Arch. Gerontol. Geriatr., 12 (1991) 133–154

AGG 00378

Aging of the liver: facts and theories *

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(Received 1 October 1990; accepted 22 October 1990)

Summary

Although most theories of aging assume that cellular functions decline with aging, many intracellular functions in the liver, such as enzyme activities, stay fairly stable in old age. This does not appear to be an antifact caused by in vitro experimental design, since in vivo pharmacokinetic data also demonstrate that most, if not all, biotransformation capacities of the liver remain stable during the aging process, if we take the decline in liver volume with age into account. Thus, many theories to explain the decline in cellular functions during aging appear to be based on erroneous assumptions.

The stability of cellular function in old age does not necessarily mean, however, that all cellular functions are identical for young and old organisms. Once unfavorable conditions, such as malnutrition, infection, etc., are involved, the response of the liver is quite different for young and old subjects, demonstrating a more efficient and versatile response in young animal livers in comparison to old livers. Large differences in enzyme activities between young and old organisms appear during stress and especially during recovery from stress. Accordingly, any aging theory needs to explain a potential difference in liver functions (such as response capability) rather than the difference in basal functions.

In contrast to rather stable intracellular functions, the uptake function of the hepatocyte surface membranes was found to be progressively decreased with age. This was shown for at least two different types of carrier systems in the surface membranes. Although the decrease of carrier unit number for these substances remains a possible causal factor, we suggest that the decline in hepatic uptake with age is at least partially the result of a gradual decrease in the mobility of surface membrane proteins, which can be shown by the fluorescence recovery after photobleaching (FRAP) technique.

Theories of aging need to be elaborated on the basis of unbiased observations on the actual manifestations of cellular aging.

Liver; Aging; Intracellular functions; Membrane function

* Paper presented in the program of the International Symposium 'Fritz Verzár Project – 2000', held from 30 September to 3 October 1990 at Ancona, Italy.

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Introduction

A number of experiments have been performed in the past to elucidate how aging affects cellular and organ functions and, further, to understand the underlying mechanisms. From these studies, as well as from theoretical considerations, a number of hypotheses to explain cellular aging processes have been proposed. However, up to now, none of them appear to be satisfactory. The elaboration of hypotheses and theories of aging is important, since it leads to future studies in a rationale direction. On the other hand, if a hypothesis has a difficulty in explaining the facts obtained from experiments, we need to reconsider it and modify the hypothesis or reexamine whether the experimental results are the real facts.

Many hypotheses are based on the assumption that cellular functions deteriorate as the age of an organism advances and attempt to explain such declines. While some parameters of cellular functions have been shown to be reduced in old organisms, the decline does not appear to be a general rule for aging cells. If most cellular functions are not decreased with age, any theory of aging to explain this decline may confront a problem. The author has been involved with studies of alterations of liver functions during aging and has noted that many, if not all, liver functions do not decline with aging, In this paper, the author will emphasize this aspect of liver aging and attempt to discuss the validity of some of aging theories based on these observations.

Biochemical parameters of hepatocyte function

Microsomal monooxygenase functions

Among various liver functions the microsomal monooxygenase system has been the most extensively investigated in relation to aging, since this enzyme system is involved in the biotransformation of many exogenous as well as endogenous substances, including drugs. Because of obvious clinical implications, many studies have been performed to examine how these drug metabolizing enzyme activities are altered during aging, mostly using rodent livers as a model. In fact, many studies have reported considerable reductions of enzyme activities and cytochrome P-450 isozyme concentrations during aging (Kato et al., 1964; Fujita et al., 1982, 1985a, c, 1986, 1990; Kamataki et al., 1985; Chiba et al., 1986; for review, see Kitani, 1986, 1988). These data have been interpreted in clinical terms as a basis for possible declines of drug metabolizing capacities in elderly humans. Furthermore, with the assumption that the function of this system declines with age, several interesting hypotheses have been proposed. These include structural alterations of microsomal membranes (Stier et al., 1982), the alteration of membrane fluidity (Schmucker et al., 1984; Lee and Yu, 1990), alteration of key enzymes (Schmucker et al., 1983) and more recently, the involvement of lipid peroxidation (Lee and Yu, 1990).

Since the endoplasmic reticulum is a complicated membrane structure consisting of proteins and lipids, it is tempting to assume some physical-chemical alteration as

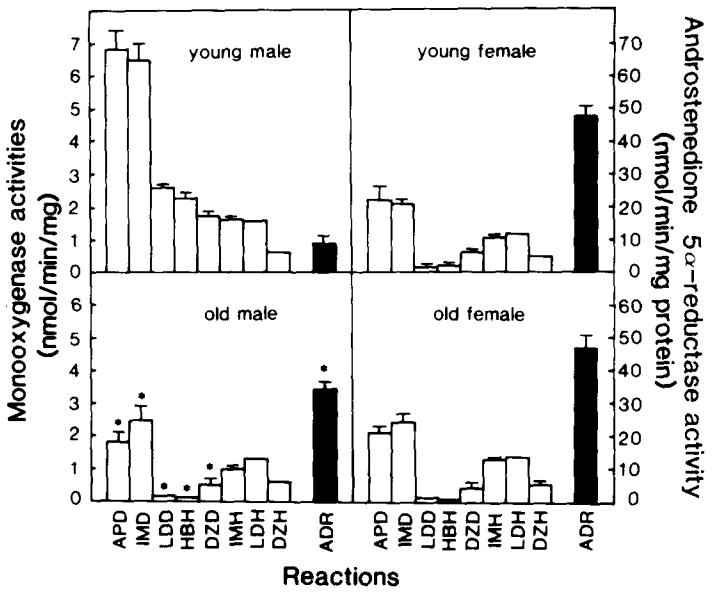

Fig. 1. Hepatic microsomal drug metabolizing enzyme activities in young (6-month-old) and old (30-month-old) male and female rats. * Significantly different from corresponding activities in young male rats. APD, aminopyrine *N*-demethylase; IMD, imipramine *N*-demethylase; LDD, lidocaine *N*-deethylase; HBH, hexobarbital hydroxylase; DZD, diazepam *N*-demethylase; IMH, imipramine hydroxylase; LDH, lidocaine hydroxylase; DZH, diazepam hydroxylase; ADR, androstendione 5α-reductase. (Reproduced with the permission of the publisher. From Fujita et al., 1986.)

the underlying mechanism for age-induced enzyme activity decreases. However, as is shown in Fig. 1, it has become increasingly clear that the age-related decline in microsomal monooxygenase activity is seen only in male rat liver (Fujita et al., 1982, 1985a, c, 1986, 1990; Kitahara et al., 1982; Schmucker and Wang, 1983; Kamataki et al., 1985; Kitagawa et al., 1985; Chiba et al., 1986; for review, see Kitani, 1986, 1988). In female rat liver (Fig. 1) (Fujita et al., 1982, 1985a, c, 1986, 1990; Kamataki et al., 1985; Chiba et al., 1986), in mouse liver (Fig. 2) of at least some strains (Kato et al., 1970; Fujita et al., 1986), and, more importantly, in monkey liver (Sutter et al., 1985; Maloney et al., 1986) the activities of this system were shown to be well preserved during aging (for review, see Kitani, 1988). Only a few exceptional reports exist which describe a minor decline in enzyme activities with age in female rat liver (Kato and Takanaka, 1968; Rikans, 1984). In human liver, too, monooxygenase activities do not decrease with aging (Boobis and Davies, 1984; Woodhouse et al., 1984; Schmucker et al., 1990). One may claim that all these studies were done in in vitro systems, so that the most important factor, namely, the physical-chemical alterations that actually determine the age differences, has been lost. On a theoretical basis such a possibility is not excluded. However, we can assess these enzyme activities in vivo by using pharmacokinetic analysis (Kitani, 1988; Wynne et al., 1989). While some studies have shown a decreased hepatic clearance of drugs in

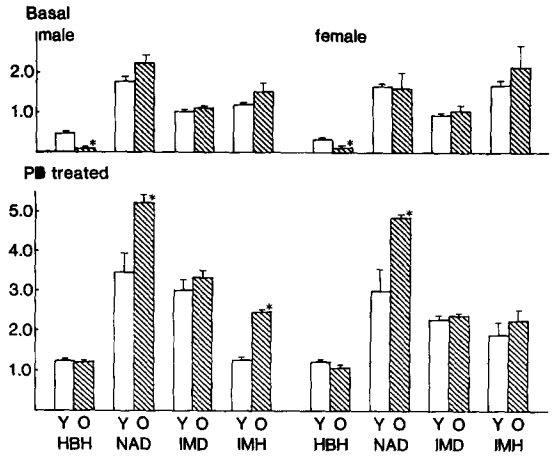

Fig. 2. Basal drug metabolism enzyme activities (upper panel) and activities after phenobarbital treatment in young (Y, 6-month-old) and old (O, 33-month-old) male and female C57Bl mice. Phenobarbital was administered by oral intubation for 1 week. 20 mg/kg/day for the first 2 days followed by 5 days at 50 mg/kg/day. * Significantly different from the corresponding activities in the young rats ($P < 0.05$). Abbreviations are the same as in Fig. 1. (Adapted from Fujita et al., 1986.)

elderly humans, suggesting that the enzyme activities of this system decline with age, many other studies did not find significant differences in clearance values between young and old subjects (Kitani, 1988). It needs to be emphasized that even in studies which showed a decline in drug clearances in the elderly, the decline was in the range that could be explained mostly by a reduction in liver size with age (Kitani, 1988; James et al., 1991).

Figure 3 shows the relation between antipyrine clearance and subject age. The subjects in this study were carefully selected to be healthy individuals who participated in the longitudinal study for aging (Vestal et al., 1975). Although the negative correlation is statistically significant, the age-associated decline in clearance is very small, only 0.4% per year. The rate of decline in liver volume has been reported to be in the same range (0.4–1.0%/year) (Wynne et al., 1989; James et al., 1991) and, since the clearance value is proportional to liver volume, we can explain the reduction in antipyrine clearance values totally by the age-dependent decline in liver volume. Accordingly we cannot expect any decline in antipyrine oxidation with age. In fact, all in vitro studies using human liver biopsy (or necropsy) materials found no significant correlation between enzyme activities and donor age (Boobis and Davies, 1984; Woodhouse et al., 1984; Schmucker et al., 1991). Thus, the contention that monooxygenase activities decline with age in human livers does not have any solid experimental proof as yet in biochemical terms.

The results from past studies which showed a significant difference in hepatic clearance values between young and old subjects appear to be explained best by the reduction of liver volume and by the presence of morbidity (Kitani, 1988) and/or frailty (James et al., 1991) in the elderly subjects. More recent studies indicate that when subjects with morbidity or frailty are rigorously excluded, the decline in

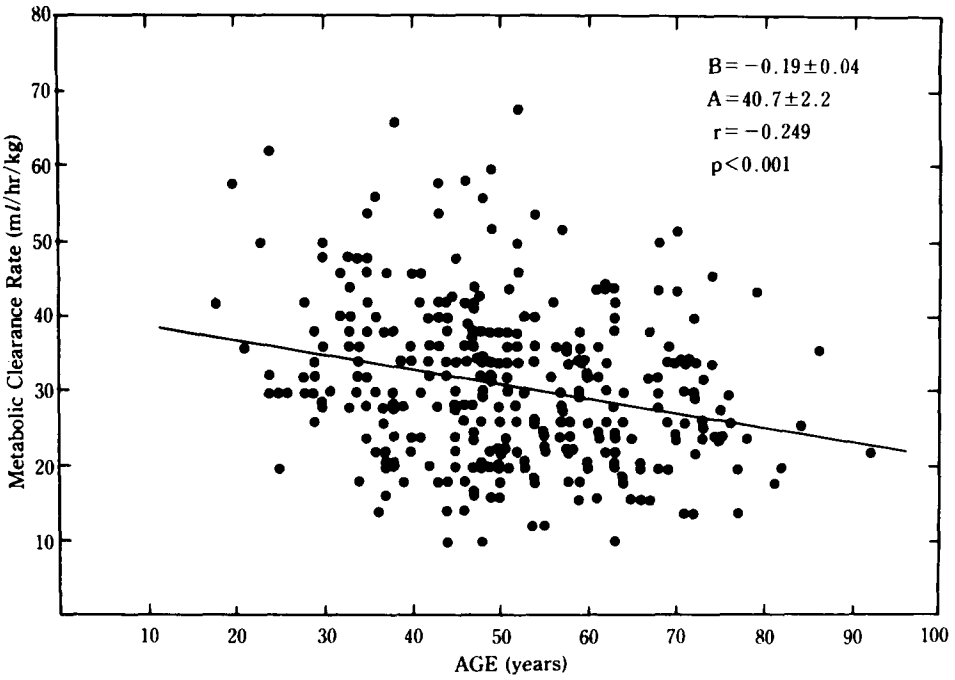

Fig. 3. Antipyrine clearance values in healthy male subjects of different ages. (Reproduced with the permission of the publisher. From Vestal et al., 1975.)

clearances in the elderly is mostly within the limit which can be explained on the basis of the decrease in liver size with age (James et al., 1991; Wynne et al., 1989).

The drastic decline in the enzyme activities of male rat liver shown in Fig. 1 has been proved to be due to the loss of a specific cytochrome P-450 isozyme form, namely, male specific cytochrome P-450, and the appearance of another (female-specific) cytochrome P-450 form during aging (Fujita et al., 1985a; Kamataki et al., 1985; Fig. 4). Thus, the events we observed in male rat liver during aging are nothing but a feminization of the isozyme population of cytochromes P-450 in male rat liver, while in female rat liver, nothing significant happens during aging, either in terms of enzyme activities or the P-450 isozyme population (Kamataki et al., 1985; Fujita et al., 1986, 1990; for review, see Kitani, 1988). The feminization of male rat liver with age in terms of drug metabolizing enzyme activities is also illustrated in Fig. 1. The enzyme activity pattern in old male rat liver is identical to that in young and old female rat livers. Since the rat is a special animal species, in which sex differences in enzyme activities in the monooxygenase system are very prominent, it is clear that observations made in male rat liver can not be extrapolated, not even to the other sex of this species, and certainly not to other animal species, such as humans. Accordingly, any theory (Stier et al., 1982; Schmucker et al., 1983, 1984; Lee and Yu, 1990) postulated to explain the underlying mechanism(s) for alterations

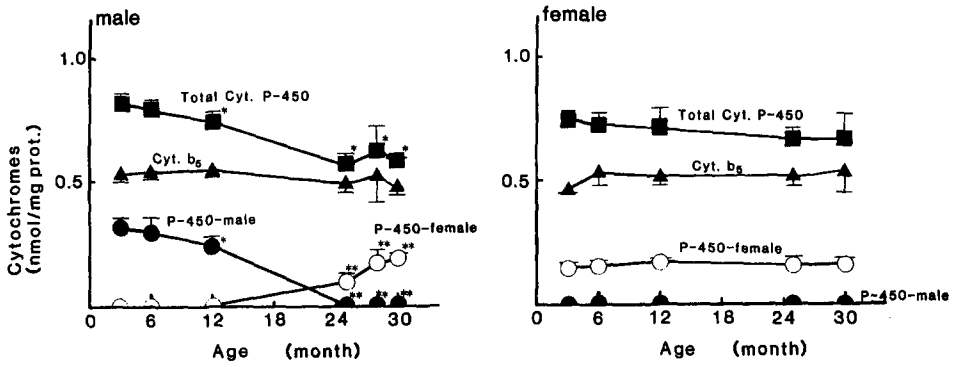

Fig. 4. Age-related changes in microsomal P-450 concentrations in male and female F-344 rats. (Reproduced with the permission of the authors and the publisher. From Kamataki et al., 1985.)

of this system in male rat liver does not have any significance in terms of overall cellular mechanisms of aging.

For example, the physico-chemical alterations of microsomal membranes have been postulated as an underlying mechanism for the age-induced decline in microsomal monooxygenase activities (Stier et al., 1982; Schmucker et al., 1984; Lee and Yu, 1990). However, since such a mechanism can merely explain the reduction in enzyme activities which occur in male rat liver only, it has no generality as a theory for the aging of the liver. In fact, Maloney et al. (1986) found an increase in microsomal membrane viscosity in aged monkey liver, whereas enzyme activities did not decline with age. Thus, these two parameters apparently do not have any causal relationship. We need to learn more from this lesson.

Phase II reactions

Information on phase II drug metabolism reactions in terms of alterations by aging can barely be found in the literature. The measurement of activities of microsomal phase II reactions, such as glucuronyltransferase activities, poses many difficulties for comparing results among animals of different ages, because apparent enzyme activities increase drastically after solubilization of membranes. We really do not know how far the in vivo system is from what we are looking at in an in vitro experiment. Even with these limitations, we can learn something from published data. Table I summarizes the results of Galinsky et al. (1986), and from these data we need to recognize that some enzyme activities in old male rat livers increase with age. Thus, the pattern of age-related alteration is not generally the same, not even in male rat liver. The enzyme activities in male rat liver which tend to decline with age are higher in male rats than in female rats at a young age and the activities which tend to increase with age are lower in males than in females when they are young. Thus, again, the change with age in enzyme activities for phase II reactions appears to be a feminization of male rat liver, as we saw for monooxygenase isozymes.

Enzyme activities in the soluble cytosol fraction, such as sulfotransferase and glutathione S-transferase, may not be as susceptible to microenvironmental alter-

TABLE I

Summary of data on phase II biotransformation reactions in male F-344 rats of different ages

Parameter	Age changes for male values	Young male vs. female values
UDP-glucuronyl transferase		
Naphthol	unchanged	M > F
Morphine	unchanged	M > F
Estrone	increased	M < F
Testosterone	unchanged	M > F
Sulfotransferase		
Phenol	decreased	M > F
Bile salt	increased	M < F
(glycolithocholate)	(sulfotransferase I↑ *)	
	(sulfotransferase II− **)	

From Kitani (1988); reproduced with the permission of the publisher. The table was adapted with the permission of the original authors (Galinsky et al., 1986) and the publisher.

* ↑: increased;

**−: unchanged.

ations of membranes as microsomal enzymes might potentially be. Age-induced alterations may be more directly assessed for these enzymes. Again, however, there are a number of data reporting a decline in cytosolic enzyme activities with age (for review, see Kitani, 1988). Again most such data are found in male rat liver. If we look at data for glutathione S-transferases (GSTs) in female rat liver, the changes are not so striking as in male rat liver (Fujita et al., 1985b; Carrillo et al., 1990). Data on mice are very similar to female rat data, showing essentially unchanged transferase activities during aging (Carrillo et al., 1989; Fig. 5). One may claim again that the reason that we do not see age differences in these enzyme activities is the bias caused by the in vitro system used. In particular, the dilution of cytosol samples for measuring enzyme activities may have produced artifactual results. This

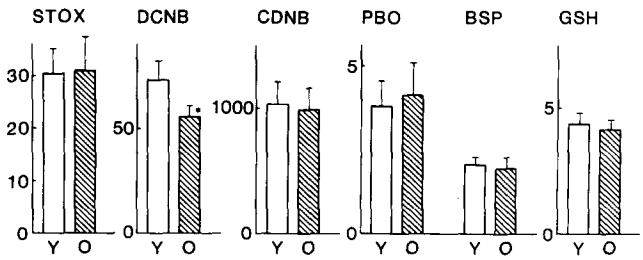

Fig. 5. Enzyme activities of GSTs toward five different substrates in liver cytosols obtained from young (Y, 8-month-old) and old (O, 27-month-old) female C57Bl mice. * Significantly different from the corresponding value in young mouse livers. BSP, sulfobromophthalein; PBO, benzalacetone; STOX, styrene oxide; DCNB, 1-2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, total glutathione. (The figure was drawn based on the data reported previously by Carrillo et al., 1989, with the permission of the publisher.)

argument is particularly important, since one theory of aging emphasizes the condensation of intracellular proteins due to the significant decrease of intracellular water content and an increase in K^+ concentration (Zs.-Nagy, 1979, 1987).

GSTs exist in liver cytosol as a family dimer enzymes, each consisting of similar or dissimilar subunits. When the subunit pattern was compared among young and old rats of both sexes, there was a striking difference between males and females in young rat livers. As predicted, however, age-associated changes in male rat liver caused a subunit pattern very similar to that of young female liver (Carrillo et al., 1991). The decline in enzyme activities with age in male rat liver was accounted for by the feminization of the isozyme pattern of male rat liver. Thus, feminization of the isozyme pattern of male rat liver could also be shown for phase II reaction enzymes in the cytosol fraction. The relative stability of enzyme activities in female rat liver does not appear to be an artifact caused by the dilution of samples, since for some substrates activities tend to decline, while for many others activities are practically identical in young and old livers (Carrillo et al., 1990). This is also true in case of mouse livers (Carrillo, 1989; Fig. 5).

The contention that enzyme activities for phase II reactions are not reduced with age is also supported by in vivo clearance data which show no difference between young and old human livers in clearance values for drugs cleared by phase II metabolism, if we exclude the frailty factor and take into account the decline in liver mass with age, which is consistently seen in human livers (Wynne et al., 1989). For example, acetaminophen clearance per unit liver volume was practically identical between young and old fit subjects (Wynne et al., 1990). In addition, the urinary clearances of the glucuronide and sulfate conjugates of acetaminophen, expressed per unit liver volume, also were not significantly different between young and old fit subjects (Wynne et al., 1990). All of these clearance values were significantly lower in unfit elderly in comparison to both young and old fit subjects (James et al., 1991; Wynne et al., 1990).

The membrane hypothesis proposed by Zs.-Nagy (Zs.-Nagy, 1979, 1987) has made a significant impact on the theory of aging. This hypothesis proposes a general decline in cellular functions during aging caused primarily by a decrease in intracellular water content and an increase in K^+ concentration. However, we need to accept the fact that many enzyme activities in old rodent and primate (including human) livers are not decreased by aging. At least, in the eyes of the author, old hepatocytes do not appear to become dehydrated enough to cause a reduction in their enzyme activities, as this theory emphasizes.

Inducibility of enzyme activities

Many enzyme activities, including those of the monooxygenase system and those of the cytosol, are induced by a variety of substances. Studies in the past have investigated the relationship between the inducibility of enzyme activities and aging (for review, see Kitani, 1988). While some studies have claimed that the inducibility of enzymes is reduced in aged animal livers, Adelman proposed an attractive hypothesis that aging causes a delay in achieving the peak activity after exposure to an inducer, but that the peak activity is not really different from that of young

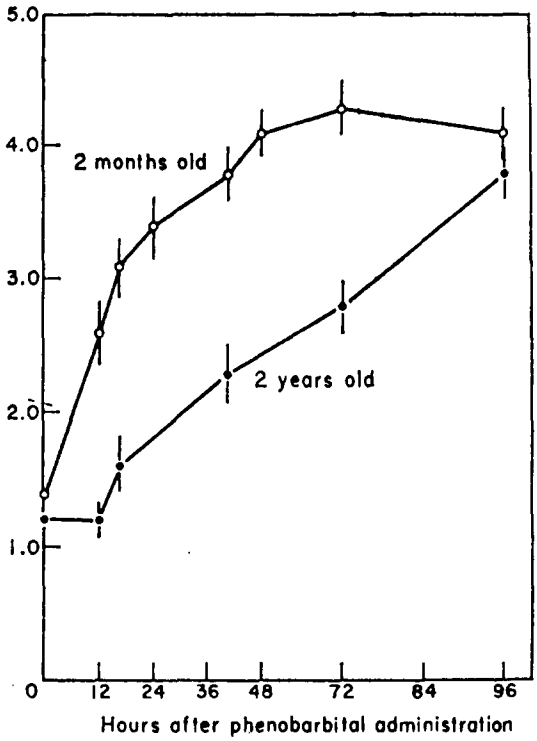

Fig. 6. Difference between young and old rat livers during the induction of NADPH cytochrome C reductase activity by phenobarbital. (Reproduced with the permission of the author and the publisher. From Adelman, 1971.)

animals (Adelman, 1970, 1971). This lag time was shown to be linearly related with animal age and was reported to be a biochemical expression of aging. This time lag theory also fits the membrane hypothesis of aging (Zs.-Nagy, 1979, 1989). While this theory beautifully explained Adelman's original data on NADPH cytochrome C reductase activity (Fig. 6), subsequent studies in his own and other laboratories yielded a number of variations in the pattern of enzyme induction in old animals, and he expanded his model to include different types of induction patterns in old organisms (Adelman, 1979). And yet, his expanded model did not include a greater inducibility in old animal organisms. As is shown in Fig. 2 for example, microsomal monooxygenase activities are not generally reduced in old mouse livers of the strain we investigated (Fujita et al., 1986). Moreover, in old mouse livers many enzyme activities were induced to levels significantly higher than corresponding peak activities in the young (Fig. 2). If we examine past studies carefully, this phenomenon (i.e., a greater inducibility in the elderly) can often be found in rodent livers (Birnbaum and Baird, 1978; Birnbaum, 1980; Kao and Hudson, 1980; Rikans and Notley, 1982) as well as in human livers (Au et al., 1985; Bonde et al., 1985). Once again, we need to be careful about generalizing biological aging phenomena by

proposing a rule. The greater inducibility of enzyme activities in old animal livers also contradicts the membrane hypothesis of aging.

Difference in liver functions between young and old animal livers

As I have briefly reviewed, as far as basal physiological data are compared, we cannot claim that values in old animal livers are generally lower. Stable basal (physiological) functions of the liver during aging, however, may not necessarily mean that liver functions are identical for young and old subjects in every circumstance. If the liver is exposed to some unfavorable stress, such as malnutrition or infection, then the functions of the liver in old subjects appear to become much lower and their recovery seems to be much slower than in young subjects.

One example is shown in Fig. 7. In this study, young and old C57BL mice of the female sex were fed a protein-free diet (PFD) for 1 week and then refed a normal diet (ND) (Carrillo et al., 1989). Animals were sacrificed at different time intervals before and during ND refeeding. Total glutathione (GSH) content as well as GST activities toward five different substrates were determined using cytosol fractions prepared from the livers of these animals. GST activities were almost identical between young and old mouse livers for 4 out of 5 substrates, as shown in Fig. 4. One week of the PFD caused a general reduction in enzyme activities for all substrates as well as a decrease in GSH content in both young and old mouse livers. As seen in Fig. 7, the reduction of enzyme activities was generally greater in old animal livers. More importantly, when young animals were refed the ND, the enzyme values exceeded control values after 2–3 days. Enzyme activities then began to decline and leveled off after 5–9 days of ND to the control levels. Thus, a clear overshoot of enzyme activities was a general phenomenon in young animals. In contrast, in old animal livers, no such overshoot was observed; enzyme activities slowly returned to control levels. Thus, the greatest difference between young and old animal livers was observed at neither basal (control) nor bottom levels caused by the PFD, but during an early recovery period during refeeding of the ND (Fig. 7).

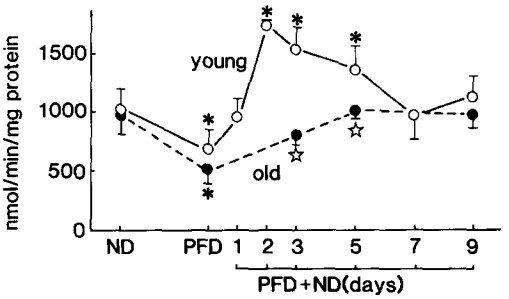

Fig. 7. Changes in activities of GSTs toward CDNB before and during diet manipulation in female C57Bl mice. * Significantly different from corresponding values in control mice on the ND only ($P < 0.05$). ☆ Significantly different from corresponding values in young mouse livers ($P < 0.05$). (Reproduced with the permission of the publisheer. From Carrillo et al., 1988.)

An overshoot of enzyme activities may be an important response of the liver to clear toxicants accumulated in the body during the PFD period. The lack of this response in old animals may mean that the functions of these detoxifying systems may become less efficient in the old organism than in the young one under conditions of stress, like eating a PFD, and that the recovery may also be generally slower. We have recently confirmed that a similar difference between young and old animal livers (i.e., the presence and absence of overshooting) exists for GSTs in rat livers for at least 3 substrates (Carrillo et al., 1990).

Similar mechanisms may be working for many enzyme activities not only in the liver but in other organs and tissues (Miyasaka and Kitani, 1989). It is too early to generalize our observations and we need to confirm that such a mechanism also exists in human livers. However, acetaminophen clearance, which is mediated by phase II reactions including GSTs, by human liver was shown to be reduced only in elderly patients who were frail (James et al., 1991; Wynne et al., 1990). These results suggest that in human liver, too, phase II reactions may be more susceptible to stresses in the elderly. Although acetanilide clearance was not reduced by frailty in the elderly, it is well known that phase I reactions are susceptible to another stress, an infection (Renton and Peterson, 1984). It is possible that many phase I reactions in the elderly are more susceptible to infections than those in the young. From the foregoing, we can propose a working hypothesis that for all practical purposes, many, if not all, enzyme activities are essentially at the same level in young and old organisms, but once the organism is exposed to unfavorable stress, the response (and recovery in particular) is quite different in young and old, and, as age advances, the recovery system may become progressively poorer, until it finally becomes totally unable to recover, leading to the irreversible breakdown of homeostasis and death.

Not only enzyme activities, but many organ functions such as cardiac output and glomerular filtration rate (GFR), that were once thought to decline during aging (Brandfonbrener et al., 1955), are now claimed to be quite stable with age (Shock et al., 1987). The apparent decline in these functions is now attributed to the presence of disease (Shock et al., 1987) rather than to age per se. This does not mean, however, in the view of the author, that the organ functions of old subjects have the same capacities as those of young ones. As long as they are physically healthy, these organs may be working as efficiently as those in young subjects, but once old subjects are exposed to disease conditions, their organ functions may not work as efficiently as in young subjects, and may show a clear tendency to decline with age.

In summary, organ and cellular functions in old organisms are not necessarily lower than those in the young. Many physiological and biochemical parameters are maintained at levels almost identical to those in the young. However, the functions in old organisms are more susceptible to stresses, so that deterioration of these functions occurs with minor stresses which would not significantly affect the functions of young organisms. A theory of aging should be elaborated to explain these potential differences between young and old subjects in response capacities of organ and cellular functions rather than trying to explain the few differences that occur during aging in basal functions in the healthy state.

Significance of retarded protein turn-over during aging; the protease inhibitor model (Ivy model) of aging

The lack of versatile response, as observed for GSTs in liver cytosol of old animals, may best be explained by a lack of rapid protein synthesis during an emergency, but it also may be basically due to the very slow synthesis rate for proteins in old animal livers. The slow rate of protein synthesis is accompanied by a slow degradation of proteins in old animal livers, so that apparent concentrations of many enzyme proteins are maintained at the same level as in young animal livers.

Although there are some data comparing protein synthesis rates in young and old animal livers, the rate of protein synthesis is a parameter not easily determined. Despite these difficulties, most data support the view that protein synthesis rates generally decline with age (Richardson and Cheung, 1982; Richardson, 1983). However, we also need to be aware that some studies revealed no difference in protein synthesis rates between young and old animal livers (for review, see Kitani, 1990).

The process of protein degradation has been less extensively studied in the past. A recent series of studies by Goto et al. (1991) on isolated mouse hepatocytes has revealed that age-associated decreases occur in rates of degradation of foreign proteins of many different species. Whether this happens with intracellular host proteins and, if it does, what kind of proteins are especially susceptible to this process need to be further investigated.

A recent study by Ivy and coworkers (Ivy et al., 1984) on brain cells that demonstrated an accumulation of ceroid-lipofuscin in young rat brains exposed to a thiol protease inhibitor (leupeptin), prompted us to examine whether such an accumulation of the ceroid-lipofuscin is induced in hepatocytes by this protease inhibitor. In collaboration with Ivy, our laboratory has successfully demonstrated that, even in the liver, sufficient amounts of the protease inhibitor can produce an accumulation of ceroid-lipofuscin (Ivy et al., 1990a, b; Kitani et al., 1990; Fig. 8). The ceroid-lipofuscin which was induced by leupeptin treatment emitted a yellow fluorescence with emission wavelengths ranging from 480 to 650 nm, as did the age pigment (Ivy et al., 1990b, c; Kitani et al., 1990). These results indicate that the perturbation of proteolysis leads to the formation of a yellow fluorescence, which contrasts with the blue fluorescence of Schiff bases which are formed by the crosslinking of malonaldehyde (from lipid peroxidation) and amine-containing compounds as originally suggested by the group of Tappel (Chio et al., 1982; Chio and Tappel, 1969). The discrepancy in the emission profile of fluorescence of the Schiff base whose emission spectrum never exceeds 500 nm and that of the age pigment has been pointed out by Eldred and coworkers (Eldred et al., 1969). Although our results do not exclude the involvement of lipid peroxidation, they clearly show that the perturbation of intracellular proteolysis is of primary importance for the formation of lipofuscin than is lipid peroxidation. Furthermore, protease inhibitor treatments produced other manifestations, such as accumulation of ubiquitin immunoreactivities which are found in normal and pathological aging in the brain (Ivy et al., 1989). Recently, we have also demonstrated that ubiquitin

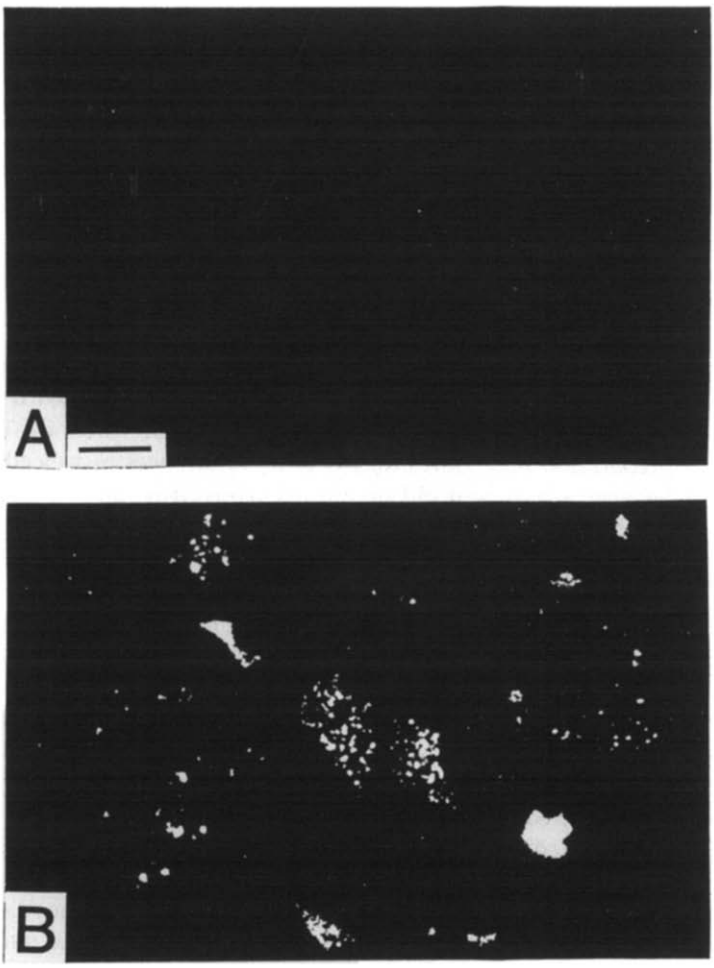

Fig. 8. Fluorescent micrographs of livers from (A) a rat treated with IP saline infusion and (B) a rat given a continuous infusion of leupeptin (20 mg/100 g BW/day for 2 weeks). In the leupeptin-treated liver (B), hepatocytes are generally enlarged in comparison to control liver, and granular structures with distinct fluorescence can be observed in most hepatocytes. Some non-parenchymal cells also contain fluorescent materials with higher intensity than hepatocytes. Bar indicates 10 μm. (From unpublished observations of M. Ohta, G.O. Ivy and K. Kitani.)

immunoreactivities build up in both leupeptin treated and aged livers (Ivy et al., 1991b). These results indicate that the slow-down of protein degradation is an important process in cellular aging in general. Interestingly, a recent study reported a significant induction of gene expression of T-kininogen (a thiol protease inhibitor) in livers of old rats (Sierra et al., 1989). This aspect of liver aging will be elaborated by Ivy et al. in this issue.

Decline in the hepatocyte surface membrane function

In the previous section, I discussed some disagreement between my view and certain implications arising from the membrane hypothesis of aging proposed by Zs.-Nagy in terms of intracellular functions in general, and protein (enzyme) functions in particular. It does not mean, however, that I want to disregard all of the points raised in his thesis. Physical-chemical alterations of microenvironments for enzymes may be quite important as determinants of intracellular functions in general, and especially for theories of aging.

A good example of agreement of our results with the theory of Zs.-Nagy will be presented. My laboratory has long been working on physiological organ functions of the liver in relation to animal age. The idea of the author is that age-induced changes in many physiological functions of organs may be undetected if we only do in vitro experiments with liver homogenates or subcellular fractions, since all of these in vitro preparation procedures may destroy differences in subcellular microenvironments between young and old organisms. Thus, it is important to examine, as much as possible, the physiological fucntions of intact organs in terms of aging and to correlate these results with the many parameters obtained from in vitro studies.

One example is the hepatobiliary transport function. It is well known that many endogenous and exogenous substances are efficiently taken up by hepatocytes and excreted into the bile by means of active transport systems. We have found that the biliary recovery of i.v. administered ouabain, a neutral cardiac glycoside, is progressively decreased with aging in both sexes of at least two strains of rats (Kitani et al., 1978; Sato et al., 1987; Ohta et al., 1988). Since the first 10-min recovery value was the most markedly affected by aging, we thought that the hepatic uptake velocity for ouabain may be decreased with aging. In fact, when this parameter was examined in an isolated hepatocyte preparation, we found a linear decline of uptake velocity for ouabain with rat age (Ohta et al., 1988). The rate of decrease with age (2.4%/month) was very close to the decline in biliary ouabain recovery in the same animals (2.8%/month, male Wistar rats). Thus, the in vivo observation of a decline in the biliary recovery of ouabain appears to be mostly, if not totally, explained by a decline in hepatic uptake velocity. When we examined the hepatic uptake velocity of another compound, taurocholic acid (an organic anion), we again observed a similar decline with age. The rate of decline was quite close to that for ouabain (Ohta et al., 1990).

The uptake processes of both ouabain and taurocholic acid are mediated by specific carriers residing in hepatocyte surface membranes, but these proteins are believed to be totally different from each other. For both substances, V_{max} for uptake velocity decreased by 50% at the age of 24 months in comparison to respective values for young (3–4 months) animals, while apparent K_m values remained unaltered during aging (Ohta et al., 1988, 1990). Thus, our observations could be explained as being due to a loss of specific carrier units for these substances. In that case, a 50% decrease in carrier unit number for both of these substances could be only a coincidence. On the other hand, we could explain these

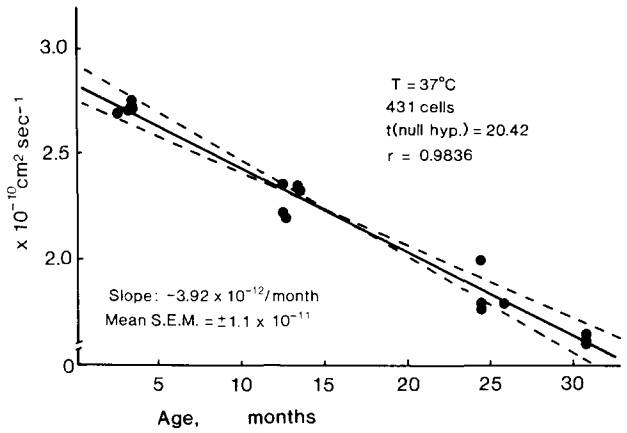

Fig. 9. The relationship between the diffusion constant of proteins in the hepatocyte surface membranes determined by FRAP (y-axis) and animal age (x-axis) in male F-344 rats. (Reproduced with modification with the permission of the publisher. From Zs.-Nagy et al., 1986a.)

observations on the basis of alterations of protein environments in membranes. In a separate series of studies, we found that the protein mobility of hepatocyte surface membranes declines in a linear fashion with age in both male and female Fischer 344 and in male Wistar rats (Zs.-Nagy et al., 1984, 1986a, b; Fig. 9). This decline was observed in mouse livers as well (Zs.-Nagy et al., 1989). The fluorescent probe we used for these studies was the autofluorescence of oxidized riboflavin bound to surface membrane proteins (Nokubo et al., 1988, 1989). Thus, the protein mobility we asssessed in these studies was not that of the specific carrier protein for either taurocholic acid or ouabain. However, it is conceivable that the mobilities of proteins working as transport carriers for these substances are also progressively restricted with the advancement of age, as were the surface membrane proteins shown by our FRAP studies.

Figure 10 shows the correlation between the lateral diffusion constant of hepatocyte surface membrane proteins as determined by FRAP and the uptake velocities for taurocholic acid (Fig. 10A) and ouabain (Fig. 10B) in three age groups of rats. Highly significant linear correlations were found for both substances. Although a linear correlation does not prove a causal relationship, these data are compatible with our working hypothesis that at least some uptake functions of surface membranes are decreased with age due to the progressively restricted mobility of the carrier proteins that form carrier-ligand complexes with these substances. While such a hypothesis needs more direct proof in future studies, physical-chemical alterations of proteins in the surface membranes, as shown for hepatocytes by FRAP, are compatible with the membrane hypothesis of aging proposed by Zs.-Nagy (1979, 1987), which emphasizes the primary role of alterations of membrane qualities in the cellular aging process. The apparent agreement between uptake studies and this hypothesis and the apparent disagreements between

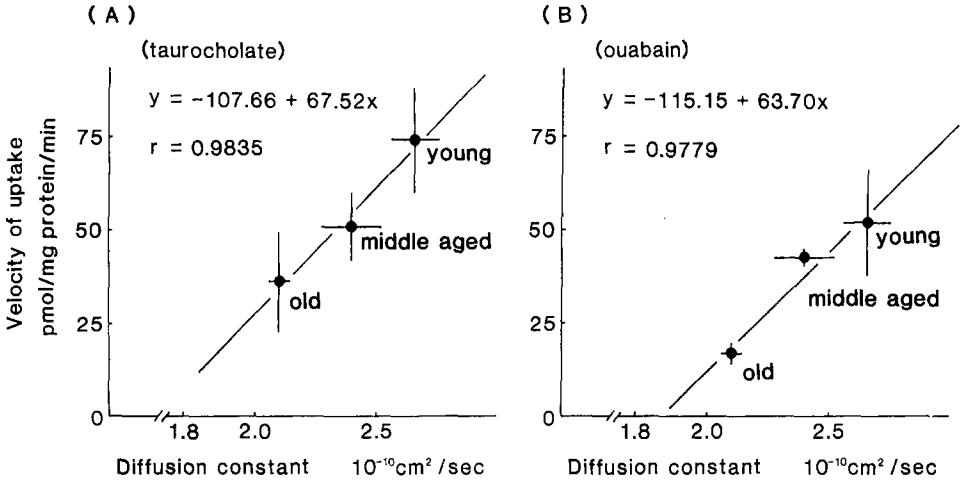

Fig. 10. The relationship between taurocholate uptake velocity at 1 μM and the diffusion constant of hepatocyte surface membrane proteins in three different age groups (A), and a similar relationship for the ouabain uptake rate at 8 μM (B). (From Ohta and Kitani, 1990; reproduced with the permission of the publisher.)

some physiological (and biochemical) studies and this hypothesis are both important, in my view, for the further elaboration of this hypothesis.

From the foregoing, the author would like to raise caution(s) for the following pitfalls in which we tend to get trapped. We need to be careful about the generalization that cellular functions (enzyme activities in particular) tend to decline with age. Many theories based on the assumption that enzyme activities decline with age do not have a rational experimental basis. Accordingly, all these theories have to be regarded as too premature to explain the cellular mechanism of aging.

On the other hand, the preponderance of comparative basal levels of cellular and organ functions between young and old subjects does not mean that these functions are always maintained in old age at the same levels as in young age. Once subjects are exposed to unfavorable conditions, the organs and cells of young and old subjects will behave quite differently. Theories to explain the cellular mechanism of aging may be pursued by examining the regulatory mechanisms that control the response and recovery of cellular functions during and after unfavorable stresses as a function of aging.

Acknowledgements

Most subjects discussed in this article are the result of collaborative studies with a number of scientists performed in our laboratory. Collaborations of the following scientists are specially acknowledged: Drs. M.-C. Carrillo, S. Fujita, G.O. Ivy, T. Kamataki and I. Zs.-Nagy.

The author expresses his sincere gratitude to Drs. G.O. Ivy and L.E. Rikans for their careful reading of the manuscript and stimulatory discussions on its contents. The skilful secretarial work of Mrs. T. Ohara and Mrs. K. Tagami is also gratefully acknowledged.

Discussion of the lecture

Cutler: I think I have to agree with you whole-heartily that one should be looking for chemical changes with age, or we should look for the maximum capacity in an induced state, etc. This was emphasized many years ago in the physiology of aging by Nathan Shock. If you want to detect physiological changes with aging in humans, you should stress the humans to a maximum capacity, since there are not too much changes in the basal physiological levels. Clearly the same concept has to be made at molecular level as well. But I have to emphasize rather a few things what we see with the aging of liver. Certainly there are functions which change with the age. Just some examples come to my mind: the accumulation of chromosome aberrations, although we do not know what they mean in terms of physiology, but this clearly happens with the animals when you stress them, e.g., by liver regeneration. The regeneration itself is much slower in the older animals as compared to the young ones. So there are several changes that seem to be occurring in liver functions with increasing age. And indeed the whole basis of the dysdifferentiation hypothesis I developed years ago was that you cannot really explain aging in terms of deficiency of proteins, or running out of them or loss of enzyme activities with increasing age, etc. Aging is much more sudden in terms of the loss of proper regulation of homeostasis control.

Damjanovich: I wish to continue the line Dr. Cutler started. Immediately after the rationale of the allosteric regulation of enzymes in 1965 by Monod, Jacob and many other people was recognized, within 1 year we succeeded in showing that some key enzymes of the liver and muscle metabolism, like the phosphorylase A and B are far more sensitive to the radiation in their regulation then in their catalytic activity. The conclusion from this fact is that some fine regulatory mechanisms may be more sensitive toward various effects, i.e., even if the basic levels of enzyme activities do not change, we may have different in vivo responses. The results depend also on the actual enzyme activity determination: if you isolate the enzyme, you may lose the information. In case you determine the enzyme activities in situ, you still have some doubts on the validity of your results, since you have to interfere with the system itself, in order to determine a given activity. In case of the membranes the situation is even more complex due to the structural implications and the transient dipoles induced by the membrane potential. Since the membrane is the primary theater of every encounter with the external world, the role of the membrane can be neglected neither in the aging process.

Kitani: Yes, I agree with you, and this is the reason why I emphasized the value of the in vivo kinetic studies, although they offer only a very rough estimate. In particular, however, the enzyme activities studied essentially do not decline in vivo with age.

Zs.-Nagy: First of all, I wish to thank Dr. Kitani, because he addressed so much attention to the membrane hypothesis of aging. This indicates that he likes the idea. As a matter of fact, we have been in collaboration in this field with him already since 1982. Regarding his enzyme studies, I have to emphasize several points. Of course, he is right that the theory has to be in harmony with the facts. He has found that a number of enyzme activities do not decline with age in the liver. I can list other enzymes (e.g., SOD, catalase, and many others) which do decline with age in the liver, and what is more, those activities have been measured together with the transcription rate and mRNA level. We know also that the overall RNA synthesis rate of the old brain cortex decreases to 40% of the young level. These facts should also be taken into consideration. For the non-declining enzyme activities, there can be a very simple explanation in the liver: if you express your enzyme activity values on a DNA basis, you will get a parameter indicating the efficiency of the given biological system in producing certain proteins from a unit weight of DNA. This way of expression would influence significantly the results in the liver, since

the liver displays a very strong tendency for polyploidization, whereas in the case of brain, it will keep the original ratios, since there is no such tendency in the nerve cells. In other words, the basic situation described by the membrane hypothesis of aging can be changed in the liver just by involving more genes in the protein production. Such a compensatory regulation may be of vital importance especially in the case of the detoxifying enzymes studied by you, since it may represent a certain reserve capacity of the liver tissue to overcome difficult situations. In my view your results do not contradict the basic concept of the membrane hypothesis of aging, just call attention to the possible compensatory mechanisms build up by various cell types.

Kitani: Yes, this is possible but you have to prove the validity of this explanation.

Miquel: I do believe that we can see the real effects of aging only if we stress the system. Therefore, I am asking whether anybody has measured the reserve capacity of young and old liver to detoxify nearly lethal doses of, e.g., paracetamol. If we apply only low doses of toxic substances, we may not reveal the loss in maximum detoxifying ability of the liver. Examples of this type of experiments are known for the nervous system: if you measure the number of ribosomes in young and old brain cells, you find practically no difference. However, if you stress the animals by forced swimming in water, the metabolism is stressed to its maximum, and in old animals it takes longer to replenish the neuronal RNA reserves than in the young ones. Other examples are known for the Ca-uptake of mitochondria, etc. Thus I believe that aging studies should pose a drastic challenge to the liver.

Kitani: The point raised by you is very complex: I can tell you something about the effects of paracetamol. Its toxicity is strongly variable in various animal models. First of all, we need to discuss liver toxicity and lethal toxicity separately. Hepatotoxicity is more directly related to the detoxifying capacity of the liver. Paracetamol is transformed into a toxic metabolite by means of the hepatic microsomal P-450 system and this metabolite is detoxified by several phase II conjugation reactions. We did some liver toxicity studies using high doses of paracetamol. Old male rats tolerated paracetamol better than young males, probably because the reduced P-450 function in old males produced smaller amounts of the toxic metabolite. Hepatotoxicity of old female rats was comparable with that of young females. In old male mice, again the hepatotoxicity was smaller than in the young ones. Since the P-450 function is comparable for young and old male mice, we need to look for some reason other than that for male rats. Finally, in old female mice, hepatotoxicity was greater than in young females. You need to realize that things are so complex depending on the animal model you choose. On the other hand, old female rats died of a much smaller dose of paracetamol than young females. It indicates an increased apparent lethal toxicity with age in female rats. It does not mean, however, that the detoxifying capacity (or reserve capacity) of the liver declines with age. Old rats died directly due to an increase in sensitivity of their CNS to paracetamol. Thus, your idea of reserve capacity tested by a loading dose of paracetamol is theoretically right, but practically difficult to do.

Ivy: Did you check whether the age-dependent decline of the P-450 system in male rats is testosterone-dependent?

Kitani: We did some experiments with old males treated with testosterone, and could see some but not complete recovery of the P-450 system. We believe that testosterone is certainly involved in the loss of this capacity of male rats, however, it is not the only factor.

Ivy: Did you look at decreases of the thiol proteinases when you analyzed enzyme activities.

Kitani: This will be a future study.

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