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ELECTROPHORETIC ANALYSIS OF BRAIN AND KIDNEY PROTEIN IN AGEING MICE

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ELECTROPHORETIC ANALYSIS OF BRAIN AND KIDNEY PROTEIN IN AGEING MICE

William James Vaughan (Ph. D. Thesis)

May 1974

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under Contract W-7405-ENG-48

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To Barbara, Laura and Brian, and also to those who have accepted the challenge of ageing research.
Electrophoretic Analysis of Brain and Kidney Protein in Ageing Mice

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Electrophoretic Analysis of Brain and Kidney Protein in Ageing Mice

William James Vaughan

Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, California

May 1974

Abstract

A major goal of ageing research is to positively identify and characterize the primary molecular events associated with senescence. Many of the current hypotheses of ageing postulate functional decreases with age which are ultimately determined by changes in structural or enzyme proteins. Since proteins are constantly being degraded and synthesized, the maintenance of protein levels requires a functioning genetic apparatus. The purpose of this study was to monitor general, steady state, gene expression by measuring protein levels. Brain and kidney proteins from young adult and senescent mice were subjected to polyacrylamide gel electrophoresis. Protein patterns were compared on the basis of the relative intensity of bands of the same migration distance. Special care was taken to avoid proteolysis and achieve total protein solubilization. Electrophoretic methods were sensitive enough to detect protein level differences when protein products were from different genomes.
For example, proteins from different species, strains, sexes and mutant mice yielded different gel protein patterns. When the genome was constant, and proteins from different organs, different regions of the brain, and different stages of growth were compared, changes were detected in gel protein patterns. Major protein levels in brain and kidney were maintained, even in 150 week old mice. Major protein levels were also maintained in various brain regions from senescent mice. These studies argue against the hypothesis that ageing is a continuation of growth and development. Also, since electrophoresis revealed no increase in the average molecular weight of mouse brain proteins with advancing age, these studies also argue against the hypothesis that a general covalent crosslinking of proteins is involved in primary aging processes (excluding crosslinkage through disulfide bonds).
Acknowledgements

To the following I would like to express my appreciation:
Giovanna Ferro-Luzzi Ames, Edward L. Bennett, Melvin Calvin, 
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Barbara Vaughan.

Special thanks to the following colleagues in ageing 
research: Richard Cutler, Gerald Hirsch, and Caleb Finch.

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Institute of Health (Grant No. 5-T 1-GM00829) and also in 
part by the U.S. Atomic Energy Commission.
### Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ATP-ase</td>
<td>Adenosine triphosphate-ase</td>
</tr>
<tr>
<td>BIS</td>
<td>N,N'-Methylene bisacrylamide</td>
</tr>
<tr>
<td>C/M</td>
<td>Chloroform/methanol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycholate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>PAMU</td>
<td>Phenol-acetic acid-water, 2/1/1, v/v/v, made 2 M in urea</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>TFMEED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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<tr>
<td>TKNS</td>
<td>50 mM Tris-HCl (pH 7.4), 25 mM KCl, 4 mM MgCl₂, 250 mM sucrose</td>
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<tr>
<td>K</td>
<td>Kilodaltons</td>
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I. INTRODUCTION

Among the characteristics shared by higher forms of life are growth, reproduction, and senescence. While many aspects of human reproduction and growth are known and can be controlled, ageing remains an enigma. Since the cause of ageing is unknown, its definition is elusive. Spirited debate has produced more definitions than researchers in this field. My own tentative definition of ageing: biochemical events in an organism responsible for decreases in its various functions with chronological age. Concurrent with an increase of chronological age and a decrease in function is a greater susceptibility to disease. The findings of ageing research should therefore be most relevant to disease prevention and control.

The current goal of ageing research is to positively identify and characterize the primary causes of ageing.

A. Problem Definition

How does one define a biological study that may help identify primary mechanisms of ageing? Which of millions of organisms does one choose? Which of the thousands of biological reactions and/or their constituents does one select? Within an organism, which organ, tissue, cell, subcellular or molecular structure involving which of the hundreds of physiological functions or thousands of biochemical functions would provide relevant ageing data?
Choosing an organism can be straightforward. An ideal organism would have at least the following advantages: (a) a short life-span during which ageing occurs (b) growth, reproduction, ageing, nutrition and disease parameters which are relevant to human studies (c) a spectrum of pathologies with increasing age so that changes due to ageing are not obscured or distorted by singular prominent diseases (d) genetic homogeneity to favor biological reproducibility (e) vigor to withstand stresses imposed by standard physiological or biochemical experiments (f) well characterized mutants (g) literature characterization of genetics, survival curves, age-related pathologies and nutritional requirements. The F1 progeny of two long-lived inbred strains of mice have most of these advantages.

Within mice, which aspects of biochemistry may be relevant to the mechanisms of ageing? The general approach in ageing research is to identify and characterize structural and functional age changes at the phenotype level, then proceed through to lower levels of organization (organ, tissue, cellular, molecular), hoping that some patterns will emerge which will point to the biochemistry involved in primary ageing mechanisms.

An aid in identifying true age changes are the following generally accepted criteria (1, 2): (a) universality-change should occur in all older members within a species or strain (b) progressiveness - a gradual and cumulative occurrence (c) intrinsicness - unavoidable by changes in environment (d) irreversibility (e) harmfulness - leading to decreased functional capacity and death.
As a further guide to experimental design, there are over 20 hypotheses of ageing, with no compelling evidence for any one of them. Among the current primary hypotheses of ageing are:

(a) Somatic Mutation (3) - With increasing age the DNA of somatic cells develop spontaneous mutations (measured by chromosomal breaks).

(b) Programmed Ageing (4,5,6) - Ageing is a continuation of the genetic program responsible for growth and differentiation. In every cell of the same type specific genes are turned off with age, and protein synthesis reduced.

(c) Error Catastrophe (7) - With increasing age, amino acid synthetases lose specificity and the frequency of amino acid mis-incorporations into all proteins increases and leads to error catastrophe.

(d) Ageing is controlled at the level of translation (8).

Each of these hypotheses deals with some aspect of gene expression which satisfied the previously mentioned ageing criteria. The fact that every species shows a characteristic maximum life span (9) clearly implicates genetic factors. To summarize gene expression (10):

DNA $\rightarrow$ RNA $\rightarrow$ Protein

(replication) (transcription) (translation)

Gerontologists have looked at various aspects of gene structure and expression: DNA and chromatin melting points (11), repetitive DNA (12), RNA to DNA hybridization (12), amounts of DNA per cell (13), rates of RNA turnover (14), rates of RNA synthesis (11), etc. The major difficulty in all of the foregoing experiments is the evaluation of the biological function of the molecules being measured.

In this sense, the study of the protein gene product is more
to the point: In each of the previous hypotheses of ageing, the postulated functional losses are ultimately determined by the proteins.

Body proteins are not static (15). Since protein turnover continues uniformly into senescence (16), specific protein levels are determined by their rates of synthesis and degradation (17). Maintenance of protein levels requires functioning genetic apparatus. Thus monitoring protein levels with age also monitors gene expression. So one general question I'm asking is: "Does gene expression, as monitored by steady state protein level measurements, change with age?"

Besides the gene expression interpretation, protein levels can also be interpreted to reflect enzyme levels, since much of tissue protein, excluding collagen, is composed of enzymes. Indeed, any ageing mechanism would require a biochemical reaction, and since most all biochemical reactions are enzyme dependent, a general enzyme hypothesis could include many other hypotheses as well as those mentioned above. In this context, structural proteins also have great importance since they can influence the milieu in which enzymes function. Although enzyme levels are just one of the many factors (other factors include substrate, activator, inactivator, inhibitor, etc. levels) that determine in vivo enzyme activities, Schimke (17) cites examples of functional losses which correlate with enzyme protein level rather than enzyme activity level.

Over 1,500 human diseases are already known to be genetically determined and in 92 of these cases a specific enzyme deficiency
has been identified (18). In other words, phenotypic functional losses, diseases, were correlated with changes in enzyme levels or activities. Just how small a change in protein level or in amino acid sequence is required before a functional loss is observed depends on the particular case. For example, the basis for the functional losses due to sickle cell anemia is a single amino acid change in hemoglobin (19). Since single enzymes are not isolated entities but constituents in a set of interdependent metabolic events, it is easy to imagine how minute changes in a single protein could lead to serious changes in major protein levels.

Since genetic diseases involving small protein changes can lead to visibly obvious functional losses, it is not unreasonable to expect protein changes to be involved in the ageing process. Schimke suggests correlating enzyme activities with enzyme levels by isolation of single enzymes (17). However, equal extraction efficiencies and proteolytic enzyme activities cannot be guaranteed when comparing young and old tissue. Age related changes in lysosomal enzyme activities have been reported (20). In addition, single enzyme studies, by their nature, are limited to a very small proportion of a tissue's protein.

In the ideal case, if a tissue had 1000 proteins, the separation, quantitation, and primary sequence determination of the 1000 proteins with age would provide an answer to the general question involving gene expression, enzyme levels and ageing.

My goal was to survey protein component levels as a function
of age by complete tissue solubilization and subsequent separation and quantitation of individual protein components. I used techniques to attain complete solubilization and also used the most sensitive techniques now available for protein separation—polyacrylamide gel electrophoresis. Protein levels were measured by the intensity of the gel protein bands. I surveyed brain and kidney protein levels from adult mice of various ages.
B. Brain Protein Studies in Ageing Mammals

Of the organs essential for survival, the brain occupies the position of highest priority. Aspects of coordination, adaptive response, and homeostasis at various levels of organization, involving every organ, ultimately require a precisely functioning nervous system. Shock (21) postulated that functional impairments in ageing humans were primarily due to a breakdown of neural functions. Since neurons do not divide in postnatal life (22), any neurons lost with age will not be replaced; thus providing a basis for age-related functional changes. In addition, the brain has, excluding collagen, the greatest amount of slowly turning over protein (23). Any slowly turning over or non-replenishing molecules would be quite likely to deteriorate and result in the decrease of functions they support.

Physiological studies have indicated that although cerebral blood flow and oxygen consumption remained constant, significant decreases in cerebral glucose utilization between a group of normal young subjects (Mean age, 20 years) and a group of normal elderly men (mean age, 70 years) were observed (24).

Morphological studies have indicated that the cerebral cortex extracellular space decreases from 20% in 3 month old rats to 10% in animals 26 months of age (25). On the cellular level, the question of whether or not neurons are lost with increasing age has been an issue for over 50 years (26). Studies on human brains generally report losses while animal studies tend to show no change (26). This disagreement may be due to the fact
that the human brains were mostly from patients with medical
problems. The animal data came from animals reared under con-
trolled conditions. Intracellular accumulation of lipofuscin
pigment has been one of the most consistent changes occurring
in neurons with increasing age (27). After 80 years of lipofuscin
observations there have, as yet, been no convincing correlations
between the lipofuscin accumulation and the loss of function
(27, 28, 29). On the subcellular level, Price et al. (30) have
reported an age-related accumulation of DNA strand breaks in
neurons.

In man there is an apparent 10% reduction in brain weight from
60 to 80 years of age (31). Although brain weights have been
reported both to increase (32) and remain constant (33) throughout
the adult life of rats, brain weights of mice were reported to
remain constant with age (31, 33, 34).

On the biochemical level, the amount of DNA in the whole
brain was unchanged throughout the adult life of mice (31, 33) and
rats (35). This means, since the DNA/cell doesn't change with
age in the brain (13), the total number of cells in the whole
brain of rats and mice remains constant with age. Brain regions
such as cerebellum and brain stem (34) in mice along with

cerebellum, brain stem and cortex in rats (33, 35) also had a
constant amount of DNA throughout adult life. The protein/DNA
ratio in whole brain remained unchanged in both rats (35) and
mice (31, 33) throughout adult life. However, comparisons of

septum and striatum (caudate) from young adult and senescent
mice revealed a 20% drop in protein/DNA with age (36). However,
total protein studies give no information on changes in neural/glial cell population ratios, or specific enzyme activities.

Specific enzyme activities have been checked for age-associated changes in whole brain and various brain regions. Table I summarizes these studies. The % difference between enzyme activities from young and old tissue (45) was calculated from young-old/young \times 100.
Table I  AGE-RELATED ENZYME ACTIVITIES IN BRAIN HOMOGENATES

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<td>mouse</td>
<td>C57</td>
<td>4 vs 28</td>
<td>0</td>
<td>DNA 33</td>
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<td>ATP-ase (Na-K activated) (E.C.3.6.1.3)</td>
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<td>C57</td>
<td>4 vs 28</td>
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<td>DNA 33</td>
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<td>Wistar</td>
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<td>Protein 37</td>
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<td>12 vs 24</td>
<td>0</td>
<td>Protein 38</td>
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<td></td>
<td>rat</td>
<td></td>
<td>7 vs 19</td>
<td>-10</td>
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<td>rat</td>
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<td>0</td>
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<td>35 yrs vs 70 yrs +80</td>
<td>Protein 40</td>
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<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-100</td>
<td>C57</td>
<td></td>
<td>12 vs 30</td>
<td>+25</td>
<td>Wet weight</td>
<td>42</td>
</tr>
<tr>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-3-2</td>
<td>C57</td>
<td></td>
<td>12 vs 30</td>
<td>-15</td>
<td>Wet weight</td>
<td>42</td>
</tr>
</tbody>
</table>
The S-100 protein (function unknown) is found in glial cells (42), and an increase can be interpreted as an increase in the number of glial cells. The 14-3-2 protein (function unknown) is found in neural cells (42). Since brain DNA remains constant with age in mice, it is not surprising to see an increase in the striatum S-100 protein (possible glial cell increase) when the striatum neural 14-3-2 protein decreases (possible loss of neurons). Of the 8 enzymes tested only monoamine oxidase and adenylate cyclase activities change; and these increase with age. While this evidence argues against a loss of gene function with age, these enzymes represent only a very limited picture of the protein gene product. Electrophoretic studies of mouse brain proteins by Grossfeld and Shooter (43) have shown a major protein band change between mice of ages 1 and 2 years. One aim of my research was to study this apparent age-related change of brain proteins.
C. **Kidney Protein Studies in Ageing Mammals**

Kidney ageing research was stimulated by Strehler's (46) presentation of Shock's kidney function data. Apparent 50% decreases occurred between 35 and 85 years of age in human renal plasma flows and glomerular filtration rates. Shock's original data (47), however, had much scatter and instances of 85 year old individuals with kidney functions equal or better than those of a 25 year old. While the use of rats reduced experimental scatter, in contrast with the human studies, no change in glomerular filtration rates between the ages of 12 and 30 months (48).

Anatomical studies are also equivocal. Observations of age-associated thickening of the renal basement membrane in humans (49) and rats (50, 51, 22) conflict with the findings of Osawa et al. (52) who found no age thickening in human basement membranes.

Total kidney weight along with total DNA and protein from rats and mice of various ages provides the following overall picture. Kidney weights in mice either increase (53, 54, 55) or remain constant (55) with advancing age. In both male (53) and female (55) mice an increase in kidney weight is accompanied by an increase in total DNA. Since the amount of DNA/nucleus remains constant with age in the kidney (13), an increase in DNA, means an increase in cell number.

When cell numbers increase with age, so does the protein/DNA ratio (53, 55). However, since renal cells do not divide after birth (56), the increase in DNA and the protein/DNA ratio could
reflect a proliferation of collagen synthesizing fibroblast cells, replacing lost renal cells (22). Total protein studies obviously cannot distinguish collagen of scar tissue from enzyme protein.

Many different specific enzyme activities have been checked for age-associated changes. A list of specific enzyme activities which have been tested in a variety of young and old whole kidney homogenates will be given. Attempts have been made to exclude inducible enzymes, since Adelman (57) and Finch (58) have shown that enzymes are not induced at the same rate in young and old animals. Also excluded will be hydrolytic enzymes which are found in elevated levels in phagocytic white blood cells (59). Since glomerulonephritis occurs in senescent rodents (60), a large population of white blood cells would be expected at the inflammation site, thus distorting renal hydrolytic enzyme levels.
Table II AGE-RELATED ENZYME ACTIVITIES IN WHOLE KIDNEY HOMOGENATES

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sex</th>
<th>Ages Compared (Months)</th>
<th>% Change</th>
<th>Change per unit</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alkaline phosphatase (E.C.3.1.3.1)</td>
<td>rat Sprague-Dawley</td>
<td>F</td>
<td>12 vs 24</td>
<td>0</td>
<td>DNA 61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat Wistar</td>
<td>M</td>
<td>6 vs 21</td>
<td>-30</td>
<td>DNA 62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57B1</td>
<td>M</td>
<td>6 vs 30</td>
<td>+25</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>6 vs 30</td>
<td>-50</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td>2. Cytochrome oxidase (E.C.1.9.3.1)</td>
<td>mouse C57B1</td>
<td>M</td>
<td>6 vs 30</td>
<td>0</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>6 vs 30</td>
<td>0</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat McCollum</td>
<td>M</td>
<td>14 vs 24</td>
<td>0</td>
<td>DNA 45</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>-10</td>
<td>DNA 45</td>
<td></td>
</tr>
<tr>
<td>3. Fumarase (E.C.4.2.1.2)</td>
<td>mouse C57</td>
<td>M&amp;F</td>
<td>12 vs 24</td>
<td>-10</td>
<td>protein 64</td>
<td></td>
</tr>
<tr>
<td>4. Lactate dehydrogenase (E.C.1.1.1.27)</td>
<td>rat Wistar</td>
<td>F</td>
<td>12 vs 24</td>
<td>0</td>
<td>protein 37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat Wistar</td>
<td>F</td>
<td>12 vs 24</td>
<td>0</td>
<td>wet weight 38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57</td>
<td>M</td>
<td>10 vs 29</td>
<td>-20</td>
<td>DNA 54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57</td>
<td>F</td>
<td>6 vs 30</td>
<td>0</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57</td>
<td>M</td>
<td>6 vs 30</td>
<td>0</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td>5. Malate dehydrogenase (E.C.1.1.3.8)</td>
<td>rat Wistar</td>
<td>F</td>
<td>12 vs 24</td>
<td>-15</td>
<td>protein 37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57</td>
<td>F</td>
<td>10 vs 29</td>
<td>-10</td>
<td>DNA 54</td>
<td></td>
</tr>
<tr>
<td>6. 5' nucleotidase</td>
<td>mouse C57</td>
<td>F</td>
<td>6 vs 30</td>
<td>-40</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57</td>
<td>M</td>
<td>6 vs 30</td>
<td>+25</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td>7. Pyrophosphatase</td>
<td>rat Sprague-Dawley</td>
<td>F</td>
<td>12 vs 24</td>
<td>+20</td>
<td>DNA 61</td>
<td></td>
</tr>
<tr>
<td>8. Succinic dehydrogenase (E.C.1.3.99.1)</td>
<td>mouse C57</td>
<td>M</td>
<td>6 vs 30</td>
<td>0</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse C57</td>
<td>F</td>
<td>6 vs 30</td>
<td>0</td>
<td>DNA 55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat McCollum</td>
<td>M</td>
<td>14 vs 24</td>
<td>0</td>
<td>DNA 45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat McCollum</td>
<td>F</td>
<td>14 vs 24</td>
<td>-10</td>
<td>DNA 45</td>
<td></td>
</tr>
<tr>
<td>9. Succinoxidase</td>
<td>rat McCollum</td>
<td>M</td>
<td>14 vs 24</td>
<td>0</td>
<td>DNA 45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat McCollum</td>
<td>F</td>
<td>14 vs 24</td>
<td>-10</td>
<td>DNA 45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat Wild</td>
<td>F</td>
<td>14 vs 33</td>
<td>-10</td>
<td>DNA 66</td>
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</tr>
<tr>
<td></td>
<td>rat McCollum</td>
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<td>14 vs 24</td>
<td>0</td>
<td>DNA 65</td>
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<tr>
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<td>rat McCollum</td>
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<td>14 vs 24</td>
<td>0</td>
<td>DNA 65</td>
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<tr>
<td></td>
<td>rat Sprague-Dawley</td>
<td>F</td>
<td>12 vs 24</td>
<td>0</td>
<td>DNA 61</td>
<td></td>
</tr>
</tbody>
</table>
On the basis of the above list, one can say that there are no large age related enzyme changes which are independent of sex and species. Keeping in mind the variabilities of enzyme assays, the data is very consistent. The above enzyme data gives only a very limited picture of gene expression as a function of age. However, taken together, the physiological, anatomical and enzyme data show no consistent, age-related trends. The following electrophoretic surveys of brain and kidney proteins were initiated with the idea that if gene expression did change with age, trends in protein levels could be detected and analyzed.
II. Methods

A. Animals

The mice used in these studies were LAF hybrid, virgin, female mice obtained at the age of 12 weeks from the Jackson Laboratory, Bar Harbour, Maine. Since environmental variables may influence longevity (67), the living conditions of the mice will be detailed. Mice were housed, 10 to a cage, in 13" x 12" x 6" high plastic cages with stainless steel wire bar lids. Bedding material was wooden shavings. Drinking water and Purina "Lab Chow" were given ad libitum. Cages and water bottles were sterilized and changed on Monday and Friday of each week. Fluorescent (warm white) lighting was turned on at 6 AM and off at 6 PM. Room temperature was maintained at 74 F with a relative humidity of approximately 50%. There was no exercise regime. Rats from other studies were housed in cages approximately 2 feet away. Environmental noise levels were quite low. As deaths occurred within a given age group, animals from different cages were combined to maintain 10 animals per cage. Mice from an initial group of 110 were killed at specific times to provide given aged organs, which were then stored in liquid nitrogen until needed.

Ninety mice were put aside for survival studies. The number surviving with time was recorded. From this data, a survival curve was plotted. The 20% survivors (see Fig. 1) were killed to provide our 151 week old tissue.
Fig 1. Survival curve for 90 female LAF1 mice, compared with the survival curve from the literature.
B. Continuous Slab Gel Electrophoresis

1. Sample Preparation of Soluble Proteins

Mice were killed by cervical spinal cord separation. All operations following excision of organs were performed near 4°C. In this preparation, blood was not perfused from the organs. Kidney was first diced into small pieces. Brain was pressed into a paste with a glass rod inside the homogenizing tube. The tissues were homogenized with a Potter-Elvehjem homogenizer for 2 minutes at 900 rpm in 0.25M sucrose, 0.01 M phosphate buffer, pH 7. The tissue weight to buffer volume ratio was 1 g/1.5 ml for kidney and 1 g/1.8 ml for brain. The homogenate was centrifuged for 2 hours at 100,000 g. Twenty μl of brain and 10 μl of kidney young and old supernatants, each with approximately 250 μg of soluble proteins were then applied to single gel slots. A dye, bromophenol blue (Allied Chemical Corp.) was added to the sample solutions to provide a migration rate marker.

2. Gel Preparation and Operation

The soluble proteins were run in a 7.5% slab polyacrylamide gel using a lucite-walled commercial unit (E-C Apparatus Corporation). The 3 mm thick gels were made from 7.5% (wt/v) Cyanogum-41 {95% acrylamide and 5% N, N'-methylene bisacrylamide (BIS)} and 0.07% N, N'-tetramethylethylenediamine (TEMED) in Tris (25mM) - glycine (190 mM) buffer, pH 8.3, and were polymerized by the addition of ammonium persulfate (0.1%). The same buffer was used in the electrode chambers. A voltage of 250 V was applied to the gel (pre-run) for 1.5 hours. Samples were then loaded and subjected to electrophoresis at room temperature, at a constant
voltage of 350 V for 2 hours. Protein migration was toward the anode. Proteins were stained in a solution 0.5% Coomassie Blue, 5% acetic acid and 25% methanol for 2 hours. Excess stain was then electrophoretically removed by applying an electric field perpendicular to the slab surface while the gel was immersed in a 5/1/1, methanol, water, acetic acid solution.

C. Tissue Powders

Organs freshly frozen or stored in liquid N2 were placed between sheets of heavy aluminum foil and smashed with a hammer into small fragments. These fragments were reduced to a fine powder in a mortar and pestle. A liquid N2-tissue slurry was then poured into a polyallomer test tube. After the liquid N2 evaporated, acetone at -20° C or below was added (10 ml acetone per 0.5 g of tissue) and, with a close-fitting teflon pestle, the slurry was homogenized for 2 minutes. After 20 ml of additional acetone were stirred in, centrifugation at 10,000 g for 10 minutes produced a pellet. Ten ml of n-butanol at -20° C was then added to the pellet. The resulting slurry was homogenized, diluted, and centrifuged as before. To the resulting pellet was added 10 ml of -20° C ether and the slurry was treated as before. The ether pellet was then dried with a N2 gas stream. The resulting powder was stored over CaCl2, in a vacuum desiccator, at -20° C.

Tissue may also be converted to powder with a chloroform/methanol (C/M) mixture (2/1, v/v) substituted for butanol. One gram of tissue requires 20 volumes of C/M.
D. Discontinuous Tube Gel Electrophoresis

1. Sample Preparation of "Insoluble" and Total Proteins
   a. Kidney - All disc samples were prepared from tissue powders. To remove soluble blood proteins, 20 mg of kidney powder were stirred (Vortex, 15 minutes) in 0.5 ml of 0.01 M Tris pH 7, and centrifuged at 4000 g for 5 minutes. The supernatant, with blood and other soluble proteins, was discarded. The pellet was again stirred with Tris buffer and centrifuged. This "insoluble" pellet was next dissolved in 0.5 ml of phenol-acetic acid-water (2/1/1, v/v/v) which was 2 M in urea (PAMU). Vortex mixing for 15 minutes produced a clear solution. Centrifuging this solution for 15 minutes at 4000 g yielded a small faint white precipitate. A dye, Pyronin Y, was added to the supernatants to provide a migration rate marker. Twenty µl of young and old kidney supernatant were added to each tube gel.
   
   b. Brain - Twenty mg of brain powder (with total brain proteins) were added to 2 ml of PAMU. Vortex mixing for 10 minutes and centrifugation for 10 minutes at 4000 g produced a clear solution with no visible pellet. Pyronin Y was added to the supernatants. Fifteen µl of young and old supernatant were added to each tube gel.

2. Gel Preparation and Operation

   Tissue powder proteins soluble in PAMU were subjected to gel electrophoresis in a commercial (Hoefer Instruments) tube type apparatus. They were not run in E-C slab unit since the gel buffer dissolves lucite. Gels were poured in tubes 6 mm in
diameter and 10 cm long. The gel procedure closely followed that of Takayama (68). The percentage of acrylamide (with 95% acrylamide and 5% BIS) used in the gels was 7.5% for kidney and 12% for brain. Gels were polymerized in a solution 35% acetic acid and 5 M urea by the addition of: 1) 0.35% ammonium persulfate (w/v) 2) 0.5% TEMED (v/v) and by heating at 50°C for 15 minutes. After pouring a gel solution to one cm below the tube top, but before heating and polymerization, 75% acetic acid (bubbled with argon to displace dissolved O₂) was carefully layered over the acrylamide solution. This is a key step. Poor gel polymerization at the origin gives poor resolution. After the acrylamide had polymerized, the tubes were rinsed with 75% acetic acid and re-filled with the same solution.

Ten percent acetic acid was used in the electrode chambers. Kidney proteins were subjected to electrophoresis at a constant current of 2.5 mamps per tube for 7 hours at 7°C. Brain proteins were run at 1.7 mamps per tube for 11 hours at room temperature. Protein migration was toward the cathode. Proteins were stained for 2 hours in a 1% Naphthol-Blue-Black water solution. Excess stain was electrophoretically removed in a 5/5/1, methanol, water, acetic acid solution.

E. SDS Discontinuous Slab Gel Electrophoresis

1. Sample Preparations

   a. Brain - Non-Perfused - Sprague-Dawley rats (2 days, 22 days, and 6 months of age) along with the C57B1/6J, Balb c, DBA and young and old LAF₁ mice were anesthetized with ether and
decapitated. The whole brain (including olfactory bulbs and medulla oblongata) was removed, rinsed in 0.9% NaCl solution and blotted dry.

Each brain was homogenized in SDS sample buffer of 0.064 M Tris, pH 6.8; 5% glycerol; 5% β-mercaptoethanol and 2% sodium dodecyl sulfate (SDS) with a Potter-Elvehjem homogenizer. The tissue weight to buffer volume ratio was 1 g to 10 ml. One volume of each homogenate was then diluted with 3 volumes of sample buffer and heated in boiling water for 2 minutes. During this time the solution becomes totally transparent. This solution was then centrifuged (5 minutes at 1000 x g), the small faint white pellet discarded, and the supernatant retained for use. Brain was also dissolved as above, substituting, however, dithiothreitol (DTT) for β-mercaptoethanol. Protein determination (69) revealed that the amount usually applied to a gel slot had 60 μg/25 μl.

Whole blood was also dissolved in SDS sample buffer (50 μl blood per 1.5 ml buffer).

b. Brain Powder - Non-perfused brains, which were converted into powder as described under Tissue Powders, were solubilized in SDS sample buffer, heated in boiling water for 2 minutes, and centrifuged at 1000 g for 5 minutes. The pellet was negligible, and 25 μl of supernatant with approximately 60 μg of protein were loaded into single gel slots. No tracking dye was used.

c. Brain-Perfused - To obtain brains free of blood some animals were perfused with 0.9% NaCl, others with 0.9% NaCl plus Dextran (Cutter Lab.). Mice were first anesthetized with approximately 0.5 g of Na-pentobarbital per kg of body weight,
administered intraperitoneally. Next, the chest cavity was opened, and a #17 needle inserted into the left ventricle. The right ventricle was then cut and 0.9% NaCl was allowed to flow into the left ventricle, then flow through the circulatory system and exit via the right atrium. This perfusion eliminated all noticiable blood from the brain. SDS sample solutions of whole brain proteins were prepared as in Brain - Non-Perfused.

d. Brain-Perfused and Dissected - After perfusion, these regions of the brain were dissected out in the following order: cortex, olfactory bulbs, olfactory tubercules, hippocampus, caudate nuclei, septum, hypothalamus, cerebellum, pons-medulla, and the remains. Following dissection the various brain regions were temporarily stored on dry ice (-79°C). As soon as possible after death and dissection, the brain regions (using the same tissue weight to buffer volume ration) were dissolved in SDS sample buffer as in Brain - Non-Perfused.

e. Brain-Soluble and Ribosomal Proteins - The final procedure adopted for the extraction of "soluble" and ribosomal proteins from mouse brain, as shown in Figure 2, closely follows the technique of Zomzely-Neurath and Roberts (70) and takes into account some observations by Dice and Schimke (71). The reason for resuspending the first 15,000 g pellet, recentrifuging, and then combining the microsomal supernatants was to increase the overall microsomal extraction efficiency. Extracting 100% of a component from both young and old tissue would allow one to attribute any age differences in extraction efficiencies. Dialysis of the DOC supernatant was necessary to remove the sucrose before ethanol precipitation.
Mouse Brain (2 g)

1) Add 18 ml of 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 4 mM MgCl₂, 250 mM sucrose (TKMS) to brain paste.

2) Homogenize in Potter-Elvehjem homogenizer (Thomas "C Type") with 5 up and down strokes at 500 rpm.

3) Centrifuge at 15,000 g for 15 min at 4°C.

Supernatant

1) Resuspend in TKMS buffer.

2) Centrifuge at 15,000 g for 15 min at 4°C.

Combine supernatants

1) Adjust Na deoxycholate (DOC) conc. to 1% by adding 10% DOC in 50 mM Tris-HCl, pH 8.2.

2) Centrifuge at 105,000 g for 2 h at 4°C.

Supernatant (has DOC soluble proteins)

1) Dialyze at room temp. for 0.5 h

2) Precipitate proteins with 95% ethanol at -20°C (10 vols)

3) Centrifuge at 4000 g for 10 min

Protein pellet

Dissolve in SDS sample buffer

"Soluble" proteins

Apply to SDS Gel

Ribosomal pellet

1) Wash in TKMS buffer.

2) Resuspend in TKMS buffer.

3) Centrifuge at 105,000 g for 2 h at 4°C.

Ribosomal pellet

Dissolve in SDS sample buffer

Ribosomal proteins

Apply to SDS gel

Figure 2 SDS sample preparation of soluble and ribosomal proteins.
f. Proteins With Known Molecular Weights - The following proteins were used as molecular weight standards (molecular weight, reference, and source of enzyme in parenthesis): cytochrome c (12,000; Sigma), lysozyme (14,300; Sigma), ovalbumin (43,500; Sigma), glutamic dehydrogenase (50,000; Sigma), albumin (68,000; Sigma), phosphorylase a (92,500; Worthington), β galactosidase (130,000; Worthington), and myosin (200,000; gift from R. Silver). The above proteins were mixed together and dissolved in SDS sample buffer. An aliquot loaded into a gel slot contained 2 μg of each in 25 μl.

2. Gel Preparation and Operation

Laemmli (76) has developed a very sensitive gel electrophoresis technique for the study of viral protein patterns. Recently, Ames (77) has modified the technique to study bacterial proteins. In turn, have adapted the technique to the study of mammalian proteins. The method uses a discontinuous SDS buffer system (76) in a thin slab of gel apparatus (78, 79).

The apparatus (prototype, Hoefer Instruments) is shown in various stages of assembly in Figure 3. Upper left shows 2 glass plates (30 cm long, 18 cm wide) which are held apart along the lengthwise edges by two 0.75 mm thick polyvinyl chloride spacers (B). After mounting on the cooling unit and sealing the bottom opening, it is between these 2 plates that the gel solution is poured and allowed to polymerize. Inserted between the plates at the top is a teflon slot former (A) with 2 wide slots and one regular-sized slot. Upper right shows a lucite support unit.
Figure 3 Apparatus for SDS Slab Gels

Upper left: glass plates; Upper right: cooling unit
Lower left: plates in position; Lower right: complete unit
-30-
equipped with a continuous flow cooling system (C, cooling port) and upper and lower electrodes (E). The largest surface (facing the front serves to cool the gel plates. Its parallel surface on the other side does likewise, so that 2 gels may be run at once.

Lower left shows pressure clamps (T) holding a pair of plates in position against a cooling surface. Where the plates extend approximately 3 cm above the cooling surface, they are pressed against a wide, U-shaped rubber gasket. When the second pair of gel plates are held similarly on the top of the unit. One plate of each pair has a 3 cm deep x 15 cm wide piece cut out from the middle of the top edge. This is the plate which is pressed against the cooling edge, and the cut out serves to expose the gel to the electrode buffer.

Electrode buffer, added to the wavy line (across the slot former), immerses the upper electrode. To illustrate the type of gel pattern obtained, a gel dried on filter paper, is shown inserted between the plates. The wide slot shows a brain protein pattern. The small slot shows some standard proteins. The arrow indicates migration toward the anode. Of course, before a gel can be run, there needs to be a lower electrode buffer in contact with the bottom of the gel. Lower right shows a chamber into which lower electrode buffer is poured. The arrow points to the required electrode buffer level. The cover, with the attached electrode leads, completes the unit.

The SDS gel procedure is similar to that of Laemmli (76), Studier (79), and Ames (77). A lower, 26 cm long, 9% separating
gel is poured first. A key requirement for a successful gel run is a good polymerization at the 9% - 5% interface. After the 9% is poured, but before polymerization, water (previously bubbled with argon to displace dissolved $O_2$) was carefully layered over the acrylamide solution.

The upper, 1 cm deep, 5% stacking gel is poured on top of the separating gel. A teflon comb of the same thickness as the gel, with teeth 0.8 cm wide, was inserted into the stacking gel after pouring. Following gel polymerization, removal of these teeth provided wells for loading the samples. Both the separating and the stacking gel used the following acrylamide mix: 97.5% acrylamide, 2.5% BIS. The separating gel was polymerized in a solution 375 mM in Tris-Base, pH 8.8; 0.1% SDS and 0.05% ammonium persulfate with the addition of 0.045% TEMED. The stacking gel was polymerized in a solution 125 mM Tris, pH 8.3; 190 mM glycine and 0.1% SDS. Sample volumes of 25 µl were delivered to gel slots from Intramedic Tubing PE50 connected to a needle and syringe.

A separate slot or the protein standards slot contained the bromophenol blue tracking dye in SDS sample buffer; no tracking dye was used in any of the brain samples. Voltages were applied as follows: 10 minutes at 50V; 25 minutes at 200 V; 1 hour, 10 minutes at 500 V, 15 minutes at 600 V and 1 hour at 650 V. After the run, gels were removed from between the glass plates and stained. Gels were stained (80) for 3 hours at room temperature in 400 ml of the following solution: 0.0375% Coomassie blue, 12.5% acetic acid, and 12.5% isopropanol 2; three hours in
400 ml of 12.5% acetic acid (repeated at least twice).

Gel photography by transmitted light requires a light box with warm white bulbs and opalized lucite and photography with a Ratten "A" red filter with exposure settings of 0.5 sec at F number 16. Ektapan (Kodak) black and white film was used. Prints were on Kodobromide number 4 contrast paper. Gels which were first dried on filter paper, then photographed with reflected light gave sharper contrast prints. Gel drying on Whatman 3 MM filter paper followed the procedure of Maizel (81).

3. Densitometer Gel Scans

Tube gels were placed in glass tubes filled with water. Slab gels were sandwiched between 2 glass plates. Separate holders accommodating the tube or slab geometries then automatically fed the gels into the unfiltered light of the Gelscan (Gelman Instruments). Optical extinction of various protein bands was measured as a function of distance traveled from the origin. Baselines were set at the lowest background in the gel, ahead of the dye front—where no proteins had been. The area under the peaks was integrated by the instrument. Using SDS gel patterns obtained from 15, 30, 45, and 60 µg of protein, the total area and the area of selected peaks were measured.
III. Results and Discussion

A. Animal Survival and General Observations

The LAFl mice are the hybrid progeny of the highly inbred C57L and A/He strains (82). The inbred strains have been developed by 50 or more generations of brother-sister mating and are therefore homozygous at well over 99.9% of the loci in their genomes (83). This favors reproducibility in biochemical experiments. A cross between 2 inbred strains gives the advantage of hybrid vigour. High genetic homogeneity does exist among the F1 offspring since the mice will accept skin grafts from other F1 mice (84).

How do we know these mice age? The survival curve for my mice is shown in Figure 1. No fighting or infectious diseases such as respiratory ailments were observed at any time during their lifespans. The median lifespan (50% survival) of 875 days may be a bit low since occasionally animals with gross tumors and in obvious pain were removed and killed. As shown in Figure 1, the age-related survival of mice in this study was higher than the best survival data in the literature (85) for this strain.

Are the deaths due to random diseases or are they a consequence of a specific disease which kills this strain before it really ages? Internal organs from 18 mice killed at 133 weeks of age were inspected at a gross level. All organs in 6 of 18 animals looked normal. Eighteen of 18 brains, 15 of 18
lungs and 11 of 18 kidneys appeared normal. Varying degrees of enlarged spleens (indicative of leukemia in this strain) showed up in 13 of 18 mice. Other old animals had ascites, ovarian, skin, liver and kidney tumors. The overall impression was, that for old mice of a given age, tumor sites and organ appearance varied from animal to animal-simulating the human case (2). Other evidence which argues against a strain-specific life-shortening factor involves lifespan comparisons between different mouse strains. Let us compare the LAFl median lifespan of 875 days with lifespans of other laboratory mice. The hybrid and inbred strains with the greatest mean lifespan (each determined with 200 or more animals) are the BC3F1 (86) and the C57B1/6 (87) with 900 days and 950 days, respectively. These average lifespan values represent the greatest one can get for a laboratory mouse over a range of "optimized" conditions.

Comparisons between mean and median lifespans are valid since they usually are very nearly the same (88). Since the lifespans of LAFl, BC3F1 and C57B1/6 are so similar, its unlikely that their lifespans are determined by any strain-specific factors not related to ageing.

Now to return to the question - "Do mice age?" The fact that mice of various strains, housed in different environments and experiencing a spectrum of diseases have approximately the same optimized lifespans and survival curves argues strongly that ageing is occurring in these animals.
B. **Soluble Proteins (Continuous Gel System)**

By operational definition, soluble proteins are those left in buffer solution after homogenization and high speed centrifugation. These *in vitro* soluble proteins are thought to represent proteins in true solution *in vivo* (89).

1. **Brain**

Figure 4 shows young and old mouse brain proteins as separated by 3 different gel electrophoresis systems. The picture illustrates the experimental evolution towards better resolution.

My initial gel studies, using the E - C slab apparatus, showed soluble brain proteins resolved into 12 bands. In the soluble protein patterns of Figure 4, there appears to be a more diffuse middle band in the old, however, the original gel showed no difference between the young (22 weeks) and the old (105 weeks) protein patterns.

One advantage of this soluble protein preparation is that many enzymes still retain their activities. A disadvantage is that preparation conditions of 4°C for 2.5 hours between killing and the gel run allows possible proteolytic degradation.

At this point I decided that there were two obvious "sensitivity" improvements I could make without changing the gel method: 1. older animals could be used, since at 105 weeks on the survival curve, 80% still were alive, and functional declines might still be small 2. choose an organ in which homoeostasis was not as vital as in the brain. I chose kidney since physiological studies by Shock (47) showed that this organ had the greatest functional decline in human ageing.
Figure 4  Comparison of brain protein resolution with 3 different techniques:  a) Soluble proteins by continuous slab gels  b) Urea (+) soluble proteins by discontinuous tube gels  c) SDS soluble proteins from SDS discontinuous slab gels.
<table>
<thead>
<tr>
<th>Soluble</th>
<th>Urea</th>
<th>SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

BRAND PROTEIN GELS

ORIGIN

XBB-744-2789
2. **Kidney**

Densitometer scans of young (15 weeks) and old (151 weeks) soluble kidney protein patterns are shown in Figure 5. Absorption peaks (arbitrary units) show various protein bands as a function of distance traveled from the origin. By comparing the scans in Figure 5 with the photo in Figure 4 we see that this technique is sensitive enough to detect qualitative differences between soluble kidney and brain proteins.

However, no qualitative differences are seen in the 20 bands of young and old soluble kidney proteins. These 2 scans depict the greatest differences I found between young and old patterns. Any quantitative comparison must consider scanning variability, blood proteins and background stains. Simply taking a gel out of the gel scanning holder, putting it back in, and rescanning can lead to a change of up to 15% in the peak heights. If we allow for a 15% scanning variability we now find the scans quite similar except for the 2 main peaks which control gels identified as albumin and hemoglobin. Due to an animal's reactions at the time of killing, the amount of blood in blood-rich organs is highly variable, even with animals of the same age. Neglecting the blood peaks, then, the remaining difference is the dip between the two blood peaks. This dip was not found on other gel runs of the same preparation suggesting that in this particular case gel destaining was not uniform. High background staining near the origin is also a problem. In spite of these gel irregularities, it is safe to conclude that there are no major age changes in the soluble kidney protein bands observed. But kidney function is
Figure 5 Densitometer scan of soluble kidney proteins from individual young and old animals.
DENSITOMETER SCANS OF MOUSE KIDNEY PROTEIN GEL PATTERNS

15 Weeks

151 Weeks

ARBITRARY DENSITOMETER UNITS

DISTANCE FROM CATHODE (cm)
membrane dependent. Approximately 80% of kidney protein is insoluble (43).

The next step, therefore, was to survey gel patterns of normally insoluble (membrane) proteins in both the kidney and brain, while at the same time removing the disadvantages of proteolytic degradation.

C. Tissue Powders

Tissues were converted to powder form to promote solubilization of the large fraction of protein which is involved in membranes and other cell structures. Proteins are insoluble in cold acetone, butanol, C/ M and ether, while lipids are soluble in butanol and C/ M. So in converting a tissue to a powder, water and lipids are removed while proteins, nucleic acids and mucopolysaccharides are retained (90). Removal of lipids from tissues allows extraction of enzymes which are usually firmly bound to lipoprotein complexes (89). Covalent bonds are not broken by such treatment and very often the activity of structural enzymes are preserved (89), although these electrophoresis studies are independent of enzyme activities. Since the preparation is carried out at temperatures below -20° C and in the absence of water, proteolytic degradation differences between young and old tissues are kept to a minimum.

This method has wide use since any tissue, such as tendon or lung, can be turned into a powder, without degradation, and then stored indefinitely at -20° C.
D. Disc Electrophoresis of Total and "Insoluble" Protein

Discontinuous (disc) electrophoresis differs from continuous gel electrophoresis by the introduction of discontinuities in buffer composition, pH and pore size in the gel (91). In the disc gels I am using, the buffer composition and pH is different between the electrode solution, the 75% acetic acid overlayer, and the gel solution. This sharpens the bands and gives an obvious improvement in resolution over the soluble technique.

1. Brain (Total Protein)

Powders of whole brain which were stirred into PAWU gave completely clear solutions, containing total brain proteins. Disc gel patterns of total whole brain proteins from individual young (14 weeks) and old (147 weeks) mice are shown in Figure 4 (Under Urea). Other individual young and old animals gave identical results. Approximately 30 bands were observed.

Resolution is obviously better than in the continuous method, which separated the soluble proteins. Degradation of a specific native protein produces a spread of lower molecular weight species. Electrophoresis of these species usually produces more diffuse gel bands than the undegraded proteins. The sharpness of the "Urea" gel bands testifies to the fact that very little proteolytic degradation occurred during the preparation. For quantitative comparison, densitometer scans were used. The basis of comparison is the relative area a particular protein peak(s) has, compared to the total area of all the peaks on a single gel scan. A lighter intensity of the leading 3 bands in the
young brain gel compared to the old is misleading. The area of these 3 peaks is 18% of the total area for young and 16% of the total area for old. I will assume, for this case, that the area under each densitometer peak is proportional to the amount of protein.

My conclusion, on the basis of the above data, is that there are no significant differences in the relative amounts of major protein bands in young and old mouse brain.

2. Kidney (Insoluble Protein)

Kidney powder was stirred in sucrose-Tris buffer to remove blood and soluble kidney proteins. The insoluble pellet stirred with PAMU gave completely clear solutions. Disc gel patterns of these insoluble kidney proteins from individual young (20 weeks) and old (150) weeks) mice are shown in Figure 6. Approximately 25 peaks are visible. There are no obvious differences between young and old patterns. Densitometer scans of insoluble kidney proteins from 2 different young (15 weeks) and old (151 weeks) mice are shown in Figure 7. These scans both have 28 bands. Arrows sometimes point to places in the scan which are not obvious peaks. These peaks were determined by visual inspection of the gels. No guessing was involved - only the peaks visible by inspection were arrowed. In spite of scanning variability, the scans are almost super-imposable. My conclusion is that there are no significant differences in the relative amounts of major insoluble protein bands in young and old mouse kidney - in agreement with the brain protein work.
Figure 6  Discontinuous tube gels of young and old "insoluble" Kidney proteins from individual animals.
WATER INSOLUBLE MOUSE KIDNEY PROTEIN GELS

Young 20 weeks

Old 150 weeks

Origin
Figure 7  Densitometer scans of young and old "insoluble" kidney proteins from individual animals.
DENSITOMETER SCANS OF MOUSE KIDNEY PROTEIN GEL PATTERNS

ARBITRARY DENSITOMETER UNITS

DISTANCE FROM ANODE cm

XBL 7110-5403
By comparing the disc gels in Figure 4 (Urea) with those in Figure 6, we see that this technique is sensitive enough to detect qualitative differences between total brain proteins and "insoluble" kidney proteins. Further resolution by running the proteins in longer gels for longer times, is not likely to increase the number of bands resolved, since diffusion, smearing and bending effects on the leading bands are obvious in Figure 6 and Figure 4 (Urea). It can be argued that changes are taking place with age but that the gels are still not sensitive to these changes. After all, the brain and kidney have many different proteins and I looked at just a small fraction. Besides limited sensitivity, another disadvantage of the soluble and disc method is that there is no characterization of the protein bands other than the amount and distance traveled from the origin. The SDS disc slab gel method to be described has some important improvements.

E. SDS Electrophoresis of Brain Proteins

Greater resolution of proteins and molecular weight characterization of protein bands are two new advantages offered by the SDS disc system. In common with the disc tube gel system, the SDS system also has the advantages of minimum proteolysis and total protein solubilization.

Before analyzing the SDS young and old brain protein patterns in Figure 4, there are 6 points to be covered: 1. sample solubility; 2. blood protein contamination; 3. protein "precipitation" at the gel origins; 4. molecular weight
approximations; 5. quantitative gel densitometry; 6. gel sensitivity.

1. **Sample Solubility**

   In the search for molecular differences between animals of various ages, positive erroneous results are often due to extraction (or fractionation) differences. For example, if only 50% of total protein is extracted from young and old tissue and a gel pattern difference is detected, one may justifiably argue that the difference lies in a selective extraction. Total extraction would avoid such difficulties. Total protein extraction requires total protein solubility.

   The importance of the solubilizing ability of the anionic SDS has been stressed by Bretscher: "the introduction of SDS as a solubilizing agent for insoluble molecules has revolutionized the study of membrane proteins" (92). Using SDS, proteins from many sources have been completely dissociated. Apparently not only protein-protein interactions are eliminated; but protein-nucleic acid and protein-lipid interactions as well. SDS completely disrupts intermolecular bonds without covalent bond changes. There are no known protein aggregates linked by noncovalent bonds that survive treatment with SDS at 100°C for 1 minute (81). Although the \( \text{PANU} \) treated protein powders gave clear solutions, no protein determinations were made. I have done protein determinations on brain homogenate and its supernatant, an aliquot of which was applied to the gel. Homogenate and supernatant gave identical protein determinations,
indicating complete solubilization of brain proteins.

2. **Blood Contamination**

At most, blood contamination would be expected to account for only 2% of the total brain volume (93). Figure 8 shows 3 individual brain preparations: 1. rinsed with NaCl; 2. perfused with NaCl; 3. perfused with NaCl-Dextran. The 2 sets to the right are repetitive. Each slot reveals over 50 protein bands in 14 cm of migration. Densitometer scans of the 3 samples revealed no differences. What is remarkable is the animal to animal and slot to slot reproducibility. In contrast to gels run in cylindrical tubes, slab gels are polymerized, run, stained and destained as a single unit. Protein patterns, as a result, exhibit more uniform mobility from well to well and fewer staining/destaining artifacts. The dark front marked "dye front" contains, as we shall see later, a series of different proteins running as a single band. The fastest moving proteins are "stacked" together.

Another control to check for blood contamination is to compare blood proteins with brain proteins. The results of this control are shown in Figure 9. While this gel doesn't have good resolution, it does have the needed information. Whole blood proteins are compared with proteins from non-perfused brain powder solubilized with a) mercaptoethanol (M) b) dithiothreitol (DTT) in the SDS sample buffer.

If the concentration of blood protein was reduced so that the fastest moving protein band in blood was the same intensity as
Figure 8  Slab Discontinuous Slab Gel. The first three slots have protein patterns from 3 different brain preparations: 1. rinsed with NaCl  2. perfused with NaCl  3. perfused with NaCl-Dextran. The 2 sets to the right are repetitious.
SDS BRAIN PROTEIN GEL

NaCl rinse

NaCl perfuse

NaCl-Dextran perfuse

Origin

Dye front

CBB-7310-6333
Figure 9  SDS slab gel comparison of proteins from blood, with brain powder proteins dissolved in SDS sample buffer using mercaptoethanol and separately, DTT.
SDS PROTEIN GEL

Whole Blood

Brain Powder
Mer. Dithio.

-54-

XBB-743-1852
a corresponding band in brain, then the amount of the slower moving blood proteins would be too small to detect. We then have one band of concern - the leading blood band, hemoglobin. This is a point to remember.

3. **Protein Precipitation at Gel Origins**

Figure 9 also contains information on the proteins precipitated at the origin. These proteins never enter the gel regardless of the running time. Are the proteins in the gel representative of those in the sample applied or is there a selective precipitation at the origin? This question must be resolved before examining young and old tissue, since, if age differences are found, one could argue selective precipitation differences at the origin. Or, if there is no change, one could argue that a difference might be in the "insoluble" origin material.

Total protein migration is achieved when proteins are dissolved using DTT instead of M in the **SDS sample buffer** (Figure 9). Brain powder was used in this case, since fresh brain in DTT - **SDS sample buffer** gives much poorer resolution, making a comparison with brain M-SDS sample buffer less effective.

With the exception of a diffuse band nearest the anode, and the intensity of the heaviest band, both in the DTT slot, the patterns are almost identical. In fact, the DTT slot was identical, except for the diffuse band near the anode symbol, with the fresh brain protein pattern run on the same gel, but not in the photo. I conclude, then, that the protein patterns within the gel are not distorted by origin precipitation and are representative
of the proteins applied to the gel.

4. Molecular Weight Approximation of Brain Proteins

Shapiro and Haizel (94) have shown that treatment of proteins with SDS and the reduction of disulfide bonds with mercaptoethanol prevents protein interaction, and that during electrophoresis all proteins migrate toward the anode, regardless of isoelectric point. In addition, Shapiro et al. (95) showed that if the logarithm of molecular weight is plotted against relative migration, a straight line can be fitted to the data. Weber and Osborn (73) have elaborated this finding using 40 polypeptides with well-characterized molecular weights. Using globular and even highly helical (troponyosin) polypeptides a smooth curve was obtained when electrophoretic migration distances were plotted against the log of the molecular weights. A straight line fit was obtained for proteins in the molecular weight range from 11,000 to 70,000. Even data for proteins such as pepsin and papain which bind very little SDS (96), fall on the line as predicted. Although proteins with molecular weights greater than 100,000 migrate faster than predicted from the straight line fit, the data does produce a predictable smooth curve.

SDS slab gel electrophoresis of cytochrome c, lysozyme, ovalbumin, glutamic dehydrogenase, albumin, phosphorylase a, β galactosidase and a mixture of all is shown in Figure 10. Running each protein in a different slot established the identity of each band in the mixture. Under the electrophoretic conditions of this particular gel, lysozyme and cytochrome c ran with the same mobility. Separation can be achieved if the pH in the
Figure 10  SDS slab gel of standard proteins: CY-C, cytochrome c; L, lysozyme; OA, ovalbumin; GD, glutamic dehydrogenase; AL, albumin; PI, phosphorylase a; GA, α - galactosidase; T, a mixture of all seven.
separating gel is increased slightly from 8.8 to 8.85. Ovalbumin, while not an ideal polypeptide standard, since it is a glycoprotein, (97) nevertheless provides a convenient marker in the 40K range. When the log of the molecular weights was plotted against the corresponding migration distances, a straight line fit was obtained, with the exception of β-galactosidase (see figure 11). Such a plot, obtained when standards are run on a slab gel, can be used to estimate the molecular weights of brain proteins run on the same gel, e.g. (see Figure 14).

Weber and Oshorn (73) conclude that with this technique electrophoretic mobility is governed solely by the MW of the polypeptides and that molecular weights of unknown proteins may be determined with an accuracy of at least ± 10%. However, more recent studies by Tung and Knight (98,99) suggest that electrophoretic mobility in SDS gels may in some cases involve charge. Therefore, my use of molecular weight standards will then be to approximate brain protein molecular weights rather than to assign absolute values.
Figure 11  Semi-log plot of the molecular weights of standard proteins listed in Figure 10 against their corresponding distances of migration from the origin.
PROTEIN STANDARDS

Distance From Origin

Mol. Wt. - Kilodaltons

10 20 30 40 60 80 100 200

10 20 25cm
6. Quantitative Gel Densitometry

Observed gel patterns are the result of light absorption by bands of protein complexed with Coomassie blue. A lengthwise densitometer scan of a gel yields a plot of absorption as a function of distance from the origin. Absorption peaks (see Figures 5, 7, 19) represent protein bands. The area under each peak was determined by machine integration. Peak areas increase linearly with protein loaded onto the gel, but deviations from linearity are possible with higher protein loads. Non-linearity can make bands quantitation quite complex. To test linearity of brain protein bands, 2 gel slots were run with each of the following protein aliquots: 15, 30, 45 and 60 μg. Densitometer scans of each of the gel slots were obtained, similar to those in Figure 19. After subtracting backgrounds, the total area under the peaks of each gel was then plotted against the amount of protein applied to the gel slot (see Figure 12). The resulting solid line shows a linear increase in area up to 45 μg of protein. At 60 μg the higher total area was 8% below that expected from a linear plot. Before quantitative comparisons, it is important to establish that the ratios of specific peak areas are independent of the amount of protein applied to the gel. This was tested by comparing their % contribution to the total area as a function of protein amount (100). Peaks in the densitometer scans of each gel were partitioned off with vertical lines (similar to Figure 19) into 11 sections which comprised 60% of the total area. The % contribution to the total area of each section was determined for different amounts of protein placed on the gel, as shown in
Figure 12 Area under densitometer tracings versus amount of protein placed on the gel.
Table III. The % value for each section remained constant with increasing amounts of protein.

Using densitometer scans from young and old brain protein patterns, quantitative comparisons are possible by taking peaks of the same migration distance and comparing their % contribution to the total area. Equal amounts of young and old brain protein were applied to gel slots. It was assumed that the molar absorbance for a specific peak did not change with age.

Some idea of reproducibility of specific peaks can be obtained by comparing % values at different protein loads; for example, the greatest variation is shown by peak number 9 in Table III where its value varies from 2.83 to 2.46 - a range of less than 15%. These are based on single scans of different gels. Rescanning of the same gel usually has less than a 5% range of peak % values.

We can estimate the minimum amount of protein we can quantitate if we make the assumption that all peaks have equal molar absorbance. Then any peak area is directly proportional to protein amount. From the value of peak 9 (Table III) at a 15 μg protein load, one can calculate that (15)(0.028)=0.42 μg of protein can be measured.

It is important to remember that the densitometer is not more sensitive than the eye. The densitometer merely quantifies an obvious visual difference in peak areas.
Table III
Quantitative Analysis of SDS Brain Protein Gels

<table>
<thead>
<tr>
<th>µg Protein added</th>
<th>Total area units</th>
<th>Band Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1490^a(100%)</td>
<td>--</td>
<td>5.03^b</td>
<td>3.47</td>
<td>9.68</td>
<td>4.75</td>
<td>3.29</td>
<td>--</td>
<td>3.77</td>
<td>2.76</td>
<td>6.46</td>
<td>12.4</td>
<td>56%</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2580 (100%)</td>
<td>1.76</td>
<td>4.46</td>
<td>3.56</td>
<td>10.8</td>
<td>4.69</td>
<td>3.47</td>
<td>3.87</td>
<td>4.11</td>
<td>2.83</td>
<td>7.92</td>
<td>13.5</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>3600 (100%)</td>
<td>1.73</td>
<td>4.91</td>
<td>3.77</td>
<td>10.9</td>
<td>4.48</td>
<td>3.66</td>
<td>3.79</td>
<td>4.38</td>
<td>2.46</td>
<td>7.76</td>
<td>12.9</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>4360 (100%)</td>
<td>1.65</td>
<td>4.55</td>
<td>3.28</td>
<td>10.3</td>
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<td>4.19</td>
<td>4.30</td>
<td>2.79</td>
<td>7.75</td>
<td>12.0</td>
<td>60%</td>
<td></td>
</tr>
</tbody>
</table>

All values were obtained by averaging single densitometer scans of 2 gels loaded with a specific amount of protein.

a - The total area units are arbitrary.

b - Values are percentages of total area contributed by each band.
6. SDS Gel Sensitivity

The previous gel patterns comparing young and old brain proteins have shown no age-related changes. Are there really no differences or is the gel method insensitive to a difference? The continuous slab and the discontinuous tube gel method were sensitive enough to distinguish between proteins of different organs, however, no positive controls have been run on the same tissue.

A series of positive controls, using whole brain, were run using the SDS slab gel technique. In each case protein differences were known to exist between the brains compared. The question was whether or not the SDS system was sensitive enough to detect possible protein level differences.

The first controls compared the brain proteins of a rat with those of a mouse. Obviously, phenotypes and genomes are quite different, so the protein product was expected to vary considerably. Adult rat (R) and mouse (M) brain proteins do indeed differ qualitatively and quantitatively (see Figure 13). Three of the largest differences are associated with arrows. Many other minor differences were obvious on the original gels. What is surprising is the degree of similarity between rat and mouse proteins. The differences were confirmed with one other rat-mouse pair.
Figure 13  SDS slab gel comparison of whole brain proteins from:  R, adult rat with M, mouse; $^{2}R$, 2 day rat with $^{22}R$, 22 day rat; $^{N}M$, normal mouse with $^{Q}M$, quaking mutant mouse. Arrows indicate band differences between the 2 animals compared. Approximate molecular weights are indicated.
The next controls kept the genome similar (rats) and followed the program of gene expression with development. Unequivocal changes in morphology and metabolism during development have been well documented (1). Although resolution was poor and background variability quite high, electrophoretic studies by Grossfeld and Shooter (43), with approximately 10 bands of brain protein, did show some indications of change with development. The question was, to what degree? In Figure 13 ten arrows indicate the more obvious differences. These differences were confirmed with another pair of brains, each brain at a different stage of development. Note also in Figure 13 that there is a difference between the adult and the 22 day old rat. Of particular interest are bands near the 12.4K marker (the 3rd and 4th bands away from the dye front) which increases in intensity with rat brain development. These are most likely the low molecular weight proteins in myelin. This increase in myelin protein complements the histological observation that the degree of myelination increases with development (1). Overall, the developmental differences were more prominent than the differences between species. The positive controls used so far were quite different so that perhaps they were not a serious test of SDS gel sensitivity.

The next pair of positive controls were brains from C57Bl/6J normal and quaking mutant mice (Jackson Lab), all the same age.
and sex. The only phenotypic difference in the mutant is a quaking of the hindquarters when it walks.

Otherwise, the physiological functions and lifespan of the mutant appear to be normal (101). On the histological level, hypomyelination of the central nervous system has been observed (101). An electrophoretic (102) study of isolated myelin found a decrease in 3 protein bands in the mutant compared with the normal mouse. I also observed a difference in 3 band intensities when normal \( N^N \) and mutant \( N^Q \) whole brain proteins were compared (see Figure 13). An SDS gel with greater resolution and band intensity, comparing proteins from 2 normal and 2 mutant brains, revealed an obvious difference in 4 bands (see figure 14). Three band intensities were lower in the mutant. During the addition of the molecular weight standards to the gel, a small amount of (1 \( \mu g \) each) of the \( \beta \)-galactosidase, phosphorylase \( a \) and albumin spilled over into sample sells N1 and Q2, providing internal markers in those wells (asterisks). These internal markers showed approximately the same mobility whether run in the standard, the normal, or the quaking samples. We can infer, then, that other SDS-soluble cell constituents (lipids, DNA, RNA, etc.) do not influence the relative protein mobilities. A further argument that lipid levels do not influence protein migration follows from the fact that quaking mice have reduced levels of brain lipid, and yet, band mobilities of internal
Figure 14  SDS gel comparison of whole brain proteins from:
two different normal C57B1/6J mice-N1, N2 with
two quaking mutant C57B1/6J mice-Q1, Q2. Arrows
indicate band differences between normal and
quaking mutant mice. Asterisks indicate presence
of molecular weight standards as internal markers.
Standard proteins and their molecular weights are
shown.
markers and other proteins appear to be the same in normal and mutant samples.

To test SDS gel sensitivity further, brain proteins from male and female Balb/c mice were compared (Figure 15). A single band intensity change was observed in the 130K range (arrow). The rationale for comparing different sexes was that the genome differs in the sex chromosomes. An additional sensitivity test compared brain proteins from C57Bl/6J and DBA mice. These mice have been reported to display genetically determined differences in learning ability (103). Again, a single band intensity change was observed (Figure 15) in the 130K range (arrow). The bracket-arrow near the origin and the arrow nearest 12.4K indicate which bands from C57Bl/6J and DBA are different from those in Balb/c and LAFl. Brain proteins from young (17 weeks) and old (127 weeks) mice were also shown in Figure 15. No difference between them was observed. In fact, the absence of a change in the young and old pair serve as a negative control--they illustrate that the previous differences were probably real and not artifacts introduced by the system.

To summarize the gel sensitivity tests - comparisons of brain proteins from pairs of animals of different species, strains, periods of development, sexes and learning ability, showed positive differences. Extensive comparisons of brain proteins from young and old mice will follow.
Figure 15  SDS gel comparison of whole brain proteins from: Balb c males with Balb c females; C57 with DBA; young with old LAFl. The arrows at 130K indicate differences between the 2 compared. The bracket-arrow near the origin and the arrow nearest 12.4K indicates bands from C57 and DBA that are different from those in Balb c and LAFl. Approximate molecular weights are indicated.
7. Young and Old Whole Brain

SDS gel patterns of whole brain proteins from 6 individual young and 6 individual old mice are shown in Figure 16. All young mice were 15 weeks old. Numbers 1, 2, 3, 4 of the old mice were 108 weeks old, while numbers 5 and 6 were 156 weeks old. Mercaptoethanol was used in the SDS sample buffer for the first 3 pair of brains, and DTT was used in the second 3 pairs.

Aside from a single band in 0-3 a quarter of the gel length away from the dye front, no changes were observed. This single band change from 0-3 was not reproducible in subsequent gel runs. The major bands in the DTT samples coincided well with those in the mercaptoethanol samples. Although resolution was poorer in the DTT samples, all of the protein left the origins.

One aspect of the crosslinking theory of ageing (104) postulates that irreversible covalent crosslinking renders the molecules more insoluble and the organism less functional. A predicted result of crosslinking is an increase in the average molecular weight of protein with age.

The gel patterns in Figure 16 provide strong evidence against crosslinking in mouse brain proteins as a function of age. Since all of the whole brain was dissolved and all of it migrated into the gel, slots 4 through 6 unequivocally represent the molecular weight distribution of all the brain proteins. The fact that young
Figure 16  SDS gel comparison of whole brain proteins from 6 individual young and old mice; young were 15 weeks of age; numbers 1, 2, 3, 4 of the old mice were 108 weeks, while numbers 5 and 6 were 156 weeks of age. Mercaptoethanol was used in the SDS sample buffer for the first 3 pair of brains, and DTT was used in the second 3 pairs.
SDS BRAIN PROTEIN GEL

Mercaptoethanol

\[ y_1 \quad o_1 \quad y_2 \quad o_2 \quad y_3 \quad o_3 \]

Dithiothreitol

\[ y_4 \quad o_4 \quad y_5 \quad o_5 \quad y_6 \quad o_6 \]
and old protein patterns appear identical, and that the proportion of high molecular weight compounds does not increase, is strong evidence against the theory of covalent crosslinking in proteins (excluding S-S crosslinkage).

The length of the separating gel shown in Figure 16 was 14 cm. Resolution was increased when the gel length was increased. Thirty additional bands were observed (in the gel, not the photograph) when the separating gel length was increased to 28 cm, as shown in Fig. 17. In Fig. 17 whole brain proteins from 4 individual young (17 weeks) and 4 individual old (127 weeks) are compared. The brains were not perfused. The mice were different individuals from those in Figure 16.

In Figure 17 there are 2 apparent band differences. The leading protein band, running faster than the 12.4K marker, is lighter in all 4 of the older animals. Recall that Figure 9 showed the hemoglobin chains had the same migration rate as the leading brain polypeptide band. Indeed, Figure 23, where blood was included reconfirms this. Hemoglobin chains have a molecular weight of 15.5K, clearly greater than 12.4K, yet hemoglobin migrated faster than cytochrome c. The hemoglobin chains, then, violate the SDS gel generalization that migration rate depends
Figure 17  SDS gel comparison of whole brain proteins from 4 individual young (17 weeks) with 4 individual old (127 weeks) LAFl mice. Arrows indicate apparent age differences. Standard proteins and their molecular weights are shown.
SDS BRAIN PROTEIN GEL

ORIGIN

Mol. Wt. (Kilodaltons)

130
92.5
68
50
43.5
12.4

MW

Young

Old

1 2 3 4

1 2 3 4

XBB-743-1849
solely on molecular weight.

The second band in Figure 17, which apparently changed with age, is marked by the arrow between 12.4 and the origin. Subsequent repetitions of the gel run, (see Figure 18) using the same samples, could confirm this band difference. The stability of the leading band (band with hemoglobin) was variable from run to run and in Figure 18 is shown as unstable in the old. Even with the good resolution and contrast of Figure 18, the young-old difference of the "2nd" band of Figure 17 is not confirmed.

So far SDS gels have compared the brain proteins from 10 individual young and 10 individual old animals; no visual qualitative or quantitative age differences have been observed. To test this lack of change, specific densitometer peaks of young and old brain proteins will be compared on the basis of their % contribution to the total densitometer area of the particular gel.

Sections 1, 19, and 21 of Figure 19 have quantitative differences greater than the maximum variability of 15% which one could expect under routine conditions. Are these differences age-related? Since both section 1 and 19 display very small peaks relative to the background, the difference is not likely due to the peak areas. It is not possible to determine the cause for the background differences. However, no trend is apparent; old background is high in section 1, but low in section 19. This should not be taken as evidence for crosslinking (shift to higher molecular
Figure 18 A subsequent repetition of the SDS gel run shown in Figure 17.
Figure 19 Densitometer scans of brain protein gels from individual young (17 weeks) and old (127 weeks) LAF1 mice. The origin is at upper left; the fastest moving protein is in section 27. Vertical lines indicate the partitioning of the total area under the curve among 27 sections. Each section has its % contribution to the total area. The "A" within a section indicates a densitometer artifact.
DENSITOMETER SCANS OF BRAIN PROTEIN GELS

ORIGIN

YOUNG

OLD

ARBITRARY MIGRATION UNITS

DENSITOMETER UNITS

ARBITRARY UNITS

Y

O

D
weights with age) since in sections 3 through 18 covering molecular weights 90K through 20K (cont.) and representing 62% of the total area, specific peak section comparisons between young and old reveal that values agree to within 8%, a remarkable agreement considering the complexities involved.

The **quantitative** difference between the young and old peaks of section 21 appears significant. However, this is the "2nd" peak of Figure 17 (indicated by arrow between the 12.4 markers and the origin), which is sometimes unstable. Instability for this peak means curvature with a faster than usual migration rate. Especially good examples of this peak's instability or displacement toward the origin is shown in Figure 22, under O-Cau. Nuc., Y and O-Hip and O-Hyp. If the peak is displaced toward the origin this means that the area values of the adjacent sections should be elevated. This is the case; section values of 22, 23 and 24 are elevated in the old. Also, in Figure 19, the sum of peak areas 20 through 24 are 7.34% for the young and 7.36% for the old. Incidentally, the gels running the same samples, but not pictured, indicate that this instability does not favor the old samples. Thus, one can account for the quantitative differences in sections 1, 19, and 21 between young and old without involving ageing effects.

Differences between young and old, in Figure 19, best described as **qualitative**, appear in sections 3 and 4. The
young gel appears stretched compared to the old. This is probably the result of positioning the gel unevenly in the gel holder. Some doubtful peaks were left without arrows. One obvious decrease in the leading peak in section 4 (old) was unconfirmed in other gels with better resolution.

Summarizing the densitometer comparisons: no quantitative differences were found between the major peaks of young and old brain protein patterns. One could argue that whole brain is too complex a system to survey, and that small differences would go undetected.

8. Young and Old Brain Regions

In an attempt to restrict cell types and reduce complexities, perfused whole brain was dissected to give: cortex, olfactory bulbs, olfactory tubercules, hippocampus, caudate nuclei, septum, hypothalamus, cerebellum, pons-medulla, and the remains. These regions are anatomically and functionally distinct so that one would expect their protein patterns to differ. Protein patterns from the various regions are shown in Figure 20. Bands with dots have different relative intensities when compared to cortex bands. The sensitivity is great enough to detect bands in each region which differ in intensity from its corresponding band in the cortex. Cerebellum showed the greatest difference from cortex. The septum and the caudate nuclei did include some cortex tissue after dissection, so its understandable that the
Figure 20  SDS gel comparison of proteins from the following brain regions: Br, whole brain; C, cortex; PM, pons-medulla; Cer, cerebellum; Hip, hippocampus; Hy, hypothalamus; CN, caudate nucleus; S, septum. When compared to cortex bands, bands with dots have different relative intensities.
SDS BRAIN PROTEIN GEL

Mol. Wt. - Kilodaltons

MW  Br  C  PM  Cer  HiP  HY  CN  S

Origin

-89-

XBB-743-1851
differences were not more pronounced. The similarities between regions of diverse functions such as cortex and hypothalamus are striking.

Brain regions were pooled from 5 young and 4 old animals. Young and old cortex, pons-medulla, and cerebellum protein patterns are compared in Figure 22. There are no detectable age differences. Lines indicate where the bands of the pons-medulla and cerebellum differ in relative intensity from those of the cortex. Cerebellum appears to have major bands (2 lines below the 43.5K marker in Figure 21) which are unique.

Young and old caudate nuclei, hippocampus, hypothalamus and septum protein patterns are compared in Figure 22. Lines indicate where the bands differ in their relative intensity from those of the cortex in the previous Figure 21. The curved bands in O-Cau. Nuc., Y and O-Hip and O-Hyp are band instabilities caused by gel conditions and are not reproducible. These are not age-related changes. Aside from the unstable bands there are no detectable age differences.

9. Young and Old Soluble and Ribosomal Proteins

One can still argue that the complexity of a given brain region is still too complex a system to survey, and that large differences in low concentration components would go undetected.

One approach toward simplification would be to isolate, from young and old tissue, a select package of protein product
Figure 21 SDS gel comparison of proteins from the following pooled young (36 weeks) and pooled old (131 weeks) brain regions: cortex, pons-medulla, cerebellum. Lines indicate where the bands of the pons-medulla and cerebellum differ in relative intensity from those of the cortex.
Figure 22  SDS gel comparison of proteins from the following pooled young (36 weeks) and pooled old (131 weeks) brain regions: Cau. Nuc., caudate nuclei; Hip, hippocampus; Hyp, hypothalamus; Septum. Lines indicate where the bands differ in their relative intensity from those of the cortex in the previous Figure 21.
which has less than a hundred components. Preferably, the stoichiometry would be close so that there would be no great range of component concentrations. Since the SDS gel method resolves bands on a molecular weight basis, another asset would be size heterogeneity. From the standpoint of the "gene expression change with age" hypothesis, the protein package should have a short half life so that it would require constant gene activity to maintain component levels. Desirable isolation characteristics for this protein package would be: 1. low proteolysis, 2. straightforward, complete extraction, 3. lack of contaminating proteins.

Enter the ribosome. Faloly and Staehelin (105) estimate 80 100 polypeptides from $2.2 \times 10^6$ daltons of protein in a single mammalian ribosome. Two-dimensional gel electrophoresis, while being able to separate all 55 known \textit{E. coli} ribosomal proteins (106), has separated just 60 from rat liver (107). Molecular weights for pea ribosomal proteins range from 10 to 77.5K (kilodaltons). The half life of brain ribosomal proteins is 21 days in senescent mice (108). Possible contaminating proteins include a 52K endoplasmic reticulum protein (109) and soluble cell proteins (110). For this reason, soluble proteins were run parallel with ribosomal proteins. Soluble and ribosomal protein patterns from young (28 weeks) and old (134 weeks) brain are shown in Figure 23.

Soluble protein patterns had over 50 bands. Four lines linking soluble young and old brain bands indicate intensity differences. The first 2 band differences (closest to the
Figure 23  SDS gel comparison of proteins from blood (Bl), whole brain (Br), molecular weight standards (MW), and from young (28 weeks) and old (134 weeks): 1. soluble whole brain proteins 2. ribosomal proteins. Lines linking young and old bands indicate differences.
origin) were not confirmed on subsequent gels with better resolution. The band difference nearest the 12.4K marker (at approximately 16 and 24K) were confirmed on subsequent gels. Both increase with age. Since the S-100 protein of glial cells has a molecular weight of 24K, an increase in this band could be due to an increase in the number of glial cells with age—thus supporting age-related glial cell observations in the literature (1). Protein yields in the ribosomal pellets were 0.38 and 0.42 mg per gram of young and old brain, respectively. Approximately 55 bands were observed in the ribosomal patterns (Figure 23). More bands are not resolved because many different ribosomal proteins have the same weight (111,112). Unequivocal identification of mammalian ribosomal proteins is beyond our present state of knowledge. Evidence, such as in vitro reconstitution (singly) of purified proteins to a partial ribosomal unit and protein synthesis resulting from this event, is required (113, 114). However, some bands are less likely than others to be ribosomal proteins. Since 100 polypeptides are in $2.2 \times 10^6$ daltons of ribosomal protein, the average protein molecular weight will be 22,000. It is reasonable then to question the origin of all the high molecular weight bands, since with just a few of these (assuming 1:1 stoichiometry of protein components), the ribosome would exceed the known observed size. Indeed, Thomas (111) finds just 2 bands (72.5 and 77.5K) over 68K in his highly purified pea ribosomes.
A band's ribosomal origin would also be dubious at a high molecular weight if it had corresponding high intensity soluble protein bands. As in Thomas' studies, other investigations have shown that a substantial portion of the total number of protein molecules in animal ribosomes have low molecular weights (115).

The most striking difference between the young and old ribosomal protein patterns in Figure 22 is the diffuse nature of all the bands in the old pattern. Diffuseness of gel bands is characteristic of protein degradation and/or high salt concentrations in the sample buffer. Since \( \text{MgCl}_2 \) was used during the ribosomal preparation, greater diffuseness in the old bands was possibly the result of a greater \( \text{Mg}^{++} \) ion concentration.

In spite of band diffuseness in the old, 2 differences were obvious between young and old ribosomal protein bands. The band nearest the origin, which decreases in the old, has a molecular weight in excess of 300K, so it is certainly not a ribosomal protein. I was not able to determine the exact molecular weight for this band because the semi-log plot of molecular weight versus migration distance is non-linear beyond 90K. A most important molecule in that size range is mammalian RNA polymerase, with a subunit weight (for mammals) of 400K.

The ribosomal band intensity difference at 30K (Figure 23) was unconfirmed on subsequent gels with lower band diffuseness.
Schimke has given evidence for heterogeneous degradation rates of ribosomal proteins (110). Heterogeneity of *E. coli* ribosomal protein populations has also been reported (116). Since ribosomal turnover continues into senescence (108), if the gene expression apparatus did become less functional with age, then almost certainly changes in relative amounts of ribosomal proteins would have been detected.
IV. Conclusions and Summary

1. Methods were developed to minimize proteolysis and to achieve total solubilization and extraction of proteins from young adult and senescent mouse tissues.

2. The sensitivity of the various gel techniques was demonstrated by revealing gel pattern differences when protein products were from different genomes. Proteins from different species, strains, sexes, and mutant mice yielded different protein patterns.

3. Sensitivities of the gel techniques were also demonstrated when the protein product was a result of different expressions of the same genome. When proteins from different organs, different regions of the brain, and different stages of growth and development were compared, changes were detected in gel protein patterns.

4. Gel patterns of whole brain and whole kidney proteins showed that major protein levels were maintained, even in senescence.

5. Major protein levels were maintained in the caudate nuclei, cerebellum, cortex, hippocampus, hypothalamus, pons-medulla and septum, even in senescence.

6. Major soluble protein levels and ribosomal protein levels were maintained in senescent mice.
7. These observations suggest that the gene functions necessary for the maintenance of steady state protein levels are not lost with age and provides strong evidence against the hypothesis that ageing is a continuation of growth and development.

8. Total solubilization and electrophoresis of whole brain and brain regions showed no apparent increase in the average protein molecular weights involved. Limited to the mouse, this is a strong argument against the hypothesis that protein crosslinkage is among the primary causes of ageing, excluding crosslinkage through disulfide bonds.

9. The advantages of complete solubilization, size separation and high resolving power of the SDS gel method, suggests that this technique will find widespread use.
V. References


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