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AGE-RELATED CHANGES IN MEMBRANE LIPID CONTENT AND ENZYME ACTIVITIES

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SUMMARY

1. Microsomal and mitochondrial fractions were isolated from livers, kidneys and hearts of 6- and 24-month-old rats and the specific activities of several membrane-bound enzymes were determined. Differences were seen in the activities of the enzymes at the different ages.

2. The phospholipid to protein ratios of the isolated fractions were determined. The phospholipid of the liver and kidney microsomal fractions was decreased in the old animals. No changes were seen in the ratio of membrane phospholipid to total lipid.

3. Kinetic analysis was performed on two membrane-bound enzymes isolated from 6- and 24-month-old animals. Altered, perhaps inhibited, forms of the enzymes were indicated.

4. It is concluded that enzyme changes observed to occur during aging are not directly correlated with *in vivo* phospholipid loss. The regulating influence of membrane conformation on the activity of bound enzymes, however, does appear to change.

INTRODUCTION

Recent studies have indicated that the activities of membrane-bound enzymes may require or be regulated by the presence of lipids in the membrane[1–3]. These studies use *in vitro* modifications in lipid to study the structural–functional relationships within membranes. Modification of lipids in membranes, particularly through the use of phospholipases and organic solvents, has indicated that many membrane-bound enzymes require phospholipid for their activity[3–6]. However, the lipid requirement of these enzymes is usually non-specific and in some cases detergents[7] and serum albumin[1] have been used to restore the altered enzyme activity. This lack of specificity indicates that lipids could regulate the activities of membrane bound enzymes by their role in maintaining membrane conformation[1,2,8].

This paper investigates the relationships between membrane lipid content and the activities of membrane bound enzymes in the tissues of young and old rats. Microsomal glucose-6-phosphatase, NADH cytochrome *c* reductase, and NADPH cytochrome *c* reductase as well as mitochondrial succinate cytochrome *c* reductase

and β-hydroxybutyrate dehydrogenase were investigated. All enzymes examined, except microsomal NADPH cytochrome *c* reductase, are thought to require membrane phospholipid for their activity[3–5,9].

METHODS

Fraction preparation

Microsomal and mitochondrial fractions were isolated from livers, kidneys and hearts of 6- and 24-month-old male Sprague–Dawley rats, maintained on standard Purina laboratory chow. All fractions were prepared from tissues pooled from a minimum of three animals. Kidneys and livers were homogenized by hand in a Potter–Elvehjem homogenizer and diluted 1:5, (w/v) with 0.25 M sucrose. Hearts were homogenized for 10 s in a Waring semi-micro blender, then homogenized using a Potter–Elvehjem homogenizer fitted with a motor driven steel pestle. The heart homogenate was diluted 1:10 (w/v) with 0.25 M sucrose. Homogenates were centrifuged at 1000 rev./min for 10 min in an International PR-2 centrifuge. Mitochondrial fractions were isolated by centrifuging the 1000 rev./min supernatant at 10000 rev./min for 15 min using a No. 30 rotor in a Spinco Model L ultracentrifuge. The mitochondrial fraction was resuspended to original volume and the centrifugal steps at 1000 rev./min and 10000 rev./min were repeated. The supernatant and loosely packed fluffy layer were discarded. The microsomal fraction was isolated by centrifuging the original mitochondrial supernatant at 30000 rev./min for 90 min.

Microsomal fractions contained no mitochondrial contamination as evidenced by the absence of succinate cytochrome *c* reductase activity. Approximately 12% of the protein in the mitochondrial fraction was due to microsomal contamination, as measured using either glucose-6-phosphatase or NADPH cytochrome *c* reductase activities.

Enzyme assays

Assays of microsomal enzymes were always carried out on freshly isolated preparations. Mitochondrial enzyme activities were measured on samples frozen as pellets for 24 h. Glucose-6-phosphatase was measured at pH 6.1 and 37 °C using acetate–succinate buffer as described by Duttera *et al.*[3] NADH cytochrome *c* reductase and NADPH cytochrome *c* reductase were measured as described by Jones and Wakil[9]. Succinate: cytochrome *c* reductase activity was measured as described by Fleischer *et al.*[5] with the final volume adjusted to 1 ml. The method of Sekuzu *et al.*[4] was used to measure β-hydroxybutyrate dehydrogenase activity.

All reagents for enzyme assays were purchased from Sigma Chemical Company (St. Louis, Mo).

Lipid extraction and analysis

Membrane lipids were extracted following the procedure of Folch *et al.*[10]. The lipid extract was washed with 0.2 vol. of 0.5% $CaCl_2$, the lower phase was collected and evaporated to dryness under a stream of nitrogen. The residue was then made to volume with chloroform and stored under nitrogen at −15 °C.

Chemical analysis

Phospholipid phosphorus was determined following wet ashing of the sample in H_2SO_4 and $HClO_4$. The method of Youngburg and Youngburg[11] was used to determine inorganic phosphate. Protein was measured by the method of Lowry *et al.*[12] using bovine serum albumin as standard.

RESULTS

Lipid content of microsomal and mitochondrial fractions in 6- and 24-month-old animals

The phospholipid to protein ratios were determined for the liver, kidney and heart microsomal and mitochondrial fractions derived from 6- and 24-month-old rats. Age-related differences were noted in this ratio in both the liver and kidney microsomal fractions (Table I). In the kidney microsomal fraction there was a decrease of approximately 42 % in the phospholipid to protein ratio. In the liver there was a decrease of 21 %. The heart microsomal fraction showed no change. The phospholipid to protein ratios of the mitochondrial fractions isolated from the three tissues were the same at 6 and 24 months.

TABLE I

PHOSPHOLIPID OF MEMBRANE FRACTIONS FROM 6- AND 24-MONTH-OLD ANIMALS

Values are expressed as μg phospholipid phosphorus/mg protein. Each value represents the average of 8–10 determinations. The standard deviations are represented.

Fraction	*Age*:	
	6 months	*24 months*
Microsomal		
Liver	14.7 ± 1.0	11.6 ± 1.1
Kidney	15.2 ± 1.3	8.8 ± 1.2
Heart	19.0 ± 1.8	18.0 ± 1.1
Mitochondrial		
Liver	8.2 ± 1.3	8.1 ± 1.1
Kidney	8.9 ± 0.8	7.9 ± 0.8
Heart	11.8 ± 1.6	11.0 ± 1.6

The ratio of phospholipid to total lipid was similar in young and old animals. The ratio of mg phospholipid phosphorus/100 mg total lipid was approximately 3.0 for liver microsomes and 2.4 for kidney microsomes. The reduction in microsomal phospholipids with age is therefore accompanied by a similar reduction in neutral lipid.

Enzyme analysis of microsomal and mitochondrial fractions in 6- and 24-month-old animals

Specific activities. Enzymes which are thought to require phospholipid for their activity were examined in both microsomal and mitochondrial fractions. In certain of these fractions there was a loss of phospholipid with age, whereas in others there was no change. There were tissue differences in the specific activities of each of

the enzymes examined. Succinate cytochrome *c* reductase, for example, was highest in the heart and lowest in the liver whereas the opposite was true for NADPH cytochrome *c* reductase (Table II).

TABLE II

SPECIFIC ACTIVITIES OF MICROSOMAL AND MITOCHONDRIAL ENZYMES

The results are expressed in nmoles of substrate hydrolysed or reduced/min per mg protein. Each value represents the average of 8–10 separate determinations. Standard deviations of the mean are given.

Fraction	*Microsomal*			*Mitochondrial*	
	NADH cytochrome c reductase	*NADPH cytochrome c reductase*	*Glucose-6-phosphatase*	*Succinate cytochrome c reductase*	*β-Hydroxybutyrate dehydrogenase*
Liver					
6 months	318 ± 40	51.1 ± 11.0	144 ± 19	39.8 ± 7.0	166 ± 32
24 months	370 ± 54	37.6 ± 7.0	99 ± 15	54.0 ± 7.6	111 ± 24
Kidney					
6 months	41.0 ± 4.8	12.0 ± 3.0	200 ± 19	98 ± 13	13.0 ± 1.9
24 months	27.0 ± 4.7	6.4 ± 1.5	117 ± 21	68 ± 14	10.2 ± 2.0
Heart					
6 months	47 ± 10	5.5 ± 1.2	*	147 ± 32	18.8 ± 2.9
24 months	64 ± 15	5.5 ± 1.2	*	147 ± 40	22.9 ± 4.5

* Heart does not contain glucose-6-phosphatase[13].

Age-related changes in enzyme activity were noted in all tissues. In the microsomal and mitochondrial fractions of kidney all enzymes decreased in specific activity with age (Table II). The largest decreases were seen in glucose-6-phosphatase and NADPH cytochrome *c* reductase activities which were reduced by 41 % and 47 % respectively. In the liver, increases and decreases in enzyme activities were noted. NADH cytochrome *c* reductase and succinate cytochrome *c* reductase activities increased by 14 % and 26 % respectively, whereas NADPH cytochrome *c* reductase activity was decreased by 27 %. No enzyme activity of the heart declined with age. However, increases in activity were seen for NADH cytochrome *c* reductase and β-hydroxybutyrate dehydrogenase.

The enzyme changes did not correlate with changes in phospholipid content. One enzyme, NADPH cytochrome *c* reductase, which is considered to be non-lipid-requiring, was reduced in both liver and kidney microsomal fractions. Phospholipid loss was noted in both. On the other hand, NADH cytochrome *c* reductase activity which is considered to be lipid-requiring was increased in liver and decreased in kidney. Also, changes in the activities of lipid-requiring enzymes were noted in the liver and kidney mitochondrial fractions in spite of the fact that no changes in lipid content were observed.

Kinetic studies. Lineweaver–Burk plots of glucose-6-phosphatase activities of liver and kidney microsomal fractions are shown in Fig. 1. The K_m of the enzyme from kidneys of 24-month-old animals was increased approximately twofold, whereas

in liver the K_m was not changed. The V for the enzyme in both tissues was reduced at 24 months. These data suggest that the mechanisms responsible for the decreased glucose-6-phosphatase activity in liver at 24 months are different from the mechanisms responsible for the decreased activity seen in kidney at 24 months.

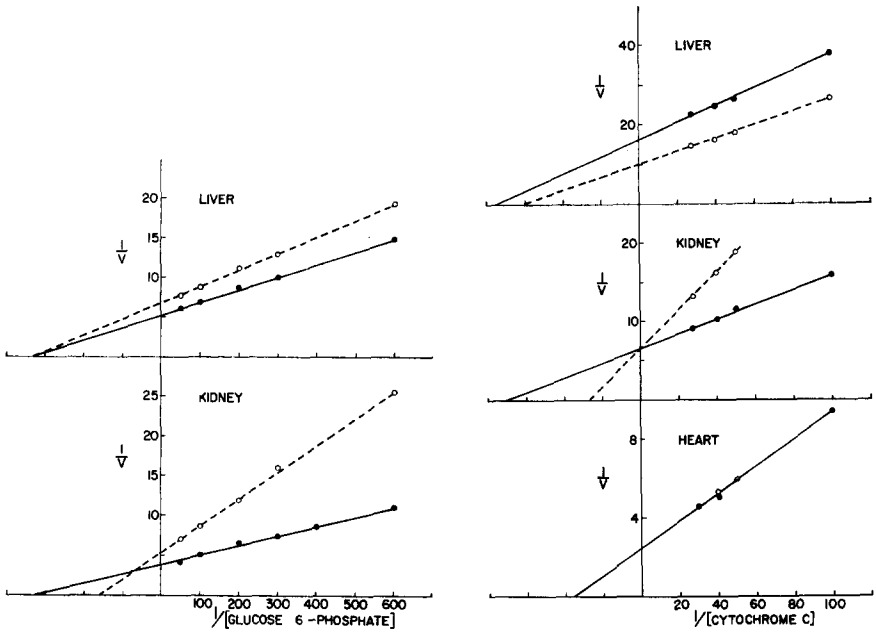

Fig. 1. Lineweaver–Burk plots of glucose-6-phosphatase activity. The velocity of the reaction is expressed as μmoles P_i released/min per mg protein. The substrate is expressed as the molar concentration of glucose 6-phosphate. ●—●, 6 months; ○---○, 24 months.

Fig. 2. Lineweaver–Burk plots of succinate cytochrome *c* reductase activity. The velocity of the reaction is expressed as μmoles cytochrome *c* reduced/min per mg protein. The substrate is expressed as the molar concentration of cytochrome *c*. ●—●, 6 months; ○---○, 24 months; ◒—◒, 6 and 24 months.

Similar analyses of succinate cytochrome *c* reductase activities in the liver, kidney and heart mitochondrial fractions are plotted in Fig. 2. The K_m of the enzyme from kidneys derived from 24-month-old animals was decreased by approximately 3-fold, whereas the V was unchanged. On the other hand, the V of the liver enzyme was increased greatly, whereas the K_m was only slightly changed. Kinetically heart succinate cytochrome *c* reductase was the same in both the 6- and 24-month-old animals. As in the microsomal fraction, the kinetic analysis of this mitochondrial enzyme revealed both tissue differences and age differences in the liver and kidney. In both the mitochondrial and microsomal fractions the presence of altered, perhaps inhibited, forms of the enzyme are indicated.

DISCUSSION

In the liver and kidney microsomal fractions the ratio of phospholipid to protein was reduced during aging. Age-related changes were also seen in the activities of microsomal and mitochondrial enzymes of rat liver, kidney and heart. Although

certain enzyme changes have been reported by others[14,15] little attempt has been made to determine the mechanisms involved in these changes. In fact, little is known of the mechanisms involved in the regulation of membrane-bound enzymes.

Membrane phospholipid appears to be of general importance in the regulation of bound enzyme activity[2,8,16], however, the exact role of the lipid is not known. The lipid may be specifically and absolutely required for enzyme activity[4], may provide the proper hydrophobic environment for enzyme activity[5] or may maintain the proper spatial orientation of the enzyme which is essential for its activity[2,8,16].

In vitro studies have shown that treatment of membranes with organic solvents or phospholipases results in loss of activity for certain enzymes[3,5,9]. The generally accepted criteria for proof of a phospholipid requirement by membrane-bound enzymes have been given by Fleischer *et al.*[5]. These criteria include a correlation between the removal of phospholipid and the loss of enzyme activity and a correlation between the rebinding of phospholipid and the restoration of enzyme activity. Roelofsen *et al.*[16], however, feel that in most cases, a true lipid requirement has not been demonstrated in that increased activity following rebinding of lipid may represent activation of remaining activity rather than a true restoration of lost activity.

Enzymes whose activities have been shown to be dependent upon lipids in *in vitro* studies do not appear to display the same dependence on lipid under conditions of *in vivo* lipid loss. Although in liver and kidney microsomal fractions the lipid-requiring enzyme glucose-6-phosphatase was reduced during aging, there was also a reduction in the activity of the non-lipid-requiring enzyme, NADPH cytochrome *c* reductase, in both tissues. Further, the lipid-requiring enzyme, NADH cytochrome *c* reductase, was reduced in the kidney but was elevated in the liver in spite of the *in vivo* lipid reduction. Several lipid-requiring enzymes of the mitochondrial fractions were altered during aging. These alterations occurred in the absence of lipid changes. Clearly, no simple relationship exists between the enzyme changes and the loss of membrane lipid even for the proposed lipid-requiring enzymes.

Kinetic analysis of glucose-6-phosphatase of liver and kidney microsomal fractions revealed that the mechanisms reponsible for the decreased activities seen at 24 months are quite different in the two tissues. For example, the kidney enzyme from 24-month-old animals has a K_m which is different from the K_m of the enzyme of the 6-month-old animals, while in the liver the K_m is unchanged. These alterations could be due to inhibition of enzyme activity by either specific inhibitor molecules or by changes in the regulation of the enzyme brought about by a change in the conformation of the membrane. *In vitro* studies have indicated that the activities of membrane-bound enzymes can be regulated by the conformation of the membrane. Zakim[1,2] has shown that modification of the membrane phospholipid alters the stability as well as the specfic activity of membrane-bound enzymes and has proposed that the structure of the membrane normally regulates the activity of membrane-bound enzymes by maintaining constraints on the enzyme molecules. Membrane constraint upon enzyme activity has also been proposed by Stetten *et al.*[17]. The age-related alterations in the kinetic properties of glucose-6-phosphatase and succinate cytochrome *c* reductase of the liver and kidney may be related to conformational changes in the membrane similar to those proposed in the *in vitro* studies of Zakim[1,2] and Stetten[17]. The possibility of a change in the number of enzyme molecules present, however, can not be ruled out, particularly in the liver.

The changes in enzyme activity which occur during *in vivo* lipid loss are different from those which would be predicted from the results of *in vitro* studies. The aging animals provide an *in vivo* system for studying the relationship between lipid changes and enzyme activity as well as to examine mechanisms involved in changes with age. The *in vivo* changes reported in this study lend support to the proposal that the membrane conformation contributes to the regulation of enzyme activity.

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