical regions in studying age and disease-related changes in the CNS.

In order to examine the possible contribution of aging to the neurodegenerative process of PD, subregional levels of dopamine and homovanillic acid were measured in post-mortem brain. The regional and subregional pattern of striatal dopamine loss in PD differed substantially from the pattern in normal aging (10).

Sex

There is an increasing amount of data concerning sex differences in the
various brain regions. Sex differences have been reported in the levels of monoamines, monoamine metabolites and related enzymes (11,12). Biochemical data from the corpus striatum, substantia nigra, red nucleus, hippocampus and frontal and parietal cortex were analyzed as a function of sex. A sexual differentiation in the neurochemical measures has been reported for 5-HIAA (5-hydroxy-indoleacetic acid) concentrations in the orbital frontal cortex, which was higher in females than in males (13).

**Brain weight**

Recent studies made it clear that in relation to brain weight, allometric measures and sex differences should be taken into account (14). Already in 1880, Bischoff (15) reported that sex differences in absolute brain weight are present at birth, but nowadays we know in addition that one should also take into account the differences in age, body weight and length (14,16-19). Brain weight is significantly influenced by various fixatives and the duration of fixation; it is known to increase during fixation in formalin and this increase is directly correlated to the fresh brain weight (20).

**Agonal state**

The agonal effects associated with death may influence the pH of the brain tissue and a number of chemical substances in the brain. Subjects who died after a long terminal illness have a lower pH in the brain, spinal fluid (CSF) and blood, and this acidosis corresponds to increased lactic acid concentrations (21,22). Lower levels of pH were found throughout the brain in cases of death following protracted illness and bronchopneumonia, as compared to sudden death (4). This is a serious difficulty for the study of human neurological disorders as bronchopneumonia is a frequent cause of death in such cases, and because the post-mortem delay is frequently long if the patient has died in a nursing home remote from an academic hospital with extensive autopsy facilities.

The stability of some enzymes has been found to be affected by the agonal state of the patient. The best characterized example is the glutamic decarboxylase (GAD; L-glutamate-1-carboxylase; EC 4.1.1.15), the enzyme catalyzing the formation of GABA from glutamate. Reduced activities of this enzyme and the enzyme tyrosine hydroxylase were reported in all brain regions of patients dying after a prolonged terminal illness (4,21). Surprisingly, GABA concentrations were not found to be affected in subcortical structures although this chemical have been recently shown to be affected by agonal state in parietal cortex.

Measuring the pH of the brain in CSF collected during rapid autopsies makes it possible to match the samples for agonal state and has therefore been introduced as a routine procedure in the Netherlands Brain Bank (NBB). In order to check whether the pH is not affected by postmortem time, we investigated whether there is any correlation between pH in brain
tissue and postmortem delay, in both rat and human brain. From the rat data it became evident that there was no significant change in the pH within a postmortem delay of 24 hours. This is well within the range of the postmortem delay of our rapid autopsies. Comparable observations were made on human autopsy material collected by the NB3 in the past three years. The pH values measured in CSF obtained by autopsy of non-demented controls and Alzheimer patients, did not change significantly with postmortem delay (23,24). From these data it was concluded that measuring the pH of either brain tissue or CSF obtained at autopsy is not influenced by the postmortem delay and is thus a crucial measure for agonal state that has to be included in brain banking routine procedures.

To prevent the use of unsuitable tissue for research we always note whether we have information on sudden and unexpected death or protracted illness and verify this at autopsy by measuring the pH in CSF. In human studies, prolonged diseases such as respiratory distress may influence a number of biochemical parameters, especially pH. Whenever possible, subjects should thus be matched for pre-terminal state. This is a particularly difficult criterium to satisfy in studies of aging, since most young donors die from accidents, suicide or drug overdose whereas older donors die from various chronic disease states. Bronchopneumonia in the terminal phase may often lead to brain hypoxia and lowering of the pH. Several brain banks have attempted to solve this problem by advising that the patient should be provided with extra oxygen until death. However, the only approach which has general applicability is to obtain the brain as soon as possible post-mortem, test the pH of the brain and discard all unsuitable specimens.

**Drug treatment**

Many patients are quite heavily medicated and the effect of this on various neurochemical substances is difficult to assess. Especially in elderly or hospitalized patients this constitutes a serious problem. Therefore it is important to compare drug treated patients with those that have not received medication for at least several months prior to death. In patients receiving opiates, for instance, a protracted terminal illness may be found, with a consequent GAD depletion (4,21).

**Seasonal variation**

Seasonal alterations have been found in the level of hypothalamic 5-HT with a minimum activity level during the months December-January and a maximum during October-November (25). Hypothalamic dopamine levels also showed seasonal variation, with two peaks, i.e. during January-February and August-September, and two nadirs, i.e. during March-June and October-December. In other brain regions the influence of biorhythms on dopamine levels was less evident.
A striking seasonal variation was observed in the volume and cell number of the human supra-chiasmatic nucleus (SCN); the volume was 2.5 times larger in October-November than in May-June and contained 2.4 times as many cells. Similar annual oscillations were found in the number of vasopressin expressing neurons in the SCN (26). The month of death is consequently a factor to consider in studies.

**Circadian variation**

Clock time of death has been found to be a significant factor for the levels of monoamines in the brain. Circadian changes were observed in NA, 5-HT and DA and their metabolites (25). There is evidence of diminished concentrations of 5-HT and DA in various brain regions in Alzheimer's disease (AD) (27), suggesting reduced activity of both systems. In a recent study, the concentrations of monoamines and various peptides in AD and vascular dementia were measured (28). The authors suggest that these changes may be important for the changes in circadian symptoms in dementia. A sleep centre in the mesencephalon has been postulated already long ago (29) and more recently it has been suggested that changes in sleep/wake rhythms may be based upon the degenerative changes in the SCN (30,31). 5-HT has been claimed to play an important role as a sleep-inducing neurotransmitter (32) and the high nocturnal activity of 5-HT favours its active role in sleep. The fact that neurotransmitters have marked circadian variations with high nocturnal activity pleads for their active and specific role in the control of sleep/wakefulness patterns.

Since a clear-cut circadian pattern was found to be present in the levels of various transmitters in the human brain with higher values around midnight (12), time of death should be considered as a factor to match for when collecting specimens for research.

**Lateralization**

Fixing one hemisphere and freezing the other is current practice in many brain banks. It prevents, however, the recognition of possibly existing left-right differences of various systems in the brain. Several functions and transmitters are asymmetrically represented in the left or right hemisphere; lateralization of NA has been demonstrated in the human brain (33) and there is evidence for a left prominence in the distribution of thyroid releasing hormone (TRH) in the ventromedial dorsal and paraventricular nuclei (34) with higher concentrations in the left side. The laterization of certain neurotransmitters makes it also difficult to relate neurochemical values to pathology. The neuronal loss in the substantia nigra of PD-patients, for instance, is asymmetrical and that has relevance to the mechanism of levodopa related motor fluctuations.

Consequently, it is preferential to sample bilaterally, or, if not possible, to mention on which hemisphere the measurements were performed.
POSTMORTEM FACTORS

Postmortem delay

The time between death and fixation or freezing of the tissue is important not only from a neurochemical point of view but also for several morphological parameters. Some substances in the brain are very unstable. Rat studies showed a striking effect of ischemia on various substrate levels. For P-creatine and ATP it is a matter of seconds for a significant drop to take place (35). Many other neurochemical substances are very stable, e.g. neuropeptides and several receptors. Several investigators have attempted to correlate brain dysfunction in PD with aberrant concentration of biogenic amines in post-mortem human brain. The interpretation of neurochemical data in this material is complicated by potential alterations in the concentrations of labile substances after death. NA, DA and 5-HT are labile in aqueous solution at body or room temperature and physiological pH. It seems probable that their post-mortem degradation would be even enhanced by catabolic enzymes. Several investigators reported post-mortem stability of neurotransmitters in the rat brain in circumstances simulating those which might attend the handling and treatment of human autopsy material of deceased PD patients (36). Rapid post-mortem increase in extracellular DA and NA has been reported in the rat brain by microdialysis studies (37,38). The post-mortem decreases in both NA and DA concentrations in human brain may be explained by an increase in extracellular levels and subsequent metabolism (13,36).

As the post-mortem delay of PD brains obtained via autopsies is an important factor, it is essential to have good estimates of the degree to which amine values change. Correlating the post-mortem delay to various chemical variables revealed that there was a significant negative correlation between this time variable and NA and normetanephrine (NM) levels. On the other hand, a positive correlation was found for the levels of the amino acids tryptophane and tyrosine (12).

The activity of the GABA-synthesizing enzyme, GAD was found to be reduced in the basal ganglia and cerebral cortex in PD patients as compared to controls (39). The subnormal GAD activity can be disease-related but can also be due to a post-mortem vulnerability of the enzyme. When the proper matching is performed, no significant differences can be observed (40).

The development of morphological methods such as immunocytochemical procedures (ICC) and in situ hybridization permits the visualization of the various neuronal systems in the brain making use of the better anatomical resolution and quantitative estimation. Some of the morphological studies have been used in PD-research to reveal the dopaminergic neurons in the mesencephalon and the neuronal loss in the substantia nigra (41). The most important finding was that the neurons
which were the most vulnerable is PD were subpopulations of pigmented neurons and that cell death might be correlated to neuromelanin (42).

ICC procedures have shown that peptides are less sensitive to the post-mortem delay; an excellent staining of vasopressin neurons in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) is obtained on tissue which was fixed 48 hours after death. A similar stability up to postmortem time of more than 60 hrs was found for vasopressin fibers (43,44). ICC studies on rat brain showed that the amount of stainable vasopressin in the SCN even doubled during a six hours post-mortem interval (45). Incubating rat brain slices in medium for 6 hours we found enhanced ICC staining and increased levels of hypothalamic VP-containing neurons in the SON, PVN and SCN as compared to brain tissue fixed immediately after death (46). These results clearly indicate that a post-mortem interval of several hours is not necessarily detrimental for ICC studies or peptide measurements on brain material which in turn enlarges the feasibility of routinely obtained autopsy material for research.

Quantitative autoradiography has been used to study the localization and regional distribution of enzymes and receptor binding sites in postmortem human brain in both normal and PD tissue. This approach appears to be particularly valuable as most binding sites appear to be quite stable in postmortem tissue (47).

The extensive dopamine loss in the striatum of PD patients, is not reflected in numerable changes of dopaminergic receptors (48). The premortem medication in PD patients, however, has been reported to affect the binding capacities of some receptors in post-mortem tissue (49).

The effect of post-mortem delay and prolonged storage of the tissue prior to performing binding assays may limit the interpretation of the disease related changes in receptor populations. The understanding of receptor changes associated with the disease may have implications for the development of therapeutic strategies by using drugs that modify receptor function. In order to be able to claim that changes in receptors found in human brain result from the disease process being studied, it is important to exclude other factors which may affect ligand binding. Autolysis of tissue due to the postmortem delay and the freezing process of the tissue may both contribute to such changes, alter the receptor and affect the binding of a ligand.

It is possible to use postmortem tissue to examine the antemortem expression of a human neuropeptide gene. Neuropeptides are known to play a major role in neural transmission in the brain and changes in these compounds occur in various neurodegenerative disorders. To be able to interpret the dynamics of neuropeptides, it is often important to investigate neuropeptide messenger RNA (m-RNA) in human brain in addition to the neuropeptide levels themselves.

Modern molecular biological techniques allow the study and quan-
Prioritization of those m-RNA's by using various hybridization techniques. Some human neurotransmitter and receptor mediated genes have been sequenced, and m-RNA expression in human post-mortem material can now be studied in tissue extracts using northern blots and in histological context using in situ hybridization histochemistry. m-RNA stability has been investigated in several studies, with some discrepancy between results. An extensive postmortem stability of total RNA has been reported in rat and human brain up to 48 hrs and 36 hrs respectively. Both the yield and the integrity of RNA stayed unchanged during the postmortem period (50). Study of the relative m-RNA levels of APP and T in the aged brain revealed no significant changes up to 50 h post-mortem (51).

Vasopressin m-RNA has been detected in rat brain by quantitative in situ hybridization (52). The hybridization signals had a modest rate of loss within 12 hrs of post-mortem time. This technique has been also applied for the study of other neuropeptide gene expression in post-mortem tissue. No significant correlations have been found between the density of the hybridization signal and parameters such as post-mortem delay, age and sex (53).

However, when the postmortem stability of arginine-vasopressin m-RNA in the rat brain was measured it appeared to be degraded postmortem more rapidly than ribosomal RNA (r-RNA) (54). The splicing pattern of m-RNA has been investigated in rat and human postmortem brain tissue, using a method based upon the polymerase chain reaction (55). There may thus be some differences between m-RNA species in post-mortem degradation; total RNA recovery does not decrease with postmortem time, whereas r-RNA and vasopressin m-RNA signals show a tendency for reduction.

The indication of an abnormal expression of the tyrosine hydroxylase gene has been reported following the use of in situ hybridization. m-RNA of tyrosine hydroxylase has been found to be present in subnormal quantity in the dopaminergic neurons of the SN of PD patients (56).

The use of the polymerase chain reaction in the analysis of m-RNA allows to measure simultaneously various types of m-RNA which have a different susceptibility to pre- and post-mortem variables (57). Surprisingly, prolonged death-refrigeration interval, was positively correlated to increase in specific RNA. Some recent results plead thus for the need for rapid autopsies.

In sum, the postmortem delay is a very essential variable. Postmortem delay of the human brain tissue obtained by autopsy, however, cannot be much shorter for obvious reasons. It would be therefore an important development for brain banking if more neurobiological techniques would be adapted such that they are suitable for tissue with a relatively long postmortem delay.

**Tissue storage**

Changes in factors such as freezing, fixation or storage time of the post-
mortem human brain may affect the interpretation of biochemical changes in the brain, as well as the potentialities of staining procedures, considerably. On the other hand, some tissue components are not very sensitive to these factors. Human brain tissue used for biochemical studies is usually rapidly frozen and slowly thawed. However, to isolate synaptosomes which are morphologically well preserved and have retained their metabolic performance, one should use the opposite procedure as snap-freezing generally yields metabolically and functionally inactive preparations (47,58).

It is noteworthy that a large number of metabolic and functional processes as well as binding capacity of various receptors are retained surprisingly well in frozen tissue. The use of synaptosomes led to the finding of reduced dopamine uptake in PD (59). It is desirable to be able to store brain tissue in such fashion as to retain its metabolic and functional integrity. One way to achieve that purpose is slowly freezing the tissue in an iso-osmotic solution, e.g. 0.32M sucrose, storing at −70°C, and defrosting it rapidly prior to the experiment.

The use of fresh tissue for binding assays has been investigated and comparison of results obtained with tissue stored at 4°C and nonfrozen neurosurgical samples shows that tissue specimens stored at 4°C exhibit smaller variations in affinity and binding capacity than samples which were stored as an homogenate. Agonist and antagonist binding assessments of frozen autopsy samples are not significantly different from those obtained with fresh tissue preparations. Valid binding studies on the muscarinic receptor can be carried out on autopsy material up to 51 hrs after death and freezing of the tissue does not significantly influence binding properties of this receptor in human cerebral cortex (60).

The construction of copied DNA (c-DNA) libraries requires non degraded m-RNA and the stability of this molecule has been previously investigated (50,61,62). These studies investigated the effect of post-mortem delay on m-RNA isolation but have not addressed the issue how dissection time or long time freezer storage affect the integrity of m-RNA and its use for multiple molecular biological studies necessary to characterize the expression of a candidate gene. Recent findings (63) have demonstrated that prolonged freezer storage and extended dissection times may compromise use of tissue when the m-RNA is to be used for in vitro expression studies of oligo deoxy Timidine primed library construction.

A prolonged storage of the dissected frozen tissue prior to receptor binding assays is a potential problem that may limit the interpretation of the effects of disease on receptor populations. No significant reductions in binding of muscarinic cholinergic receptor have been established after storage of 1 year (64) and no change in GABA receptors was reported with a storage time up to 64 months (65) whereas dopamine receptors were found to be degraded (66).

Fixation in formalin causes an increase in brain weight and the subse-
quent washing in water introduces a systematic error in brain weight, e.g. larger brains gain more weight than small brains. However, brains from younger individuals do not gain more in weight than brains from elderly individuals when the difference in fresh brain weight between the two groups was taken into account. Similarly, the increase in brain weight during fixation is not sexually dimorphic when the fresh brain weight is taken into account (20). Conventional formaldehyde fixation for one month results in excellent vasopressin and oxytocin staining of the hypothalamic SON, PVN and SCN neurons (67,68). However, this procedure was found not suitable for studying the extrahypothalamic fibres of these peptidergic neurons for which thick glutaraldehyde-parafomaldehyde sections are preferable. Our experience with rat material is that storage in glutaraldehyde-parafomaldehyde fixative preserves immunoreactivity of vasopressin (VP), oxytocin and alfa-MSH for more than a year. Since immersion in this fixative does not fully penetrate the human brain, smaller tissue blocks have to be fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde for one week. Subsequently the blocks can be frozen and stored in sealed plastic at -80 °C and cryostat sections can be made for immunocytochemistry. This procedure gives good staining of VP fibers in the human brain (44). On the other hand, vasopressin immunoreactivity of hypothalamic neurons was still present in material which has been fixed and stored for more than 50 years (43).

All the observed changes in postmortem fixed brain mentioned in this section have clear consequences for brain banking procedures. In the past 10 years our group has used a constant fixation procedure in order to minimize the systematic errors and variables. Brains obtained at autopsy are fixed for 1 month in 10% buffered formalin at room temperature before further washing and embedding in paraffin blocks (67).

Neurobiological research on the human brain brings up problems associated with the storage of large amounts of tissue and the necessity for using a battery of fixatives compatible with modern immunocytochemical and related techniques. A recently reported method enables fixation of previously fresh-frozen and stored post-mortem tissue (69). This method involves fixing, during slowly thawing under controlled, cryoprotected conditions and is compatible with good histological quality and the preservation of enzymatic activity and immunoreactivity of various neural antigens. It offers considerable advantages in the storage of large amounts of tissue, from which multiple samples can be taken and processed under fixation while being optimized for a variety of methods. Unfortunately, using this method no immunoreactive staining can be carried out for dopamine beta hydroxylase, GAD, GABA, glutamate or serotonin.

This new method is important for routine brain banking as it allows an archive of brain material to be built up that can be re-investigated as new techniques become available that may have different fixation requirements.
SUMMARY AND CONCLUSIONS

An increasing number of intracerebral processes can be studied on autopsy material of deceased PD patients. The availability of this material, whether fresh, frozen or fixed, makes it possible to develop methodologies for studying the neuroanatomical and neurochemical aspects of the disease. Recently, it has also become possible to correlate functional changes with neurochemical and neuroanatomical changes.

The common knowledge that some compounds and structures are damaged irreversibly within seconds or minutes after death led to the widespread idea that autopsy material would not be suitable for basic research purposes and would not supply the necessary answers on the various fundamental questions regarding processes occurring in normal or diseased brain. However from recent published data in which autopsy material has been routinely used, it becomes evident that this is a misconception. There is an increasing number of reports based on the use of normal and pathological human brain tissue obtained by autopsies in spite of the fact that there is a worrying continuous decline in autopsy rate which causes international serious concern among scientists (70). Furthermore, it also became evident that when using the proper fixation procedures, sufficient structural integrity is retained in the tissue to allow morphometrical studies (71,72). Electron microscopic examination of synaptosomal preparations from postmortem human brain showed them to be only slightly less pure than preparations from fresh tissue although there was some degree of damage (58).

Agonal state affects the stability of brain compounds and causes brain hypoxia. This forms a tremendous difficulty for the study of human neurological and psychiatric diseases as one of the frequent causes of death is bronchopneumonia which leads to brain hypoxia and results in pronounced lactic acidosis. The Netherlands Brain Bank has succeeded to partly circumvent some of the serious problems encountered in providing human tissue for research by performing rapid autopsies with an average short postmortem delay.

FUTURE OPTIONS

In PD patients the changes in levels of neurotransmitters and regional brain metabolism are considered to reflect the decrement in striatal dopamine neurotransmission. Future neurochemical studies should be aimed at understanding the effects of dopamine depletion on other neurons and neurotransmitter systems, thought to be involved in PD. This can form the basis for new therapeutic strategies to supplement dopamine replacement.

Another future line should be aimed at identifying the origin of the selective vulnerability of the dopamine neurons in PD patients which may
result in therapeutic strategies to arrest the progression of or actually prevent the disease.

Collecting post-mortem tissue and CSF from well documented PD patients makes accurately characterized and high quality specimens available to scientists investigating this disease. The findings of this research have important implications for clinically based studies of PD, in particular those concerned with drug development and epidemiology. Until PD can be better characterized, e.g. by a special biological marker, and in order to further investigate the nature of PD, large clinico-pathological series are needed. A network of Brain Banks is a necessary prerequisite for such studies.

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REFERENCES


