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Author

Vaughan, William J.

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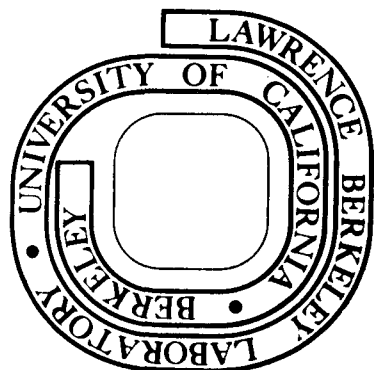
William J. Vaughan and Melvin Calvin

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ELECTROPHORETIC ANALYSIS OF BRAIN PROTEINS
FROM YOUNG ADULT AND AGED MICE

William J. Vaughan, School of Optometry

and

Melvin Calvin, Laboratory of Chemical Biodynamics
University of California, Berkeley, California 94720

Running title: Brain Gels

KEY WORDS

Electrophoresis

Mouse brain

Protein levels

Gene expression

ABSTRACT

The purpose of this study was to electrophoretically examine proteins from mice of different ages to determine whether general changes occurred in protein levels during senescence and to relate the data to specific ageing hypotheses involving proteins. Proteins from whole brain, brain regions, soluble and ribosomal brain fractions, from young adult and senescent mice, were totally solubilized and subjected to polyacrylamide slab gel electrophoresis. The resulting gel patterns were then compared by examining the intensity of gel bands of the same migration distance. Positive controls were used to establish the high sensitivity of the electrophoretic method. For example, band intensity differences were observed when brain proteins from different species, different periods of growth and development, normal and mutant mice, and different brain regions were compared.

Virtually all band intensities and therefore corresponding brain protein concentrations were unchanged when patterns from senescent mice were compared to patterns from younger adult mice. These results provide evidence against ageing hypotheses involving: 1) gene function losses, 2) progressive programmed ageing, and 3) protein crosslinking.

The few, select, protein band changes that did occur provide evidence that: 1) the volume of the brain vascular system decreases with age, and 2) the brain S-100 protein concentration increases with age.

INTRODUCTION

Ageing studies at the cellular level have taken many approaches. A general question still persists: Do ageing effects occur at the cellular level? This question will be answered by age-dependent studies of cell structure and function.

A major intracellular function/activity is gene expression. The following summarizes how information within the nucleotide sequence of DNA is ultimately expressed through proteins [22,23,38]:



Clearly, many aspects of gene expression could be studied to monitor cell function. Gerontological studies of gene structure and expression have included, among others: DNA and chromatin melting points [20], repetitive DNA [7], RNA to DNA hybridization [7], Fl histone/DNA ratios [37], amounts of DNA per cell [10], rates of RNA turnover [24], rates of label incorporation into RNA [20]. The major difficulty in all of the foregoing experiments is the correlation of observed molecular changes with actual functional losses. In short, many such changes can be described as "molecular changes in search of ageing effects." For example, the relevance of an age-dependent change in repetitive DNA is obscure if that portion of DNA does not code for proteins or is never transcribed.

In this sense, the study of the protein gene product is more to the point, since ageing very likely involves functional changes which are enzyme dependent. In each of the following hypotheses: somatic mutation [6], programmed ageing [1,19,21], error catastrophe [29], translational ageing [2], protein crosslinking [3], and the more general, failures of regulatory

mechanisms in protein synthesis [18], the postulated functional losses are ultimately determined by proteins.

There are many ways of comparing proteins from young and old animals. Working with the total protein of a tissue, one can ask if age-dependent changes in the amount of protein per unit wet weight or per cell have occurred. Other comparisons, using the rate of uptake or loss of radioactive amino acids in total protein, could provide some insight into the protein synthesis/degradation aspect of gene expression. However, interpretations of radioactive label studies are often complicated by transport aspects of metabolism and by label reutilization [15]. However, total protein studies obviously lack the sensitivity to detect large changes in individual proteins that are present in tissues at low concentrations. Comparisons of individual proteins in young and old tissue homogenates are often based on enzyme activities per unit protein or per unit DNA [40]. Yet, any age-dependent differences noted in a given enzyme activity, besides reflecting the level and quality of a given enzyme, also depends on the concentration of activators, inhibitors, coenzymes, substrates, products and ions in the tissue homogenates [40], thus making changes quite difficult to interpret.

To reduce complexity, many investigators have undertaken fractionation studies of young and old tissue homogenates to compare component proteins in such fractions as nuclei, chromatin, mitochondria, and ribosomes. Subsequent interpretation of age dependent differences in component protein is simplified -- at least conceptually. Artifactual age dependent protein differences can easily be experimentally produced since equal extraction efficiencies and proteolytic enzyme activities cannot be guaranteed when comparing young and old tissue. While age-related differences due to changes in extraction or proteolysis may in themselves be important, such changes could generate false age-related differences in extracted protein levels and lead to false interpretation.

Further efforts to simplify comparisons have sought to isolate specific proteins from young and old tissue homogenates. Specific enzyme activities, peptide maps, amino acid composition and amino acid sequence are parameters which can be compared. However, the possibility of selective fractionation and proteolysis still exists. In addition, single protein studies, by their nature, are limited to a very small proportion of a tissue's protein.

Our approach to the proteolytic, fractionation and complexity problems was as follows. Greater sensitivity than former studies which compared total protein and greater breadth than the specific enzyme studies can be achieved by electrophoretic separation of the total complement of brain proteins into a hundred bands of different molecular weights and intensity levels. During brain homogenization enzyme inactivation and total protein solubilization was achieved, thus eliminating fractionation and proteolytic difficulties. Although, to reduce complexity, ribosomal fractions were also examined, fractionation and proteolytic problems were re-introduced.

The rationale of the present investigation was, while minimizing proteolysis and achieving complete protein solubilization, to survey brain proteins from mice of different ages. High resolution gel electrophoresis was used to determine whether general changes occurred in brain protein levels during senescence. Specific protein levels are determined by their rates of synthesis and degradation [30]. Since protein degradation continues into senescence [25,26], the maintenance of protein levels requires a genetic apparatus capable of synthesizing new protein. By measuring tissue protein levels (gene product), with increasing age, we are thus monitoring gene function and, therefore, cell function.

Correlations of loss of function with changes in protein levels have been cited by Schimke [30], thus it would not seem unreasonable to expect band intensity changes in protein gels of aged tissue. Indeed, electrophoretic

studies of mouse brain proteins by Grossfeld and Shooter [17] had shown a major protein band change between mice of ages 1 and 2 years. One aim of our research, using much more sensitive gel techniques, was to repeat and characterize this apparent age-related change of brain proteins.

METHODS

Virgin, LAF1 female mice were obtained at the age of 12 weeks from the Jackson Laboratory, Bar Harbor, Maine. A group of 90 mice were put aside for survival curve determination. Mice from another group were killed between the ages of 127 and 134 weeks to provide old brain tissue. Young adult brain tissue was obtained from mice between the ages of 17 and 36 weeks. Studies comparing mutant and normal brains used C57 B1/6J mice (Jackson Laboratory). For convenience, studies comparing brains in different stages of development (two days and 22 days) used female Sprague-Dawley rats instead of mice.

Samples were completely solubilized before being loaded onto the gels. Whole brain, brain regions, soluble proteins, ribosomal proteins and a set of proteins of known molecular weights were individually dissolved in sodium dodecyl sulfate (SDS) sample buffer. This buffer consisted of 0.064M Trizma HCl¹, pH 6.8; 5% glycerol; 5% β -mercaptoethanol and 2% SDS (w/v).

Animals used for whole brain studies were anesthetized with ether and decapitated. After removal, whole brain (including olfactory bulbs and medulla oblongata and blood) was rinsed in 0.9% NaCl solution and blotted dry. Each brain was individually homogenized in SDS sample buffer using a tissue weight to added buffer volume ratio of 1g to 10ml. One volume of each homogenate was then diluted with 3 volumes of sample buffer, stirred,

¹Trizma HCl - hydrochloride salt of tri(hydroxymethyl)aminomethane from Sigma Chemical Co.

then heated in boiling water for 2 minutes. During this time the solution becomes totally transparent. Centrifugation for 5 minutes at 1000 g produced a very small faint white pellet. This small pellet was not likely protein since protein determinations [14] on the supernatant and the initial homogenate indicated that brain proteins were completely solubilized.

Animals used for regional brain studies were anesthetized with 0.5g of Na-pentobarbital per kg of body weight, administered intraperitoneally, and by cardiac perfusion with 0.9% NaCl, all noticeable blood was removed from the brain. To minimize proteolysis during brain dissection, the various regions were temporarily stored on dry ice (-79°C). Brain regions from 5 young and 4 old mice were pooled. The pooled regions were then dissolved, using the procedure described for whole brain. Whole blood was also dissolved in SDS sample buffer (50µl blood per 1.5ml buffer).

Soluble and ribosomal proteins were extracted from 5 pooled young and 5 pooled old mouse brains closely following the technique of Zomzely-Neurath and Roberts [41]. One gram of brain was homogenized in 9ml of 50mM Trizma-HCl (pH 7.4), 25mM KCl, 4mM MgCl₂, 250mM sucrose (TKMS), then centrifuged at 15,000g for 15 min at 4°C. Soluble and ribosomal proteins were in the supernatant (microsomal fraction). To increase overall microsomal yield, the first 15,000g pellet was resuspended in TKMS, again centrifuged, and the microsomal supernatants combined. Adding Na deoxycholate (DOC) to the microsomal supernatants (to 1%) and centrifuging (100,000g for 2 hours), produced DOC soluble proteins and ribosomal pellets. The DOC supernatant was then dialyzed, its protein precipitated with 10 volumes of 95% ethanol and subsequently centrifuged. After drying, the resulting pellet was re-solubilized in SDS sample buffer. Ribosomal pellets were directly solubilized in the same buffer.

The following proteins were used as molecular weight standards:
cytochrome c, 12,400 daltons (12.4K); ovalbumin, 43K; glutamic dehydrogenase,

50K; albumin, 68K; phosphorylase a, 92.5K; galactosidase, 130K; myosin, 200K. The above proteins were mixed together and dissolved in SDS sample buffer. An aliquot loaded into a gel slot contained 2 μ g of each protein in 25 μ l.

Since all samples were completely solubilized, all protein components are represented in the aliquot loaded onto the gels. Protein samples were then subjected to discontinuous SDS polyacrylamide gel electrophoresis [12], in a thin slab [33]. The gel apparatus (Hoefer Scientific Instruments, San Francisco) sandwiches a 0.75mm thick slab gel between 2 glass (30cm long, 18cm wide) plates. A slab gel handles 10 samples in 10 separate slots. Each slot was loaded with a young or old brain sample which contained approximately 50 μ g of protein in volumes up to 40 μ l. Typically, 600 dc volts were applied across the slab gel for 5 hours. Gel bands were stained by Coomassie blue (Sigma) reacting specifically with proteins. Proteins which migrate into the gel are representative of those loaded since there is no evidence for selective precipitation of specific proteins at gel origins [36]. Gel patterns resulting from the electrophoresis of equal amounts of young and old protein were compared visually on the basis of the intensity of bands of the same migration distance. Densitometer scans were not more sensitive than the eye. The densitometer merely quantifies an obvious visual difference in peak intensity. Where bands are of uneven quality (ribosomal bands in fig. 6) visual comparisons are still possible by selecting two bands in the young, taking the ratio of their intensities and comparing this ratio to the ratio derived from comparable bands in the old. Although SDS gels generally resolve proteins as a function of molecular weight, our intention was to compare only band intensities--not to look for age differences in gel band mobility. Gel densitometer scans were used to insure that the intensity of each band increased linearly with protein loaded onto the gel [36]. Further details of the gel system and operational procedures are available elsewhere [36].

RESULTS

Survival data indicated a median lifespan of 125 weeks (fig. 1) which was greater than the highest value reported in the literature for LAF1 mice [27]. This median value may still be less than optimum since it includes animals which were removed and killed because of gross tumors.

Whole brain weighed 0.42g in 12, 104 and 156 week old mice. Brain protein was approximately 100mg/g wet weight of whole brain for the three ages.

In general, the SDS slab gels resolved between 80 and 100 bands in gels 28 cm in length. Relative band mobility was generally a function of the protein molecular weight.

The sensitivity of the gel technique was shown by positive controls--cases in which protein differences were known to exist between the brains compared. In fig. 2, qualitative and quantitative differences between adult rat and mouse whole brain proteins are shown. Four of the greatest differences are arrowed; many additional minor differences were obvious on the original gels. The differences were confirmed with one other rat-mouse pair in many other gel runs. Protein changes during growth and development have been well documented [35]. Rat brain proteins from two different periods of development are compared in fig. 2. Arrows indicate the more obvious protein level differences. These same differences were confirmed with another pair of rat brains in other gel runs. The developmental differences were more prominent than the species differences. The next pair of positive controls were brains from C57B1/6J normal and quaking mutant mice. Quaking mutants have been shown to be deficient in myelin proteins [16]. In agreement, our results show that three band intensities in fig. 2 were lower in the

mutant. These same differences were confirmed with another mutant-normal pair.

In all whole brain comparisons in fig. 2, unequivocal band intensity differences were observed. Using the same gel procedure, comparisons of whole brain proteins from mice of different strains, sexes, and learning ability also showed positive differences [36]. Each brain region, morphologically and functionally distinct, exhibited positive, reproducible differences when compared to cortex (figs. 4 and 5). Pronounced differences were also observed between whole brain, soluble, and ribosomal proteins (fig. 6). Sensitivity was sufficient to detect changes in protein concentrations, as measured by gel band intensities, in all positive controls tested. No data has been excluded in which changes were absent in positive controls. Negative controls are also required to show that changes are not readily introduced by the gel procedure itself. Running the same protein sample on the same gel should show no change. For example, the relative band intensities in the two slots which ran young ribosomal proteins in fig. 6 are identical.

Brain proteins from young adult and senescent mice are compared in figs. 3 through 6. In fig. 3, whole brain proteins from 4 individual young (17 weeks) and 4 individual old (127 weeks) mice are compared. In terms of relative intensity of bands of the same migration distance, two differences were observed between young and old. The arrow nearest the origin points to apparent age differences, not reproducible on subsequent gels; however, the leading "arrowed" band, in front of the 12.4K marker, is lighter in the old. Later gels (fig. 6) which ran blood as a control, suggest that this leading band is hemoglobin (Hb). The chains of Hb have a molecular weight of 15.5K, markedly greater than cytochrome c (12.4K), yet Hb migrated faster than cytochrome c.

The Hb chains, then, violate the SDS gel generalization that migration

rate depends solely on molecular weight [39]. Excluding the Hb band, there are no detectable age differences.

Pooled young (36 weeks) and old (131 weeks) cortex, pons-medulla, and cerebellum protein patterns are compared in fig. 4. Lines indicate where the bands of the pons-medulla and the cerebellum have different relative intensities when compared to cortex bands of the same migration distance. Cerebellum appears to have major bands (2 lines below the 43K marker in fig. 4) which are unique. There are no detectable age differences.

Young and old caudate nuclei, hippocampus, hypothalamus and septum protein patterns are compared in fig. 5. Lines indicate where the bands differ in their relative intensity from those of the cortex in the previous fig. 4. Since caudate nuclei and the septum did include some cortex tissue after dissection, it is understandable that the differences were not more pronounced. The curved bands in O-Cau.Nuc., Y and O-Hip. and O-Hyp. (fig. 5) and band instabilities caused by gel conditions and are not reproducible. Again there are no detectable age differences.

Soluble and ribosomal protein patterns from young (28 weeks) and old (134 weeks) brain are shown in fig. 6. Soluble proteins had over 50 bands. The four lines linking the bands from soluble young and old brain proteins indicate intensity differences. Although the first two band differences (closest to the origin) were not confirmed on subsequent gels with better resolution, the band differences nearest the 12.4K marker (at approximately 16 and 24K) were confirmed on higher resolution gels. Both band intensities increase with age.

Protein yields in the ribosomal pellets were 0.38mg/g for young brain and 0.42mg/g for old brain. Approximately 55 bands were observed in the ribosomal protein patterns illustrated in fig. 6. The bands are more diffuse in the old pattern.

Diffuseness of gel bands is characteristic of proteolytic degradation

and/or high salt concentrations in the sample buffer. Since $MgCl_2$ was used during the ribosomal preparation, greater diffuseness in the old was probably the result of a greater Mg^{++} ion concentration. By selecting two bands in the young, taking the ratio of their intensities and comparing this ratio to the ratio derived from comparable bands in the old, two differences are apparent between young and old. The ribosomal band intensity difference at 30K was unconfirmed on subsequent gels with lower band diffuseness. The band difference very near to the origin, too great (in excess of 300K) to be a ribosomal protein, indicates an intensity decrease in the old.

DISCUSSION

Our results, which show no change in brain weight or whole brain total protein concentration, are in agreement with previous reports on mouse brain [13,28,34]. As discussed previously, total protein studies lack the sensitivity to detect large changes in individual proteins that are present in tissues at low concentrations.

Gene Function, Gene Product and Ageing

Whole brain protein patterns in fig. 3, excluding the Hb difference, indicate that major brain protein concentrations are maintained, even in senescence. Additional data, comparing whole brain protein patterns of 15 and 156 week old mice agreed with the above observation. Densitometer comparisons found no differences between the major peaks of young and old patterns [36]. Combining all data, 10 individual young and 10 individual old whole brain patterns were indistinguishable from each other [36]. Brain region protein patterns (figs. 4 and 5) also indicated that major protein concentrations were maintained in older mice. One could argue that whole brain and brain regions are too complex to survey, and that despite the

success with positive controls, many small protein differences could go undetected. Hundreds of different brain proteins have been detected [31] and many thousand more may exist.

Simplification and a more complete survey would result if a select package of young and old protein product were isolated and compared. Ribosomes which are crucial for protein synthesis have approximately 100 proteins [11]. Protein turnover is size dependent, larger proteins degrade more quickly [9,15]. Ribosomal protein turnover continues into senescence [25]. Therefore, if gene function was lost with age, it would be reasonable to expect: 1) a decrease in the yield of isolated ribosomes, and 2) a greater proportional decrease in the band intensities of the larger proteins in the older ribosomal sample. However, the yield of ribosomal protein did not decrease with age, nor did the proportional band intensities change in the older ribosomal protein patterns.

Excluding the Hb bands in fig. 3 and the high molecular weight, non-ribosomal band in fig. 6, there was no evidence for decreased concentrations of major protein bands in older animals. General, steady state (constitutive) protein concentrations were maintained even into senescence, in spite of continual degradation, thus providing evidence against the idea that ageing effects are the result of a major failure in gene function.

As mentioned previously, protein levels are determined by their rates of synthesis and degradation. Menzies and Gold [25] have observed that proteins of rat brain ribosomes had degradation half-lives of 7 days and 18 days for young and old respectively. Since both our yield and gel patterns of ribosomal proteins were constant with age, and since the degradation half-life slowed to 18 days, the rate of brain ribosomal protein synthesis must also have been slower. One can conclude, therefore, that an age-related change occurred in gene expression which

was involved in brain ribosomal protein synthesis. A slowdown in protein synthesis could result in a loss of adaptability in a stress situation--leading to a higher mortality rate. Another viewpoint would be that slower protein synthesis was simply due to less demand or a more efficient metabolism in the old, and that the capacity for youthful protein synthesis rates was still there. In light of this apparent decrease in ribosomal protein synthesis, it is interesting to note that the only reproducible difference between the young and old ribosomal protein bands is the band nearest the origin (fig. 6). This band, which decreases in the old, has a molecular weight in excess of 300K. Very few protein subunits are greater than 300K [8]. Some RNA polymerases have subunit weights of 400K [8].

If this band is interpreted to be an RNA polymerase involved in transcription, its decrease with age could provide a basis for a decrease in the rate of ribosomal protein synthesis discussed above. Caution should be included in this interpretation since fractionation and proteolytic differences could have generated this band difference.

Ageing and Growth

Our studies show that while protein pattern changes are quite dramatic during growth and development (fig. 2), no major changes occur in the adult--even in senescent mice. Our data, therefore, present strong evidence against the hypothesis that mammalian ageing results from a continuation of the progressive genetic program involved in growth.

Crosslinking and Ageing

Total solubilization and electrophoresis of whole brain, brain

regions, soluble and ribosomal proteins revealed no apparent increase in the average protein molecular weights. Although our method would destroy disulfide crosslinks, other intermolecular covalent crosslinks should have remained unperturbed. Although these studies are limited to mouse brain proteins, the lack of an age-related size increase is an argument against the hypothesis that protein intermolecular crosslinkage is among the universal effects of ageing.

Brain Vascular Volume

As mentioned in the results, non-perfused whole brain protein patterns shown in fig. 3 indicate that the leading band, Hb, decreases in older brains. Brain regions, dissected from perfused brains, do not show this band difference (figs. 4 and 5). Silini and Andreozzi have shown that Hb concentrations in mouse blood were highest in their oldest (921 days) mice [32]. It follows, then, that the Hb decrease probably relates to a brain blood volume decrease with age. This age-related blood volume difference is significant, reproducible, and, since it may reflect a decrease in the volume of the brain vascular system, could provide a basis for brain functional losses with age. Studies which report age changes as intrinsic to brain cells should take into account the consequences of a possible decreased vascular volume. However, the blood volume difference noted could be due to an age-related change in the response of mice to ether. Therefore, this result should be checked using a different method of killing, decapitation without ether, for example.

S-100 Glial Cell Protein

Two reproducible, age-related band differences in the soluble

fractions of brain proteins appear in fig. 6 at approximately 16K and 24K. Both band intensities are greater in the older fraction. Since the S-100 protein of glial cells has a molecular weight of 24K [5], S-100 proteins may be in the band at 24K. An increase in this band could be due to an increase in the number of glial cells with age-- thus supporting the observation that glial cell densities increase with age [4]. A greater concentration of S-100 per glial cell could also explain this increase.

An overall increase in S-100 concentration with age agrees with the immunochemical assays of Cicero et al. [5]. Further evidence, such as amino acid analysis, is required before the 24K band can be positively identified as S-100 protein. Which protein(s) are involved in the 16K band increase is an open question.

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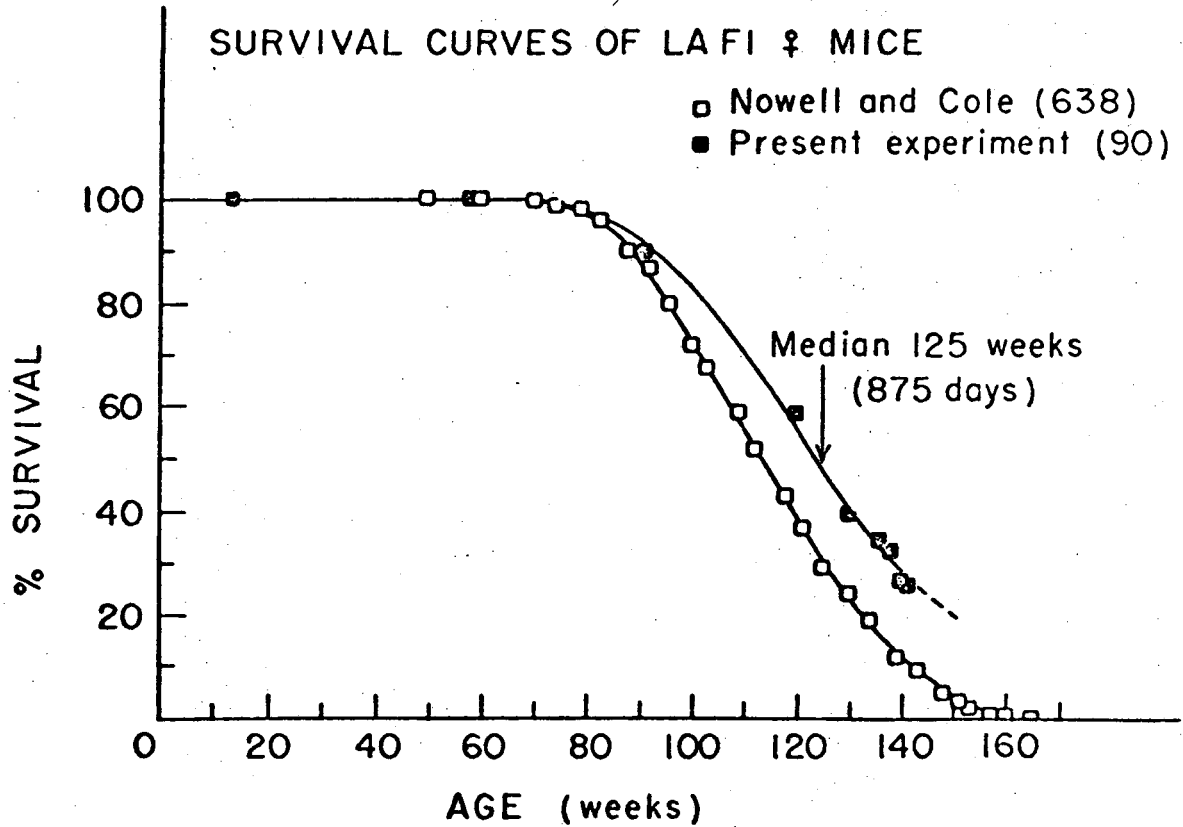
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FIGURE CAPTIONS

- Fig. 1 Survival curve for 90 female LAF1 mice, compared with a survival curve from the literature.
- Fig. 2 SDS slab gel comparison of whole brain proteins from: R, adult rat with M, mouse; R^2 , 2 day rat with R^{22} , 22 day rat; M^N , normal mouse with M^Q , quaking mutant mouse. Arrows indicate band differences between the two animals compared.
- Fig. 3 Gel comparison of whole brain proteins from four individual young (17 weeks) with four individual old (127 weeks) LAF1 mice. Arrows indicate apparent age differences.
- Fig. 4 Gel comparison of proteins from the following pooled young (36 weeks) and pooled old (131 weeks) mouse brain regions: cortex, pons-medulla, cerebellum. Lines indicate where the bands of the pons-medulla and cerebellum differ in relative intensity from those of the cortex.
- Fig. 5 Gel comparison of proteins from the following pooled young (36 weeks) and pooled old (131 weeks) mouse brain regions: caudate nuclei, hippocampus, hypothalamus, septum. Lines indicate where the bands differ in relative intensity from those of the cortex in the previous fig. 4.

FIGURE CAPTIONS (cont.)

Fig. 6 Gel comparison of protein standards (MW), and mouse proteins from blood (Bld), whole brain (Br), and from pooled young (28 weeks) and old (134 weeks): 1) soluble whole brain proteins 2) ribosomal proteins. Lines linking young and old bands indicate apparent differences.



XBL 7110-5379

Fig. 1

SDS BRAIN PROTEIN GELS



Fig. 2

XBB 752-1545

SDS BRAIN PROTEIN GEL

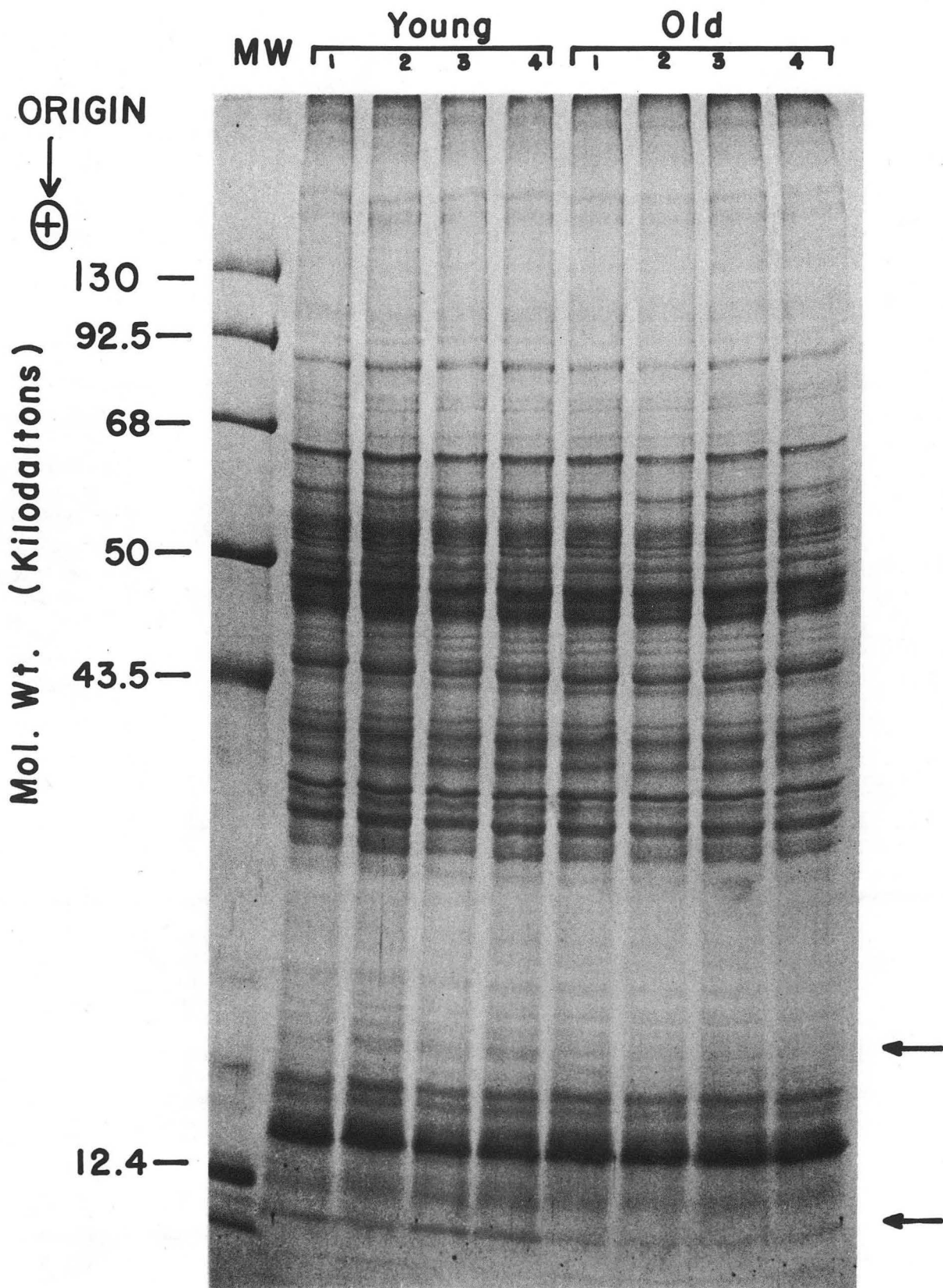


Fig. 3

XBB 742-915

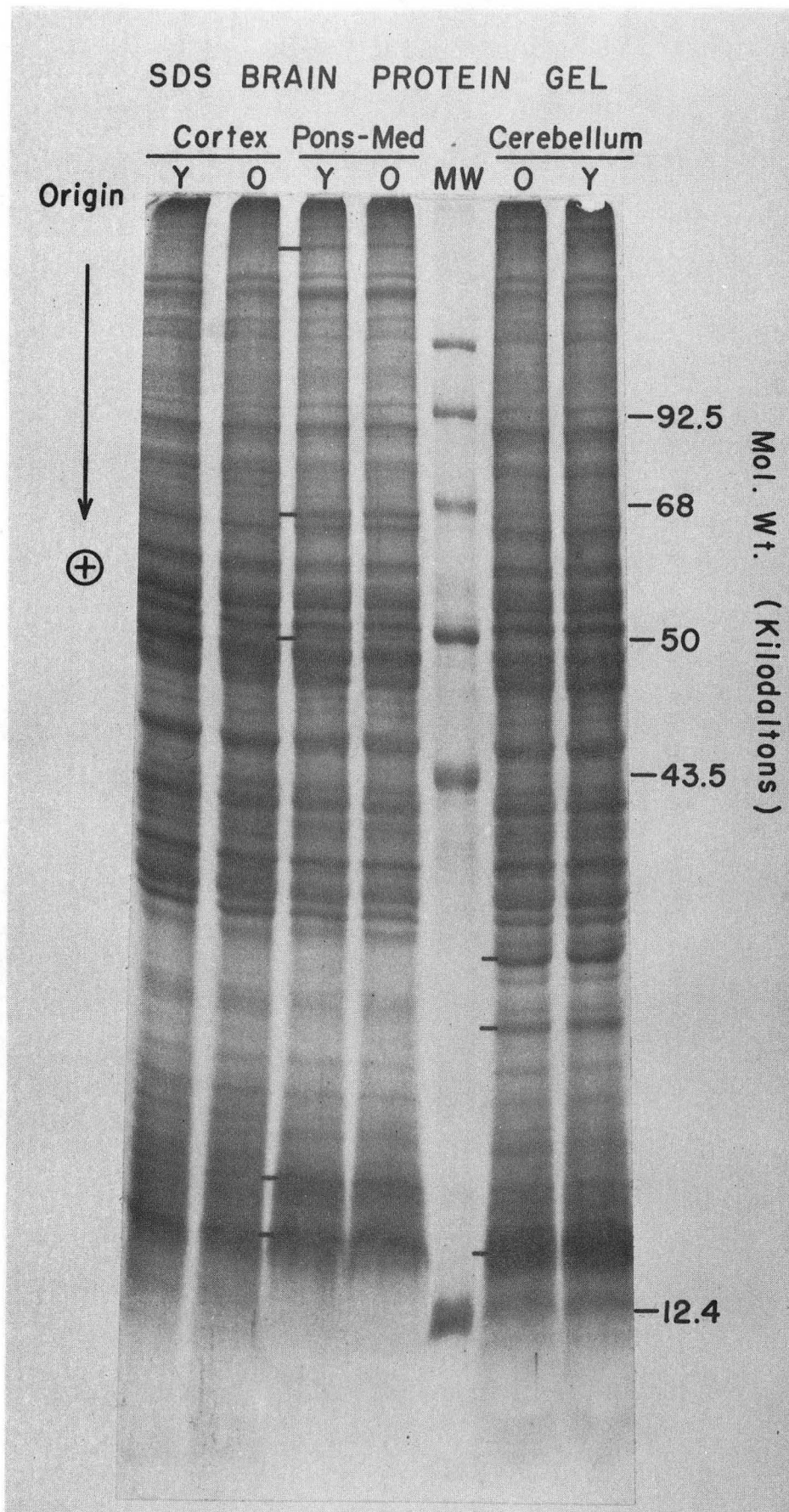


Fig. 4

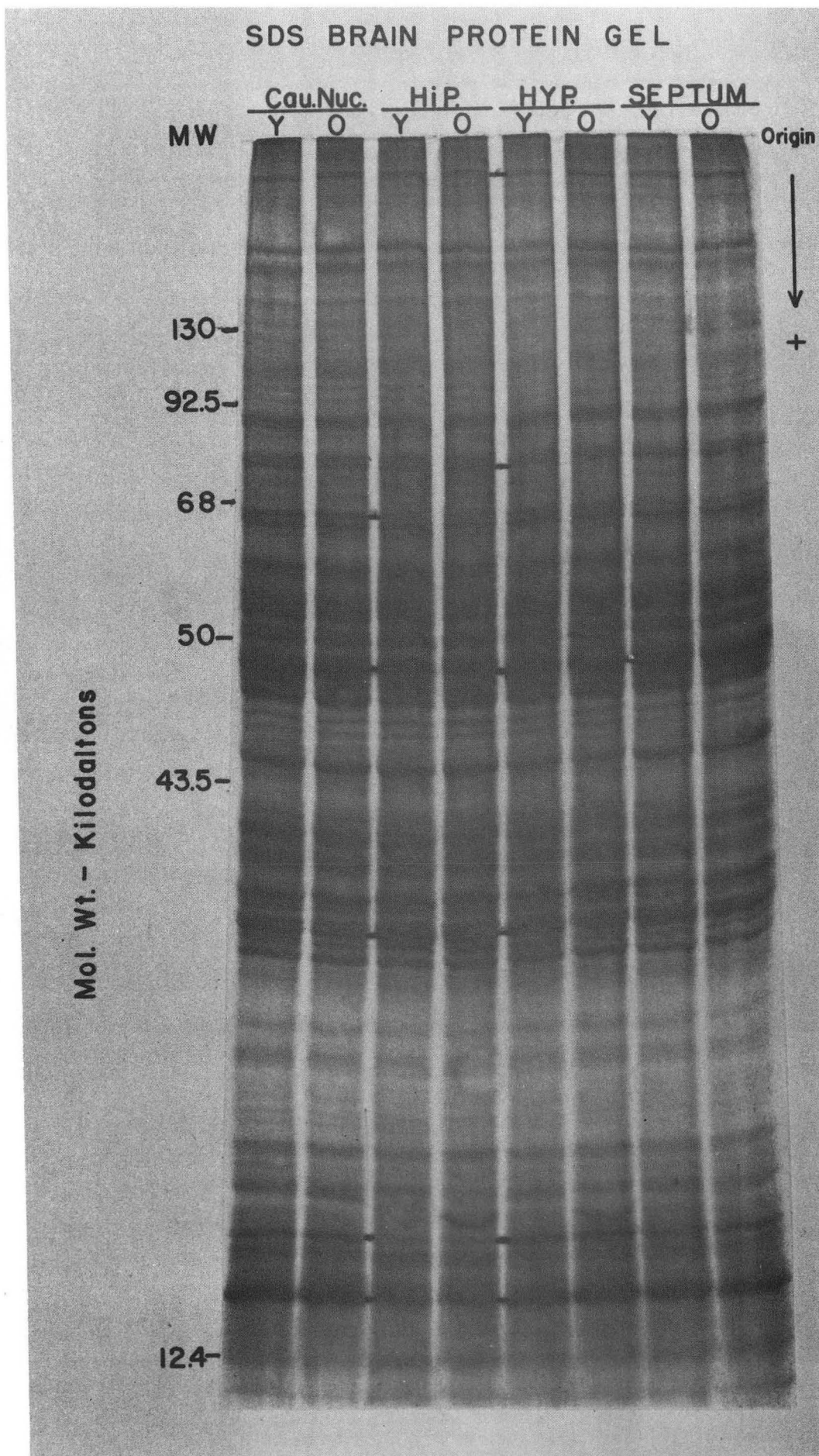


Fig. 5

XBB 743-1887

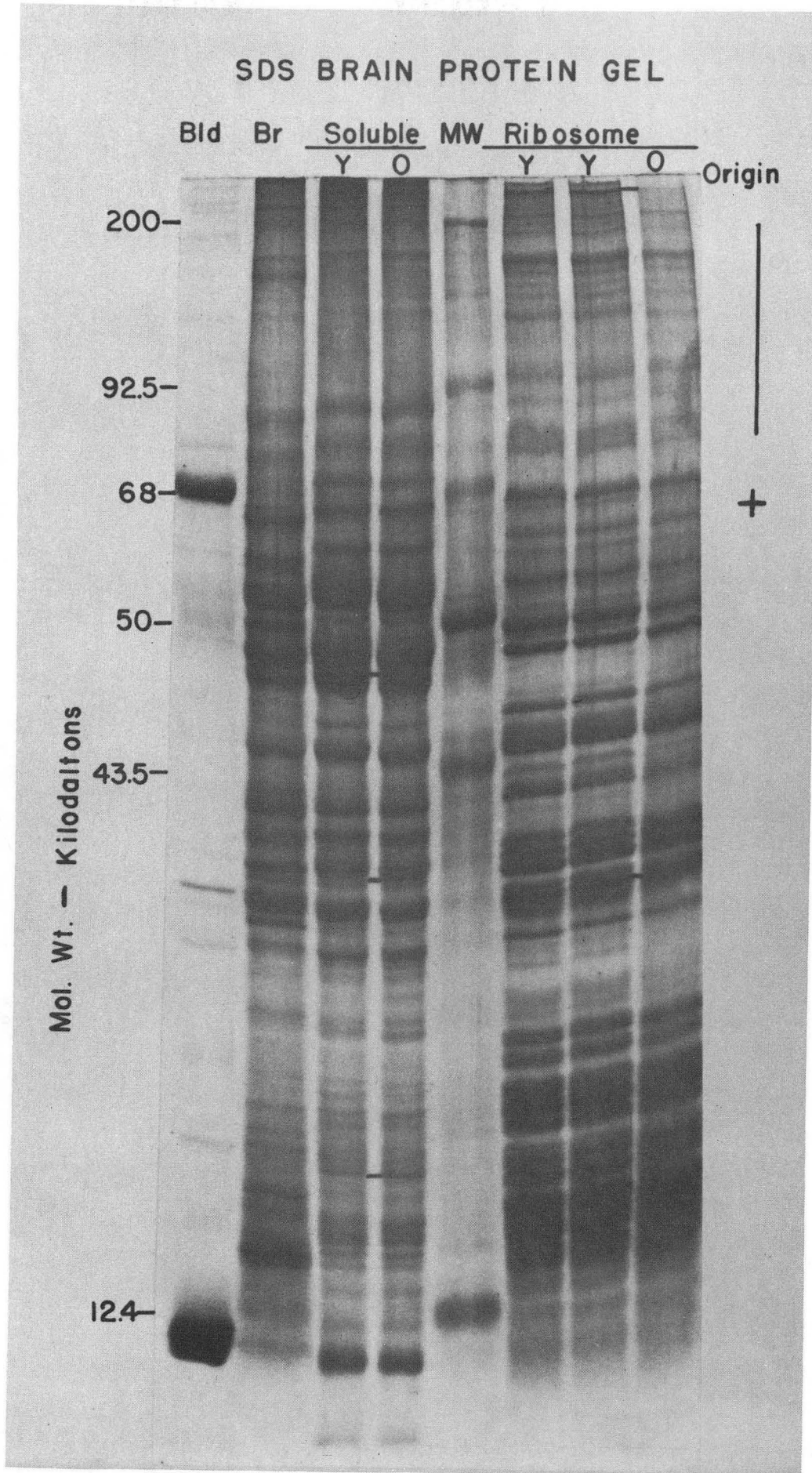


Fig. 6

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TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720