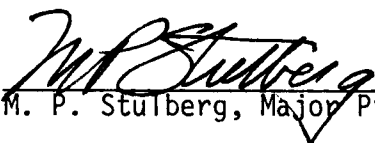
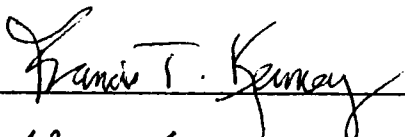


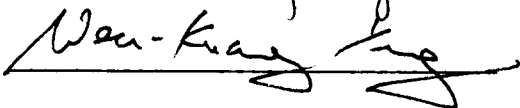
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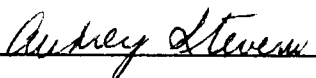
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M. P. Stulberg, Major Professor

We have read this dissertation
and recommend its acceptance:


Francis T. Kemmer


Wan-Kuang Tseng


Audrey Stevens


Nathaniel R. Rios

Accepted for the Council:


Vice Chancellor
Graduate Studies and Research

THE FIDELITY OF ALBUMIN BIOSYNTHESIS IN AGED C57B1 MICE

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

James R. Burke

December 1981

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DEDICATION

This dissertation is dedicated to my wife, Diana, without her love and support it would not have been possible.

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ABSTRACT

The fidelity of albumin biosynthesis in 3- and 30-month-old C57B1 male mice was assessed. Albumin was isolated from serum and liver by immunoaffinity chromatography and monitored by double immunodiffusion throughout isolation. Two-dimensional gel electrophoresis was used to detect possible alterations in the isoelectric point and size of albumin from serum and liver as a function of age. No differential heterogeneity in albumin was found, thus no amino acid substitutions involving charge changes or modification of molecular weight occurred with aging of the animals. The hydrophobic properties of fragmented and alkylated albumin were examined by Triton X-100 polyacrylamide gel electrophoresis and found to be unaltered with age, thus neutral amino acid substitutions had not occurred. The molecular conformation and net charge of albumin, assayed by gel sieving electrophoresis at acidic and basic pH, were identical in the 3- and 30-month-old mice. In contrast to the albumin data, a striking quantitative difference between the serum of young and old animals was observed in what was tentatively identified as transferrin.

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CHAPTER I

INTRODUCTION

The evidence that animals age is everywhere around us. The mechanism(s) by which this process occurs, however, remains unelucidated. The goal of this dissertation is to assess the fidelity of macromolecular biosynthesis in aging mice by analyzing the protein product of gene expression. The synthesis of a protein is the result of a large number of cellular processes, any or all of which could play a role in the aging process. Some of the mechanisms and factors proposed to be involved in aging include (a) genetically determined (programmed) aging; (b) somatic cell mutation, possibly even including error-prone repair; (c) decreased fidelity of transcription or translation; (d) stochastic events such as damage caused by free radicals; (e) posttranslational modifications, either enzymatic or nonenzymatic (1).

It is unlikely that any single mechanism is wholly responsible for aging, but it is reasonable to assume that the impaired physiology associated with aging may be caused, or accompanied, by the accumulation of altered cellular products of biosynthesis. Numerous proteins have been found to be altered as a function of age, but the nature and results of the alterations require further evaluation.

Mouse serum albumin has been chosen as the protein to be examined. Mice are useful models of human aging because they are similar to humans in both physiology and biochemistry, but have a much reduced life-span (2). C57Bl mice, the strain used, have a mean life-span of 28 months (3).

Our studies compare albumin from 3- and 30-month-old C57Bl male mice. Any change correlated with age should be readily apparent in a 30-month-old mouse. Three-month-old mice are capable of reproducing and have achieved their adult weight (2).

Our methods of analysis do not favor any one model of aging, but, rather, are designed to detect age-related changes regardless of the cause. Albumin is examined for changes in charge and molecular size by two-dimensional gel electrophoresis. Two-dimensional gels can resolve proteins differing by a single charge (4), so even slight age-related changes in the charge of a protein are detectable. However, only about one-third of all amino acid substitutions will result in a change in charge (5). Most amino acids have neutral side-chains so a large amount of variation could go undetected if one relies only on two-dimensional gels. Triton X-100 polyacrylamide gel electrophoresis separates proteins on the basis of small differences in their hydrophobicity (6). This technique has been used to separate histone variants differing only by neutral amino acid substitutions (7, 8). Both two-dimensional and Triton X-100 gels will separate proteins that differ in posttranslational modifications as well as amino acid substitutions. Changes in conformation may also play a role in the alteration of protein with age (9). Gel sieving electrophoresis is capable of resolving proteins differing only in shape (10), and is used to detect any age-related conformational change in albumin.

CHAPTER II

LITERATURE REVIEW

The subject of this dissertation is an assessment of the fidelity of mouse albumin biosynthesis as a function of age. A review of the literature encompassing age-related errors in translation; the occurrence of altered enzymes and pertinent information on the properties of albumin are presented.

Leo Szilard (11) proposed in 1959 that aging might be due to the accumulation of damage in DNA. L. Orgel (12, 13, 14) suggested that errors in protein synthesis could be responsible for aging. Orgel pointed out that a misincorporation of amino acids into proteins involved in transcription or translation is potentially much more serious than an error in a structural protein or enzyme of intermediary metabolism. An error in a structural protein or enzyme of intermediary metabolism will be diluted out by functional molecules with the worst result being a decrease in total function. Proteins involved in transcription or translation can, however, produce an exponential increase in the frequency of errors because of their role in the synthesis of additional cellular macromolecules (one can extend Orgel's hypothesis by including proteins involved in replication). An error in an RNA polymerase molecule, for example, may result in errors in all of the RNA molecules transcribed by that polymerase. The error-laden messenger RNAs may then be translated producing further malfunctioning proteins. The end result of this compounding of errors, suggests Orgel, is an error catastrophe and death.

No claim has been made that the error catastrophe hypothesis is the single mechanism of aging, nor has it ever been demonstrated to occur. The great utility of the hypothesis is that it proposes a testable prediction. Namely, aging will result in the accumulation of error containing proteins.

Induced Error-Catastrophe

It is reasonable to assume that if there is a natural rate of aging and it is related to the fidelity of cellular biosynthetic processes, it should be possible to accelerate the aging process (i.e., induce an error catastrophe) by increasing the error load. Harrison and Holliday (15) attempted to increase the rate of aging by feeding amino acid analogues and streptomycin to Drosophila melanogaster larvae. The mean life-span of the flies was considerably shortened by the treatment as predicted by the error catastrophe hypothesis. Using a similar protocol, Dingley and Smith (16) found no life-shortening effect. The difference between the two experiments is that Dingley and Smith used adult flies while Harrison and Holliday treated larvae. It was proposed that feeding amino acid analogues to larvae Drosophila either induces the formation of morphological abnormalities or causes errors in proteins synthesized by the larvae which must function throughout adult life. Regardless of the reason, amino acid analogues fed to adult Drosophila do not precipitate an error catastrophe.

Holliday (17) examined the phenomenon of senescence in fungi in light of the error catastrophe hypothesis. In Podospora and Aspergillus,

prolonged vegetative growth results in colonies that stop growing and produce an intense brown pigment. The control of senescence (i.e., cessation of growth) has been shown by heterokaryon analysis to be cytoplasmic. Holliday proposed that an error catastrophe would be expected to be cytoplasmic because that is where the translation machinery resides. Muggleton and Danielli (18) had previously shown that the type B "life-spanning" phenotype in Amoeba proteus is also a cytoplasmic phenomenon. With this information available, Holliday attempted to induce an error catastrophe by growing fungi on medium containing amino acid analogues or high concentrations of magnesium. The colonies did senesce at earlier times, as measured by smaller colonies formed, as a result of the treatment.

Lewis and Holliday (19) studied a mutant of Neurospora crassa, Leu 5, that undergoes senescence. Leu 5 is a temperature sensitive mutant which grows normally at 20-25°C but ceases growth, within three to four days, when grown at 37°C. The Leu 5 mutation is in a leucyl-tRNA synthetase which misaminoacylates leucine tRNA at 37°C but not at the permissive temperature. A defective tRNA synthetase is the classic example proposed by Orgel (14) as the inducer of an error catastrophe. They examined glutamic dehydrogenase, as a representative protein, and found that more antiserum was necessary to precipitate a given number of units of enzyme activity for the Leu 5 mutant grown at 37°C than when Leu 5 is grown at 20-25°C. The presence of enzymatically inactive, but immunologically cross reacting glutamic dehydrogenase is consistent with, but not proof for, the error catastrophe hypothesis. No

demonstration was provided that the results with glutamic dehydrogenase are representative of other cellular proteins or that senescence in Neurospora is the result of the accumulation of inactive proteins.

Branscomb and Galas (20) attempted to induce an error catastrophe in Escherichia coli by increasing the basal error rate with streptomycin. Streptomycin increases the occurrence of ribosome-mediated misincorporation of amino acids into proteins. The appearance and subsequent increase in thermolabile β -galactosidase was used as a measure of the change in the error rate. The thermolability of enzymes is reported to increase with random amino acid substitutions (19). Branscomb and Galas observed that E. coli grown in medium containing low levels of streptomycin had a high percentage of thermolabile β -galactosidase in addition to abnormally shaped cells and a slowed growth rate. Prolonged growth in low levels of streptomycin, however, never resulted in the death of the colony. The cultures regained their original growth rate and the amount of thermolabile β -galactosidase decreased. The mechanism by which these E. coli adapted to streptomycin was not determined.

Edelman and Gallant (21) and Gallant and Palmer (22) have questioned whether an error catastrophe is inevitable when the natural error rate is significantly increased. They suggest, as have many others (13, 23, 24), that a stable, albeit higher, error frequency may be possible. Flagellin in E. coli normally contains no cysteine so incorporation of (^{35}S) sulfate into flagellin may be used as an index of the error rate [(^{35}S) sulfate incorporation into methionine was blocked]. Bacteria were grown in streptomycin and followed for eight generations. Initially the increase

in error frequency approximated Orgel's exponential prediction, but in the fourth generation a constant value was reached. The cells were 100% viable at this level of infidelity in flagellin (53 times the basal error rate) and the growth rate was slowed only 20%. Of greater significance was the demonstration that following streptomycin removal the error frequency returned to its original low level. No error catastrophe occurred. R. F. Rosenberger et al. (25) have questioned the conclusion of Gallant and his associates (21, 22) on the grounds that they underestimated the basal error rate and chose an extracellular protein as an index of intracellular conditions. Supporting this contention are the findings by Goldberg (26) that abnormal proteins are preferentially degraded in E. coli. It is not known whether abnormal extracellular proteins are similarly degraded. It is possible that the stress on the E. coli, caused by intracellular errors, was significantly less in Gallant's experiments than was indicated by his measure of error, the extracellular protein flagellin.

Except for Drosophila melanogaster, all of the organisms used to study aging mentioned thus far must be manipulated experimentally before they display signs of aging. Ultimately, results on induced errors in procaryotes, or simple eucaryotes, must be questioned as models for aging in higher eucaryotes simply because these organisms do not normally exhibit senescence.

In Vivo and In Vitro Amino Acid Misincorporation

The fidelity of protein synthesis is determined by the correct placement of amino acids into a polypeptide chain. The components

involved in translation are many and varied but an overall view of the accuracy of the process can be obtained by examining a well-characterized polypeptide or protein. Kurtz (27) using a cell-free protein synthesizing system from 2- and 31-month old mice found no significant difference in the leucine:phenylalanine incorporation ratio with poly(U) as a template. Poly U codes for polyphenylalanine so any incorporation of leucine is due to misincorporation. Mariotti and Ruscitto (28) using a similar system prepared from rat liver have shown an increasing misincorporation ratio with age (3-month versus 12-month-old animals). These contrasting results emphasize the difficulties encountered when comparing systems far removed from natural conditions (poly U-directed phenylalanine incorporation), different animals (mice or rats), and in one case ages nonrepresentative of old animals (twelve-month-old rats).

The results of a more relevant series of experiments that have cast doubt on Orgel's error catastrophe hypothesis were conducted by Hirsch, Popp and associates (29). Human hemoglobin contains no isoleucine so the authors proposed that its appearance as a function of age would serve as an index of amino acid misincorporation. Hemoglobin was isolated from blood samples of people living in the Marshall Islands. The age of the blood donors ranged from 20 to 60 years old and no increase in the amino acid misincorporation frequency with age was noted. Marshall Islanders exposed to radioactive fallout served as positive controls and demonstrated that the technique was sensitive to small increases in the isoleucine substitution frequency. A possible reservation about the study is the age of the participants. Only one

person was as old as 60 years; perhaps the only one old enough to be at the exponential portion of the error curve predicted by Orgel (13, 14). Interestingly, this 60-year-old had a significantly higher substitution frequency, 7.15×10^{-5} , compared to a mean for the other persons of 3.2×10^{-5} . Another reservation about the study is the use of hemoglobin as an index for the substitution frequency of amino acids into other proteins. Goldberg and St. John (114), summarizing the data from a number of sources, have shown that many hemoglobin variants are rapidly degraded in vivo. A number of these mutants differ from native hemoglobin in only a single amino acid residue. Preferential degradation of error containing hemoglobin could have caused Popp et al. (29) to underestimate the misincorporation frequency.

Hirsch, Grunder and Popp (30, 31) have also studied amino acid analogue incorporation into protein as a function of age. The frequency of (^3H)- α -aminoisobutyric acid (AIBA) incorporation to [^{14}C]-leucine incorporation showed no statistically significant differences in organs from adult (10 month) or aged (25 month) mice. Dimethyl sulfoxide, used as a positive control to demonstrate the validity of the technique, increased the AIBA/leucine incorporation ratio. Ogrodnik et al. (32), measuring methionine and ethionine incorporation into mouse liver ribosomal proteins, found a decrease in discrimination as a function of age. Results of amino acid analogue incorporation experiments reflect only errors in a single tRNA synthetase, thus these data must be interpreted with this limitation in mind. Foote and Stulberg (33), in a more ambitious experiment, have demonstrated that tRNA isolated from

various organs of mature (10-12-month) and aged (29-month) mice functioned identically, as measured by rate, extent and fidelity, in an in vitro protein synthesizing system.

Another approach that has provided results which are difficult to reconcile with a simple error catastrophe theory is the examination of cellular proteins by two-dimensional gel electrophoresis. Two-dimensional gels separate on the basis of a protein's isoelectric point as well as its molecular weight. Wilson et al. (34) analyzed rat cervical sympathetic ganglia proteins in 2-12-month and 21-24-month-old animals. The ganglia were excised and incubated for one hour with (^{14}C) leucine, homogenized, and the high speed supernate run on two-dimensional gels. Of the approximately 500 protein spots on the gel, none was found to be different between the young and old rats. A problem with this work was the use of 24-month-old rats as being representative of old animals. Englehardt et al. (35) examined the two-dimensional gel pattern of proteins synthesized by presenescent and senescent human foreskin fibroblast cultures. They report that only two proteins differed significantly from over 500 proteins examined. No protein was found to be present on one gel but absent on the other. A possible reservation about these studies is the difficulty of comparing 500 spots on different gels. If only a small fraction of the proteins are altered in charge and the difference is slight (e.g., one charge unit) it would be easily possible to overlook the change.

The most convincing studies that cast doubt on the aging catastrophe hypothesis are the results of Holland et al. (36) and

Tomkins et al. (37). In both studies the investigators compared viral infection in middle (phase II) and late (phase III) passage WI 38 fibroblast cultures. Human fibroblast cultures exhibit a limited capacity to replicate in vitro and the number of divisions a culture is capable of making is inversely proportional to the age of the donor of the fibroblasts (38). Phase II cells are still capable of dividing. Phase III cells are delayed in reaching confluency compared to Phase II cells. Human fibroblast cultures are considered by many to be a model of cellular aging. If a cell's translation apparatus is impaired by the aging process a comparison of the products of viral infection in phase II and phase III should highlight these differences. The results, however, showed that virus infected phase II or phase III cells produce equivalent numbers of viral progeny, identical kinetics of virus production, specific infectivity (plaque forming units/mg virus protein), kinetics of thermal inactivation, mutation rate, plaque size and viral protein products as analyzed by SDS-polyacrylamide gel electrophoresis (36, 37). It is difficult to imagine, if an error accumulation process occurs with age, how the properties of virus synthesized by middle or late passage cells can be identical.

Altered Enzymes

The activity of many enzymes has been examined as a function of age. Some enzymes exhibit a decreased specific activity when measured in cellular or tissue homogenates. Gershon and Gershon (39) found that the specific activity of isocitrate lyase declined steadily throughout

the life span of Turbatrix aceti. Immune precipitation of an equal number of units of isocitrate lyase activity from the young and old animals revealed that more antiserum was required to precipitate the "old" enzyme than the "young." Heat lability studies of the enzyme showed no difference as a function of age. The Gershons concluded that isocitrate lyase in aged T. aceti is made up of two populations of molecules: one population is active and identical with the enzyme from young animals, the other population is catalytically inactive but retains the ability to be precipitated by anti-isocitrate lyase antibody.

Reiss and Rothstein (40) further characterized isocitrate lyase from T. aceti. They found that the enzyme is composed of five isozymes and all show a reduction in specific activity as a function of age. Other parameters such as K_m , behavior toward inhibitors and molecular weight were unchanged. Reiss and Rothstein, in contrast to the Gershons' (39), proposed that the isocitrate lyase from aged nematodes is composed of active and partially active molecules rather than active and inactive ones. They based their opinion on data from an isocitrate lyase affinity column using the competitive inhibitor oxalate as the lyase specific ligand. Enzyme isolated from old T. aceti binds equally as well to the column as does enzyme from young T. aceti. If inactive enzyme were present, they suggest, it would not be expected to bind to oxalate.

A multitude of enzymes have been found to be altered with age in T. aceti (41-47). Triose phosphate isomerase activity has been found to be decreased in homogenates of old T. aceti compared to the activity

found in homogenates from the young (44). Upon purification, however, the specific activities of the young and old enzyme preparations were identical. Immune titration of the "young" and "old" enzyme showed no differences.

Rothstein has suggested a model to explain how some proteins can be altered with age (e.g., isocitrate lyase) and others can have reduced activity but no evidence that the protein is altered (e.g., triose phosphate isomerase). Rothstein has proposed (9, 48, 49) that altered enzymes appear as a function of age because aging slows protein turnover. Proteins are synthesized in catalytically efficient conformations but the decreased turnover rate allows the proteins from the old animals to decay into catalytically less efficient forms. The process is avoided in young animals because the proteins undergo turnover before this can occur. M. B. Rotman (50) has shown using single molecule fluorometric assays of β -galactosidase that enzyme inactivation need not be an all or none process. Stored solutions of enzyme were found to have a much wider, and decreased, range of activity than do freshly prepared preparations. Applying Rothstein's proposal to isocitrate lyase, he would say the lyase is a protein capable of a metastable conformation. The metastable conformation is able to react with antibody and is either partially or totally inactive. Triose phosphate isomerase, on the other hand, is incapable of a metastable conformation so it is present only as an active or denatured molecule. Denatured protein is not able to react with antiserum nor does it behave like the active enzyme during isolation procedures so it does not lower the specific activity of the purified protein.

The Rothstein hypothesis for the formation of altered enzymes is flexible; it can explain either decreased or unchanged enzyme activity, but it also offers a testable prediction. Protein turnover must be slowed with age. Prasanna and Lane (51) and Reznick and Gershon (52) have examined the rate of protein turnover in the soluble proteins of I. aceti and found that turnover is slowed. Sharma et al. (53) found that the half-life of enolase, an altered enzyme, increased from 58 hours in 5-day-old I. aceti to 161 hours in 22- to 30-day-old animals. So, at least in I. aceti, that condition for Rothstein's proposal is met.

Another testable condition of the altered enzyme hypothesis is the appearance of a conformationally altered species for an enzyme. Sharma and Rothstein (46) examined enolase for changes in conformation and found that the enzyme from the aged nematodes has an extra titratable tryptophan and two titratable tyrosines not found in enzyme from the young animals. When the titratable groups of the enzyme were analyzed in the presence of denaturants, no difference between the young and old animals was found. Circular dichroism revealed slight differences in the secondary structure of enolase which disappeared upon incubation of the protein in 6M guanidine hydrochloride. "Old" enolase was also found to be more thermolabile than "young." All of these results are consistent with the proposal that enolase isolated from aged I. aceti is conformationally different from the same enzyme isolated from young I. aceti. In a subsequent paper (47), Sharma and Rothstein demonstrated that the difference in enolase is probably not due to covalent change. After unfolding the protein in 1.25 M guanidine hydrochloride and then

refolding, the recovery of activity was essentially quantitative and both the young and old form of enolase had been converted to an identical third form (as assayed by circular dichroism, thermal inactivation, immunotitration, and the kinetics of protease induced deactivation).

Further support for the altered enzyme hypothesis is found by examining the enzymes in the lens of the eye. If decreased turnover plays a role in the appearance of altered enzymes, then the lens should be an ideal site to search for proteins with altered properties because the lens has a very slow rate of protein turnover (54). The lens contains a complete glycolytic pathway and Krebs cycle and the activity of most of these proteins declines with age. Ohrloff et al. (55) have shown that rat lens aldolase and rabbit lens phosphofructokinase decrease in specific activity and increase in immune cross-reacting material as a function of age. Many other proteins of the lens are found to be modified with age but often the change is due to posttranslational modifications (54). The age-altered proteins we have discussed have not been changed in charge or molecular weight so it seems that most of the changes in the lens are of a different type.

Except for the specialized case of the lens, we have not referred to any literature on altered enzymes in mammals. The evidence for age-altered proteins is more equivocal than in Turbatrix aceti. Holliday and Tarrant (56) examined glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the fetal lung fibroblast line MRC-5. A heat labile component was found for both enzymes in late passage cells that is absent at earlier passages. Use of 5-fluorouracil, a base

analogue, hastens the appearance of the heat labile component and also causes premature senescence of the cell line. Holliday and Tarrant interpret the data as being supportive of the error catastrophe hypothesis, but it also agrees with Rothstein's concept of altered enzymes. A possible problem with these results is that they were obtained using cell homogenates rather than purified enzymes. Pendergrass et al. (57) have examined glucose-6-phosphate dehydrogenase, in a different strain of human diploid fibroblasts than that used by Holliday and Tarrant, and found neither an increase in thermolability nor an increase in cross-reacting material. Pendergrass' results were also obtained with a homogenate so they too are suspect. Racz et al. (58) has shown that the concentration of endogenous NADP can alter the heat lability of glucose-6-phosphate dehydrogenase from erythrocytes, emphasizing the problems associated with performing heat lability studies on homogenates. Kahn et al. (59) tested glucose-6-phosphate dehydrogenase in cells from adult human liver and found no difference in either immune titration or thermolability using partially purified enzyme. Prior to purification Kahn found that the enzyme from the aged liver was more thermolabile than the young. Schofield and Hadfield (60) found a small percentage (9 to 16%) of glucose-6-phosphate dehydrogenase activity to be thermolabile in mouse liver but this percentage was constant from 12 to 32 months so its role in aging is questionable. In summary, glucose-6-phosphate dehydrogenase is not altered with age.

Gershon and Gershon (61) performed immunotitration studies on fructose-1,6-diphosphate aldolase from 3- and 31-month-old mice. They

found aldolase cross-reacting material in the aged liver but not the young. Following purification, the specific activity of aldolase from aged mice is half that of the aldolase from the young mice. A. Weber et al. (62) examined aldolase from rat liver and found no evidence for any age-related alteration. Petell and Lebherz (63) have solved this discrepancy by demonstrating that mouse liver aldolase is altered during isolation or storage rather than in vivo. The alteration, cleavage of tyrosine from the carboxyl terminus, causes a decrease in enzyme specific activity and is completely preventable by isolating aldolase in the presence of protease inhibitors.

Rothstein, Sharma and associates (64, 65) examined rat muscle phosphoglycerate kinase and enolase as a function of age. They found that phosphoglycerate kinase is altered with age but muscle enolase is not. Purified muscle phosphoglycerate kinase from aged rats is less sensitive to heat than young, has a slower reacting sulfhydryl, and requires more antiserum for 50% inactivation. An interesting, but unexplained finding, is that purified phosphoglycerate kinase from young and old rats has the same specific activity despite the change in immunotitration. Isoelectric focusing revealed no difference in charge and amino and carboxyl terminus amino acid analysis were identical for the "young" and "old" enzyme. Phosphoglycerate kinase is a mammalian enzyme that is altered with age in a way that closely resembles the pattern of age-altered enzymes found in Turbatrix aceti.

Many enzymes are not identical when isolated from different tissues but rather are present as isozymes. Superoxide dismutase (SOD)

isolated from cytoplasm does not have different isozymic forms (66). Examination of SOD in different tissues should, therefore, provide information on whether an enzyme can be altered in some tissues and not others. SOD is also interesting because it is the primary defense against free radical damage caused by superoxide (67). Free radical damage has been proposed as one of the causative factors of aging (1). Reiss, Gershon and others (67, 68, 69) have examined SOD in various tissues of rats and mice to see if it is an age-altered enzyme. Rat and mouse heart, liver and brain cytoplasmic SOD are altered with age; they show a decrease in catalytic activity per antigenic unit with age. The isoelectric focusing pattern and antigenic identity, assayed by double immunodiffusion, are unchanged. It remains to be seen if the alteration of an enzyme in a single tissue means that it will be altered in all tissues, as seems to be the case with SOD. An examination of human erythrocyte SOD indicates no change with age (70). The effect of the change in SOD activity on the aging process is unknown.

Some proteins in mammals are altered with age. The cause, nature and role that the alteration plays in the aging process has not been ascertained. Rothstein's proposal that altered enzymes occur because the protein turnover rate is slowed is consistent with the information available from I. aceti, but insufficient information is available on protein turnover in mammalian systems.

Albumin

Albumin is the most abundant protein in serum. It has a molecular weight of approximately 66,000 daltons, an acidic isoelectric point,

and the ability to bind a wide range of compounds including fatty acids, drugs, steroids and bilirubin. Unlike most other serum proteins, albumin does not perform a single function but serves many roles: osmotic pressure regulation; transport of fatty acids and bilirubin binding (71). No function relies totally on albumin because fourteen cases of analbuminemia in humans have been reported with no apparent clinical disorders associated (72). Nagase and his associates have even established a strain of rats that has no albumin (72). Albumin is not usually thought of as a glycoprotein but C. E. Guthrow et al. (73) have shown that approximately 8% of human serum albumin is nonenzymatically glucosylated in vivo.

The amino acid sequence of bovine and human serum albumin has been known since 1975 (71). Using cloned messenger RNA, Sargent et al. (74), recently reported the amino acid sequence of rat serum albumin. The amino acid sequence of mouse serum albumin, unfortunately, is unknown, but the amino acid composition has been determined (75). A great deal of homology exists among albumins from closely related mammals including antigenic cross-reactivity (71, 76) so generalizations may often be made.

Brown proposed a structure for bovine serum albumin which divided the molecule into nine loops (71). The loops are held together by disulfide bonds; albumin has 17 cystines. Proteolytic and chemical cleavage of albumin has confirmed the assignment for the disulfides proposed by Brown. The stability of the individual regions of the albumin molecule are such that even following cleavage, the fragments retain their ability to react with antialbumin antibody (76, 77).

In addition to its 17 disulfide bridges from cystine, albumin has a single free sulfhydryl (71). Artifactual dimerization of albumin through the free cysteine sulfhydryl has been reported, but dimer formation is minimized by storing albumin in the presence of reducing agent (78). The cysteine sulfhydryl, even in vivo, is not usually free but rather is involved in a disulfide bond with plasma cysteine or glutathione (79).

The structure of albumin, despite the constraints imposed by the disulfide bridges, is capable of undergoing reversible pH-induced conformational changes (71, 76). An acid expansion of the molecule occurs between pH 2 and 4, and a change in electrophoretic mobility between pH 4 and 6. The change from the normal form (N) to a faster migrating form (F) has been explained as an unmasking of previously unexposed charged groups (71). Salaman and Williamson (80) have examined the isoelectric point of albumin and several other proteins by isoelectric focusing in the presence and absence of 8M urea. They found that the isoelectric point of bovine serum albumin is approximately one pH unit higher in the presence of urea than in its absence. This difference has been shown to be the result of bound fatty acids (81), and urea containing isoelectric focusing gels remove bound fatty acids from albumin (82). The isoelectric point of none of the other nine proteins examined was affected by the presence of urea.

Albumin is synthesized in the liver by hepatocytes with a hexapptide extension on its amino terminus (83). This signal sequence in rat proalbumin is highly basic, three of its six amino acids are

arginine, and the sequence is cleaved prior to the secretion of albumin from the cell (84).

Following secretion the half-life of albumin in the mouse is 2.5 days (85). In this study of albumin turnover with age, Quinn et al. found that the half-life of albumin remained constant from the age of 3 to 28 months. At 30 months, however, the half-life in male and female mice was found to be doubled. The finding that the rate of albumin turnover is decreased by half only in the oldest animals studied is intriguing, but must be viewed with caution. The experiments were performed by injecting mice intravenously with radioiodinated albumin and the loss of label followed by whole body counting. Whole body counting only allows one to follow the label, ^{131}I ; the actual fate of the protein is unknown. Many physiological changes could affect the rate of loss of labeled albumin without being a true change in protein turnover (e.g., a change in kidney permeability to protein).

Overview

The literature summarized demonstrates that an increasing number of altered proteins, correlated with aging, are being discovered in eucaryotes. Though seemingly not ubiquitous, this phenomenon deserves further study. Thus far, analyses have included heat lability, specific activity, antigenicity, kinetics, substrate specificity and electrophoretic mobility of crude and a few purified enzymes. Very few experiments have included all, or even a significant number of these tests. It is quite possible, therefore, that many more altered enzymes may exist than

have been detected. The nature of the modification leading to proteins with altered activity or altered physical-chemical properties also requires additional examination, as does the role of altered protein in the aging process.

C57Bl mouse albumin has been chosen for this study of age-related changes in amino acid content, posttranslational modification or variability in molecular shape. Our choice of albumin was based on a number of factors. Albumin is easily purified from serum and liver. Its antigenic structure has been well-characterized and provides reasonable hope that altered molecules will remain isolatable by immune techniques. A comparison of albumin from serum and liver allows a determination to be made if alterations are present at the time a protein is synthesized or appear following secretion. The choice of albumin also makes it feasible to examine if error containing protein is synthesized whether it is eliminated prior to secretion. If an altered albumin had been found, the integrity of albumin messenger RNA could be examined by cell-free translation; procedures for the isolation of albumin messenger RNA are well-documented (86, 87).

CHAPTER III

MATERIAL AND METHODS

Tissue Fractionation Procedures

Three- and 30-month-old C57Bl mice were obtained from the Charles River Breeding Laboratories. The mice are anesthetized with ether and bled by cardiac puncture with a 1 cc syringe. The blood is pooled and allowed to clot on ice for 4 hours. Cells are pelleted by centrifugation at $750 \times g$ for 30 minutes at 3°C . Serum is recovered and 2-mercapto-ethanol, phenylmethylsulfonylfluoride (PMSF) (Pierce Chem. Comp., Rockford, Il.), and leupeptin (Sigma Chem. Comp., St. Louis, Mo.) added to respective final concentrations of 0.01 M, 0.02 M and $15 \mu\text{g/ml}$. The serum is stored in liquid nitrogen.

Livers are removed from the mice, with care not to rupture the gall bladder, and are rinsed with phosphate buffered saline (PBS) [8 g of NaCl; 0.2 g of KCl; 1.15 g of Na_2HPO_4 ; 0.2 g of KH_2PO_4 and 800 ml of water (pH 7.2)]. The livers are blotted dry and stored in liquid nitrogen until needed.

Livers are fractionated by a modification of the procedure of Peters (88). All operations of the following procedures are at 4°C . The mouse liver is removed from liquid nitrogen, weighed, macerated with a razor blade, and washed extensively with PBS containing 0.001 M ethylenediaminetetraacetic acid (EDTA) and 0.002 M PMSF. The washed liver is then suspended in 2.5 volumes of 0.25 M sucrose; 0.001 M EDTA;

0.002 M PMSF; 15 $\mu\text{g/ml}$ leupeptin and homogenized using a motor driven homogenizer with a Teflon pestle (5 strokes). Following homogenization the sample is centrifuged at $5,000 \times g_{av}$ for 10 minutes and the supernate recovered. The supernate is dialyzed against 0.2 M Tris-HCl pH 7.5; 0.001 M EDTA; 0.01 M 2-mercaptoethanol. The dialysed supernate is treated with 0.5% w/v Triton X-100 (Sigma Chem. Comp., St. Louis, Mo.) for 30 minutes to disrupt albumin-containing vesicles and centrifuged at $150,000 \times g_{av}$ for 30 minutes. The supernate is frozen at -80°C and the pellet is discarded.

DEAE Sephadex A-50 Column Chromatography

Albumin is the most acidic protein present in serum in appreciable quantities, thus its isolation can be readily accomplished using anion exchange chromatography (71).

Serum albumin is isolated by a modification of the procedure of Winter et al. (89). Serum samples are chromatographed on a 0.9×40 cm column of DEAE Sephadex A-50 (Pharmacia Fine Chem., Piscataway, N.J.) which was previously equilibrated with 0.2 M sodium phosphate pH 5.75 (starting buffer). The buffer is prepared as a five-fold concentrate by mixing 0.9 M NaH_2PO_4 with 0.09 M Na_2HPO_4 . When diluted five-fold the pH of this buffer is 5.75. The column is poured and run at room temperature. Serum samples are prepared by diluting 200 μl of serum with 50 μl of phosphate buffer concentrate. The sample is applied by gravity flow. Flow is regulated to $10 \text{ ml/cm}^2/\text{hr}$ by adjusting the height of the buffer reservoir and the elution is carried out with

starting buffer. The column effluent is passed through a Beckman BD spectrophotometer and the absorbance at 280 nm is recorded. Two ml fractions are collected in a fraction collector at 3°C. The presence of albumin in a fraction is detected by placing aliquots of each fraction in a double immunodiffusion plate and reacting with rabbit antimouse albumin (Miles Lab., Elkhart, In.). The albumin containing fractions are pooled and frozen at -80°C.

Immunoaffinity Column Preparation

Epichlorohydrin-Activated Sepharose 4B

Sepharose 4B (Pharmacia Fine Chem., Piscataway, N.J.) is treated with epichlorohydrin (ECD) (Eastman Organic Chem., Rochester, N.Y.) according to the procedure of Porath et al. (90) with slight modification. One liter of Sepharose 4B beads is washed with water on a Buchner funnel. The beads are then mixed with 1 liter of 1 M NaOH containing 5 g of sodium borohydride and 20 ml ECD and heated for 1 hour at 60°C in a hood. The beads are washed with hot distilled water until the pH reaches 7.0 and then treated with 1 liter of 2 M NaOH containing 5 g of sodium borohydride and autoclaved for 1 hour at 120°C. If prior treatment has been correct, the beads will not caramelize upon autoclaving. The autoclaved beads are washed with 2 liters hot 1 M NaOH containing 0.5% w/v sodium borohydride and then with 2 liters of ice-cold 2 M NaOH containing 0.5% w/v sodium borohydride. The Sepharose is transferred to a beaker containing crushed ice and glacial acetic acid is added until the pH reaches 4.0. The beads are then washed with 2 liters of

hot water followed by 2 liters of ice-cold water. The ECD-Sepharose 4B is stored at 4°C in 0.2 M potassium phosphate pH 6.8 containing 0.01 M sodium azide.

Immunoabsorbent Preparation

Antibody coupled to ECD-Sepharose 4B is prepared according to a modification of the procedure of Cuatrecasas (91). Serum is made 50% saturated in ammonium sulfate [31.3 g/100 ml] to precipitate the immunoglobulin G (Ig G). The precipitate is collected by centrifugation at $15,000 \times g$ for 30 minutes at 3°C and the pellet resuspended in 0.1 M potassium phosphate pH 6.8 in a volume equal to that of the original serum sample. Ig G solutions are stable for extended periods of time when stored at -20°C.

The ECD-Sepharose 4B is washed on a sintered glass filter with water to remove the azide. The beads are removed from the filter and suspended 1:1 in water. In a hood, cyanogen bromide (CNBr) (Pierce Chem. Comp., Rockford, Il.) is finely divided and added to the beads at a concentration of 4 g CNBr/30 ml of bead suspension. The reaction is stirred gently and the progress of the reaction followed with a pH meter. The pH of the reaction is kept between 10 and 11 by the dropwise addition of 4 M NaOH. When the pH is stable the reaction is complete (6 to 8 minutes). The beads are washed with water and Ig G added to the activated beads to a final concentration of Ig G of 10 to 20 mg/ml. The antibody is coupled overnight at 4°C; the tube containing the coupling reaction is tumbled continuously to assure maximum interaction between the beads and the Ig G. At the completion of the coupling the

unreacted CNBr-activated sites are filled by the addition of 1/5 volume of 1 M glycine (Pierce Chem. Comp., Rockford, Il.). The glycine is allowed to react for 3 hours at 3°C with tumbling. Noncovalently bound material is removed by washing the Ig G-ECD-Sepharose 4B with 3 M potassium thiocyanate (Aldrich Chem. Comp., Metuchen, M.J.) followed by washing with copious amounts of water. The beads are stored at 4°C in 0.1 M potassium phosphate pH 6.8 containing 0.02% w/v sodium azide.

Mouse Albumin Isolation by Immunoaffinity Chromatography

Nonspecific Adsorption

A nonspecific (i.e., not antialbumin) immunoaffinity matrix is prepared by coupling mouse Ig G to ECD-Sepharose 4B. This material is used as a precolumn to remove any proteins present in the liver or serum samples which bind nonspecifically to Ig G-ECD-Sepharose 4B (92).

Liver or serum samples in 0.1 M potassium phosphate pH 6.8 (10-50 mg of protein, as estimated from the absorbance of the sample at 280 nm assuming a molar absorption coefficient of 1) are incubated with 1 ml of packed beads of nonspecific Ig G-ECD-Sepharose 4B. The sample is incubated at 4°C for 4 hours with tumbling. At the completion of the incubation period the supernate is removed and used for the subsequent isolation of albumin.

Albumin Isolation

The supernate of the precolumn is incubated with the anti-mouse albumin-ECD-Sepharose 4B beads at 4°C with continual tumbling. After a 4 hour incubation period the sample is removed from the cold and

transferred to a 1 cc syringe whose outlet has been plugged with glass wool. Polyethylene tubing is attached to the syringe needle to control the flow rate. After packing the column, the nonbound proteins are eluted by extensive washing (25 to 50 bed volumes) with 0.1 M potassium phosphate pH 6.8. The albumin is eluted with 5 to 10 bed volumes of 8 M urea; 0.2 M potassium acetate pH 4.0 and collected in a plastic tube [to minimize protein loss through adsorption (92)]. The albumin is dialysed against two 2 liter changes of 0.02 M potassium phosphate pH 6.8 at 4°C over a 24 hour period. If necessary, samples are concentrated to 1 to 2 mg/ml by a Millipore ultrafiltration unit (Millipore Corp., Bedford, Ma.). Concentrated samples are stored at -80°C.

Assay for Optimal Elution Conditions

Many different elution conditions can be used to elute proteins from immunoaffinity columns. The use of a radioactive tracer simplifies the comparison of various elution conditions (93). Mouse serum albumin, purified by DEAE Sephadex A-50, is labeled by reductive methylation using tritiated sodium borohydride (94) and mixed with unlabeled serum to determine the effectiveness of different elution procedures.

Double Immunodiffusion Assay and Stain

Double immunodiffusion (Ouchterlony) plates (Miles Lab., Elkhart, In.) are used to determine the presence of antigen in fractions collected from column chromatography. They are also used for the detection of more than one antibody-antigen system (95). This is especially useful when comparing antigens for immunologic identity.

Twenty μ l of sample is placed in one of the outer wells of an Ouchterlony plate and 25 μ l of rabbit antimouse albumin antiserum (Miles Lab., Elkhart, In.) (diluted 1:4) is placed in the center well. The plate is incubated at room temperature until a precipate line forms (about 24 hours). Antiserum and sample not involved in the precipitation reaction must be rinsed away if subsequent staining is necessary. Rinsing is accomplished by washing the gel with numerous changes of PBS over a period of several hours. Plates are stained with 0.2% w/v Amido black (Eastman Organic Chem., Rochester, N.Y.); 25% isopropanol; 10% glacial acetic acid for a period of 6 hours at room temperature. Destaining is accomplished by several changes of a solution containing 10% glacial acetic acid and 25% methanol.

Electrophoresis

Slab gels were run on a Hoeffer vertical slab gel electrophoresis apparatus Model SE 500 (Hoeffer Sci. Instr., San Francisco, Ca.) and tube gels in a Hoeffer GT tube gel electrophoresis unit. The power source for all electrophoresis experiments was a Buchler 3-1500 constant power supply.

Isoelectric Focusing Gel Electrophoresis

Isoelectric focusing (IEF) gels are polyacrylamide gels that establish a stable pH gradient when subjected to an electric field. The gradient is formed by the presence of a series of amphoteric ions that differ slightly in their isoelectric point. IEF gels are used to

detect changes in the isoelectric point of proteins; they are sensitive enough to detect as little as a single charge change in a protein molecule.

Preparation and running of gels. IEF is performed according to the procedure of O'Farrell (4) with some modification. IEF gels are made in glass tubes (125 mm $\times$ 2.5 mm inner diameter) treated with Column Coat (Miles Lab., Elkhart, In.) and sealed at the bottom with Parafilm. The gel mixture, prepared in a side-arm flask, is composed of 9 M urea; 2% w/v NP-40 (Shell Chem., New York, N.Y.); 2% w/v Servalyte pH 4-6 (Serva Fine Chem., Hicksville, N.Y.); 4% total acrylamide [from a stock containing 28.38% w/v acrylamide (Pierce Chem. Comp., Rockford, Il.) and 1.62% w/v bis-acrylamide (Eastman Org. Chem., Rochester, N.Y.)]. The flask is swirled at room temperature to dissolve the urea and the solution degassed for 3 minutes using lab vacuum. Ammonium persulfate 0.01% w/v and 0.14% v/v TEMED (Eastman Org. Chem., Rochester, N.Y.) are added to initiate polymerization. The ammonium persulfate stock solution is prepared fresh biweekly. Approximately 0.5 ml of gel solution is added to each tube and the gel gently overlaid with 8 M urea. Following polymerization, approximately 1 to 2 hours, a sharp interface is visible between the gel and the urea overlay. The 8 M urea is removed and replaced by 20 μ l of lysis buffer. Lysis buffer is composed of 2% w/v NP-40; 2% w/v Servalyte pH 4-6; 5% v/v 2-mercaptoethanol and 9 M urea. The lysis buffer is overlaid with a small amount of water. After 1 to 2 hours, the lysis buffer, water and Parafilm at the base of the tubes are removed and the tube bottoms covered with dialysis membrane. Special

care must be taken to prevent air from filling the space between the dialysis tubing and the gel. The tubes are placed in the electrophoresis apparatus and 20 μ l of lysis buffer is added to each gel. The tubes and upper electrode reservoir are filled with extensively degassed 0.02 M NaOH. The lower electrode reservoir is filled with 0.01 M phosphoric acid. IEF gels are run at 20°C. Gels are prefocused at 200 V for 15 minutes, 300 V for 30 minutes, and 400 V for 30 minutes. Following prefocusing the upper reservoir is emptied and sample added to the tubes. Samples are prepared in 8 M urea and diluted 1:1 with lysis buffer. The samples are then overlaid with 20 μ l of lysis buffer diluted 1:1 with water. The tubes and upper reservoir are filled with degassed 0.02 M NaOH and the gels focused for 12 hours at 400 V and 1 hour at 800 V (a total of 5600 V-hours). Following electrophoresis the gels are extruded from their tubes and the acidic end of the gel marked with India ink for orientation. IEF gels are either stained or frozen for subsequent use in a two-dimensional gel. Gels are frozen in ethanol-dry ice following 30 minutes equilibration at room temperature in 5 ml of sodium dodecyl sulfate (SDS) (Pierce Chem. Comp., Rockford, Il.) sample buffer. SDS sample buffer contains 0.0625 M Tris-HCL pH 6.8; 2% w/v SDS; 10% w/v glycerol and 5% v/v 2-mercaptoethanol.

pH gradient determinations are performed using a Bio-Rad pro-pHler (Bio-Rad Lab., Richmond, Ca.). Carbamylated protein is used as a standard on IEF gels to make intergel comparison more meaningful. Carbamylation of protein results in the modification of the ϵ amino group of lysine. The resultant carbamino group is uncharged. By

varying the extent of the reaction a series of modified protein species can be produced which differ by a single charge. Electrophoresis of a carbamylated protein preparation on an IEF gel results in a "train" of spots with the isoelectric points differing by a single charge.

Carbamylated proteins are prepared essentially by the same procedure as that used by Anderson and Hickman (96). Creatine phosphokinase (CPK) from rabbit muscle (Sigma Chem. Comp., St. Louis, Mo.) is dissolved in 8 M urea at a concentration of 2 mg/ml and heated at 95°C. Aliquots of the solution are removed after 0, 2, 4, 6, 8, 12, 15, 18, 30, and 60 minutes and pooled after icing. These times have been chosen because they provide an equal mixture of all the carbamylated species. The pooled standards are then aliquoted into smaller tubes and stored at -20°C for future use. Twelve μ l of the pooled carbamylated CPK is added to each IEF gel.

Staining of isoelectric focusing gels. IEF gels are stained by a modification of the procedures of Vesterberg (97). The gels are stained at room temperature with slow shaking for 20 hours. The stain is prepared by adding 75 ml methanol; 186 ml water; 30 g trichloroacetic acid; 9 g sulfosalicylic acid (Pierce Chem. Comp, Rockford, Il.); and Coomassie Brilliant Blue R250 (Pierce Chem. Comp., Rockford, Il.) to a final concentration of 0.1% w/v. Destaining is accomplished by frequent changes of a solution containing 250 ml ethanol; 80 ml glacial acetic acid and 650 ml water.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is used as a second dimension after isoelectric focusing. SDS-PAGE separates on the basis of molecular weight and serves as a complement to separation based on isoelectric point provided by IEF. SDS-PAGE is also used independently to determine molecular weight.

Preparation and running of gels. SDS-PAGE is performed according to a modification of the procedure of Laemmli (98). The resolving gel is prepared by mixing the following components, to the final concentration indicated, in a side-arm flask: 9% total acrylamide [from an acrylamide stock solution containing 30% w/v acrylamide; 0.8% w/v bis-acrylamide]; 0.375 M Tris-HCl pH 8.8; 0.1% w/v SDS. The solution is degassed for 3 minutes using lab vacuum and polymerization initiated by adding 0.067% w/v ammonium persulfate and 0.067% v/v TEMED. Ammonium persulfate stock solutions are prepared fresh biweekly. The resolving gel is overlaid with water (approximately 0.5 ml) and the gel polymerized at room temperature for 1 to 2 hours. Following polymerization the water is removed and the surface of the gel rinsed with fresh water. The stacking gel is poured directly on the air dried resolving gel surface. The stacking gel is prepared by mixing the following components, to the final concentration indicated, in a side-arm flask: 4.5% total acrylamide [from an acrylamide stock solution containing 30% w/v acrylamide; 0.8% w/v bis-acrylamide]; 0.125 M Tris-HCl pH 6.8; 0.1% w/v SDS. House vacuum is used to degas the solution for 3 minutes and 0.125% w/v ammonium persulfate and 0.125% v/v TEMED are added to initiate

polymerization. The gel is overlaid with water and polymerized at room temperature for 1 to 2 hours. SDS polyacrylamide gels to be used as a second dimension are filled with stacking gel to the bottom of the bevel of the notched glass plate. Total stacking gel height is 2.5 cm. Following polymerization the water overlay is removed and the surface of the gel rinsed with water. The surface of the gel is allowed to air dry and a thawed IEF gel is placed in the trough formed by the bevel of the notched glass plate. One percent LGT agarose (Miles Lab., Elkhart, In.) in SDS sample buffer is used to cement the IEF gel in place. SDS polyacrylamide gels are run at 50 mA/slab gel. The dimensions of the slab are $140 \times 150 \times 1.5$ mm. The running temperature is 10°C and the electrode buffer is 0.025 M Tris; 0.192 M glycine; 0.1% w/v SDS (pH 8.3). Fifty μl of 0.1% w/v bromphenol blue (Aldrich Chem. Comp., Metuchen, N.J.) is added to the upper buffer reservoir to serve as a tracking dye. After electrophoresis the bromphenol blue front is marked with India ink as is the right hand corner of the gel to serve as an orientation point.

Standards for SDS-PAGE are proteins of known molecular weight that are run in adjacent lanes to the unknown samples. Molecular weight markers used are: bovine serum albumin 66,000 daltons; ovalbumin 45,000 daltons; pepsin 34,700 daltons; β lactoglobulin 18,400 daltons; lysozyme 14,300 daltons (Sigma Chem. Comp., St. Louis, Mo.). Lysozyme migrates with the front in a 9% SDS polyacrylamide gel. The molecular weight standards are dissolved in SDS sample buffer at a concentration of 0.5 mg/ml for each protein. SDS sample buffer contains 0.0625 M Tris-HCl pH 6.8; 2% w/v SDS; 10% w/v glycerol; 5% v/v 2-mercaptoethanol and

0.02% w/v bromphenol blue. The protein standard mix is heated in boiling water for 3 minutes and cooled in ice before use. Twenty-five μ l of standard is used per lane.

Staining of SDS polyacrylamide gels. SDS polyacrylamide gels are stained by the procedure of Fairbanks et al. (99). Changing of the destaining solution is avoided by absorbing the free stain from the destaining solution with charcoal retained in a nylon mesh bag.

Slab gels are photographed and dried using a gel drying apparatus like that described by Maizel (100). To prevent cracking, the destaining solution is made 1% v/v glycerol for 5 minutes prior to drying the gel.

Triton X-100, Acetic Acid,
Urea Gel Electrophoresis

Triton X-100, acetic acid, urea gel electrophoresis separates proteins based on differences in hydrophobicity (6).

Preparation and running of gels. Gels are prepared according to the procedure of Zweidler (6). Both slab and tube gels can be used. All glassware is rinsed with 0.01 M 2-mercaptoethanol to remove any traces of oxidizing agent left after detergent washing. The gel is prepared in a side-arm flask by mixing the following components to the final concentrations indicated: 12% total acrylamide [prepared from an acrylamide stock solution containing 30% w/v acrylamide and 0.2% w/v bis-acrylamide]. The stock solution is stirred for 30 minutes with 3 g of mixed bed resin [AG 501-X8 (Bio-Rad Lab., Richmond, Ca.)] and 1 g of activated charcoal and filtered through a glass-fiber filter before use;

5 M urea; 0.006 M Triton X-100 [prepared from a 0.3 M stock made by dissolving 18.75 g of Triton X-100 in 83 ml of water] and 5% acetic acid. The mixture is degassed for 3 minutes using lab vacuum and polymerization initiated by adding 0.08% w/v ammonium persulfate and 0.5% TEMED. The gel is overlaid with 5% acetic acid and the gel polymerized for 2 to 4 hours at room temperature. After polymerization the overlay is removed and the gels placed in the electrophoresis chamber. Fifty μ l of 5 M urea; 0.006 M Triton X-100; and 5% acetic acid is added to each tube or well and the electrode reservoirs filled with 5% acetic acid. The gels are then preelectrophoresed to constant wattage. One hundred μ l of 5 M urea; 0.006 M Triton X-100; 5% acetic acid and 0.5 M cystamine hydrochloride (Aldrich Chem. Comp., Metuchen, N.J.) is then added to each tube or well and preelectrophoresis continued for an additional 30 minutes at 4 mA/cm^2 gel surface. The top reservoir is then emptied and refilled with fresh 5% acetic acid. Reduced and alkylated, CNBr-fragmented mouse albumin is then added to the surface of the gel in sample buffer. Sample buffer contains 8 M urea; 5% 2-mercaptoethanol; 0.025 M methylamine (Aldrich Chem. Comp., Metuchen, N.J.); 1% v/v thiodiglycol (Pierce Chem. Comp., Rockford, Il.) and 0.01% pyronin Y (Aldrich Chem. Comp., Metuchen, N.J.). Albumin is cleaved with CNBr, reduced and alkylated by the procedure of Franklin et al. (101). Sample volume is limited to 1 to 5 μ l/mm² well or tube surface. The sample is electrophoresed at 4 mA/cm^2 of gel surface until the pyronin Y front reaches the end of the gel. The front is marked with India ink.

Staining of Triton X-100, acetic acid, urea gels. Triton X-100 gels are stained by the method of Fairbanks et al. (99). Gels are destained by diffusion in 5% acetic acid; 25% methanol and the destaining solution is replaced as necessary. Stained gels are scanned using an Isco 1310 linear transport gel scanner (Isco Sci. Instr., Lincoln, Ne.) with a 0.25 mm slit width and a 545 nm filter. Scanning speed is 30 cm/hr.

Gel Sieving Electrophoresis

Gel sieving electrophoresis provides information concerning the shape as well as the net charge of proteins. The molecular charge is determined in native gels in contrast to IEF where the gels contain 8 M urea and thereby nullify structural effects on net charge.

Preparation and running of gels. Buffers used for both high and low pH discontinuous native gels are prepared as described by Maizel (100). Concentrations of acrylamide and bis-acrylamide used are those described by Johnson (10). Gels are poured in 130 × 5 mm tubes coated with Column coat and sealed at one end with Parafilm. The height of the stacking and resolving gels are 20 and 100 mm respectively. Concentrations of acrylamide range from 5 to 10% and bis-acrylamide is added at a concentration of 0.184 g/100 ml of gel. Three gels are poured for each concentration of acrylamide: one is used for albumin from the 3-month-old mice; one for albumin from the 30-month-old mice and one gel for a sample of mixed young and aged albumin. Samples, made to a 10% w/v sucrose concentration, are underlaid onto the gels. Bromphenol blue is

used as a dye front marker for the high pH gels and methyl green (Eastman Org. Chem., Rochester, N.Y.) for the low pH gels. Gels are run at 2 mA/gel and 10°C until the dye front is close to the end of the gel.

Staining of native polyacrylamide gels. Native gels are stained by the procedure of Fairbanks et al. (99). Gels are destained in 10% acetic acid. Stained gels are scanned using an Isco 1310 linear transport gel scanner with a 0.25 mm slit width and a 545 nm filter. Scanning speed is 30 cm/hr.

CHAPTER IV

RESULTS AND DISCUSSION

The purpose of this dissertation is to search for age-related alterations in albumin from the serum and liver of male C57Bl mice. The experimental results have, therefore, been interpreted mainly by emphasizing comparisons between albumin from the adult and aged mice.

Isolation of Albumin

A primary consideration in the search for age-modified proteins should be the choice of an appropriate isolation procedure. If the age-altered protein does not behave identically with the native molecule, it is likely that the modified protein will be lost during purification. A pertinent example where this principle was overlooked is Schofield's examination of mouse serum albumin as a function of age (102, 103). Schofield employed six consecutive column chromatography steps to isolate serum albumin. Needless to say, he found no difference. If the aim of an experiment is simply to purify a protein it is an acceptable compromise to lose a small percentage of the desired protein in return for a many-fold purification; in the search for altered proteins the fractions discarded may well contain the protein of interest. In addition care must be taken during isolation to prevent the introduction of artifacts.

Petell and Lebherz (63) have convincingly demonstrated that in vitro proteolytic cleavage of the carboxyl terminus during processing

and storage of preparations can mimic age-related alterations in mouse liver aldolase. To avoid artifactual proteolysis, isolation of albumin is performed in the presence of proteinase inhibitors, Phenylmethylsulfonyl Fluoride (PMSF) and leupeptin. Limited proteolysis of the amino terminus is readily detected in mouse albumin because the amino terminal amino acid is glutamic acid (104). Removal of glutamic acid is detectable by IEF because of loss of the ionized carboxyl side-chain and its associated negative charge. The amino acid which occupies the carboxyl terminus of mouse albumin is unknown, however precautions mentioned above eliminate this possible artifact.

Qualitative Identification of Albumin

To determine if albumin is present in a sample the double immunodiffusion assay is used. Rabbit anti-mouse serum albumin was purchased from Miles Laboratories. Double immunodiffusion techniques are used to assess the specificity of an antiserum (95). A single precipitin line is present when mouse serum is incubated against anti-mouse albumin (Figure 1). The presence of a single band indicates that the antiserum is monospecific. Purified serum albumin from 3- and 30-month-old mice also reacts with the antiserum and has a reaction of identity with the serum precipitate line. The antigenic identity of serum albumin from 3- and 30-month-old mice must also be identical because they both show identity with the precipitate line from serum. Double immunodiffusion of a liver supernate also reveals a single precipitin line indicating monospecific antiserum (Figure 2). Albumin from 3- and 30-month-old mouse liver has a reaction of identity with

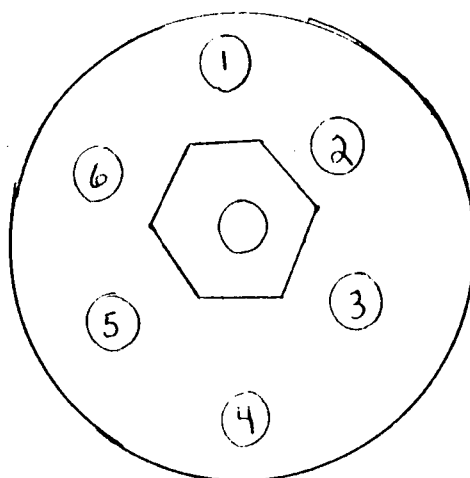


Figure 1. Immunospecificity of rabbit antimouse albumin tested by double immunodiffusion against C57Bl serum. Center well: 25 μ l of antimouse albumin; Wells 1, 3, 5: 3-month C57Bl serum; Well 2: 3-month C57Bl immunopurified serum albumin; Well 4: 30-month C57Bl immunopurified serum albumin; Well 6: 3-month and 30-month immunopurified serum albumin.

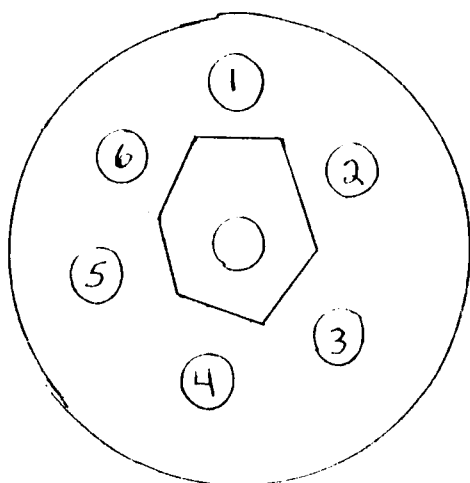


Figure 2. Immunospecificity of rabbit antimouse albumin tested by double immunodiffusion against C57Bl liver homogenate. Center well: 25 μ l of antimouse albumin; Wells 1, 3, 5: 3-month C57Bl liver homogenate; Well 2: 3-month C57Bl immunopurified albumin from liver; Well 4: 30-month C57Bl immunopurified albumin from liver; Well 6: 3-month and 30-month immunopurified albumin from liver.

the liver supernate precipitate line. As with serum albumin, the antigenic identity of liver albumin from 3- and 30-month-old mice must be identical because the 3- and 30-month mouse liver albumin both show identity with liver supernate albumin.

DEAE Sephadex-A50 Isolation of Mouse Serum Albumin

Albumin is the most acidic protein found in serum in appreciable quantities (71). Winter et al. (89) made use of this fact to isolate a mutant form of human serum albumin. Albumin, because of its highly negative charge, will transiently bind to DEAE Sephadex-A50 at acidic pH whereas other serum proteins elute much earlier. A representative elution profile of serum from 3-month-old C57Bl mice, using Winter's procedure (89), is shown in Figure 3. Albumin, peak III, is retained longest on the column, and was identified by double immunodiffusion and the appearance of a 66,000 dalton band on SDS-PAGE (not shown). Peaks I and II were found not to contain albumin when assayed directly prior to concentration. However, analysis of pooled and concentrated peaks I and II showed immunodiffusion identity with serum albumin, and SDS-PAGE revealed a 66,000 dalton band. Changing elution conditions (pH and buffer) did not eliminate albumin in peaks I and II. Thus albumin is present nonspecifically in fractions containing high protein concentrations and protein-protein interaction may be responsible for this phenomenon.

Immunoaffinity Isolation of Albumin

The difficulties encountered in purifying serum albumin by anion exchange chromatography could be compounded when isolating albumin

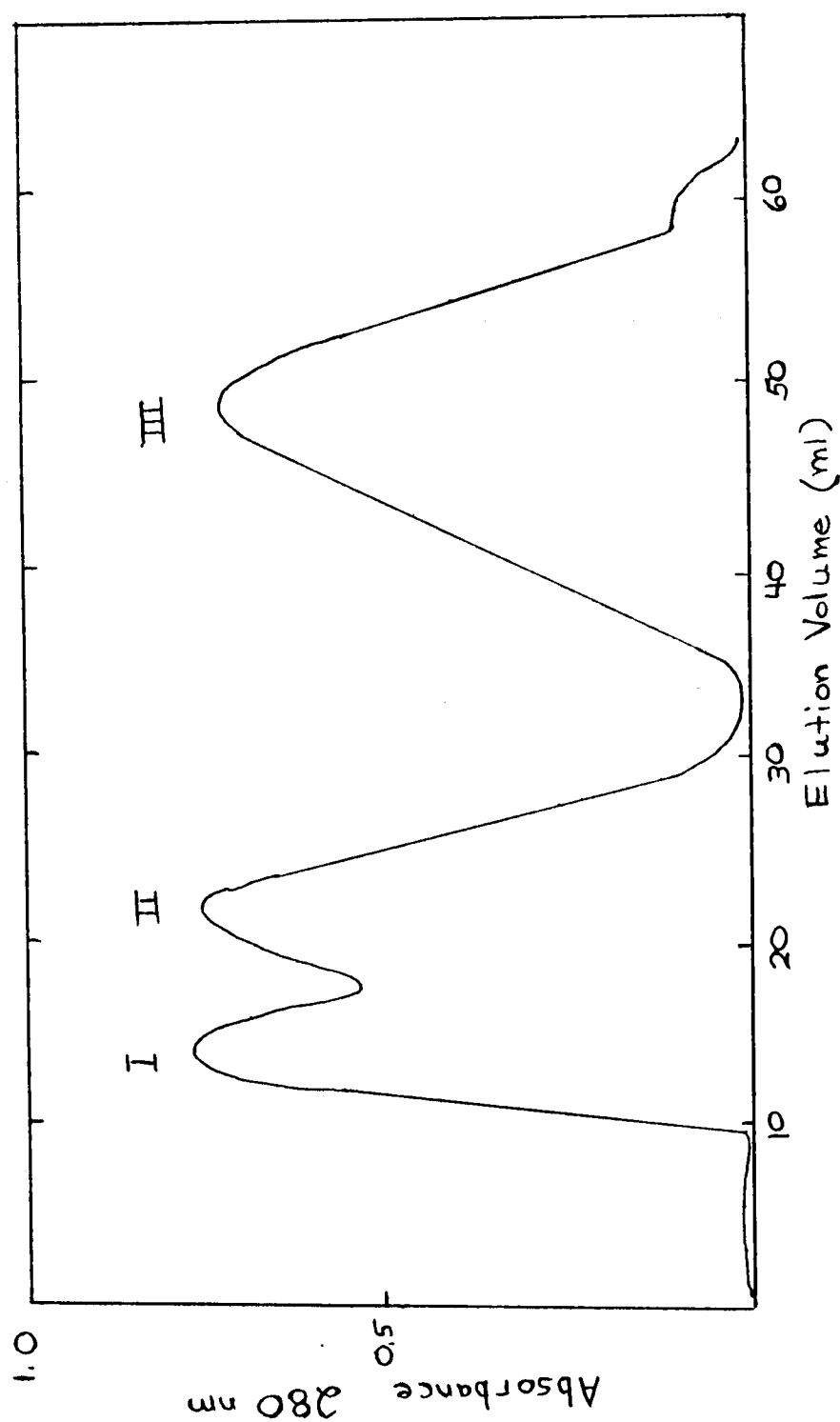


Figure 3. Elution profile of 3-month-old C57B1 mouse serum from DEAE-Sephadex-A50. Elution buffer 0.2 M sodium phosphate pH 5.75. Peak numbers are shown above peak.

from liver due to smaller amounts of albumin and the presence of many more cellular proteins. A solution to this isolation problem is the use of an immunoaffinity column. As discussed in the literature survey, an albumin molecule contains several antigenic determinants and these determinants are retained despite cleavage of the molecule (76, 77). The significance of these findings for the present purpose is that the individual antigenic sites are independent of the structure of the albumin molecule as a whole. If the sites were not independently stable, fragmentation of the molecule would be expected to destroy antigenicity. Johanson (105) has found that even following reduction of the disulfide bonds in a fragment, the conformation upon reoxidation closely resembles native albumin in terms of ligand binding and antigenicity. This suggests that error-containing albumin should still be isolatable by immunological procedures because of the stable antigenic structure of the unmodified regions. Immunoaffinity isolation is also preferable to ion-exchange chromatography because the amount of other proteins in a preparation does not interfere with the isolation of an immunologically-bound protein.

Conditions for elution of albumin from antialbumin-ECD-Sepharose 4B affinity column are shown in Table I. The percentage albumin eluted by a given treatment is determined using (^3H)-labeled albumin as a tracer. The most effective eluant tested was 8 M urea in 0.2 M sodium acetate pH 4.0. This eluant is used for all of the following studies. Peters and Reed (93) report that high pH (e.g., 1 M ammonium hydroxide) converts proalbumin to albumin so conditions involving high pH are

TABLE I
ELUTION OF MOUSE ALBUMIN FROM IMMUNOAFFINITY COLUMN

Eluant	Elution of albumin ^a
5 N acetic acid	59%
1 M ammonium hydroxide	36
8 M urea in 0.2 M potassium phosphate pH 6.8	30
8 M urea in 0.2 M potassium acetate pH 4.0	83

^aAssayed by recovery of ³H-labeled mouse albumin added as a tracer.

avoided. To avoid carbamylation of the albumin by the urea used in the eluant (4, 106), the albumin-containing eluate is dialysed immediately to remove the urea.

Analysis of Isolated Albumin

Two-Dimensional Gel Electrophoresis

The two dimensions of IEF and SDS-PAGE will separate proteins on the basis of isoelectric point and molecular weight respectively (4). Albumin has an acidic isoelectric point, so to obtain maximum resolution, a narrow range acidic ampholyte solution is used (Servalyte pH 4-6). A typical pH gradient profile is shown in Figure 4. It is apparent that the pH gradient generated is neither perfectly linear, nor confined to the pH range of 4 to 6. A sharp rise occurs at the cathodal (basic) end of the gel and this has been attributed to the scarcity of ampholytes capable of buffering in the region of pH 7 (107).

The irregular nature of the pH gradient makes comparison between gels difficult, if not impossible. An internal reference is needed for comparison. Anderson and Hickman (96) have suggested using a protein which has been carbamylated to varying extents as an IEF gel standard. Proteins are easily carbamylated by heating in urea and each carbamino group formed results in the loss of a single positive charge in the protein molecule because the ϵ amino group of lysine and the free amino terminal are modified to the uncharged carbamino group. A carbamylated protein has a more acidic isoelectric point than the unmodified protein. By varying the extent of carbamylation, an IEF standard is prepared where

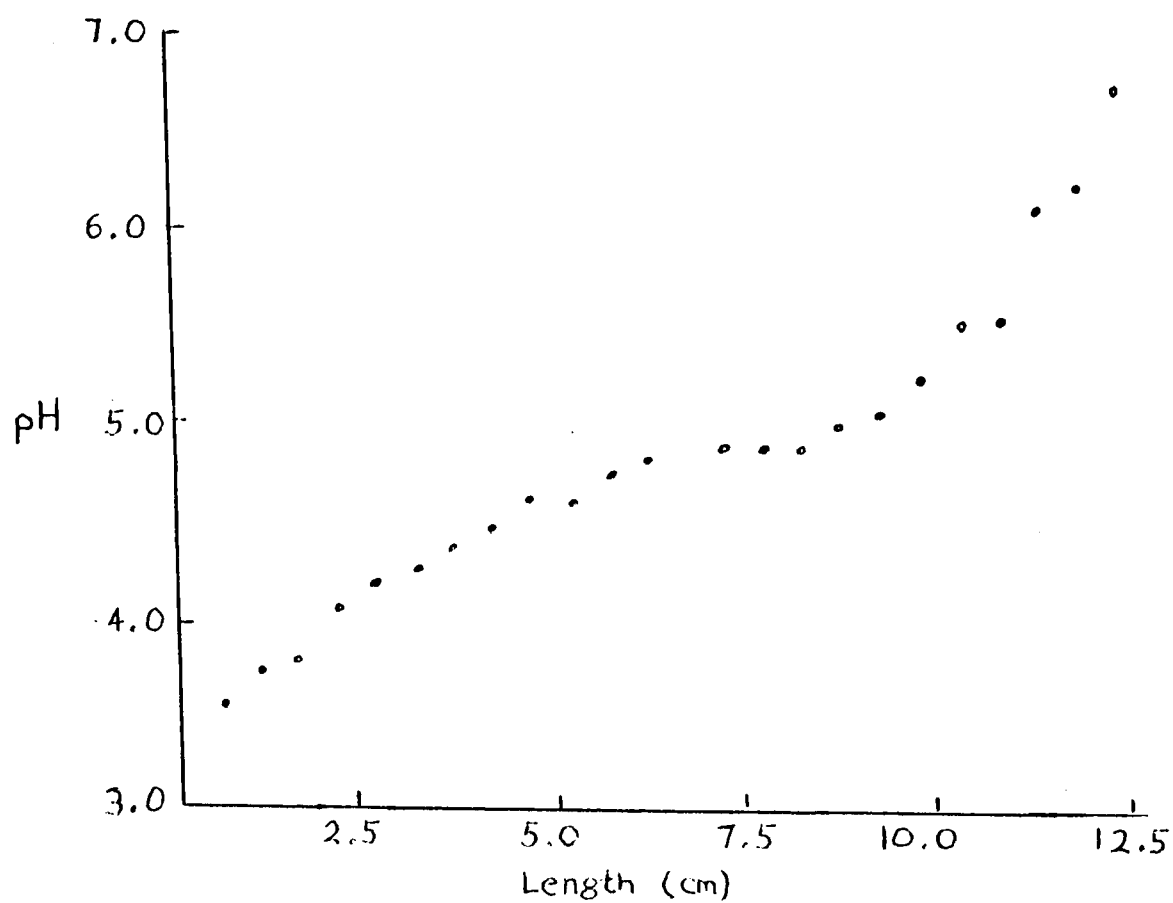


Figure 4. pH gradient profile of polyacrylamide isoelectric focusing gel. The gel was run for 5600 V-hrs and pH determined using a Bio-Rad pro-pHler.

each species differs by a single charge. The spacing between bands of the carbamylated creatine kinase (CPK) standard are not uniform, despite the fact that they differ by a single charge (Figure 5). Two factors are involved in the uneven spacing. First, the pH gradient is not perfectly linear. Second, and more importantly, proteins behave as complex buffers (4, 106). If, for example, the isoelectric point (pI) of a protein is near the pK of a dissociating group of an amino acid side chain, the addition of a single charge will result in a small change in the pI because the protein is a good buffer in this region. As the pI becomes further removed from the pK a small change in charge results in a large change in pI. The distance between bands differing by a single charge is also affected by the total number of ionizable groups a protein contains. A protein with a large number of titratable groups is less influenced by the addition of charge than a protein with fewer titratable groups and the same pI (4). A direct correspondence between change in charge and alteration in pI is, therefore, not possible.

Problems sometimes arise when trying to number the various carbamylated species of CPK because the pattern is blurred, or the minimally carbamylated proteins are present at low concentrations. This problem is minor because gels can be oriented and compared by the unequal, but constant, spacing of the carbamylated CPK bands.

Immunopurified serum albumin from 3- and 30-month-old C57Bl mice behaves identically on two-dimensional gels (Figure 5 and 6). There is no change in the charge of serum albumin as a function of age. Albumin focuses as a horizontally elongated band over CPK spots 7 and 8

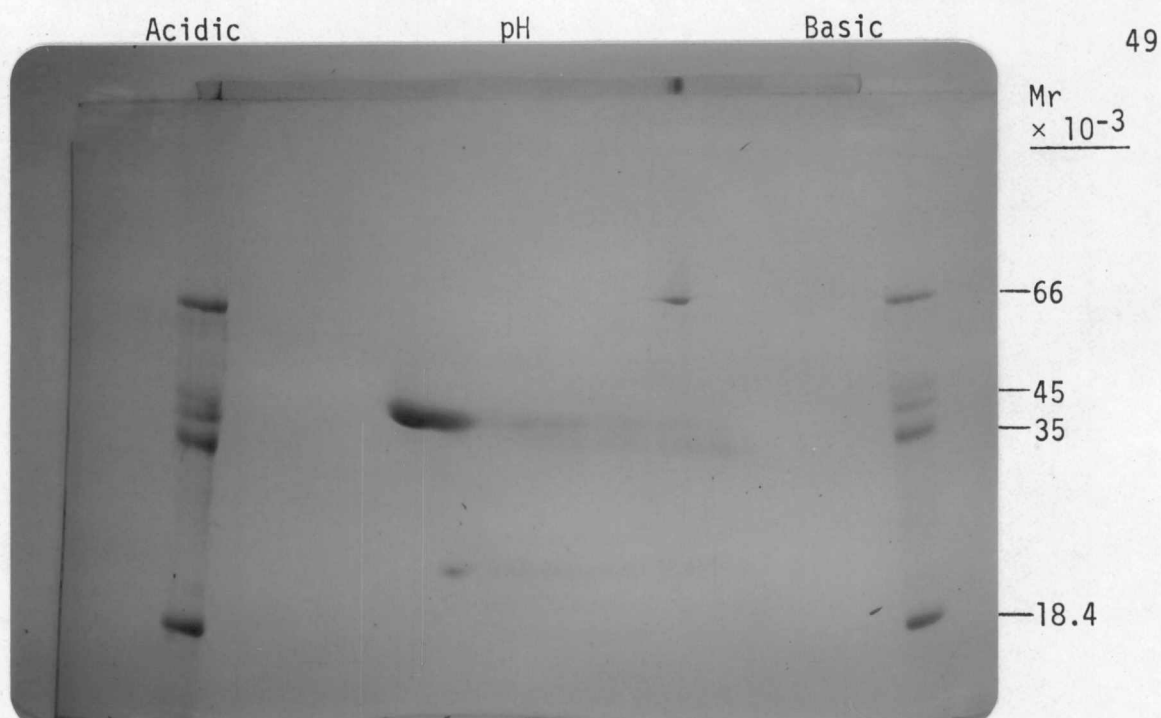


Figure 5. Two-dimensional gel electrophoresis of 3-month-old C57Bl mouse immunopurified serum albumin with carbamylated CPK standard.

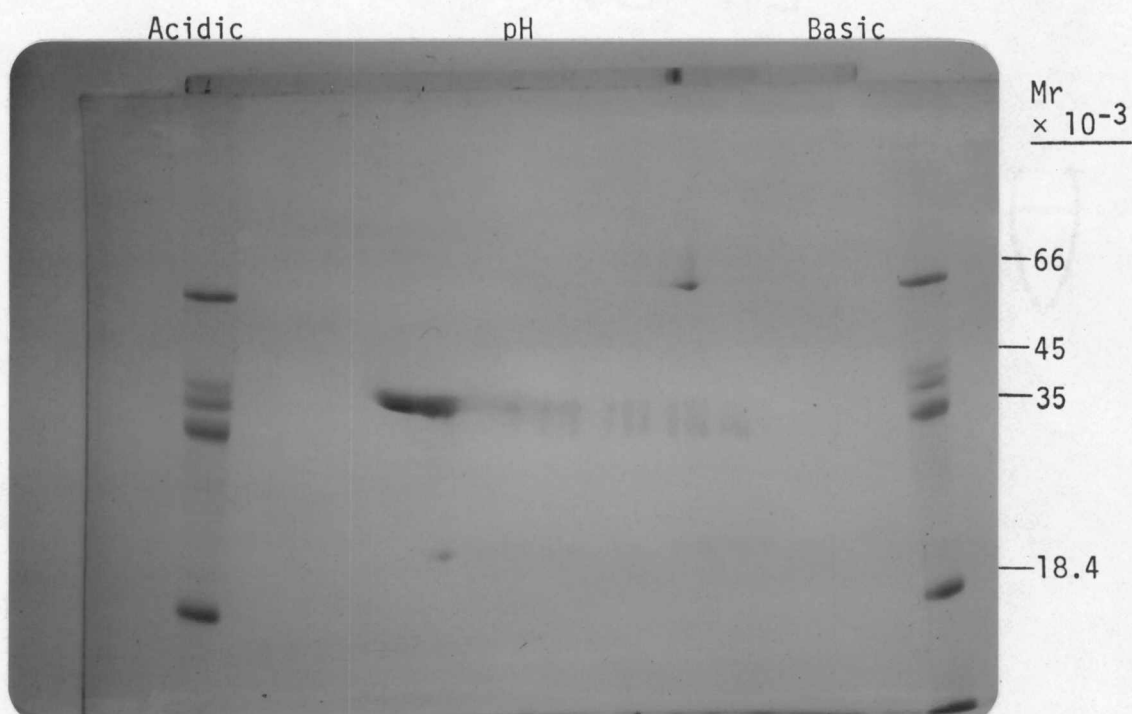


Figure 6. Two-dimensional gel electrophoresis of 30-month-old C57Bl mouse immunopurified serum albumin with carbamylated CPK standard.

at a molecular weight of 66,000 daltons. An examination of the elongated spot reveals that it is eccentrically shaped suggesting the presence of more than one species of protein. A stained IEF gel, containing the same albumin samples as the two-dimensional gels, clearly shows the presence of two bands (top of Figures 5 and 6). The stained IEF gel also confirms that the species are not present in equal concentration; the more basic form is present at a higher concentration. The presence of more than one pI for albumin has been reported many times (79, 80, 82). Spencer and King (82) measured the percent distribution between the two forms in bovine serum albumin and found it to be 80:20 which appears consistent with the present results. Scanning the gels to determine the distribution was not possible in the present case because the bands are too close together. Spencer and King have also demonstrated that the difference between the forms is a single charge and calculated that the difference in charge is 0.07 pH units. The origin of the heterogeneity in pI is unknown, but has been ascribed to oxidation of the free cysteine sulfhydryl with charged low molecular weight thiols (79), deamidation of asparagine or glutamine (80), and glucosylation of the ϵ amino group of lysine (73).

The albumin spots are poorly resolved for two reasons. First, the gels were purposely overloaded with protein so that minor bands would be visible. The second factor is the nature of albumin itself. Albumin stained with Coomassie blue streaks, especially in the horizontal, IEF, dimension. The information obtained from the stained IEF gel and the pattern shown on the second-dimension SDS polyacrylamide gel complement

each other. The use of a stained IEF gel allows the SDS polyacrylamide gel to be overloaded to detect minor spots while still maintaining sufficient resolution in the IEF gel to detect changes in the distribution of the pI isomers. The increased resolution in the IEF gel is due to its larger diameter (2.5 mm) compared to the second dimension slab (1.5 mm width). O'Farrell (4) has shown, using the same size gels, that 0.1 μ g is the maximum amount of protein a single band can contain without increased spot dimensions when transferred from the IEF gel to the second-dimension.

A comparison of albumin isolated from the livers of 3- and 30-month-old C57B1 mice by two-dimensional gels reveals no age-related differences (Figures 7 and 8). Albumin from liver, like serum albumin, focuses as an elongated spot corresponding to CPK units 7 and 8. The spot for liver albumin is less eccentrically shaped than that of serum albumin.

Albumin is synthesized with a hexapeptide extension on its amino terminus which is cleaved prior to secretion (83). Peters and Reed (93) have used an immunoaffinity column prepared from antibody against serum albumin to isolate proalbumin (albumin with the amino terminal extension) but no discrete proalbumin spot is visible on the second-dimension SDS polyacrylamide gels (Figures 7 and 8). The stained IEF gel contains a faint band on the basic side of albumin which approximately corresponds to the position expected for proalbumin. Proalbumin, in the rat (the mouse sequence is unknown), has a more basic isoelectric point than albumin because three of the six amino acids in the leader sequence are basic amino acids (arginine) (84). The shift in pI between albumin and

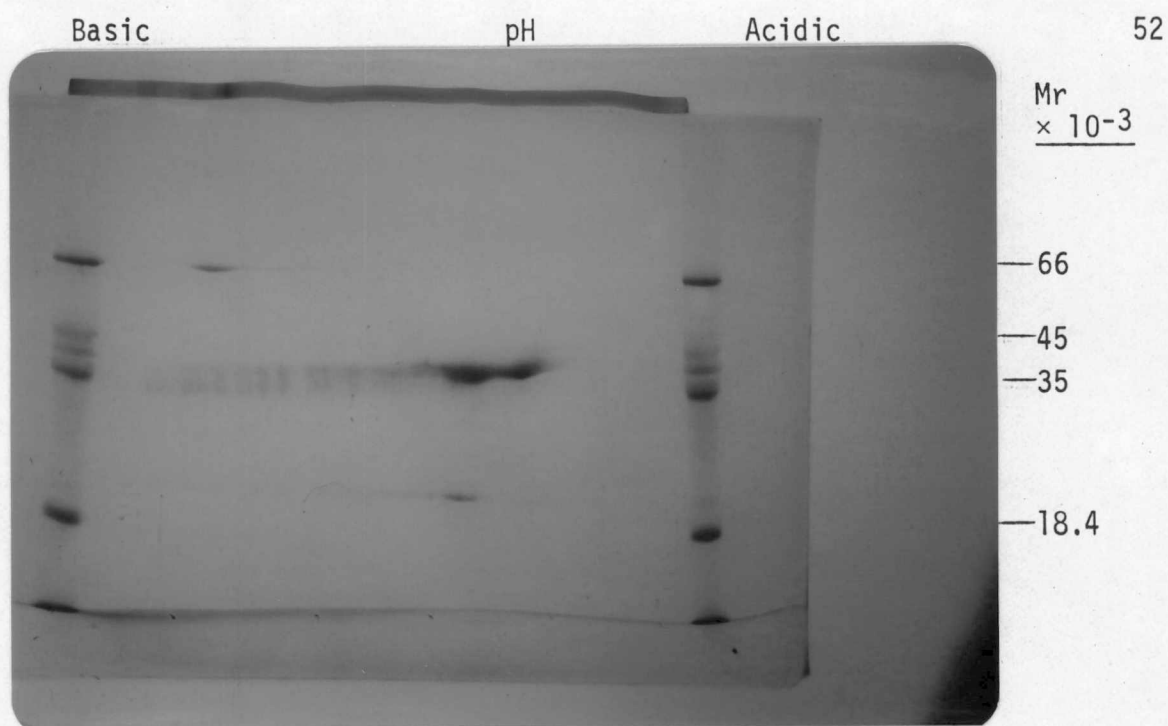


Figure 7. Two-dimensional gel electrophoresis of 3-month-old C57Bl mouse immunopurified albumin from liver with carbamylated CPK standard.

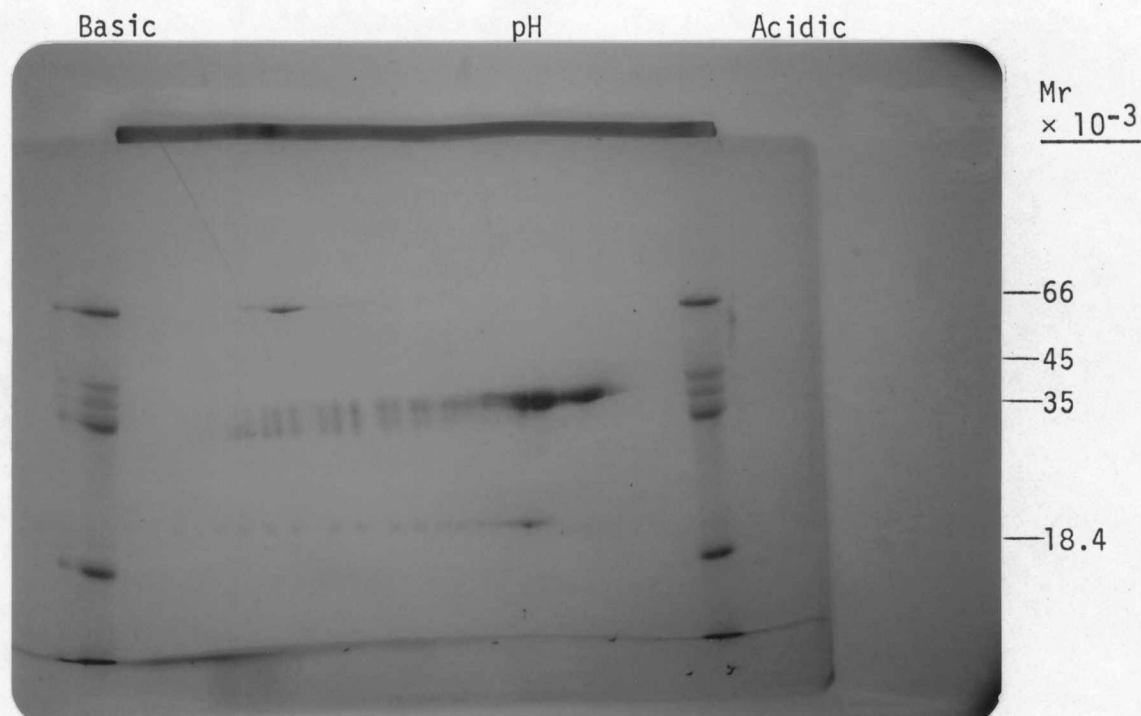


Figure 8. Two-dimensional gel electrophoresis of 30-month-old C57Bl mouse immunopurified albumin from liver with carbamylated CPK standard.

proalbumin is 0.3 pH units (93). The apparent absence of proalbumin from the second-dimension may be due to loss of protein during equilibration (stained gels are not equilibrated) and/or masking of the proalbumin spot by the horizontal streak of the main albumin bands.

Total liver supernate from adult and aged mice have also been compared on two-dimensional gels (Figures 9 and 10). Proalbumin is quite evident on both gels. It is present on the basic side and has a slightly higher molecular weight than albumin. Albumin is present in liver at a concentration of 1 mg/g liver (88), proalbumin is found at a concentration of 60 μ g/g liver (93) so the albumin spot should be at least 16 times the size of the proalbumin spot, assuming no contamination by serum albumin. The vertical multibanded lane adjacent to the molecular weight standards at the basic side of the SDS gel is due to the large number of proteins in the liver supernate with near neutral pI's.

An examination of total serum by two-dimensional gel electrophoresis reveals a significant difference between 3- and 30-month-old mice (Figures 11 and 12). Serum from the aged mice has a horizontal series of spots having a molecular weight of approximately 75,000 daltons that is absent from the young adult serum. The discrete spacing and slight increase in molecular weight as the pI becomes more acidic suggests that a single protein with varying degrees of modification is responsible for the pattern. To determine if the protein is truly absent from the young adult serum, a two-dimensional gel was run with a greater amount of serum protein. Albumin was removed from the sample by precipitating the nonalbumin serum proteins with ammonium sulfate so that more sample

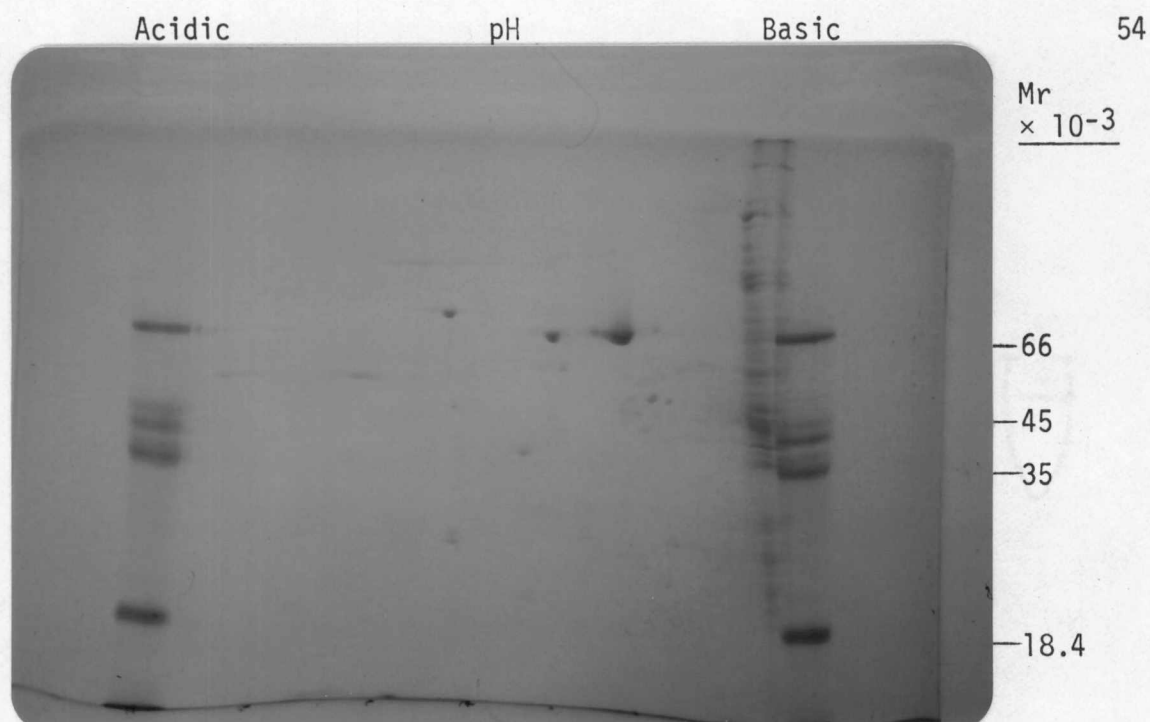


Figure 9. Two-dimensional gel electrophoresis of 3-month-old C57B1 liver supernate.

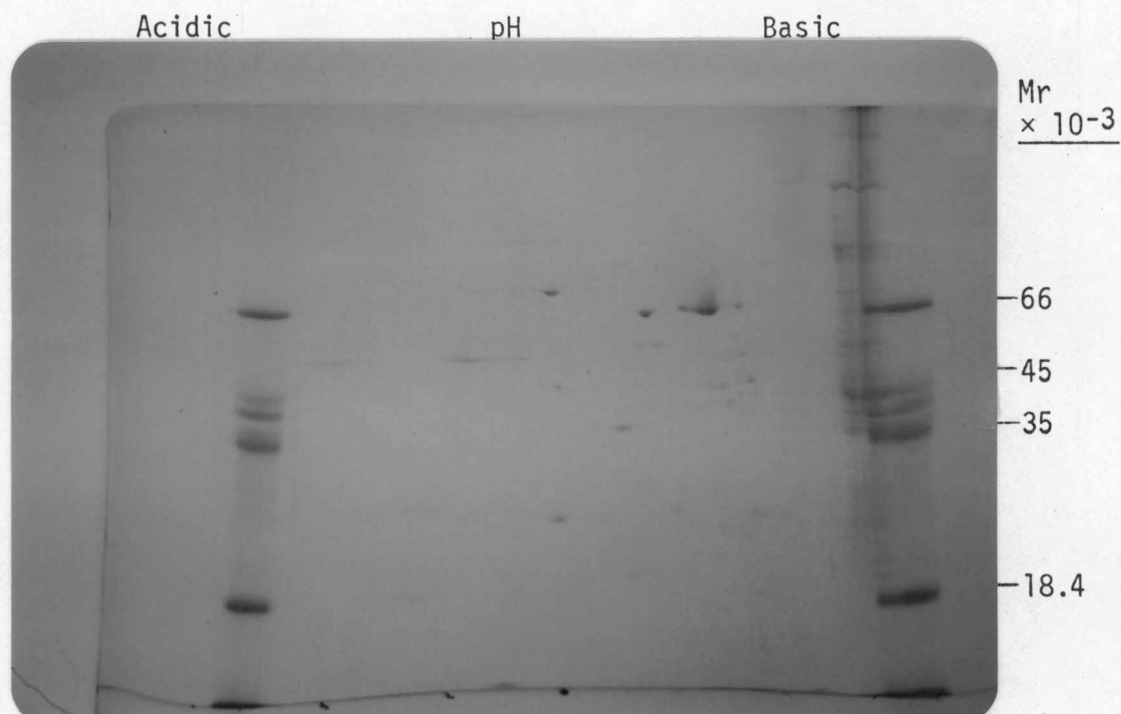


Figure 10. Two-dimensional gel electrophoresis of 30-month-old C57B1 liver supernate.

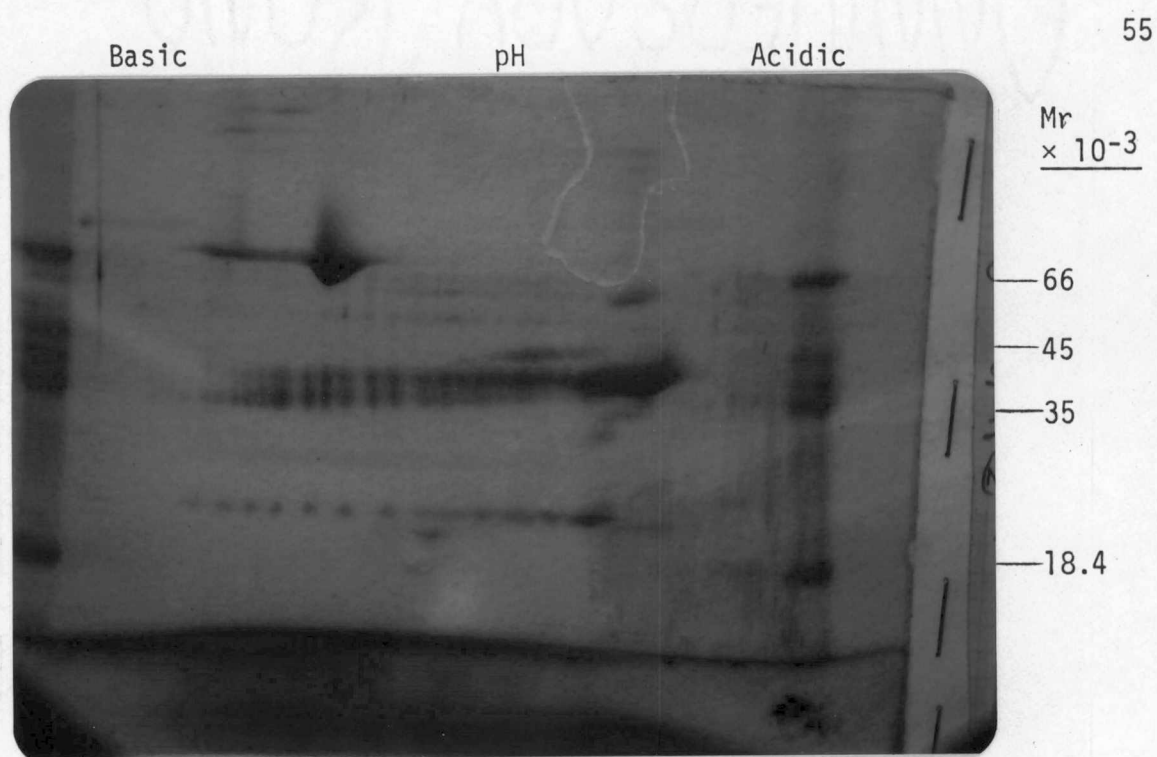


Figure 11. Two-dimensional gel electrophoresis of 3-month-old C57B1 serum with carbamylated CPK standard.

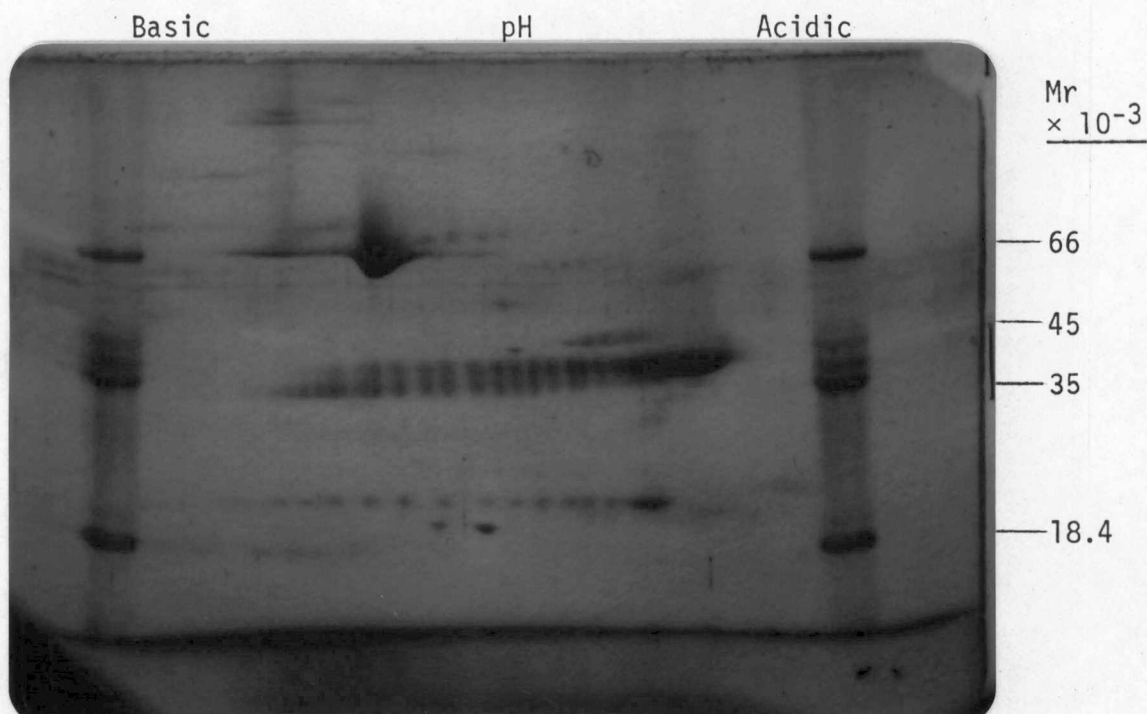


Figure 12. Two-dimensional gel electrophoresis of 30-month-old C57B1 mouse serum with carbamylated CPK standard.

could be added to the gel without albumin overloading the region of interest. The two-dimensional gel of the ammonium sulfate precipitated serum proteins shows that the protein is present in the serum of 3-month-old mice (Figure 13). A normal serum component that could be responsible for the type of pattern seen is transferrin. Transferrin is a glycoprotein with a molecular weight of 75,000 daltons and an acidic pI (108). The charge heterogeneity of transferrin is due to the addition of different numbers of sialic acid residues which make the pI of the protein more acidic and slightly increase the molecular weight. Transferrin is the major transporter of acid-soluble iron (i.e., nonheme iron) in blood. Elevated transferrin levels have been reported as a result of chronic iron deficiency and the increased level of transferring correlated with the severity and length of duration of iron deprivation (108). If the age-related increase in transferrin is confirmed, the cause and mechanism of transferrin induction should be examined.

Triton X-100 Gel Electrophoresis

Approximately two-thirds of all amino acid substitutions will not result in a change in the charge of a protein (5), so a large amount of variation could go undetected if one relied only on two-dimensional gels. To detect this variation a separation procedure based on some parameter other than charge is required. Triton X-100 polyacrylamide gel electrophoresis separates proteins based on differences in hydrophobicity. Zweidler (6) has explained the resolution of proteins on Triton X-100 gels as being due to a reduction in electrophoretic

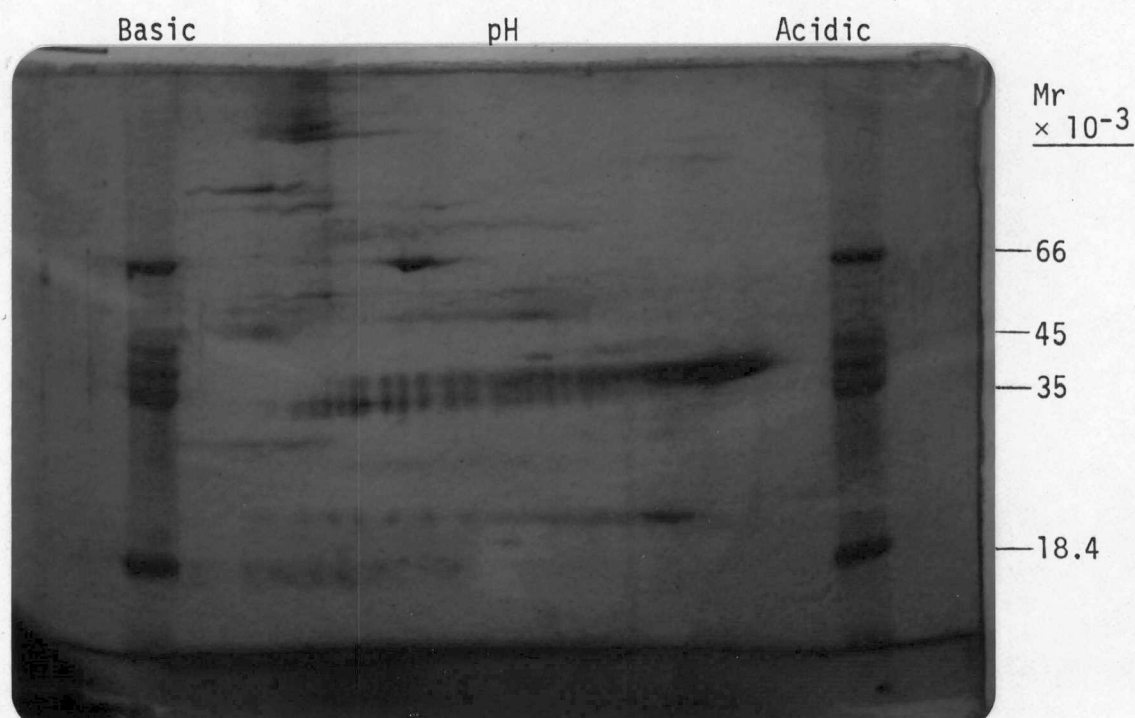


Figure 13. Two-dimensional gel electrophoresis of 2.1 M ammonium sulfate precipitable protein from 3-month C57Bl serum with carbamylated CPK standard.

mobility as a result of mixed micelle formation between the nonionic detergent and hydrophobic regions of the proteins. Slight differences in hydrophobicity are detectable by this procedure. Franklin and his associates have used Triton X-100 polyacrylamide gel electrophoresis to separate histone variants differing only in neutral amino acids (7, 8). Nonionic detergent polyacrylamide gel electrophoresis has also been used to detect variants of human albumin (101, 109).

In order to maximize the interaction between the Triton X-100 and albumin, the albumin is fragmented by cleavage at methionines with cyanogen bromide. Following cleavage the fragments are separated by reduction of the cystines holding them together and heterogeneity of the sulfhydryl is minimized by alkylation. When immunopurified serum albumin from 3- and 30-month-old mice is prepared in this way and electrophoresed in Triton X-100 gels, there is no difference in the hydrophobicity of albumin as a function of age (Figure 14). Analysis of fragmented and alkylated albumin from adult and aged liver also shows no age-related change (Figure 15).

The identical Triton X-100 polyacrylamide gel pattern for adult and aged albumin also indicates that there is not an increase in the oxidation rate of methionine to methionine sulfoxide with age. Methionine sulfoxide is not cleaved by cyanogen bromide (110), so if the amount of methionine oxidation is increased an alteration of the electrophoretic pattern would be expected. SDS-polyacrylamide gel electrophoresis of the fragmented albumin also confirms that the extent of oxidation of methionine is not increased (i.e., albumin fragment size is identical in young and aged mice) (not shown).

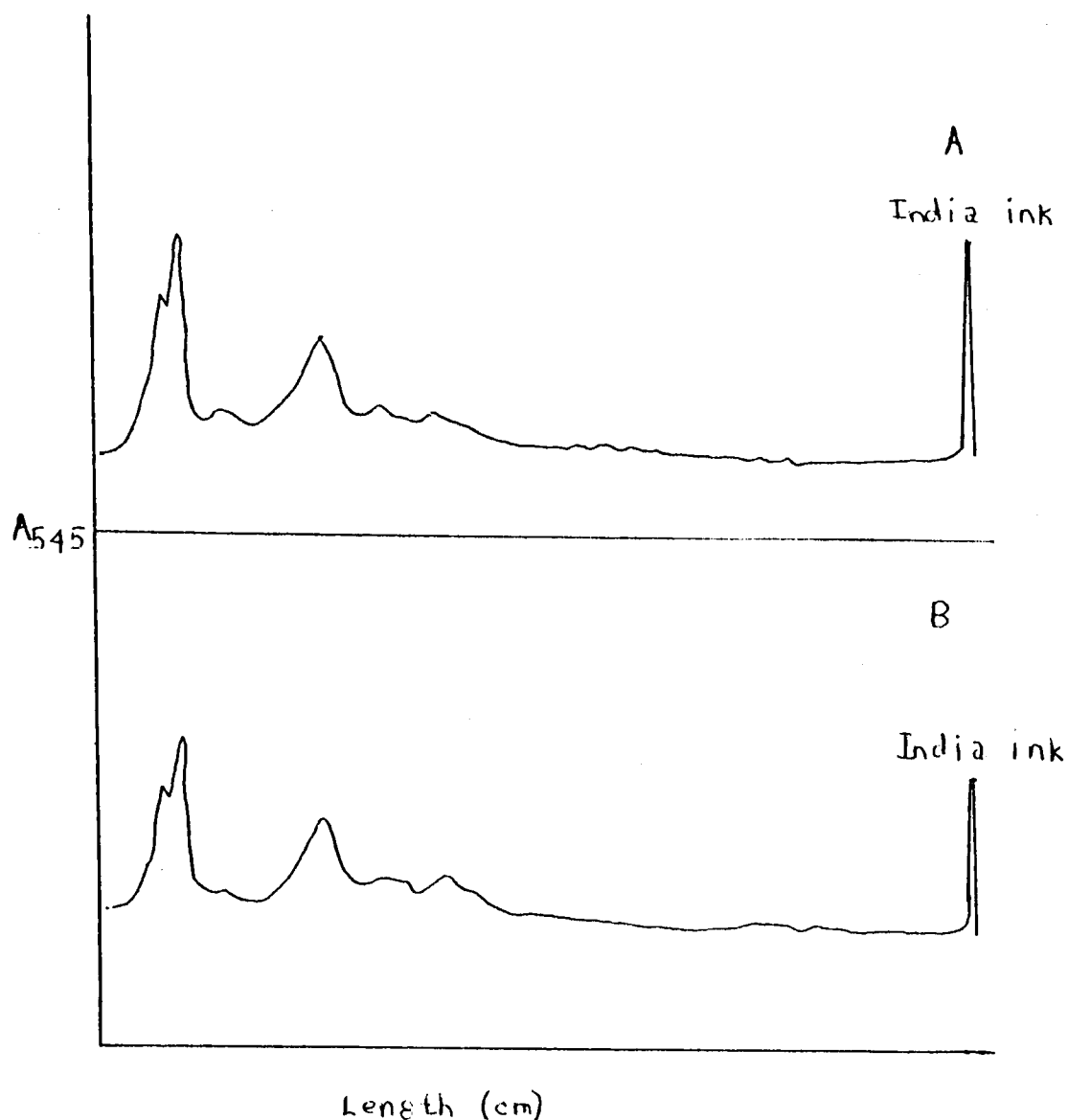


Figure 14. Absorbance profile of stained Triton X-100 polyacrylamide gels containing serum albumin. A: Alkylated fragments of immunopurified serum albumin from 3-month-old C57B1 mice. B: Alkylated fragments of immunopurified serum albumin from 30-month-old C57B1 mice.

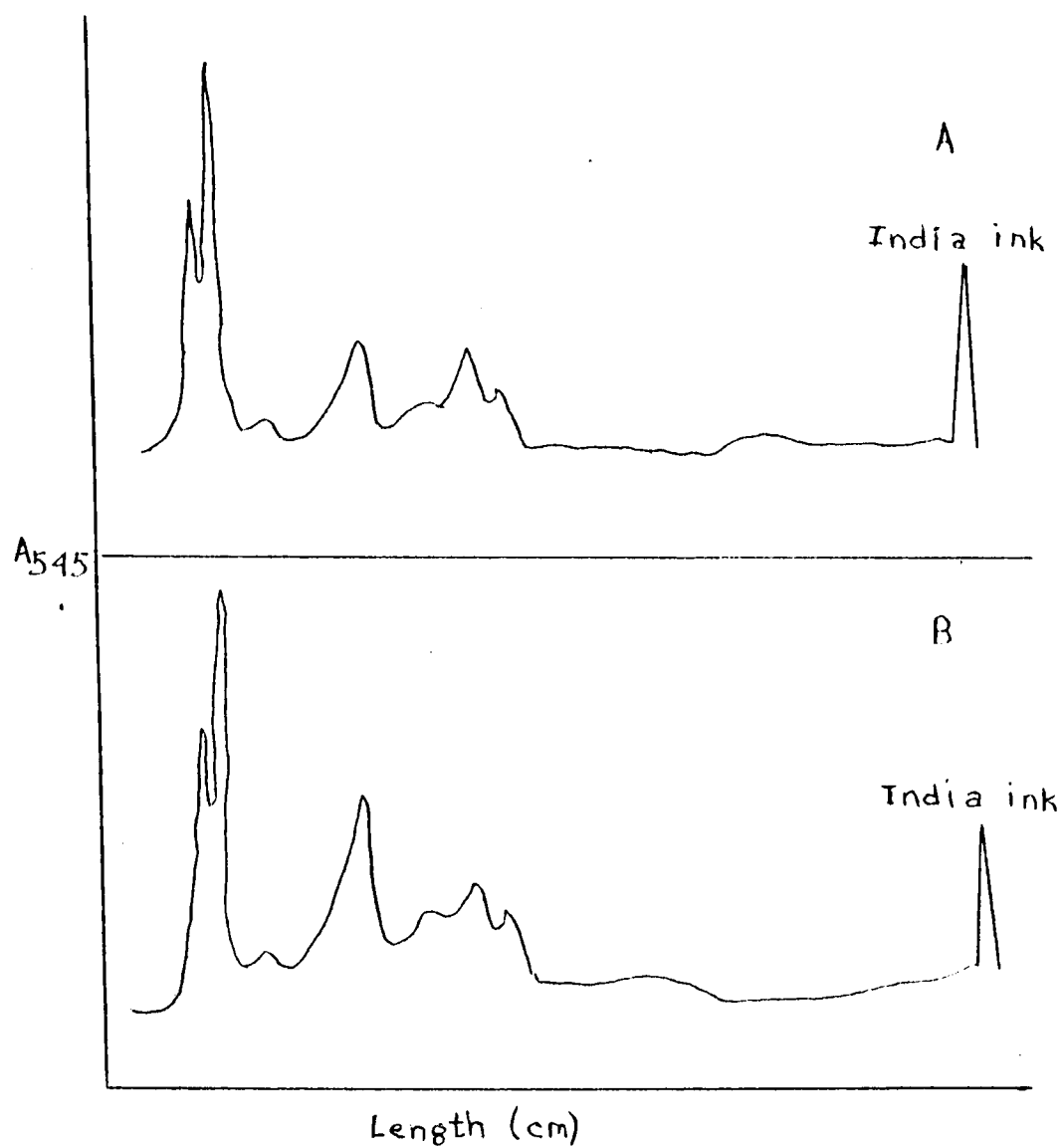


Figure 15. Absorbance profile of stained Triton X-100 polyacrylamide gels containing liver albumin. A: Alkylated fragments of immunopurified albumin from the liver of 3-month-old C57B1 mice. B: Alkylated fragments of immunopurified albumin from the liver of 30-month-old C57B1 mice.

Gel Sieving Electrophoresis

A parameter unconsidered by either two-dimensional or Triton X-100 gel electrophoresis is molecular shape. Rothstein has proposed that age-altered proteins differ from their unmodified counterparts in conformation (9, 48, 49). None of the procedures used to detect altered proteins (e.g., immune titration, heat lability, spectrophotometric analysis) separate altered from nonaltered proteins. Spectrophotometric analysis is particularly limited because it provides information on the average properties of the sample, despite the fact that the sample may be heterogeneous (i.e., contain altered plus nonaltered molecules). Gel sieving electrophoresis, on the other hand, can separate molecules differing in shape (10). Gel sieving electrophoresis has been used to confirm protein polymorphism suggested by thermolability studies, but that was undetected by traditional native gel electrophoresis (10). This is an intriguing finding because some age-altered enzymes have been reported to have heat-labile components (43, 65). Johnson, using gel sieving electrophoresis, has detected protein variants that differ in shape, but not charge (111), and also variants differing in shape and charge (112).

Ideally, electrophoresis separates proteins on the basis of net charge; electrophoresis in a polyacrylamide gel is not an ideal situation. The polyacrylamide matrix retards protein migration, and the smaller the pores (i.e., higher percentage of acrylamide or increased acrylamide: bis-acrylamide ratio), the greater the degree of retardation. Gel sieving electrophoresis turn this potential drawback into an asset,

whereby net charge and molecular shape can be determined in a single experiment. The net charge measured by native gel sieving electrophoresis is not equivalent to the total charge measured by isoelectric focusing under denaturing conditions. Proteins because of their complex three-dimensional shape are capable of masking charge.

The migration of a protein in a polyacrylamide gel can be described by the equation (113):

$$\log R_f = K_R (\%T) + \log \left(\frac{M_o}{u_f} \right)$$

where R_f = the mobility of the protein relative to the dye front; K_R = retardation coefficient; $\%T$ = percent acrylamide; M_o = free electrophoretic mobility; u_f = electrophoresis buffer constant. This equation is a linear equation of the form $y = m(x) + b$, where m = the slope of the line and b = the y-intercept. Thus, the migration of a protein may be expressed in terms of two experimentally obtainable quantities: the retardation coefficient (K_R) which is a measure of the protein's shape and the free electrophoretic mobility (M_o) which is a measure of net charge. The buffer constant (u_f) is ignored because the same buffer is used for all gels compared. By electrophoresing a protein in a series of gels of different acrylamide concentrations a plot of $\log R_f$ versus percent acrylamide ($\%T$) may be drawn (Figure 16). The slope of the line is equal to the retardation coefficient (K_R) and the y-intercept is a measure of the free electrophoretic mobility (M_o). The y-intercept provides a measure of the free mobility of a protein because it estimates the mobility of a protein in the absence of any interference by the

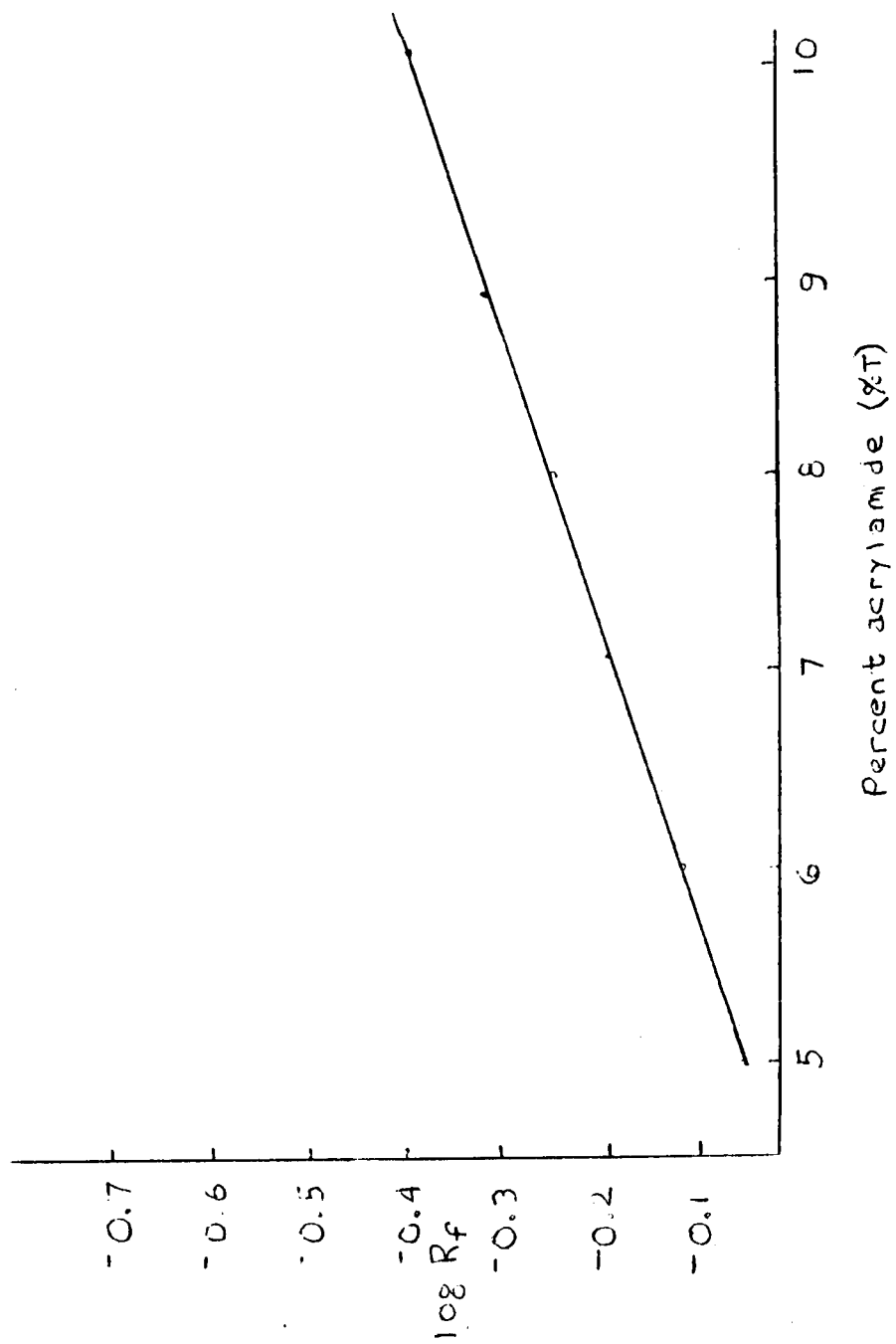


Figure 16. Basic pH gel sieving electrophoresis of a mixed sample of immunopurified serum albumin from 3- and 30-month-old C57B1 mice.

polyacrylamide matrix (i.e., 0% acrylamide). To minimize error, Johnson (10) suggests using the mid-Y value (the R_f observed at the average acrylamide concentration used) as a measure of the free electrophoretic mobility instead of the y-intercept. The mid-Y value is used because it is less sensitive to errors in the slope of a line than is the y-intercept.

In order to compare electrophoresis runs from several experimental samples, very demanding standardization and normalization procedures are necessary. This difficulty is circumvented by electrophoresing all samples to be compared in one electrophoresis run. At each acrylamide concentration there is an albumin sample from the 3-month-old mice, from the 30-month-old mice, and a sample containing a mixture of both.

The results of a typical basic pH gel sieving electrophoresis experiment are shown in Figure 17. The line drawn is based on data collected from scans of the stained mixed sample gels. A similar plot could be drawn for liver albumin (not shown). The retardation coefficient (K_R) and the determination of the free electrophoretic mobility (mid-Y) for liver and serum albumin are virtually identical (Table II). Serum and liver albumin from adult and aged mice has also been examined by gel sieving electrophoresis at acidic pH and found to be unchanged with age (Table III). The purpose of changing the pH is to compare the alteration induced in the shape of albumin by changing the protein's net charge. There is no apparent change in the shape of albumin in response to the change in pH (compare K_R in Table II and Table III).

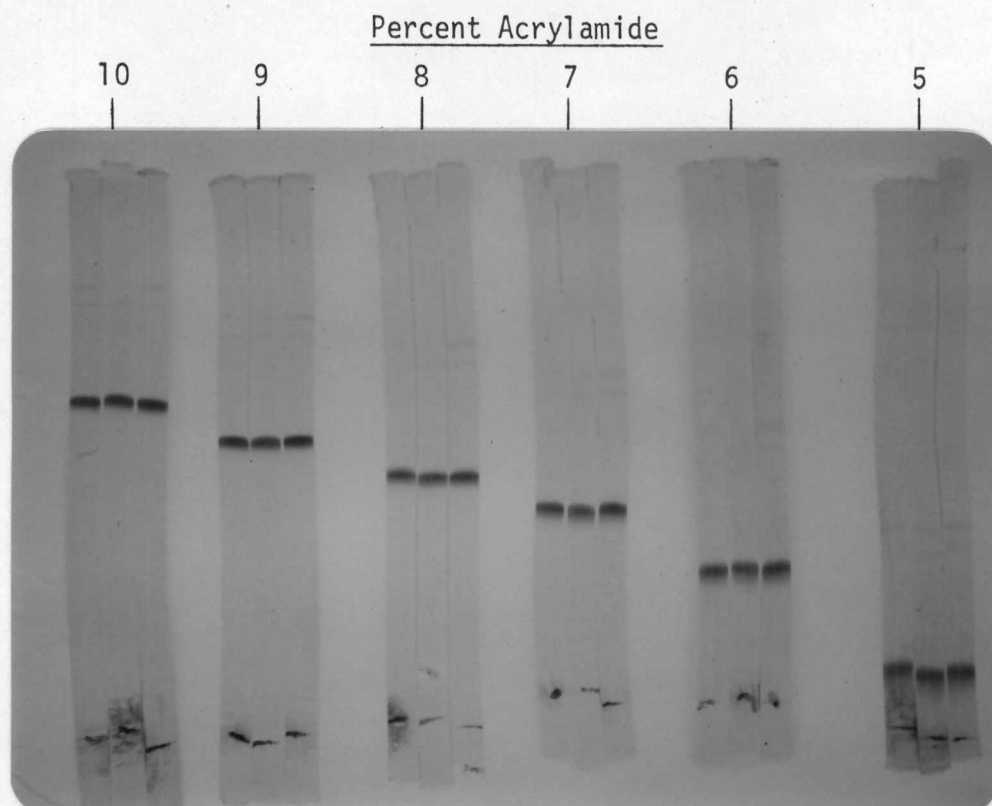


Figure 17. Migration of immunopurified C57Bl serum albumin in 5 to 10% polyacrylamide at basic pH. The first gel of each set contains albumin from 3-month-old C57Bl mice; the second albumin from 30-month-old mice; and the third a mixed sample from the 3- and 30-month-old animals.

TABLE II
BASIC pH GEL SIEVING ELECTROPHORESIS IN 5 to 10% ACRYLAMIDE

	K_R	mid-Y
Immunopurified C57B1 serum albumin	0.067	0.603
Immunopurified C57B1 liver albumin	0.067	0.608

TABLE III
ACIDIC pH GEL SIEVING ELECTROPHORESIS IN 5 to 10% ACRYLAMIDE

	K_R	mid-Y
Immunopurified C57B1 serum albumin	0.068	0.231
Immunopurified C57B1 liver albumin	0.066	0.251

CHAPTER V

SUMMARY

As outlined in the introduction, infidelity of protein biosynthesis could be the result of numerous mechanisms of biological aging. It was assumed that altered products of macromolecular synthesis could, if present in sufficient quantity, contribute significantly to senescence.

Albumin from the serum and liver of 3- and 30-month-old C57Bl mice was isolated by immunological techniques and compared by various procedures for age-related differences in the fidelity of *in vivo* synthesis.

In comparing the albumin of young and aged mice: (1) the isoelectric focusing analysis demonstrated that no amino acid substitutions or post-translational changes had occurred that involved net charge changes in the protein; (2) SDS-PAGE analyses provided evidence that no premature termination or read-through occurred during translation leading to molecular size alterations; (3) chemical fragmentation and subsequent nonionic detergent gel electrophoresis demonstrated no differences due to alterations by neutral amino acid substitutions; (4) the exacting procedure of gel sieving electrophoresis with varying parameters of pH and pore size served to demonstrate that no alterations had occurred due to protein conformational changes.

Thus within the limits of sensitivity of detection, no differences were observed between albumin of young and old animals. This conclusion is strengthened by data from liver albumin where altered molecules

might be detected prior to elimination during the processes of intracellular transport and secretion, but no altered molecules were detected.

Whatever molecular mechanisms are ultimately responsible for the aging process, they do not seem to include alterations in the genetic information system as exemplified by the biosynthesis of albumin. If alterations exist at levels undetectable by the procedures utilized, it is difficult to rationalize how they could play a role in aging and ultimately the death of the animal.

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VITA

James Robert Burke was born in Brooklyn, New York on February 2, 1953. He graduated from Manhattan College with a Bachelor of Science degree in Biology and English in 1976.

In 1977 he entered The University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences. He received the Doctor of Philosophy degree with a major in Biomedical Sciences in December 1981.

He is married to the former Diana Massaia of Brooklyn, New York.