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Lateral mobility of proteins and lipids of cell surface membranes during aging: do the data support 'The Membrane Hypothesis of Aging'?

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Abstract

Many previous studies regarding the change with age in surface membrane fluidity of different cell types, including hepatocytes, as determined by the fluorescence anisotropy method, are in conflict, demonstrating decreased, unchanged or even increased fluidity with age. In contrast, the results of our series of works using the fluorescence recovery after photobleaching (FRAP) technique, which measures protein lateral diffusion coefficients of hepatocyte surface membranes (Dp), have demonstrated that Dp generally declines in a linear fashion with age in hepatocytes of all animal strains and species examined. The major coworker (I. Zs.-Nagy) of these studies insists that our observations support his original hypothesis, 'The Membrane Hypothesis of Aging' (MHA), the primary assumption of which is that changes in cell surface membranes with age cause a general decline in intracellular enzyme activities. However, while it seems clear that cell surface membrane changes do occur with age, a number of past observations including those from the laboratory of this author, provide strong evidence that intracellular enzyme activities do not generally decline with age. This paper presents the data in detail, along with the author's view that the results do not support the main assumption of the MHA, but are more likely related to alterations in membrane functions with age.

Keywords: Lateral mobilities of proteins; Hepatocyte surface membranes; FRAP; Intracellular enzyme activities; Aging

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1. Introduction

Many investigators have insisted that the physical–chemical properties of intracellular membranous structures may be altered during aging, leading to alterations of their physiological functions. Indeed, many studies have shown results which are consistent with such a notion. For example, lipid fluidities of cell surface membranes as well as synaptosomal membranes have been shown to decrease with age (Hegner and Platt, 1975; Nagy et al., 1983; Cimino et al., 1984; Yu et al., 1992). However, when we look at the past literature carefully, the results are quite variable, showing decreased (Hegner and Platt, 1975; Nagy et al., 1983; Cimino et al., 1984; Yu et al., 1992), unchanged (Nokubo, 1985) and even increased (Armbrecht et al., 1982) fluidities of membranes during aging. Most of these past studies have been performed by the use of the fluorescence anistropy method or other techniques [e.g. electron spin resonance (ESR)] using lipid fluorescent probes, most frequently diphenylhexatriene (DPH) and have measured a so-called microviscosity, a reciprocal parameter to the fluidity of the lipid domain of membranes.

In contrast with these rather inconsistent results in the past, our group has consistently reported a linear decline in the lateral diffusion coefficient of proteins (Dp) of hepatocyte surface membranes in a number of different animals (Zs.-Nagy et al., 1986a,b, 1989, 1993a,b; Kitani et al., 1988). Rather than using fluorescence anistropy, our group used a fluorescence recovery after photobleaching (FRAP) technique. More recently, we have also observed a similar linear decline with age in the lateral diffusion coeffcient of lipids (Dl) in hepatocyte surface membranes (Zs.-Nagy and Kitani, 1996). We have further shown that a decrease in Dp with age also occurs for other cell types, namely striated muscle cells of mice (Zs.-Nagy et al., 1998). The main worker in our FRAP studies, I. Zs.-Nagy who proposed the Membrane Hypothesis of Aging (MHA) has insisted that the above data are a partial basis for his hypothesis. This theory holds that physical–chemical alterations of cell surface membranes lead to intracellular dehydration and an excessive potassium influx causing a decrease in intracellular protein mobility which then leads to a general decline in intracellular enzyme activities with age (Zs.-Nagy, 1991, 1994).

The purpose of the present paper is first to summarize the results of our past observations on protein diffusion of hepatocyte surface membranes (Zs.-Nagy et al., 1986a,b, 1989, 1993a,b; Kitani et al., 1988) and then to introduce our recent new observations on lipid lateral diffusion for hepatocytes (Zs.-Nagy and Kitani, 1996) as well as on protein diffusion in skeletal muscle cells (Zs.-Nagy et al., 1998) as examined by the FRAP technique. Second, and more importantly, this paper will critically discuss the validity of the Membrane Hypothesis of Aging (MHA) of Zs.-Nagy (1991, 1994) especially whether intracellular enzyme activities generally decline with aging. Finally, the paper will discuss the possible physiological significance of our observations from our FRAP studies.

2. Materials and methods

The animals used are mainly from two different sources. Animals in our first studies were raised in the aging farm of the Tokyo Metropolitan Institute of Gerontology (TMIG), Tokyo Japan. The husbandry conditions are described in detail elsewhere (Nokubo, 1985). Another source for our recent studies are Harlan Sprague Dawley (Indianapolis, IN) for Fischer 344 (F-344) 6JNia and BN/Bi RijNia rats and Charles River Laboratories (Kingston, NY) for C57BV6JNia mice which were raised under a contract with the National Institute on Aging in the United States and purchased and imported to the National Institute for Longevity Sciences (NILS), Obu, Aichi prefecture Japan. All these animals were primarily animals raised as specific pathogen-free (SPF) animals. Some other animal species such as *Peromiscus leucopus* and *Mus musculus* are from the animal facility of the Gerontology Research Center (GRC), NIA, Baltimore, and were raised in conventional conditions. Animals which were given various treatments [e.g. dietary restriction (Zs.-Nagy et al., 1993b; Zs.-Nagy and Kitani, 1996) spironolactone (Sp) (Kitani et al., 1988), and various other medications] were all originally from SPF farms but were brought to our clean conventional animal facility at TMIG or NILS, generally a few days prior to the start of any treatment.

The FRAP apparatus which was used in our studies was constructed by Zs.-Nagy in 1982 in the TMIG (Zs.-Nagy et al., 1984), Tokyo, Japan. Although several additional modifications, especially on parts of the computer apparatus were subsequently made because of the rapid progress in this area in the past 15 years, the major parts of the apparatus are essentially the same as in the original one. This FRAP machine was moved to Tokyo University Medical School in 1992 and then again to NILS, in 1995 and has been used throughout the past 15 years. Principles and detailed structures of this FRAP instrument have been reported elsewhere (Axelrod et al., 1976; Zs.-Nagy et al., 1984, 1988).

The concept of the FRAP method is shown in Fig. 1. The small dots on the figure symbolize some protein (or lipid) components of the cell membrane with a suitable fluorescent compound. By measuring the fluorescence intensity ($F(t)$) in a relatively small area (3 μm total diameter), one can obtain the average concentration of the label in the membrane provided that the label is not present outside the

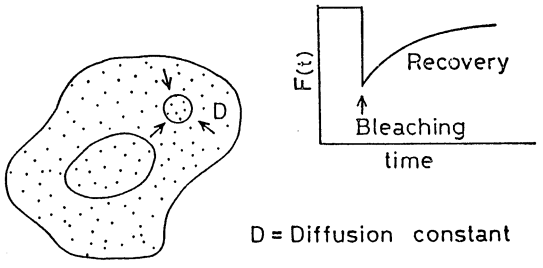

Fig. 1. The concept of the FRAP method. Reproduced from Zs.-Nagy et al. (1984) with the permission of the Publisher. For details see the text.

membrane. If the measured area is illuminated with a much stronger light pulse, the fluorescent compound undergoes an irreversible, light-induced decomposition called photobleaching. However, the fluorescence intensity in the bleached area will show a recovery in time subsequent to the bleaching, since the non-bleached labeled molecules from the environment outside of the monitored area will diffuse in and bleached molecules will diffuse out of the bleached (and measured) area (Zs.-Nagy et al., 1984). The speed of this recovery of fluorescence intensity depends on the lateral diffusion coefficient (D) of the labeled molecule. D can be calculated from the recovery curve by means of known mathematical formulas (Axelrod et al., 1976).

Although the principle of the FRAP technique is rather simple, the proper utilization of such an instrument excluding all the possible sources of errors and artifacts is by far not easy. At the same time the system must be computerized in order to assure an automatic data collection and a bias-free computation of the diffusion coefficients. The composition and function of our FRAP instrument has been described elsewhere (Zs.-Nagy et al., 1984, 1988).

There are two unique aspects of our own FRAP system. One is that in our studies of hepatocytes we used compact tissues rather than isolated cell preparations. The detailed procedures and validities of our unique method have been discussed in the past (Zs.-Nagy et al., 1984, 1988). Second, for hepatocyte membrane protein studies we used an autofluorescence which was enhanced in its intensity by a prior treatment with hydrogen peroxide solution (Zs.-Nagy et al., 1988). The characteristics and the origin of this autofluorescence were intensively studied. It turned out that this fluorescence comes from oxidised riboflavin bound to membrane protein(s) and is abundantly available for FRAP studies (Nokubo et al., 1988, 1989). Lipid lateral diffusion coefficients (Dl) were determined by the use of an external probe, *N*-4-nitrobenzo-2-oxa-1,3-diazolylphosphatidylethanolamine (NBD-PE, Molecular Probes, USA) (Zs.-Nagy and Kitani, 1996).

Studies from our laboratories for enzyme activity measurements discussed in this manuscript are all previously published works. The technical details can be found in each paper quoted.

3. Results

Fig. 2 shows one example of our series of studies for protein lateral diffusion (Dp) (Zs.-Nagy et al., 1993b). During the observation period, the fluorescence intensity is very stable. Once it is bleached by a 10 000-fold stronger (in energy) laser beam, the intensity is decreased considerably (Zs.-Nagy et al., 1984, 1988). However, after a brief bleaching period, the fluorescence intensity in the monitored area is gradually recovered. The recovery curve is the function of Dp. The diffusion coefficient can be automatically computed by the computer program (Zs.-Nagy et al., 1984, 1986a).

Fig. 3 shows a typical change with age of Dp in male F-344 rats (Zs.-Nagy et al., 1986a). The change of Dp during aging was surprisingly linear, resulting in a highly

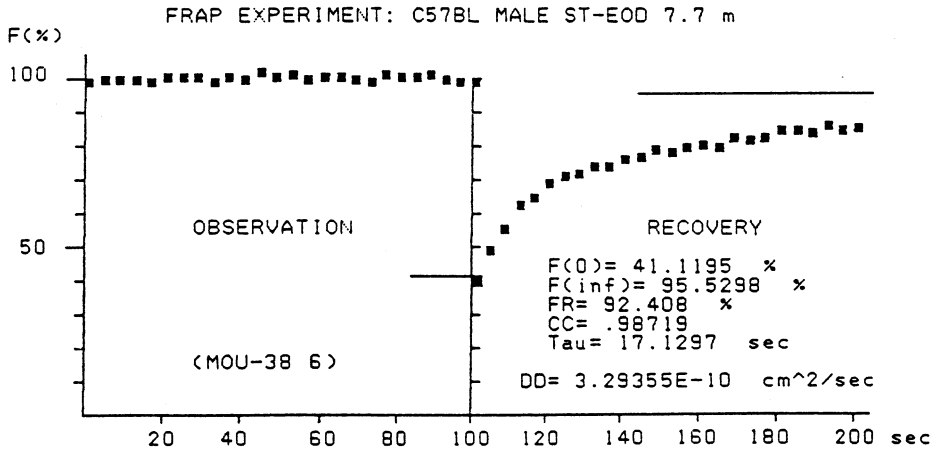

Fig. 2. The computer plot of the FRAP experiment. CC-correlation coefficient of fit to the reciprocal equation of Yguerabide et al. (1982). Reproduced from Zs.-Nagy et al. (1993b) with the permission of the Publisher.

significant linear correlation between Dp and animal age. Fig. 4 shows a similar linear relationship in C57BL mice of both sexes (Zs.-Nagy et al., 1989). Fig. 5 shows results obtained from *Peromiscus leucopus*, a long-lived rodent species (maximal life span is 8 years +), and *Mus musculus* also (a wild type mouse strain with a relatively short life span, 1.5 year). The slope value is much lower in a

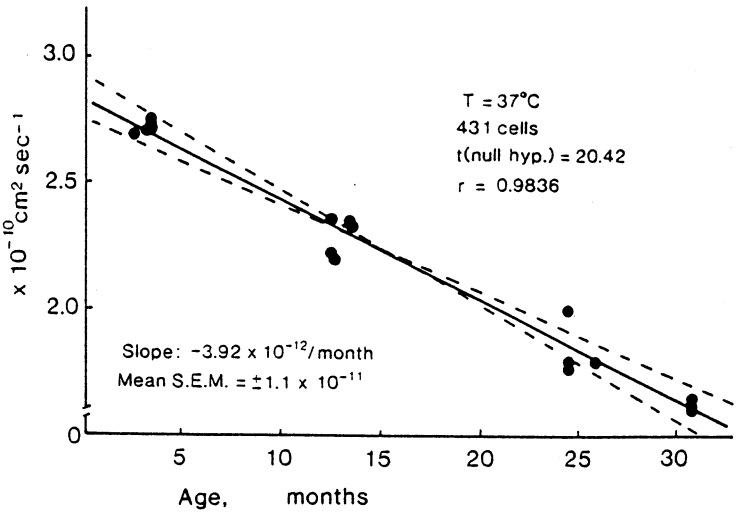

Fig. 3. The age-dependence of the diffusion coefficient of proteins (ordinate) in the hepatocyte surface membrane of male F-344 rats as measured by the FRAP method. Reproduced from Zs.-Nagy et al. (1986a) with the permission of the Publisher.

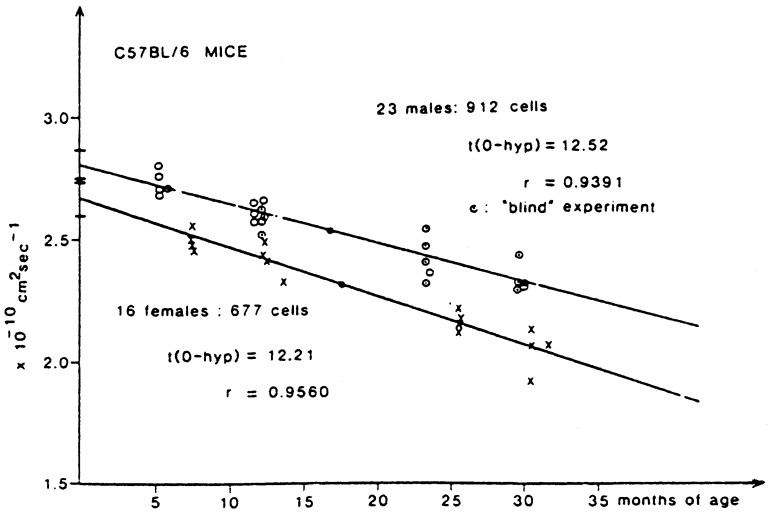

Fig. 4. The age-correlation of the values of individual averages of the lateral diffusion coefficient of cell membrane proteins (D) of hepatocytes as revealed by linear regression analysis for male (O) and female (X) mice. Reproduced from Zs.-Nagy et al. (1989) with the permission of the Publisher.

long-lived rodent but, interestingly, the intercepts at the Y axis were almost identical for the two rodent species (Zs.-Nagy et al., 1993a).

Table 1 summarizes our past observations on Dp in various animal species. Although the slope values were variable, the linear decline with age of Dp was a universal observation.

Fig. 6 summarizes our recent study on Dl of hepatocyte surface membranes in F-344 rats. Again the decline in Dl was very linear with age, although the absolute Dl value was one order of magnitude greater than Dp (Zs.-Nagy and Kitani, 1996).

Fig. 7 shows our recent observation on Dp of striated muscle cell surface membranes in male C57BL mice (Zs.-Nagy et al., 1998). In this study, an external probe (Con A Fl) was used. Again, we found a very linear decline with age for Dp.

4. Discussion

4.1. Steady decline in Dp and Dl with age

Our series of studies using the FRAP method have shown that the Dp as well as the Dl of hepatocyte surface membranes undergoes a steady decline with age in a linear fashion (Zs.-Nagy et al., 1989, 1993a,b, 1998; Zs.-Nagy, 1994; Zs.-Nagy and Kitani, 1996). These observations are in contrast with past reports obtained by the use of the 'fluorescence anisotropy' technique. For example, some past studies have shown a decline in lipid fluidity of hepatocyte membranes with age, while some others reported an increase (Armbrecht et al., 1982). Results from our own

laboratory have shown that the membrane fluidity decreases with age in hepatocytes from male rats, while in females, the fluidity increased up to middle age and then declined thereafter (Nokubo, 1985). Many different explanations for these discrepant results are possible, but one in particular seems most likely: fluorescence anisotropy is not a reliable method for measuring a lipid fluidity in hepatocyte surface membranes and possibly other membrane preparations as well.

Our FRAP studies, in contrast, have consistently shown that hepatocyte surface membrane qualities (in terms of both protein and lipid lateral diffusions) decline in a linear fashion with age in all rodent species tested, suggesting that this change is a universal phenomenon, at least in rodents. Further, our recent study on striatal muscle cells (Fig. 7) (Zs.-Nagy et al., 1998) as well as our very recent observations on cortical neuronal cells (Zs.-Nagy et al., 1999) suggest that the linear decline in Dp of surface membranes is a phenomenon not only for hepatocytes but for some other cell types also.

Interestingly, both Dp and Dl can be increased by dietary restriction (DR) (Zs.-Nagy and Kitani, 1996), however, there appear to be some upper limits for the effect of DR. For example, Dp of hepatocyte surface membranes in 27 month old mice could be raised by 16% by means of an every other day (EOD) regimen for 3.5 months, however, DR performed for 15 months was unable to further raise Dp (Zs.-Nagy et al., 1993b). Dl in 30 month old F-344 rats could be raised by life-long DR (EOD feeding) to the level of 17 month old rats but not to younger levels. Pretreatments with pharmaceuticals can modify Dp as will be discussed later. These

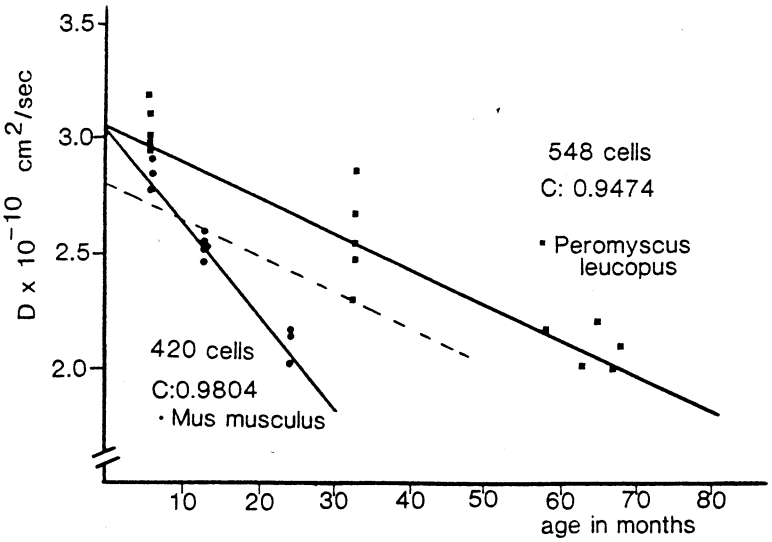

Fig. 5. Comparison of the negative linear age-correlation of the protein diffusion coefficient (D) in males of the two wild strain mice. Note that the differences between the slopes are highly significant. Dotted line indicates the age-correlation of this parameter for C57BL/6 male mice published before Zs.-Nagy et al. (1989). Reproduced from Zs.-Nagy et al. (1993a) with the permission of the Publisher.

Table 1
Linear regression analyses for lateral diffusion coefficients of proteins in hepatocyte surface membranes (Y axis, 10^{-10} cm^2/s) and animal age (X axis, months) in different groups of rodents as examined by the FRAP system in the author's laboratory[a]

Animals	$I \pm$ S.D.	$-S \pm$ S.E.M.	References
Fischer 344 rats			
M	2.83 ± 0.08	0.039 ± 0.002	(Zs.-Nagy et al., 1986a)
F	1.69 ± 0.10	0.018 ± 0.002	
Fischer 344 rats			
M	2.85 ± 0.15	0.038 ± 0.005	(Zs.-Nagy et al., 1986b)
Wistar rats			
M	2.77 ± 0.11	0.027 ± 0.004	(Kitani et al., 1988)
C57BL/6 mice			
M	2.81 ± 0.06	0.016 ± 0.001	(Zs.-Nagy et al., 1989)
F	2.67 ± 0.07	0.020 ± 0.002	
C57BL/6 mice			
M	2.74 ± 0.06	0.013 ± 0.001	(Zs.-Nagy et al., 1993b)
Peromyscus leucopus			
M	3.13 ± 0.16	0.016 ± 0.002	(Zs.-Nagy et al., 1993a)
F	3.14 ± 0.06	0.015 ± 0.001	
Mus musculus			
M	3.10 ± 0.08	0.042 ± 0.003	(Zs.-Nagy et al. 1993a)
F	3.09 ± 0.05	0.038 ± 0.001	
BN/Bi rats			
M	2.75 ± 0.17	0.029 ± 0.004	(Kitani et al., 1998)
F	2.71 ± 0.15	0.017 ± 0.002	
Fischer 344 rat			
F	2.62 ± 0.08	0.026 ± 0.001	(Kitani and Zs.-Nagy, unpublished data)

[a] $D = (I - S \cdot X)10^{-10}$ cm^2 s^{-1}. M, males; F, females.

modifications may provide some clues for understanding the physiological significance of these parameters.

In summary, a steady decline with age in Dp and Dl for surface membranes as revealed by our FRAP studies is compatible with the first tenet of the MHA: the physical–chemical qualities of cell surface membranes are altered by aging (Zs.-Nagy, 1991, 1994). Do these changes then lead to a general intracellular enzyme activity decline?

4.2. Intracellular enzyme activities during aging

Could a decline in the lateral diffusion coefficients of both proteins and lipids as have been shown above be the proof of the MHA? As is shown schematically in Fig. 8, the MHA by Zs.-Nagy attempts to explain the assumed general decline of intracellular enzyme activities (Zs.-Nagy, 1991, 1994). Fig. 9 summarizes some of

our previous work on hepatic microsomal P-450 dependent enzyme activities in rats of both sexes (Fujita et al., 1986). In rat livers, these enzyme activities are generally several to 10-fold higher in male rats than females, when they are young. As they get older, enzyme activities generally decline in male rat livers, which are consistent with the MHA. However, in female rat livers, there is no tendency of a decline with age for their enzyme activities, showing identical enzyme values for young and old rats (Fujita et al., 1986).

In male rat livers,, the activity of androstendione 5α-reductase (which is not a P-450 enzyme but is an important enzyme in the hepatic microsome for the metabolism of androgens) increases several-fold with age (Fig. 9), revealing a complete feminization of P-450 (and related) enzyme patterns of male rat liver enzymes with age (Fujita et al., 1986). In C57BL mouse livers, P-450 enzyme activities stay essentially unchanged with age (Fujita et al., 1986). These enzymes are those located in microsomal membranes. Thus, the general decline in intracellular enzyme activities assumed by the MHA does not hold for P-450 enzymes in female rat and mouse livers of both sexes (Fujita et al., 1986). Even for male rat livers, it does not hold, since androstendione 5α-reductase activity increases several-fold with aging in male rat livers (Fujita et al., 1986).

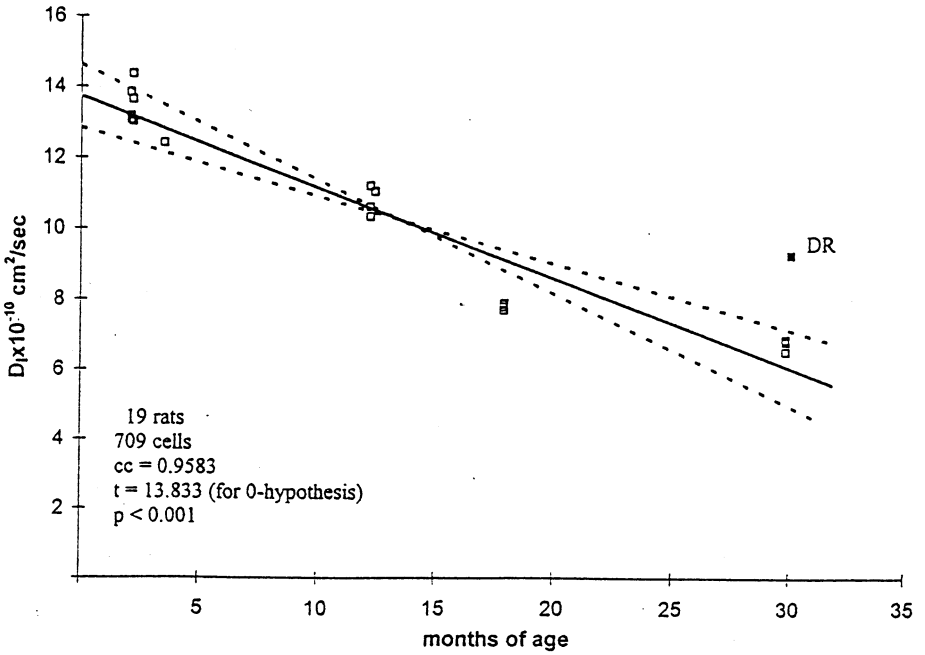

Fig. 6. The age dependence of lipid lateral diffusion coefficient (D1) in ad lib fed animals. Each open square symbol indicates the average value of one rat. Dotted lines indicate the possible scatter of the regression line calculated from the S.D. of the intercept. Black square indicates the average value obtained in the dietary restricted group, being significantly different from the age-matched control. Reproduced from Zs.-Nagy and Kitani (1996) with the permission of the Publisher.

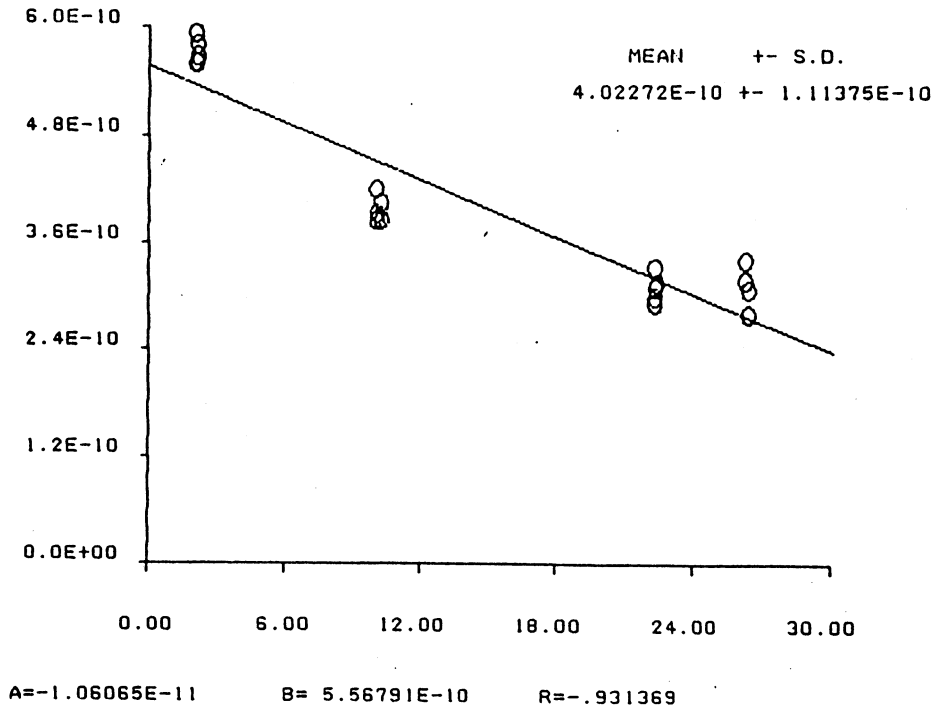

Fig. 7. The correlation of age X axis, in months with Dp values Y axis, in cm^2/s in male mice for the Con-A-receptors in the quadriceps femoris muscle. Each circle represents the average of one animal. A, the slope of the line/month of age; B, the intercept value; R, the correlation coefficient. Reproduced from Zs.-Nagy et al. (1998) with the permission of the Publisher.

According to the MHA, intracellular cytosolic enzyme activities should also generally decline with age (Zs.-Nagy, 1994). Glutathione *S*-transferase (GST) is a typical cytosolic detoxifying enzyme. Activity changes of GSTs with age in female F-344 rats (Carrillo et al., 1990, 1991) and C57BL mice (Carrillo et al., 1989) are shown in Figs. 10 and 11 respectively. Although in the activities toward one substrate (DCNB) among five tested in female mice, there was a mild (and significant) decline with age, enzyme activities were essentially unaltered with age for the other four substrates (Fig. 11) (Carrillo et al., 1989). Similarly, there was no significant difference between young and old female rat livers (Fig. 10) (Carrillo et al., 1990, 1991). It is clear from these results that a decline in GST enzyme activities with age does not hold in general terms. Another study from a Dutch group also confirmed unaltered enzyme activities with age for GST activities in BN/Bi rat livers (Rogiers et al., 1991). They also showed that there is essentially no differences in the levels and proportion of subunit monomers for GST proteins between young and old animal livers (Rogiers et al., 1991).

Catalase (CAT) is an important (mainly cytosolic) enzyme needed for chain reactions for the detoxification of free radicals. Richardson and coworkers have

shown a steady decline for CAT activities as well as its messenger RNA levels with age in male rat livers (Semsei et al., 1989). However, again, there is a distinct sex difference for this change, since CAT activity increases with age in female rat livers (Fig. 12) (Rikans et al., 1991). The sex difference in CAT enzyme activity change with age in rat livers was also confirmed by our group (Carrillo et al., 1992). Discrepancies in age-related changes for hepatic drug metabolism summarized in a previous extensive review by the author clearly show that no generalization is possible for the trend for age-related changes for these enzyme activities (Kitani, 1988, 1991).

In the discussion after a lecture by the present author in one symposium (Kitani, 1991), Zs.-Nagy defended the MHA by stating that hepatocytes are special because of their increasing tendency for polyploidization. He also raised SOD and CAT in the liver as an example of the declining tendency of activities with additional evidence for decreasing mRNA levels for these enzymes (Semsei et al., 1989). The declining tendency for SOD activities in the liver is really not consistently seen in the past literature, as discussed in detail by the author (Kitani, 1991). Further his notion that CAT activities in rat liver decline with age can hold only for males but not for females as discussed above.

Are liver enzymes exceptions for the rule of the MHA? Figs. 13–15 summarize our own observations on SODs, CAT, and glutathione peroxidase (GSH Px) activities in several different brain regions of F-344 rats (Carrillo et al., 1992).

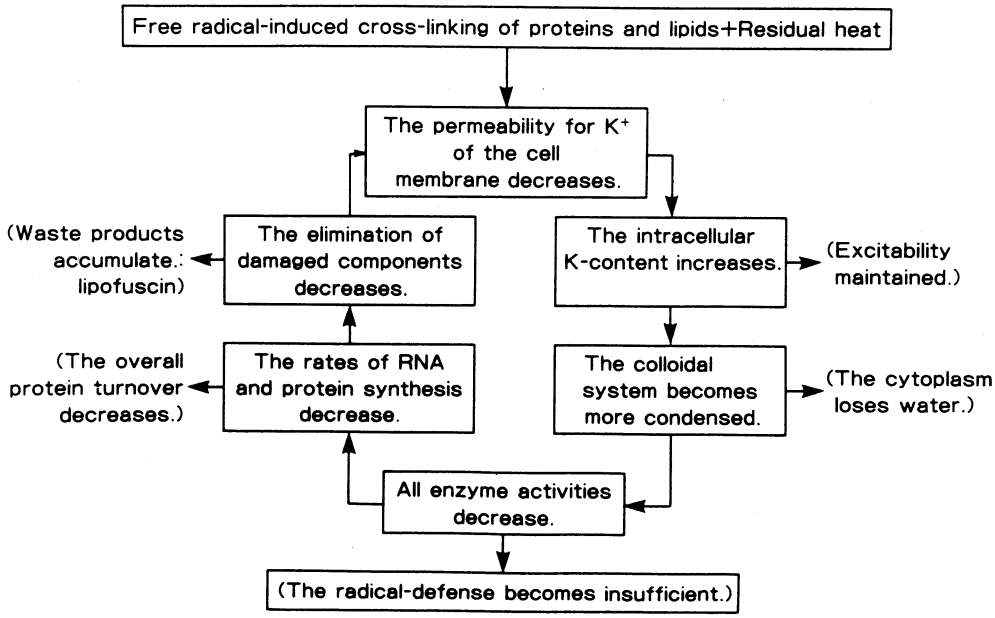

Fig. 8. A summarized conceptual outline of the membrane hypothesis of aging proposed by Zs.-Nagy. The arrows indicate causal interrelationships between the events described. Reproduced from Zs.-Nagy (1991) with the permission of the author and Publisher.

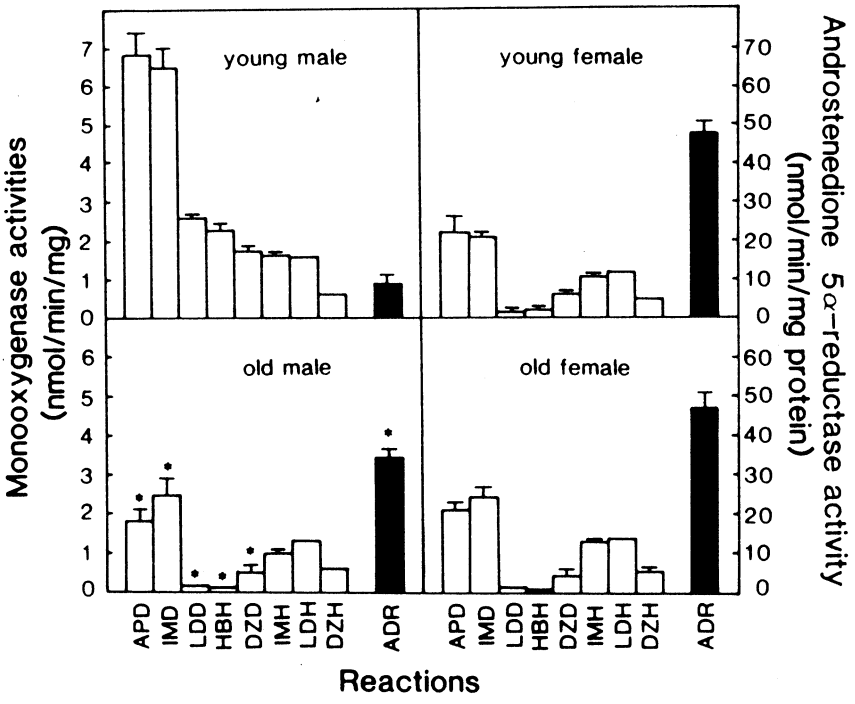

Fig. 9. Hepatic microsomal drug metabolizing enzyme activities in young (6 month old) and old (30 month old) male and female rats. *Significantly different from corresponding activities in young male rats. APD, aminopyrine *N*-demethylase; IMD, imipramine *N*-demethylase; LDD, lidocaine *N*-demethylase; HBH, hexobarbital hydroxylase; DZD, diazepam *N*-demethylase; IMH, imipramine hydroxylase; LDH, lidocaine hydroxylase; DZH, diazepam hydroxylase; ADR, androstendione 5α-reductase. Reproduced from Fujita et al. (1986) with the permission of the Publisher.

Except for SOD activities in male rat brains which were several-fold greater in old animals than young ones, enzyme activities stayed essentially unchanged with age. It should be noted that we found no decrease with age for any of these enzyme activities in different brain regions. Williams et al. (1995) further have shown that mRNA levels of SOD are several-fold elevated in several brain regions such as hippocampus, *S. nigra*, etc. in old male rats (Williams et al., 1995), which exactly correspond to our observations as shown in Fig. 13 (Carrillo et al., 1992). Further, SOD activities in heart and other striated muscles are reported to be generally greater in old animals than in the young.

One criticism which is always raised by Zs.-Nagy is that in vitro measurements of enzyme activities are not valid because of the dilution of samples, which causes a loss of the most important in vivo factor, the intracellular dehydration with age and differences in cation concentrations among animals of different ages as well. This important point is well taken by the present author. Fig. 16 shows one response to such a criticism. Acetaminophen is metabolized in the liver by means of glucuronidation and sulfation. The determination of these metabolites in urine allows

an in-vivo measurement of enzyme activities for glucuronosil transferase and sulfatase in the human liver (Wynne et al., 1990) Although the interindividual variation is wide in each group, there is essentially no difference in enzyme activities between young and old fit subjects (Wynne et al., 1990) Only when old subjects became frail, did these enzyme activities become much lower than in the former two groups. Much information obtained from clinical pharmacokinetic studies also strongly suggests that in vivo drug metabolizing enzyme activities in the liver do not decline with aging, as long as subjects are healthy (see Kitani, 1988).

The arguments raised above that enzyme activities do not decline with age in liver and brain cells also apply more generally. Let us take kidney functions with aging as an example. The long standing belief that glomerular filtration rate (GFR) steadily declines with aging in humans (Rowe et al., 1976) has been totally revised by a recent report of the Baltimore Longitudinal Study (BLS) (Lindeman et al., 1985). In their recent report, it has been dearly shown that GFR does not decline with age per se, rather only the accompanying morbidities causing GFR decline (Lindeman et al., 1985). GFR is a physical process (a filtration) involving no biochemical reactions. However, renal tubular reabsorption processes are strong, biochemically active processes requiring metabolic energy. We need to recognize again, that as long as they maintain their health, elderly subjects ususally do not develop glucosuria after meals or even after a glucose loading test. This clearly indicates that the reabsorption process for glucose is functioning perfectly well in

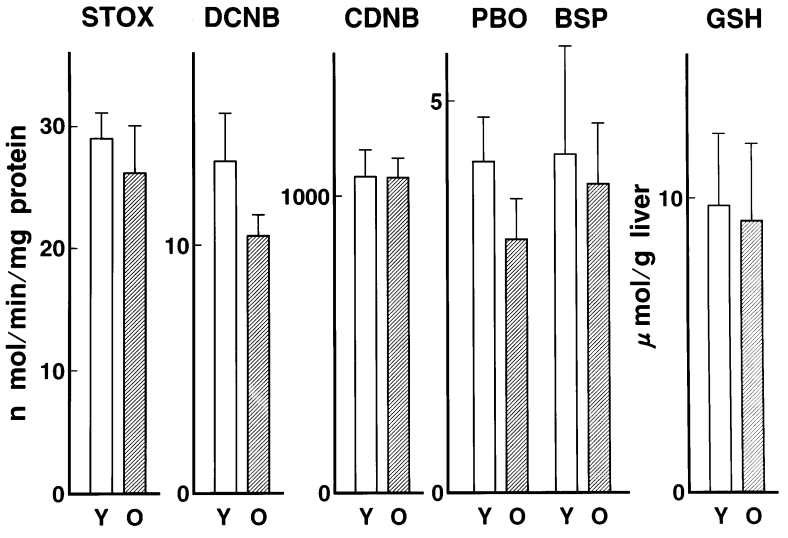

Fig. 10. Enzyme activities of GSTs toward five different substrates and glutathione concentration in liver cytosol obtained from young (Y, 8 month old) and old (O, 25-month-old) female F-344 rats. STOX, styrene oxide; DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene; PBO, benzal-acetone; BSP, sulfobromopthalein sodium tetrahydrate; GSH, reduced glutathione. This Figure was made from the data reported in Carrillo et al. (1990).

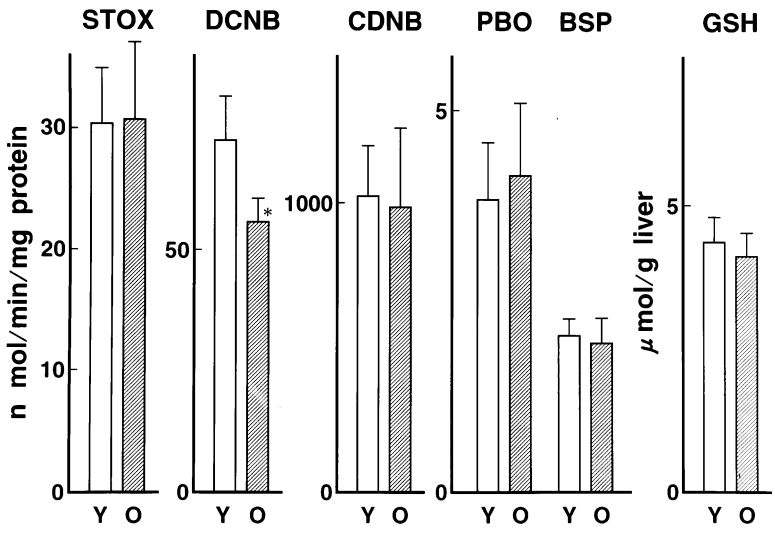

Fig. 11. Enzyme activities of GSTs toward five different substrates and glutathione concentration in liver cytosols obtained from young (Y, 8 month old) and old (O, 27 month old) female C57 BL mice. *Significantly different from the corresponding values in young mouse livers. This Figure was made from the data reported in Carrillo et al. (1989). Abbreviations are the same with those in Fig. 10.

the healthy elderly as in their younger counterparts even under the stress of a so-called loading test. If aging causes a steady decline in enzyme activities, there should be a tendency toward physiological glucosuria at least on a glucose loading test, and this does not occur in the healthy elderly.

What the originator of the MHA (Zs.-Nagy) should provide in the future is ample (and convincing) evidence that intracellular enzyme activities generally decline with age, which at present does not exist. It is important to express the view of the author of this manuscript that there is no intention to negate the notion that membrane structures are susceptible to aging processes. On the contrary, the present author is one of the coauthors for many studies showing that at least surface membranes of cells are significantly susceptible to aging processes (Zs.-Nagy et al., 1986a,b, 1989, 1993a,b, 1998, 1999; Kitani et al., 1988; Zs.-Nagy and Kitani, 1996). However, the interpretation of such observations must be done very carefully. Second, the author does not intend to insist that all intracellular enzyme activities are intact during aging. Rather, the author is of the opinion that levels of many enzymes for protein synthesis and protein degradation tend to decline with aging. However, just because these opposing processes change in harmony with each other, most intracellular protein concentrations including enzymes and their activities stay essentially unchanged with aging. Further, there is evidence that some intracellular enzyme activities tend to increase with age as discussed above.

From the foregoing, the author concludes that the attempt to explain the aging process on the basis of general decreases in intracellular enzyme activities does not help our further understanding of the aging process.

4.3. The physiological significance of a general decline in the diffusion coefficient of proteins and lipids with aging

We have clearly shown that Dp and Dl of cell surface membranes steadily decline with age (Zs.-Nagy et al., 1986a,b, 1989, 1993a,b, 1998, 1999; Kitani et al., 1988; Zs.-Nagy and Kitani, 1996). However, the author of the present article does not regard these observations as proof for the MHA proposed by Zs.-Nagy as discussed above. In order to understand the physiological significance of these observations, the author will provide several past observations possibly related to this subject.

Fig. 17 shows results of our previous study examining the effect of age on hepatic uptake rates of ouabain (Ohta et al., 1988) as well as taurocholic acid (Ohta and Kitani, 1990) using isolated hepatocyte preparations obtained from rats of different ages in comparison to Dp of hepatocyte surface membranes. It is clear that these hepatic functions steadily decline with age. Ouabain (as well as taurocholic acid) is known to be efficiently taken up by hepatocytes and excreted into the biliary system once it is i.v. injected in rats. Uptake processes are clearly saturable suggesting that these are the carrier-mediated processes (Eaton and Klaassen, 1978, 1979), although neither the carrier protein(s) nor the energy-source for ouabain uptake has been clarified. Aging decreased the apparent V_{max} value for ouabain but apparent K_m values were not different between young and old rats (Ohta et al., 1988). An ordinary interpretation of such data is a decrease in the number of carrier units with no qualitative alteration for carrier proteins. However, this interpretation holds only when the mobility of carriers is not altered, since, the V_{max} is a function of carrier number and carrier mobility (de Pont and Bonting, 1977). Accordingly,

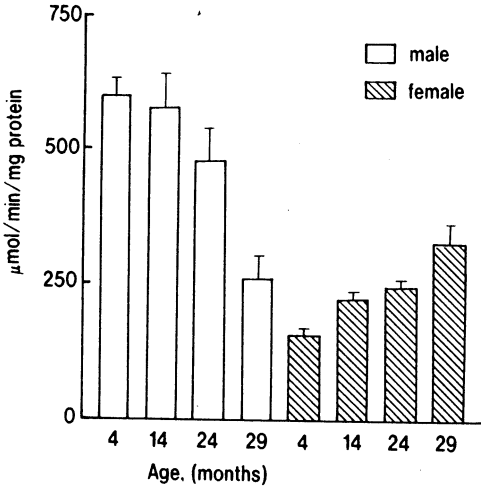

Fig. 12. Age-related changes in catalase activities in livers of Fischer 344 rats of both sexes. While activities in male rat liver tend to decline with age, in female rat liver the opposite tendency (i.e. an increase with age) is clearly observed. Reproduced from Rikans et al. (1991) with the permission of the authors and Publisher.

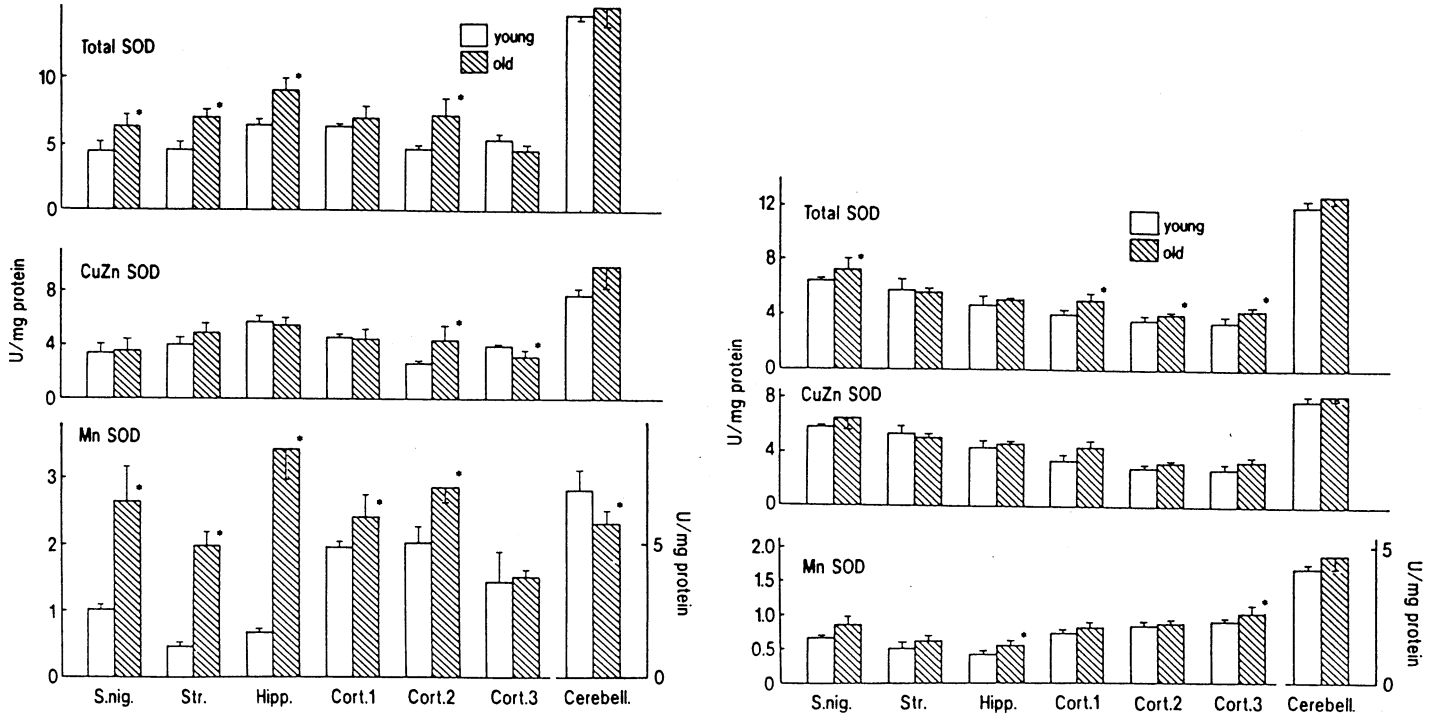

Fig. 13. Enzyme activities of superoxide dismutase (SOD) in different brain regions of young and old male (left panel) and female (right panel) rats. White columns indicate values for young animals and shadowed columns indicate values for old animals. Cort 1, 2 or 3 indicate cerebral cortex of frontal, parietotemporal and occipital regions, respectively. *Significantly different from the corresponding values in young rats ($P < 0.05$, t-test). Reproduced from Carrillo et al. (1992) with the permission of the Publisher.

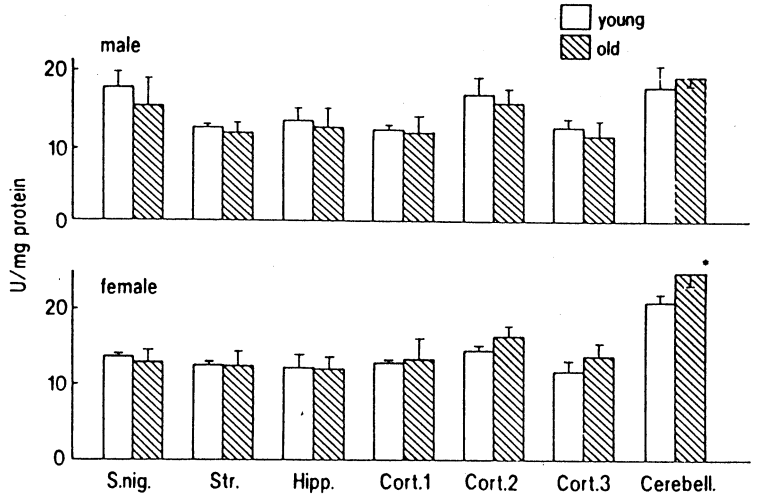

Fig. 14. Enzyme activities of catalase (CAT) in different brain regions of young and old rats of both sexes. White columns indicate values for young animals and shadowed columns indicate values for old animals. Cort, 1, 2 or 3 indicate cerebral cortex of frontal, parietotemporal and occipital regions, respectively. *Significantly different from the corresponding values in young rats ($P < 0.05$, t-test). Reproduced from Carrillo et al. (1992) with the permission of the Publisher.

another possibility which the author has raised is that the decreased mobility of surface membrane proteins of hepatocytes in old rats could be at least a partial cause for the decrease in the hepatic uptake rate (and V_{max}) (Kitani et al., 1988). When Sp was administered orally for 4 days prior to the study, both Dp as well as ouabain biliary excretion for the first 10 min after i.v. injection increased in rats of three different ages; however, the correlation between the Dp and the excretion was preserved (Kitani et al., 1988) (Fig. 18). We have recently confirmed that Dl was also increased by Sp pretreatment (Kitani and Zs.-Nagy, 1998). Further, estradiol which is known to decrease the uptake rates for ouabain and many other organic anions by the liver (Rosario et al., 1978) was shown to decrease the Dl as well as the Dp of hepatocyte surface membranes (Kitani and Zs.-Nagy, 1998).

Table 2 summarizes effects of aging and Sp and estrogen treatments on Dp (and Dl) so far examined by ourselves and others. It is clear that there are parallel changes for Dp (and Dl) and hepatocyte functions caused by these manipulations (i.e. increase by Sp and decrease by aging and estradiol).

The effect of Sp on Dp (and Dl) requires special attention in terms of liver physiology. At least two carefully performed studies in the past have reported a decrease in the lipid fluidity of hepatocyte surface membranes as determined by the fluorescence anisotropy method (Miner et al., 1983; Smith and Gordon, 1988). It has been generally believed that surface membrane lipid fluidity regulates the mobility of membrane proteins and consequently their functions (Kimelberg, 1977). If this is true, Sp should decrease the protein mobility of surface membranes leading to the decrease in hepatic uptake as well as subsequent biliary excretion of various

Table 2
Summary of effects of aging, spironolactone, and estradial pretreatment on Dp, M, and hepatocyte functions in rats as survey from the past literature[a]

	Lipid fluidity	DP	D1	Hepatic uptake	Biliary excretion
Aging	↓ (Hegner and Platt, 1975; Nokubo, 1985) ↑ (Armbrecht et al., 1982) – (Nokubo, 1985)	↓ (see Table 1)	↓ (Kitani and Zs.-Nagy, 1996; Kitani et al., 1988)	↓ (Ohta et al., 1988)	↓ (Kitani et al., 1988)
Spironolactone	↓ (Miner et al., 1983; Smith and Gordon, 1988)	↑ (Kitani et al., 1988)	↑ (Kitani and Zs.-Nagy, 1996)	↑ (Eaton and Klaassen, 1978)	↑ (Klaassen, 1974; Kitani et al., 1988
Estradiol	↓ (Smith and Gordon, 1988)	↓ (Kitani and Zs.-Nagy, 1996)	↓ (Kitani and Zs.-Nagy, 1996)	↓ (Stacey, 1986)	↓ (Gummucio and Valdiniesso, 1971)

[a] ↓, decrease; –, unchanged; ↑, increase.

organic compounds. In contrast, there are a number of studies including those of the author himself showing an enhancing effect of Sp for hepatic uptake and biliary excretion of these substances (Klaassen, 1974; Eaton and Klaassen, 1978, 1979; Kitani et al., 1988). Thus, the decrease in lipid fluidity caused by Sp pretreatment as shown by the fluorescence anisotropy method (Miner et al., 1983; Smith and Gordon, 1988) does not explain any of the data on the Sp effects on hepatocyte functions. Dp (and Dl) as determined by FRAP, on the other hand, provides a reasonable agreement on the relationships between physical-chemical qualities of surface membranes and physiological functions of hepatocytes (Kitani et al., 1988).

From the foregoing, the author suggests that Dp (and Dl) as determined by FRAP has a more reasonable basis for explaining cellular functions, at least those related to surface membranes (such as hepatic uptake). The above argument is, however, only speculative as to the physiological significance(s) of Dp

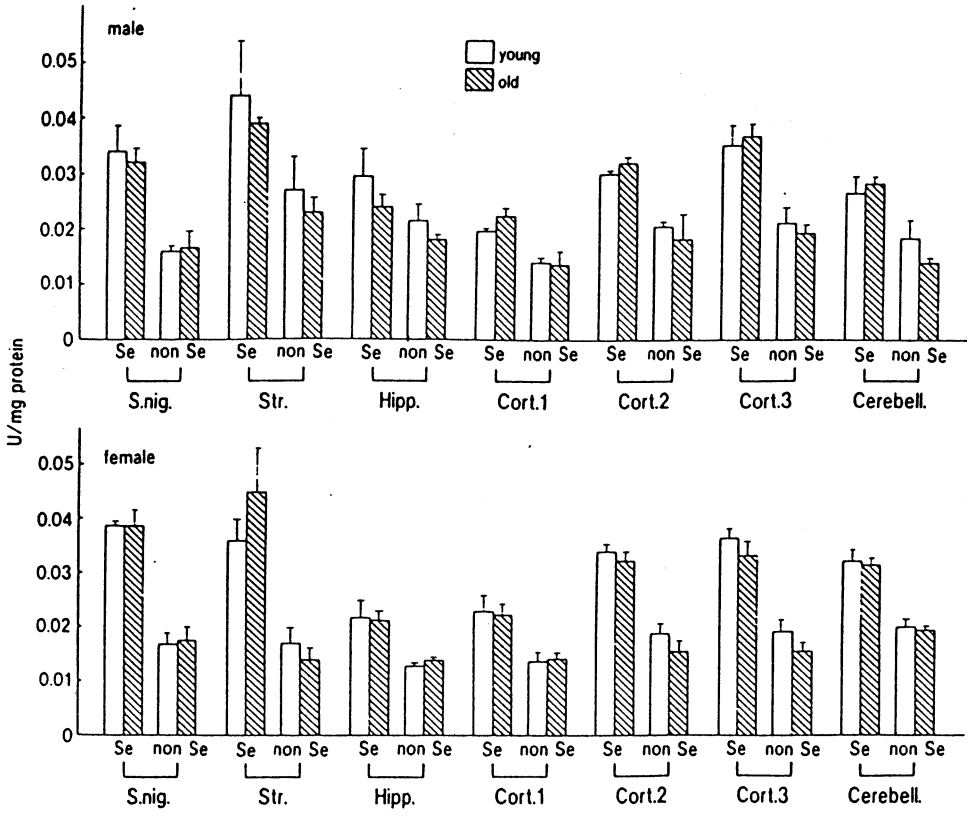

Fig. 15. Enzyme activities of glutathione peroxidase (GSH-Px) in different brain regions of young and old rats of both sexes. White columns indicate values for young animals and shadowed columns indicate values for old animals. Cort, 1, 2 or 3 indicate cerebral cortex of frontal, parietotemporal and occipital regions, respectively. Reproduced from Carrillo et al. (1992) with the permission of the Publisher.

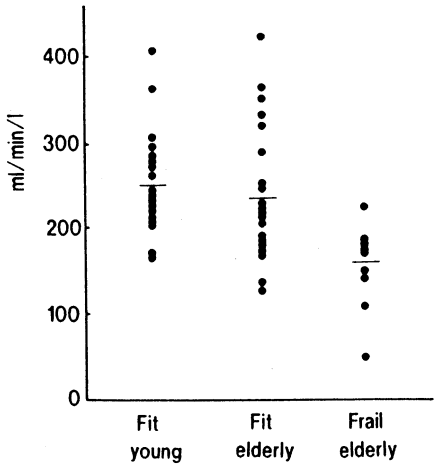

Fig. 16. Paracetamol (acetaminophen) clearance per unit volume of liver in three groups ($P < 0.01$, ANOVA). Reproduced from Wynne et al. (1990) with permission of the authors and Publisher.

and Dl. The real physiological significance of lateral mobilities of proteins (Dp) and lipids (Dl) of cell surface membranes should and can only be explored in the future.

In conclusion, our FRAP studies have dearly shown that there is a general decline with aging in lateral mobilities of proteins and lipids not only for hepatocytes but also for other cell types, which agrees with the first part of the MHA that

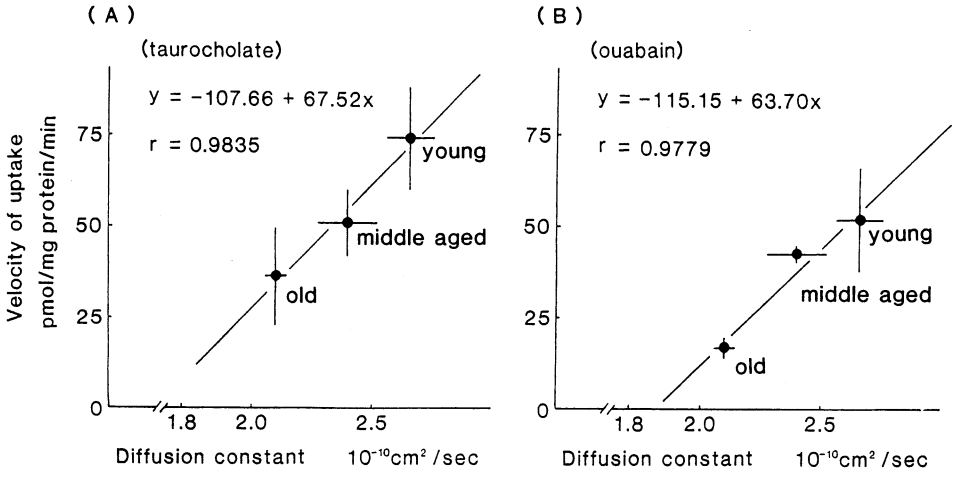

Fig. 17. The relationships between uptake velocity for taurocholate (A) at 1 μM and for ouabain at 8 μM (B) and the diffusion coefficients of hepatocyte surface membrane proteins in three different age groups. Y (young, 4 months old), M (middle aged, 10–12 months old) and O (old, 24–26 months old). Reproduced from Ohta and Kitani (1990) with the permission of the Publisher.

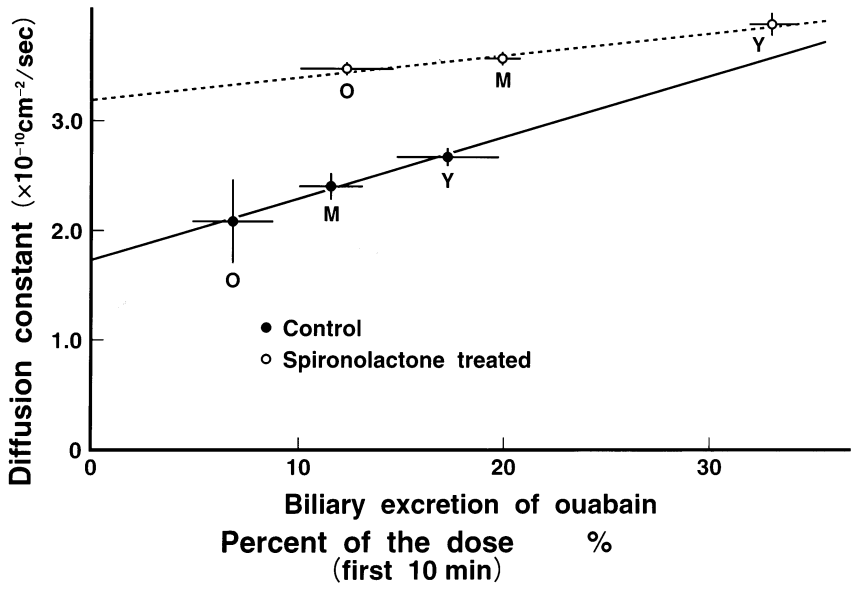

Fig. 18. Comparison of the diffusion coefficients of hepatoctye plasma membrane proteins and ouabain excretion into the bile (first 10 min value) in rats of different ages with and without Sp pretreatment. Y (young, 4 months old), M (middle aged, 10–12 months old), and O (old, 24–26 months old). Reproduced from Kitani et al. (1988) with the permission of the Publisher.

there occur changes in physical-chemical qualities of cell surface membranes with aging. However, the attempt of this hypothesis to explain any general decline with aging in intracellular enzyme activities does not have any solid experimental basis, since past literature does not provide any convincing evidence supporting the notion that all intracellular enzyme activities decline with aging. Accordingly, the interpretation of our FRAP studies must be limited and should not be extrapolated to support the MHA. The MHA, first of all, must provide solid experimental evidence that all intracellular enzyme activities generally decline with aging in order to survive as a theory of aging in the future.

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