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REVERSAL OF AGE-RELATED CHANGES IN MICROSOMAL ENZYME ACTIVITIES FOLLOWING THE ADMINISTRATION OF TRIAMCINOLONE, TRIIODOTHYRONINE AND PHENOBARBITAL

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SUMMARY

1. Microsomes were isolated from the livers of 3- and 24-month-old Fischer rats, and specific activities were determined for glucose-6-phosphatase, NADPH cytochrome *c* reductase, NADPH diaphorase, NADH cytochrome *c* reductase, NADH diaphorase and NADH neotetrazolium reductase.

2. In agreement with other reports the specific activities of glucose-6-phosphatase and NADPH cytochrome *c* reductase decreased in the old animals, and NADH cytochrome *c* reductase increased.

3. Chronic administration of low doses of triamcinolone almost doubled the specific activity of glucose-6-phosphatase in old animals, but caused essentially no change in young animals. In contrast, treatment with phenobarbital halved this activity in young animals, but was essentially without effect in old animals. Treatment with triiodothyronine caused a marked stimulation of this activity in both young and old animals.

4. There was very little change in the specific activity of NADPH cytochrome *c* reductase or NADPH diaphorase following these treatments.

5. NADH cytochrome *c* reductase, NADH neotetrazolium reductase and NADH diaphorase activities were greatly decreased in both young and old animals by administration of all three compounds.

6. Age-related differences in the activity of these enzymes were either diminished or reversed following each treatment. Old animals were as responsive to these stimuli as young animals, and the highest and lowest specific activities observed for each enzyme during the course of all experiments were very similar for young and old animals of the same sex. These observations suggest that age-related differences in these enzyme activities are probably caused by a different metabolic or endocrine state in young and old animals or their hepatocytes, and not by a deterioration of the integrity or control of the synthesis of these proteins.

INTRODUCTION

Three enzyme activities of rat liver, localized almost exclusively in the endoplasmic reticulum [1–3], have been reported to change with advanced age; glucose-6-phosphatase [4, 5] and NADPH cytochrome *c* reductase [5] activity decreased while NADH cytochrome *c* reductase activity increased [5]. Dallner et al. [6] reported that during the development of these activities in rat liver, the specific activity of glucose-6-phosphatase increased rapidly to a level above that found in the adult, and then subsequently decreased. NADPH cytochrome *c* reductase activity reached the adult level during the first day and then remained constant, whereas NADH cytochrome *c* reductase activity did not attain the adult level during the first two weeks. While it has been determined that the appearance of hormones or metabolites or both triggers the appearance and proliferation of many enzymatic activities during development [7], the reason for these changes during the aging process has not been established.

Two experimentally distinguishable explanations may be offered. The age-related changes in the specific activities of these enzymes could be caused by a senescent deterioration of the hepatocyte itself. This could involve any combination of the integrity of the genome, or the integrity or control of the transcriptional and translational process. The result would be a constituitive alteration of either the enzymes themselves, or the protein(s) directly involved in their activity. The other possibility is that the changes in enzyme activity could be caused by an altered cellular level of hormones or metabolites, or an altered cellular response to these stimuli. If the first explanation is correct, young and old animals treated with compounds known to alter the activity of each enzyme would maintain a consistent difference in enzyme activity. For the second, experimental conditions might be found in which the age-related changes were reversed.

In an attempt to distinguish between these alternatives we have therefore examined the effects of the administration of triamcinolone, triiodothyronine or phenobarbital on these enzyme activities in young and old animals. Glucocorticoids have been reported to cause a stimulation of glucose-6-phosphatase [4, 8, 9]; thyroid hormones have been reported to increase glucose-6-phosphatase [10] and NADPH cytochrome *c* reductase [11, 12], and decrease NADH cytochrome *c* reductase [13]; phenobarbital has been reported to cause a lowering of glucose-6-phosphatase [14] and an induction of NADPH cytochrome *c* reductase [15]. We report here results which suggest that the age-related changes in these enzymes are caused by an altered cellular level of hormones or metabolites, or an altered cellular response to these stimuli, rather than a general deterioration of the synthesis or control of these enzymes. Some of the results have been presented in a preliminary communication [16].

MATERIALS AND METHODS

Rats

Fischer rats were maintained, without mortality before 24 months of age, under specific-pathogen free conditions at Hilltop Laboratories, Scottdale, Pa. Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and water were provided ad

libitum, and food was removed 14–16 h before rats were sacrificed by being stunned and exsanguinated.

Chemicals

Calf thymus DNA, bovine serum albumin, cytochrome *c* (Type III), barium glucose 6-phosphate, NADPH and 3,3',5-triiodo-L-thyronine were purchased from Sigma Chemical Co., St. Louis, Mo. NADH was obtained from Fermco Laboratories, Chicago, Ill., phenobarbital from Merck and Co., Inc., Rahway, N.J., triamcinolone diacetate (Aristocort) from Lederle Laboratories, Pearl River, N.Y., and neotetrazolium chloride from Nutritional Biochemical Corp., Cleveland, Ohio.

Isolation of microsomes

Microsomes were prepared by a method similar to that of Ernster et al. [17], except that the homogenized liver was filtered through 110-mesh nylon cloth to remove connective tissue, and microsomes were washed by resuspension in 0.25 M sucrose and recentrifugation at 50 000 rev./min in a Spinco 50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). All operations were carried out at 0–4 °C.

Chemical determinations

Protein was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard, and DNA by the method of Burton [19] with calf thymus DNA as a standard. Phosphate was measured according to Chen et al. [20]. The method and extinction coefficient of Munro and Fleck [21] were used to estimate RNA.

Enzymatic assays

All solutions for chemical and enzymatic assays were passed through a 0.45-μm Millipore filter to avoid microbial contamination. Enzyme activities were determined for individual animals at two different tissue concentrations, and only fresh tissue was used.

Glucose-6-phosphatase was assayed at 37 °C by the methods of deDuve et al. [22] and Swanson [23] in 100 mM Tris–maleate, pH 6.6, 1.0 μM EDTA, and 10 mM sodium glucose 6-phosphate.

Cytochrome *c* reductases were measured at 25 °C according to the method of Ernster [24]. Assays were carried out in 50 mM potassium phosphate, pH 7.4, 0.1 μM KCN, 50 μM cytochrome *c*, and 100 μM NADH or NADPH. The reduction of cytochrome *c* was followed on a Beckman DB spectrophotometer (Beckman Industries, Inc., Fullerton, Calif.), and an extinction coefficient for (reduced – oxidized) cytochrome *c* of 18.5 ml/μmole per cm at 550 nm [25] was used to calculate results.

NADH diaphorase and NADPH diaphorase were measured at 25 °C by the method of Dallner et al. [6]. Assay conditions were the same as for cytochrome *c* reductase except that 0.66 μM $K_3Fe(CN)_6$ replaced cytochrome *c* as substrate. Oxidation of both pyridine nucleotides was followed on a Beckman DB spectrophotometer and results were calculated from an extinction coefficient (reduced – oxidized) of 6.22 ml/μmole per cm at 340 nm [26].

NADH neotetrazolium reductase was measured at 25 °C in a modified and direct assay procedure. Incubations containing 50 mM potassium phosphate, pH 7.4, 0.1 μM KCN, 0.75 mM neotetrazolium chloride, and 0.8 mM NADH were continuously monitored for formazan formation by following the change in absorbance at 505 nm, using a Beckman DB spectrophotometer. This method was made possible by carefully sonicating the neotetrazolium in buffer, then passing it through a 0.45-μm Millipore filter. This method was easier than the discontinuous method of Lester and Smith [27], and noticable formazan precipitation did not occur during this assay. Results were correlated to the amount of NADH oxidized by following formazan formation at 505 nm in incubations run to equilibrium with limiting amounts of NADH. By this same procedure exactly two moles of cytochrome *c* were reduced for each mole of NADH added to the microsomal incubations.

RESULTS

Untreated animals

Figs 1 and 2 and Tables I and II describe several of the chemical and enzymatic characteristics of liver homogenates and microsomal preparations from 3- and 24-month-old male Fischer rats. Values were also determined for female rats (data not shown) and will be cited only when a marked difference was observed between the sexes.

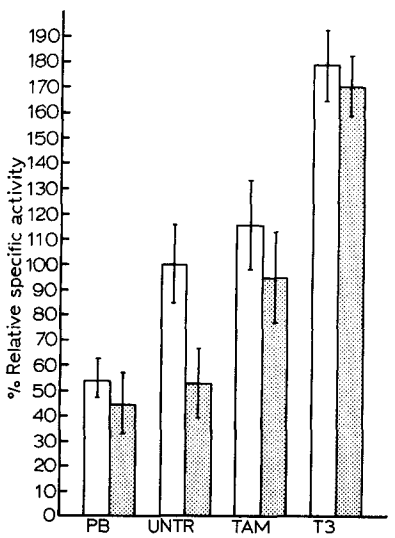

Fig. 1. Relative specific activity of glucose-6-phosphatase in hepatic microsomes from treated and untreated, young and old, Fischer rats. For each enzyme, the specific activity was calculated relative to a value of 100% for untreated 3-month-old males. (Actual value is 4.99 $\pm$ 0.08 μmoles P_i released/20 min per mg protein). Open bars represent young and stippled bars represent old untreated (UNTR) rats or rats treated with triamcinolone (TAM), triiodothyronine (T3) or phenobarbital (PB). Treatments were given as follows: TAM, 7 days $\times$ 100 μg/100 g body weight per day; T3, 7 days $\times$ 40 μg/100 g body weight per day; and PB, 4 days $\times$ 5; 8; 8; 8 mg/100 g body weight per day.

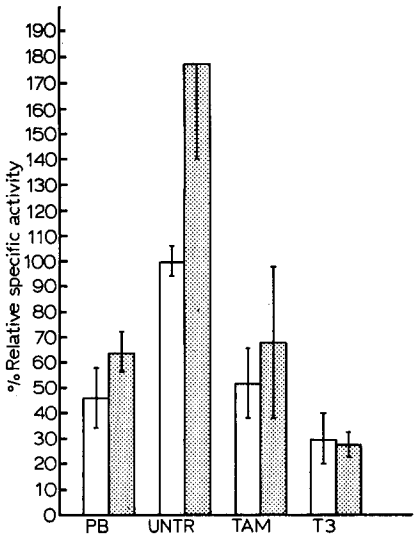

Fig. 2. Relative specific activity of NADH cytochrome *c* reductase in hepatic microsomes from treated and untreated, young and old, Fischer rats. For each enzyme, the specific activity was calculated relative to a value of 100% for untreated 3-month-old males. (Actual value is 1.26 ± 0.07 μmoles cytochrome *c* reduced/min per mg protein). Open bars represent young and stippled bars represent old untreated (UNTR) rats or rats treated with triamcinolone (TAM), triiodothyronine (T3) or phenobarbital (PB). Dose and duration of each treatment are described in the legend to Fig. 1.

In old animals the specific activity of glucose-6-phosphatase (Fig. 1) was decreased by 47%, and NADPH cytochrome *c* reductase and NADPH diaphorase activity (Table I) were decreased by 23–26%. In contrast, NADH cytochrome *c* reductase activity (Fig. 2) was increased by 78% in old male rats (a 15% increase was obsserved in old females). Similar results have been reported by other investigators [4, 5]. Two other enzymes in the NADH electron transport chain were also assayed. The specific activity of NADH neotetrazolium reductase (Table I) was not altered with age, and there was a 23% reduction of NADH diaphorase in old males; no difference was observed, however, in females. Both microsomal protein and RNA per g liver (Table II) were similar in young and old animals (5–6% lower in old male animals, but slightly higher in old females). The ratio of liver weight to body weight (data not shown) was also relatively constant with age in Fischer rats. DNA per g liver was, however, 35% lower in old animals. This was unexpected because increases in liver polyploidy with age have been well established [28].

Treated animals

Duration and dose of treatments. To study the effects of triamcinolone, triiodothyronine and phenobarbital, each treatment was continued until a maximum change was observed in the activity of glucose-6-phosphatase, as determined by preliminary experiments. The 5–8 days of treatment were sufficient time for the majority of microsomal proteins to attain a new steady-state level because the average half-life of microsomal proteins is approximately two days [29–32]. Treatment was also continued well beyond the period during which age-related differences have been

TABLE 1

ENZYMATIC CHARACTERISTICS OF LIVER MICROSOMES FROM TREATED AND UNTREATED MALE FISCHER RATS

Each value represents the mean and standard deviation of at least three animals assayed individually and on different days. Numbers in parentheses represent the percentage of the mean for treated animals compared to untreated animals of the same age. Dose and duration of each treatment are described in the legend to Fig. 1. Units are defined in Materials and Methods; determinations were carried out as described in the text.

Assay	Age of animals (months)	Untreated animals (100%)	Animals treated with		
			Triamcinolone	Triiodothyronine	Phenobarbital
NADH diaphorase (μmoles NADH oxidized/min mg protein)	3	3.09 ± 0.33	1.15 ± 0.27 (37%)	1.49 ± 0.09 (48%)	1.14 ± 0.35 (37%)
	24	2.37 ± 0.13	1.13 ± 0.53 (48%)	0.881 ± 0.41 (37%)	0.735 ± 0.21 (31%)
NADH neotetrazolium reductase (μmole NADH oxidized/min per mg protein)	3	0.274 ± 0.06	0.158 ± 0.03 (58%)	0.095 ± 0.02 (35%)	0.212 ± 0.06 (77%)
	24	0.270.270 ± 0.05	0.195 ± 0.11 (72%)	0.093 ± 0.01 (34%)	0.240 ± 0.03 (89%)
NADPH cytochrome *c* reductase (μmole cytochrome *c* reduced/min per mg protein)	3	0.137 ± 0.03	0.154 ± 0.01 (112%)	0.161 ± 0.02 (118%)	0.174 ± 0.05 (127%)
	24	0.106 ± 0.02	0.125 ± 0.01 (118%)	0.107 ± 0.01 (101%)	0.149 ± 0.03 (141%)
NADPH Diaphorase (μmole NADPH oxidized/min per mg protein)	3	0.111 ± 0.03	0.106 ± 0.07 (95%)	0.130 ± 0.05 (117%)	0.146 ± 0.04 (132%)
	24	0.083 ± 0.03	0.093 ± 0.03 (112%)	0.112 ± 0.04 (136%)	0.118 ± 0.06 (143%)

observed in the temporal response of enzymes to compounds which do not have direct effect on their activity [33, 34].

A dose of triamcinolone was employed (100 μg/100 g body weight per day) which, from preliminary experiments, did not cause a weight loss in young animals. This was to prevent severe alterations in fluid balance; an unwanted side effect caused by higher doses of this potent anti-inflammatory and diuretic glucocorticoid analog. Triiodothyronine, the more potent of the thyroid hormones, was administered at

TABLE II

CHEMICAL CHARACTERISTICS OF LIVER AND LIVER MICROSOMES FROM TREATED AND UNTREATED MALE FISCHER RATS

Each value represents the mean and standard deviation of at least three animals assayed individually and on different days. Numbers in parentheses represent the percentage of the mean for treated animals compared to untreated animals of the same age. Dose and duration of each treatment are described in the legend to Fig. 1. Microsomal protein and RNA were corrected for recovery of glucose-6-phosphatase.

Assay	Age of animals (month)	Untreated animals (100%)	Animals treated with		
			Triamcinolone	Triiodothyronine	Phenobarbital
Protein (microsomes, mg/g liver)	3	41.4 ± 5.5	28.8 ± 4.0 (70%)	34.4 ± 1.3 (83%)	47.8 ± 2.6 (115%)
	24	39.2 ± 5.3	30.0 ± 5.2 (77%)	36.7 ± 2.2 (94%)	48.3 ± 1.0 (123%)
RNA (microsomes, mg/g liver)	3	4.55 ± 0.18	4.68 ± 0.44 (103%)	5.79 ± 0.88 (127%)	5.06 ± 0.30 (111%)
	24	4.28 ± 0.14	5.17 ± 0.39 (121%)	6.67 ± 1.6 (156%)	6.40 ± 0.33 (150%)
DNA (homogenate, mg/g liver)	3	1.84 ± 0.11	1.25 ± 0.28 (68%)	1.67 ± 0.48 (91%)	0.976 ± 0.27 (53%)
	24	1.20 ± 0.14	1.01 ± 0.25 (84%)	1.75 ± 0.38 (146%)	0.934 ± 0.29 (78%)
Recovery of glucose-6-phosphatase in microsomes from homogenate	3	44%	50%	48%	54%
	24	36%	46%	45%	42%

40 μg/100 g body weight per day. This dose was several-fold above the total triiodothyronine in blood (see Discussion) and several-fold above a pharmacological replacement dose [35]. Phenobarbital was administered at 5; 8; 8; 8 mg/100 g body weight per day. The low initial dose was to prevent prolonged periods of sleep for old and female [36, 37] rats, which have a poor barbiturate tolerance.

Effects of treatments

Fig. 1 is a comparison of the specific activity of microsomal glucose-6-phosphatase in treated and untreated male rats. Treatment with phenobarbital caused a marked (46%) reduction of this activity in young animals, in agreement with reports of other investigators [14], but had essentially no effect in old animals. Treatment with phenobarbital for longer periods of time did not cause an additional decrease in this enzymatic activity, and higher doses were not used for the reasons previously explained. In contrast to the changes observed with phenobarbital, a low dose of triamcinolone caused a marked (80%) increase in the activity of glucose-6-phosphatase in old animals, but was essentially without effect in young. Higher doses of cortisone, dexamethasone or triamcinolone are required to cause a marked induction of glucose-6-phosphatase in young rats [4, 8, 9]. Triiodothyronine treat-

ment caused a major induction in both young and old animals; however, the induction in old animals (3.25-fold) was proportionally greater than the induction in young animals (1.8-fold). The result of each treatment was to diminish markedly the magnitude of the age-related difference observed in untreated animals. In female rats (data not shown) glucose-6-phosphatase activity was actually higher in old animals after treatment with either phenobarbital or triamcinolone.

The specific activity of NADH cytochrome *c* reductase in treated and untreated animals is shown in Fig. 2. The activity of NADH cytochrome *c* reductase, unlike glucose-6-phosphatase, was decreased by every treatment, and a proportionally smaller decrease was always observed for young animals. Treatment with phenobarbital or triamcinolone dimished the (78%) age-related difference in the specific activity of this enzyme observed in untreated male rats. The age-related change of this activity was reversed in male rats treated with triiodothyronine; in female rats (data not shown) the age-related increase observed in untreated animals were reversed following each treatment.

Specific NADH diaphorase and NADH neotetrazolium reductase activities (Table I) were also reduced by each compound tested. Treatment with triiodothyronine produced more than a 52% reduction in the activity of both these enzymes, however, it should be noted that each treatment did not cause identical changes in both of these enzymes. This was most clearly seen for treatment with phenobarbital; there was a 19–23% reduction in NADH neotetrazolium reductase activity and a 63–69% decrease in NADH diaphorase activity. This, in addition to the changes in NADH cytochrome *c* reductase activity following each treatment, clearly indicate that these three NADH reductases were not responding to these treatments in a concerted manner.

The specific NADPH cytochrome *c* reductase and NADPH diaphorase activities in treated and untreated rats are also shown in Table I. Both of these enzymes were induced by all compounds tested with the exception of NADPH diaphorase activity in young male rats treated with triamcinolone and old female rats (data not shown) treated with triiodothyronine. In all cases the greatest relative induction of these enzymes was produced by treatment with phenobarbital. The most striking aspect, however, of all these treatments was observed in young male rats; none of these treatments caused more than a 27% increase in the specific activity of NADPH cytochrome *c* reductase. Because of the numerous reports [29, 33, 38–43] of a much greater induction following treatment with phenobarbital, this observation was further investigated. The low response of NADPH cytochrome *c* reductase in male Fischer rats to treatment with phenobarbital appears to be a strain variation of this animal and caused by neither the dose of phenobarbital nor the handling and bedding used for these animals (Gold, G. and Widnell, C. C., unpublished).

Chemical characteristics of rat liver were also affected by these treatments (Table II). DNA per g liver, which was 35% lower in untreated, old male animals, was approximately the same in young and old rats treated with each of these compounds. There was a proliferation of smooth endoplasmic reticulum following treatment with phenobarbital [44, 45] as judged by an increase in microsomal protein per g liver in both young and old animals. Treatment with triamcinolone or triiodothyronine decreased microsomal protein in both young and old animals, and microsomal RNA was increased after administration of each compound.

DISCUSSION

Age-related decreases in glucose-6-phosphatase activity have been reported for rat liver [4, 5] and rat and mouse kidneys [5, 46]. In this study the age-related decrease in hepatic glucose-6-phosphatase activity was observed only in untreated animals. Treatment with triiodothyronine increased this activity in both young and old animals, but proportionally more in old animals. Phenobarbital treatment reduced this activity in young animals but not old. Conversely, treatment with triamcinolone (at the specified dose) elevated glucose-6-phosphatase activity in old but not young animals. After each treatment the glucose-6-phosphatase activity in young and old rats was approximately the same.

It has been reported [8] that high doses of dexamethasone produced a greater induction of glucose-6-phosphatase in young rats than old. In our hands treatment of these animals for seven days with 250 μg/100 g body weight per day of triamcinolone produced an induction of glucose-6-phosphatase activity in both young and old rats very similar to that seen with triiodothyronine; however, the animals lost 30–40% of their body weight. In addition, it was not possible to cause an increase in glucose-6-phosphatase activity above that seen in the animals treated with triiodothyronine nor a decrease below that seen in the untreated old animal or the phenobarbital-treated animals. The variation of a constituitive enzymatic activity within what seems to be specific upper and lower limits has also been observed for other enzymes [47].

The age-related increase in the specific activity of NADH cytochrome *c* reductase was only observed consistently in untreated rats. This increase in activity was either diminished or reversed following each treatment. NADH diaphorase and NADH neotetrazolium reductase were also reduced following all treatments, but the activity of these two NADH reductases and NADH cytochrome *c* reductase did not change in an identical manner. This provides additional evidence that the individual reductase activities of this electron transport chain may vary independently [6].

The specific activities of these enzymes have been expressed per mg microsomal protein. Since the enzymes are localized in the endoplasmic reticulum, and since the microsomal fraction is essentially uncontaminated by other subcellular organelles, this expression of data reflects the relative enzymatic composition of the endoplasmic reticulum. If these activities are expressed per g liver, or per liver, or per cell, the changes in activity are not necessarily the same because the regulation of this organelle involves alterations in its quantity in addition to its composition (see ref. 48). Enzymatic data expressed in these other terms, therefore, reflect differences in cell or organ function.

The relationship between activities in treated young and old animals shows that the relative composition of these enzymes may be regulated within similar limits despite the differences observed in untreated, young and old animals. Chemical characteristics of hepatic microsomes from young and old rats were, in general, similar. Failure to correct for loss of tissue during preparative procedures could, however, lead to a conclusion of major losses in microsomal protein and RNA with age. The age-related decrease in DNA per g liver was seen only in untreated rats, and, as with the age-related differences in enzymatic activity, was diminished or reversed after each treatment.

The reason why these age-related differences are only observed consistently in

untreated rats has not been established. The level of blood corticoids is the same in young and old humans [49] and rats [50]; the level of blood thyroxine is also the same in young and old rats [51]. In collaboration with Dr P. R. Larsen, we have measured the circulating levels of triiodothyronine in 3-month-old and 24-month-old male Fischer rats [52]. Values of 0.690 $\pm$ 0.05 and 0.687 $\pm$ 0.26 ng/ml serum were obtained for groups of three young and old animals, respectively. It is therefore unlikely that the age-related changes in enzymatic activity are caused by differences in the serum levels of either thyroid or glucocorticoid hormones.

It has been reported that the turnover of glucocorticoids [50] is decreased in old animals. This could explain the increased response of old animals observed following the administration of triamcinolone. The effects of treatment with triiodothyronine, however, are unlikely to be explained simply by different rates of turnover of the hormone, since there is evidence against this in rats [51] and since glucose-6-phosphatase was stimulated to the same absolute level in both young and old animals. A greater physiological response to thyroxine, as judged by oxygen consumption has been reported for thyroidectomized old rats when compared to young [35]. It is thus conceivable that differences may exist in the cellular responsed to hormones in old animals. Experiments to test such a possibility are in progress in this laboratory.

It is clear that the age-related changes in the specific activity of glucose-6-phosphatase and NADH cytochrome *c* reductase may be reversed by appropriate treatment. The nature of the responses suggests that no major deterioration occurred with the age in either the control mechanisms which determine the level of these activities or the capacity of the endoplasmic reticulum to accomodate such changes.

It has been suggested [53] that decreases in enzymatic activity with age may be caused by the accumulation of altered enzyme protein as a result of a change in the integrity of the genome or transcriptional or translational process. The results described here do not provide support for this concept, but suggest instead that the age-related changes in these microsomal enzymes are likely to be caused either by different levels of hormones and/or metabolites in the cell or by a modified cellular response to these stimuli. These changes could, therefore, be a continuation of developmental changes, and of a common or similar origin.

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