Abnormal lipid composition of microsomes from cirrhotic rat liver – does it contribute to decreased microsomal function?

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Abstract. We determined to what extent a change in the lipid composition of the smooth endoplasmic reticulum contributes to altered microsomal function in cirrhosis. Rats were rendered cirrhotic either by chronic exposure to phenobarbital/CCl_4 (MCIR) or by bile duct ligation (BCIR). Microsomal function was tested in vivo by the aminopyrine breath test (ABT), then microsomes were prepared and their phospholipid and cholesterol composition analysed. ABT was reduced by 35 and 41% in BCIR and MCIR, respectively. Cholesterol in microsomes was increased in both cirrhotic groups. (BCIR + 154%, MCIR + 75%) while total phospholipid content was not affected. As shown in other membrane systems, the phospholipid/cholesterol (PL/XOL) ratio showed an excellent inverse correlation with fluorescence anisotropy determined by diphenylhexatriene fluorescence polarization ($r = -0.896$). The PL/XOL ratio was significantly correlated with aminopyrine N-demethylation in vivo ($r = 0.649$). Alterations in the composition of phospholipid groups (an increase in sphingomyelin in both cirrhotic groups, and a decrease in phosphatidylcholine and an increase in phosphatidylethanolamine in BCIR) also contributed to increased membrane rigidity. We conclude that altered membrane fluidity contributes to diminished microsomal function but that other factors must also be involved since the PL/XOL ratio explained only 42% of the variance in aminopyrine N-demethylation.
Key words. Fluidity; cytochrome P450; aminopyrine; cholesterol; phospholipids.

Abnormal lipid composition has been described in plasma membranes [1] and microsomes [2, 3] of rats with cirrhosis of the liver, as well as after acute CCl_4-intoxication [4, 5]. A decrease in membrane fluidity is associated with altered function of different membrane-bound cell functions [6]. We have observed in the past that during the development of bile duct ligation-induced biliary cirrhosis in the rat, membrane-bound functions such as aminopyrine N-demethylation deteriorate more rapidly than non-membrane related functions such as galactose phosphorylation, and that the deterioration shows a different pattern [7].

To what extent such membrane lipid alterations could affect membrane fluidity and microsomal function has not yet been explored in vivo, however. We therefore measured microsomal function by aminopyrine N-demethylation in vivo in two models of cirrhosis in the rat, namely biliary cirrhosis induced by bile duct ligation, and micronodular cirrhosis induced by chronic exposure to phenobarbital/CCl_4. The alterations in aminopyrine demethylation were compared with the phospholipid and cholesterol contents of microsomal membranes. In a subset of the experimental group, membrane fluidity was assessed by steady-state diphenylhexatriene fluorescence polarization.

Materials and methods

Male Sprague-Dawley rats were obtained from the Süddeutsche Versuchstierfarm, Tuttlingen, FRG. Coprostanol, sphingomyelin, phosphatidylcholine and phosphatidylinositol were purchased from Supelco; phosphatidylserine was from Serva, 1,6-diphenyl-1,3,5-hexatriene (DPH) and cholesterol from Sigma.

Biliary cirrhosis was induced by ligation and excision of the bile duct [8]; sham-operated animals served as controls. This group was studied 4 weeks after surgery. Micronodular cirrhosis was induced by chronic exposure to phenobarbital and carbon tetrachloride according to the method of McLean et al.[9] as described from our laboratories [10]. The animals were studied 10 days after the last treatment, when the acute effects of carbon tetrachloride and phenobarbital were not present any longer [10, 11]. Untreated animals served as controls. Since there was no difference between the sham-operated animals and the untreated controls, results from these two groups were pooled and will be reported as one control group.

Animals were characterized by the aminopyrine breath test as previously described from our laboratory [7]; its results will be reported as ABT_{AUC}, the area under the curve of $^{14}CO_2$-radioactivity in breath between O and 120 min after administration of ^{14}C-dimethylaminopyrine (1.5 μCi) intraperitoneally. After the aminopyrine breath test, the animals were anesthetized with pentobarbital (50 mg/kg i.p.), a blood sample was obtained from the vena cava inferior, and the liver perfused in situ with ice-cold saline. Liver and spleen were removed and weighed. Then, liver microsomal fractions were prepared by differential centrifugation as previously described [11].

NADPH cytochrome c reductase activity was determined in liver homogenates and in microsomal fractions according to Phillips and Langdon [12]. Cytochrome P450 content was determined by differential spectrophotome-

try[13]. Protein concentration was determined according to Lowry[14] using serum bovine albumin as standard.

Microsomal lipids were extracted by the procedure of Bligh and Dyer[15] using coprostanol as an internal standard for cholesterol determination.

Individual phospholipids were separated and quantitated by high-performance thin-layer chromatography by a modification of the method of Vitiello and Zanetta[16] on silica gel 60 plates 20 × 20 cm (Merck, Catalog No. 5641) in a saturated HPTLC double chamber (CAMAG) using methylacetate : n-propanol : $CHCl_3$: methanol : KCL (25 : 25 : 25 : 10 : 9 v/v) as the mobile phase. After development, the plates were dried at 120 °C for 10 min; they were cooled to room temperature, dipped into staining solution for 10 s, and then dried at 120 °C again. The staining solution was prepared from 7.5 g copper acetate in 24 ml phosphoric acid (85%) made up to 250 ml in water[17]. The individual spots were quantitated by scanning densitometry at 547 nm using a CAMAG TLC II scanner interfaced with a Hewlett-Packard 9000 Pc 300. Cholesterol was quantitated by gas-liquid chromatography on a Perkin-Elmer 900 gas chromatograph on 3% OV-17 on 80/100 Chromosorb WHP (Supelco)[18]. Fatty acids of phosphatidylcholine were quantitated by scraping off the corresponding spots from HPTLC plates, extracting them according to Bligh and Dyer[15] and preparing methyl esters[19]. Then the methyl fatty acids were quantitated by capillary gas-liquid chromatography using C-17 : 0 as an internal standard[20].

Fluorescence anisotropy of microsomal membranes was measured at 37 °C in a Shimadzu RF-540 spectral fluorescence photometer at an excitation and emission wavelength of 360 and 430 nm, respectively. The microsomes had been stored at − 20 °C; the solutions were thawed, homogenized and brought to OD 250 at 360 nm (measured in a Pye-Unicam spectrophotometer) with Hank's buffered salt solution. To each cuvette, 20 µl of a 0.2 mM solution of diphenylhexatriene in tetrahydrofurane were added. After an incubation of 15 min at 37 °C, fluorescence intensity (I) was measured in quadruplicate at 5-min intervals. The average of these four measurements will be reported as

$$r(G) = \frac{I_{vv} - G \cdot I_{vh}}{I_{vv} + 2 \cdot G \cdot I_{vh}} \quad (1)$$

where G is the correction factor I_{hv}/I_{hh}[21]; the subscripts v,h refer to vertical and horizontal polarization of the excitation and emission light, respectively.

All results are reported as mean ± 1 standard deviation; differences between the three experimental groups were analysed by analysis of variance followed by Student's test with the Bonferroni correction, where ANOVA indicated the presence of significant differences[22]. Regression analysis was carried out by the method of least squares[22]. $p < 0.05$ was considered statistically significant.

Results

In both experimental groups, cirrhosis was present at both macro- and microscopic levels. The organ weights are reported in table 1; body weights were significantly smaller in both experimental groups as compared to controls. Liver weights were elevated in bile duct ligated animals only. As an expression of the presence of portal hypertension, spleen weights were increased in both experimental groups.

The results of the estimation of serum bile acids and of the aminopyrine breath test are also given in table 1. While ABT_{AUC} was significantly decreased to a similar extent in both cirrhotic groups, serum bile acid levels were elevated significantly more in the bile duct-ligated animals.

In agreement with previous publications from our laboratories[1,7] the microsomal preparations from both cirrhotic models had similar qualities to control microsomes, as judged by the activity of the microsomal marker enzyme, NADPH cytochrome c reductase (table 2). The relative specific activity of this marker (activity microsomes/activity homogenate calculated from the values given in table 2) averaged 3.3 ± 1.1, 3.6 ± 0.8 and 4.3 ± 2.5 in microsomes from control, bile duct ligated and CCl_4-cirrhotic rats, respectively (n.s.). In contrast, cytochrome P450 content was decreased in both experimental groups, significantly more so in the bile duct-ligated as compared to the CCl_4-cirrhotic animals (table 2).

Table 1. Organ weights (g), serum bile salt concentrations (µmoles/l) and results of the aminopyrine breath test (ABT_{AUC}) in bile duct-ligated rats (BCIR), rats with cirrhosis induced by CCl_4/phenobarbital (MCIR) and control animals (CON). Means ± 1 SD are given; the groups were compared by analysis of variance, followed by t-tests with the Bonferroni correction if ANOVA was found to be significant.

	BCIR	MCIR	CON
n	12	17	19
Body weight	537 ± 67	567 ± 71	631 ± 74[b,c]
Liver weight	28.8 ± 10.6	20.1 ± 4.7	20.7 ± 3.6[a,b]
Spleen weight	3.1 ± 1.6	2.5 ± 0.6	1.1 ± 0.3[b,c]
Serum bile acids	57.5 ± 30.3	15.1 ± 10.9	1.2 ± 1.2[b,c]
ABT_{AUC}	20.9 ± 6.2	19.1 ± 6.2	31.9 ± 4.9[b,c]

[a]: $p < 0.005$ BCIR vs MCIR; [b]: $p < 0.005$ BCIR vs CON; [c]: $p < 0.005$ MCIR vs CON.

Table 2. Characteristics of microsomal preparations obtained from bile duct-ligated rats (CIR), rats with cirrhosis induced by CCl_4/phenobarbital (MCIR) and control animals (CON). NADPH cytochrome c reductase activity (CCR) is given in nmoles · min^{-1} · mg $protein^{-1}$, cytochrome P450 content in nmoles/mg protein. Means ± 1 SD are given; the groups were compared by analysis of variance, followed by t-test with the Bonferroni correction if ANOVA was found to be significant.

	BCIR	MCIR	CON
n	12	17	19
CCR homogenate	7.7 ± 1.9	12.6 ± 7.0	12.6 ± 6.9[a]
CCR microsomes	25.6 ± 7.9	45.1 ± 26.5	54.2 ± 17.1[a]
Cytochrome P450	0.31 ± 0.08	0.72 ± 0.36	1.07 ± 0.25[b]

[a]: no significant differences by ANOVA; [b]: $p < 0.01$ BCIR vs MCIR, BCIR vs CON and MCIR vs CON.

Table 3. Lipid composition of microsomal membranes from bile duct-ligated rats (BCIR), rats with cirrhosis induced by CCl_4/phenobarbital (MCIR) and control animals (CON). Phospholipids and cholesterol are indicated in μg/mg protein, the individual phospholipids as percentages of the total. Means ± 1 SD are given; the groups were compared by analysis of variance, followed by t-tests with the Bonferroni correction if ANOVA was found to be significant.

	BCIR	MCIR	CON
n	12	17	19
Phospholipids	336 ± 117	361 ± 101	389 ± 109[a]
Sphingomyelin	4.7 ± 1.6	3.4 ± 1.5	1.9 ± 0.8[c,d]
P-Choline	66.0 ± 7.4	75.4 ± 5.3	78.8 ± 4.4[b,c]
P-Serine	4.3 ± 1.3	4.5 ± 1.1	3.1 ± 1.1[c,d]
P-inositol	9.2 ± 3.3	9.1 ± 3.0	8.4 ± 2.3[a]
P-ethanolamine	14.3 ± 4.2	9.1 ± 3.0	7.9 ± 2.6[b,c]
Cholesterol	40.3 ± 10.9	27.8 ± 6.0	15.9 ± 5.3[b,c,d]

[a]: no significant differences by ANOVA; [b]: $p < 0.005$ BCIR bc MCIR; [c]: $p < 0.005$ BCIR vs CON; [d]: $p < 0.005$ MCIR vs CON.

Table 4. Fatty acid composition of phosphatidylcholine in the different groups. Mean ± 1 SD are given. Differences between the groups were evaluated by analysis of variance.

	BCIR	MCIR	CON
n	5	7	5
14:0	1.8 ± 0.5	3.5 ± 3.2	3.8 ± 1.6
16:0	21.8 ± 3.7	17.0 ± 4.4	17.0 ± 2.2
16:1	3.3 ± 0.5	1.1 ± 0.5	1.6 ± 0.8[a,b]
18:0	21.0 ± 4.2	20.7 ± 5.4	25.3 ± 2.6
18:1	7.6 ± 2.1	8.2 ± 5.3	5.6 ± 2.9
18:2	5.2 ± 1.7	7.5 ± 1.2	5.7 ± 2.1
18:3	1.1 ± 0.3	0.9 ± 0.8	0.3 ± 0.2
20:0	1.1 ± 0.5	1.4 ± 0.8	1.6 ± 1.2
20:1	2.6 ± 1.3	1.8 ± 0.8	2.5 ± 2.3
20:2	0.4 ± 0.6	0.4 ± 0.2	0.3 ± 0.2
20:3	3.7 ± 2.4	11.1 ± 5.6	18.0 ± 1.0[b]
20:4	1.8 ± 1.1	0.7 ± 0.9	1.0 ± 0.8
22:0	0.9 ± 0.3	0.6 ± 0.4	1.0 ± 0.6
24:0	0.7 ± 0.2	0.5 ± 0.4	1.4 ± 0.9

[a]: $p < 0.005$ BCIR vs MCIR; [b]: $p < 0.005$ BCIR vs CON.

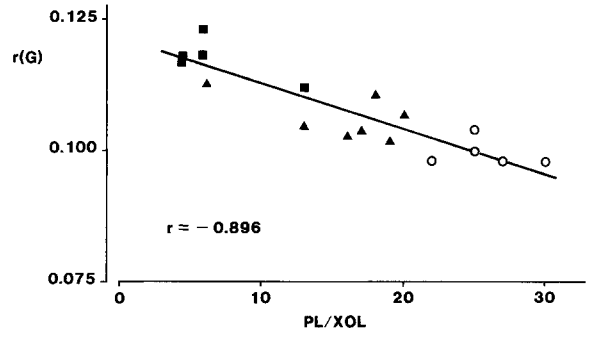

Figure 1. Relationship between fluorescence anisotropy of DPH, expressed as r(G) and the ratio of total phospholipids/cholesterol (PL/XOL) in microsomal membranes of rats with cirrhosis induced by bile duct ligation (■), by chronic treatment with phenobarbital and CCl_4 (▲), and controls (o). The regression equation was $y = 0.121 - 0.849 \cdot x$ ($r = -0.896$).

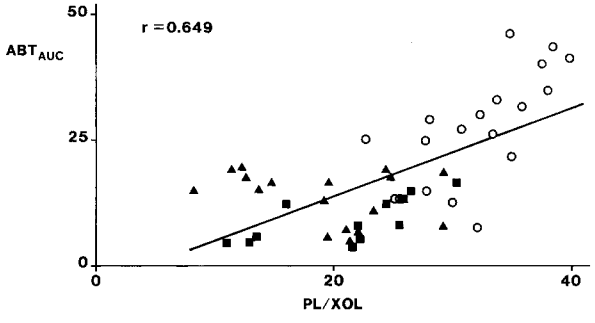

Figure 2. Correlation between microsomal function in vivo measured as aminopyrine N-demethylation (ABT_{AUC}) and the ratio of total phospholipids/cholesterol (PL/XOL) – an expression of membrane fluidity – in microsomal membranes of rats with cirrhosis induced by bile duct ligation (■), by chronic treatment with phenobarbital and CCl_4 (▲) and controls (o). The regression equation was $y = 16.02 + 0.48 \cdot x$ ($r = 0.649$).

The phospholipid and cholesterol composition of microsomal membranes is reported in table 3. While total phospholipid content was not affected by either treatment, cholesterol content was significantly increased in both experimental groups compared to controls, and significantly more so in bile duct-ligated than in CCl_4-cirrhotic animals. Both cirrhotic groups had a higher sphingomyelin and phosphatidylserine content than control animals. Phosphatidylcholine was significantly decreased only in bile duct-ligated animals. This group also exhibited a significant increase in phosphatidylethanolamine content.

The fatty acid composition of phosphatidylcholine isolated from microsomal membranes is given in table 4; no major differences, in particular no increase in saturated fatty acids, were found. Bile duct-ligated rats had marginally increased contents of 16:1 but decreased contents of 20:3 fatty acids compared to the control group.

Steady-state fluorescence anisotropy of DPH was measured in the three experimental groups of one series comprising 5, 7 and 5 bile duct-ligated, CCl_4-cirrhotic and control animals, respectively. The corresponding r(G) averaged 0.118 ± 0.010, 0.106 ± 0.004 and 0.100 ± 0.003; this difference was highly significant by ANOVA ($p < 0.0001$). All groups differed from each other at a $p \ll 0.01$. As described in other membrane systems, both the ratio of total phospholipids/cholesterol (see fig. 1) and the ratio phosphatidylcholine/sphingomyelin ($y = 0.123 - 0.00698 \cdot x$; $r = -0.856$: data not shown) correlated closely with DPH fluorescence anisotropy.

Since fluorescence polarization anisotropy could be measured only in a subset of the data, different aspects of microsomal function were correlated with the phospholipid/cholesterol ratio since it showed the closest correlation with DPH fluorescence polarization anisotropy. The ratio was quite closely correlated with ABT_{AUC} (fig. 2; $r = 0.649$). The correlation with microsomal cytochrome P450 content was weaker ($r = 0.532$, data not shown) and absent for NADPH cytochrome c reductase activity ($r = 0.233$, data not shown).

Discussion

Our results demonstrate an increase in cholesterol content of microsomal membrane fractions in two models of

cirrhosis in the rat; from this, as well as from the increase in sphingomyelin content, one would expect these membranes to be more rigid. This was confirmed by measurement of fluorescence anisotropy of diphenylhexatriene. In the case of bile duct-ligated animals, the lipid changes leading to more rigid membranes in cirrhosis were further aggravated by a loss of phosphatidylcholine and a compensatory increase in phosphatidylethanolamine. Accordingly, membranes of bile duct-ligated rats were more severely affected than membranes of rats rendered cirrhotic by chronic exposure to CCl_4. Membrane 'fluidity', as assessed by the ratio of total phospholipids/cholesterol or that of phosphatidylcholine/sphingomyelin, correlated with microsomal function as assessed by aminopyrine N-demethylation in vivo.

The loss of phosphatidylcholine in bile duct-ligated rats is in accordance with results obtained by Bengochea et al. 8 days after bile duct ligation [23]. In contrast to our results, these authors also reported a loss of phosphatidylethanolamine and sphingomyelin as well as a decrease in cholesterol content. While the different pattern of phospholipid content could be due to the different time points investigated (8 vs 28 days in the present study) the discrepancy with respect to cholesterol is surprising, since elimination of cholesterol, which is predominantly via bile, is completely blocked after bile duct ligation. Indeed, Kawata et al. found an increase in cholesterol of hepatic microsomes after 3 weeks of bile duct ligation [2]. It is tempting to speculate that the marked elevation of serum bile acid levels in this model of chronic cholestasis/biliary cirrhosis is related to the loss of phosphatidylcholine due to the detergent action of bile acids. Indeed, a correlation between bile acid concentration and loss of cytochrome P450 has been described after short-term bile duct ligation [24].

Acute administration of CCl_4 is thought to exert its membrane damaging effect via lipid peroxidation: James et al. have demonstrated a selective loss of polyunsaturated fatty acids, predominantly of phosphatidylserine, with no change in phospholipid composition [25, 26]. This is associated with a rigidizing effect on deep parts of the lipid bilayer [27]. Little is known about the effects of chronic exposure to CCl_4 on microsomal membrane lipid composition. Yahuaca et al. have demonstrated an increased phospholipid/cholesterol ratio to be responsible for decreased activity of Na, K-ATPase in plasma membranes of rats rendered cirrhotic with CCl_4 [1]. Similarly, the microsomal UDP-glucuronyltransferase activity is reduced in acute CCl_4-poisoning; this change correlates with an increased membrane rigidity [4].

We have previously demonstrated that aminopyrine N-demethylation in vivo decreases in a monotonous fashion in bile duct-ligated animals, suggesting that this could reflect an alteration in membrane characteristics [7]. This contention is corroborated by the present findings. No such data are available in the CCl_4-model. The correlation between aminopyrine N-demethylation and membrane 'fluidity' suggests that the more rigid membranes are involved in the pathogenesis of decreased liver function in cirrhosis of different etiologies. It has to be pointed out, however, that the phospholipid/cholesterol ratio could explain only 42% of the variation in aminopyrine N-demethylation ($r = 0.649$). Clearly, other mechanisms must be contributing to the decreased microsomal function in cirrhotic liver. These mechanisms could include altered microvascular exchange, altered hemodynamics, decreased hepatocellular volume, decreased activity of the rate-limiting enzyme of aminopyrine N-demethylation and/or decreased endoplasmic reticulum in liver cirrhosis; indeed, correlations with various other determinants of drug metabolism in models of cirrhosis have been previously published by our laboratories [7, 11, 28].

Future investigations will have to establish the reason for the altered lipid composition of microsomal membranes in models of liver cirrhosis. Such studies could be of importance in as much as they may point a way towards novel therapeutic modalities for improving the function of the diseased liver.

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Preferential excretion of glycated albumin in C57BL-Ks-J mice: Effects of diabetes

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Abstract. Urinary excretion of glycated albumin was quantitated in genetically hyperglycemic mice (C57BL-Ks-J, db/db mice), a model for non-insulin-dependent diabetes mellitus, and compared with their non-diabetic littermates. The data indicated a preferential excretion of glycated albumin in non-diabetic mice. This phenomenon of 'editing' of glycated albumin is decreased significantly in diabetic mice. Quantitative measurements of overall excretion of glycated albumin suggested that the loss of editing in diabetic mice is due to the dilution of glycated albumin by the unmodified albumin which is excreted in large amounts in diabetic mice. Therefore, the loss of editing observed in this model resembled the one we characterized in insulin-dependent diabetic humans and a streptozotocin-diabetic rat model[3].

Key words. C57BL-Ks-J mice; db/db mice; glycated albumin; urinary excretion.

A growing body of experimental evidence suggests that the mammalian nephron selectively excretes glycated albumin into urine. This manifests as a marked increase in the percent of glycated urinary albumin which can reach levels 16 times higher than that of plasma glycated albumin in healthy humans[1-3]. This phenomenon of editing, which is the ratio of percent glycated albumin in urine to glycated albumin in plasma, is lost suddenly in diabetic humans and in streptozotocin-diabetic rats[1,3]. In contrast to the abrupt loss of editing in diabetes, we have also observed a gradual reduction in editing of glycated albumin as a function of age[3] and postulated two different models for this phenomenon associated with diabetes and aging. In diabetes, the attenuation in editing is due to the dilution of glycated albumin by the unmodified albumin, which is excreted in large amounts. On the other hand, in aging nephron, the reduction in editing is the result of decreased filtration of more negatively charged glycated albumin[3,4] into the urine.

The diabetic mutant mouse (C57BL-Ks-J strain, db/db) is widely used as a model for non-insulin-dependent diabetes mellitus, and this mouse has been shown to manifest symptoms like those in adult onset diabetes in man, with obesity and hyperinsulinemia being characteristic features[5]. While it has been shown that diabetic mice develop microvascular complications associated with diabetes[5], the ability of the nephron to selectively discriminate glycated from unmodified proteins has not been studied. The present study was undertaken to see whether the loss in editing observed in streptozotocin-diabetic rats is also manifested in these diabetic mice and, also, to evaluate the possible mechanism(s) underlying such a reduction.

Methods

Diabetic mice (11–12 weeks old) and their non-diabetic littermates belonging to C57BL-Ks-J strain were obtained from the Jackson Laboratory, Bar Harbor, ME. Animals were housed in a light cycled room and cared for in accordance with National Institutes of Health and Institutional Animal Care and Use Committee Guidelines. Degree of hyperglycemia was monitored by quantitating glycated albumin and glycated hemoglobin (HbA1C) as described below.

Collection and processing of blood and urine from diabetic and non-diabetic mice. Blood was collected into heparinized tubes by cardiac puncture. Twenty-four-hour urine collections were done using metabolic cages (Nalgene, Rochester, NY). Two groups of diabetic and two of non-diabetic mice (consisting of four mice per cage) were used in the present study. This grouping of animals was necessary to obtain sufficient volumes of urine for biochemical analyses. Urine was collected on ice in containers containing sodium azide to prevent growth and the total volumes of urine in 24 h measured. Known volumes of urine (2–3 ml) were dialyzed against distilled water to remove salts and other low molecular weight compounds and lyophilized.