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Biochemical Analysis of the Pinocytotic Process, I

Isolation and Chemical Composition of the Lysosomal and the Plasma Membrane of the Rat Liver Cell

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Summary: Plasma membranes and the membranes of secondary lysosomes were both isolated for the biochemical analysis of the pinocytotic process. As a first approach the chemical compositions of both membranes were determined by analysis and classified as:

a) *Lipids:* A high amount of sphingomyelin and cholesterol compared with other subcellular organelles is present in both membranes. These two lipid classes together with a high content of saturated fatty acids in the phospholipids may contribute to the membrane stability of both membranes. The other lipid classes differ markedly.

b) *Proteins:* The amino acid composition of the total protein of both membranes is quite similar, whereas the protein profiles in polyacrylamide gel electrophoresis are completely different from each other.

c) *Carbohydrates:* The secondary lysosomal membrane is characterized by a carbohydrate content twice as high as that of the plasma membrane.

The chemical composition of the two membranes shows some similarities particularly with respect to the lipid constituents. Two hypotheses are discussed: Either the secondary lysosomal membrane originates from the plasma membrane in part or the structural similarities reflect similar functional properties in particular with regard to permeability properties.

Zusammenfassung: *Biochemische Analyse pinozytotischer Vorgänge, I: Isolierung und Zusammensetzung der Plasmamembran und der Membran sekundärer Lysosomen aus Rattenleber.* Als Modellsubstanz für die Pinozytose wurde Triton WR-1339 gewählt. Zur biochemischen Analyse dieses Prozesses wurden beide beteiligten Membranen, die Plasmamembran und die Membran von sekundären Lysosomen gleichzeitig isoliert und analysiert. Die chemische Zusammensetzung beider Membranen ergab folgendes Bild:

a) *Lipoide:* Im Vergleich mit anderen subzellulären Membranen fällt bei beiden Membranen ein relativ hoher Sphingomyelin- und Cholesteringehalt auf. Das wird im Zusammenhang mit dem bei der Fettsäureanalyse festgestellten — besonders in der Lysosomenmembran — hohen Anteil von gesättigten Fettsäuren auf die besonderen Stabilitätsansprüche beider Membranen zurückgeführt. In den übrigen Lipoidklassen bestehen keine deutlichen Übereinstimmungen.

b) *Proteine:* Die Aminosäurezusammensetzung des

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Enzymes:
Acid phosphatase, orthophosphoric monoester phosphohydrolase (EC 3.1.3.2)
Cytochrome oxidase, ferrocytochrome *c*: oxygen oxidoreductase (EC 1.9.3.1)
Glucose-6-phosphatase, D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9)
5′-Nucleotidase, 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5).

Gesamtproteins beider Membranen zeigt große Ähnlichkeiten, während die Polyacrylamid-Gelelektrophorese völlig verschiedene Proteinmuster ergibt.

c) *Kohlenhydrate:* Die sekundäre Lysosomenmembran enthält alle untersuchten Kohlenhydratkomponenten in doppelt so hoher Konzentration wie die Plasmamembran.

Die chemische Analyse beider Membranen zeigt manche Ähnlichkeiten, besonders im Lipoidanteil. Bei einem gleichzeitigen Membranumbau wird einmal die Entstehung der sekundären Lysosomenmembran aus der Plasmamembran diskutiert. Andererseits können die wenigen strukturellen Gemeinsamkeiten auch Ausdruck ähnlicher funktioneller Anforderungen an die Membranzusammensetzung im Hinblick auf die Permeabilität sein.

Extracellular macromolecules which have to be degraded by intracellular digestion cannot diffuse into the cell, but according to the present concept enter the cell by the process of pinocytosis or phagocytosis[1]. This process includes the invagination and segregation of the plasma membrane to form the pinocytotic vesicles which have been observed with the electron microscope[2,3]. The intracellular digestive system is located in the lysosomes[4]. The catabolic processes occur after the primary lysosome has been fused with the pinocytotic vesicle containing the exogenous material to form the secondary lysosome (for terminology see DE DUVE[4]). This sequence of events and the precise origin of the membrane of the pinocytotic vesicle have been derived mainly from morphological observations. In the view of this theory the membrane of the secondary lysosome is formed, at least in part, by the plasma membrane introduced into the cell during the pinocytotic process.

Various substances such as dyes[4], albumin[5] and hemoglobin[6] have been used for studies of the pinocytosis. We chose the detergent Triton WR-1339. This nonhemolytic detergent allows the easy isolation of the secondary lysosomes[7]. This and subsequent papers will report on investigations studying the relations between the plasma membrane and the secondary lysosomal membrane by biochemical methods. Here we wish to report the chemical composition of the membrane of the secondary lysosome in comparison with the plasma membrane. A detailed chemical analysis of the membrane of secondary lysosomes has not yet been reported.

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Results

48 h after Triton WR-1339 application to adult rats, liver plasma membranes and secondary lysosomal membranes were prepared simultaneously. The fractionation procedure yielded 0.46 mg of plasma-membrane protein per g fresh weight of liver and 0.36 mg of lysosomal-membrane protein comprising 44% of total isolated lysosomal protein.

1. Control of the purity of cell fractions

The purity of the cell fractions was checked by marker enzymes. Table 1 shows that the lysosomal fraction contained no detectable mitochondrial contamination and the plasma membrane only 0.17% as assayed by cytochrome *c* oxidase activity. Contamination by endoplasmic reticulum amounted to 3.1% and 16.1% of lysosomal and plasma membrane fractions respectively as measured by glucose-6-phosphatase activity.

2. Lipid analysis

Lysosomal membranes contained 0.256 µmol of lipid phosphorus/mg of protein, plasma membranes 0.301 µmol/mg of protein. The distribution of phospholipids is shown in Table 2.

The fatty acid composition of the glycerophospholipids is summarized in Table 3.

Table 1. Assays of marker enzymes for cell fractions. Enzyme activities are given in μmol of substrate utilized per mg of protein per min. The numbers in parentheses represent the number of fractions analyzed.

	5′-Nucleotidase	Acid phosphatase	Cytochrome c oxidase	Glucose-6-phosphatase
Plasma membrane	1.15 (33)	0.120 (4)	0.0006 (6)	0.036 (24)
Lysosomes	0.158 (5)	0.915 (14)	0	0.007 (10)
Mitochondria	0.024 (14)	0.012 (8)	0.567 (11)	0.012 (5)
Microsomes	0.107 (22)	0.051 (8)	0.002 (5)	0.225 (24)
Homogenate	0.054 (3)	0.048 (4)	—	—

Table 2. Phospholipid distribution in lysosomal and plasma membranes in % of total lipid phosphorus (n = number of fractions analyzed).

	Lysosomal membrane ($n = 4$)	Plasma membrane ($n = 8$)
Lysophosphatidylcholine	—	2.6 ± 0.6
Sphingomyelin	32.9 ± 0.9	20.5 ± 0.8
Phosphatidylcholine	33.5 ± 4.0	34.8 ± 1.0
Phosphatidylinositol/phosphatidylserine	8.9 ± 1.0	17.4 ± 0.5
Phosphatidylethanolamine	17.9 ± 2.5	22.4 ± 1.0
Solvent front (cardiolipin, phosphatidic acid etc.)	6.8 ± 0.4	2.3 ± 0.4

Table 3. Fatty acid composition of phospholipids in % (tr. = traces).

	Lysosomal membrane			Plasma membrane		
Chain length: No. of double bonds	Phosph. ethanol-amine	Phosph. inositol/ phosph. serine	Phosph. choline	Phosph. ethanol-amine	Phosph. inositol/ phosph. serine	Phosph. choline
14:0	tr.	tr.	—	—	0.5	—
16:0	22.1	14.8	38.3	15.7	5.6	28.3
16:1	3.3	3.1	3.8	0.7	1.1	0.3
18:0	21.1	44.8	20.0	27.0	45.2	25.3
18:1	14.1	15.4	10.5	7.0	5.4	9.3
18:2	13.5	4.4	10.5	13.3	4.8	23.7
18:3	—	—	—	1.2	0.9	—
20:4	13.5	12.9	10.9	15.9	20.5	12.8
20:5	3.6	4.6	1.9	4.6	3.5	0.3
22:5	2.1	tr.	—	7.9	8.5	—
22:6	3.6	tr.	2.2	7.9	8.5	—
Sum of polyunsaturated	36.6	21.1	27.9	46.6	40.7	36.8

With regard to the fatty acid composition of the phospholipids an important difference is obvious. The sum of the polyunsaturated long chain fatty acids is higher in the plasma membrane, whereas in the lysosomal membrane the saturated fatty acids prevail. The neutral lipids were analyzed by the determination of the ester groups after separation by thin-layer chromatography. Their concentrations are expressed in μmol per μmol of phospholipid phosphorus (Table 4). An unidentified component of the neutral lipids of the lysosomal membrane with an R_F value between those of cholesterol and the triglycerides in the solvent system petroleum ether/ether/acetic acid 70:30:1 was detected. It gave strong positive reactions with vanillin and anisaldehyde, indicating a steroidal compound.

Table 4. Distribution of the neutral lipids in μmol per μmol of phospholipid phosphorus (n = number of fractions analyzed).

	Plasma membrane ($n = 6$)	Lysosomal membrane ($n = 9$)
Monoacyl glycerides	0.146	0.098
Diacyl glycerides	0.099	0.059
Triacyl glycerides	0.152	0.033
Esterified cholesterol	0.168	0.139
Cholesterol (total)	0.903	0.520

The cholesterol/phospholipid-phosphorus ratio was 0.52 in the lysosomal and 0.903 in the plasma membrane.

3. Amino acid analysis and polyacrylamide gel electrophoresis

The amino acid composition shown in Table 5 is not significantly different in the two membranes. Disc-electrophoresis, performed with the alkali soluble proteins of the plasma membrane, resulted in 13 well separated bands, whereas the alkali soluble proteins of lysosomal membranes could not be separated with this system. The protein profile of both membranes (Fig. 1) obtained by a modification[8] of the polyacrylamide gel electrophoresis according to TAKAYAMA[45] was completely different.

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Table 5. Amino acid composition of total plasma membrane- and lysosomal membrane protein. Values given in mol per 100 mol of amino acids.

Amino acid	Plasma membrane	Lysosomal membrane
Asp	9.9	9.8
Thr	5.7	5.7
Ser	7.8	8.2
Glu	13.1	10.4
Pro	5.8	6.6
Ala	8.5	7.1
Cys*	2.0	3.3
Met	1.6	1.4
Ile	4.6	4.4
Leu	9.7	6.9
Val	5.7	8.6
Tyr	2.6	3.8
Phe	4.1	4.3
Lys	5.3	5.8
His	1.7	2.1
Arg	3.3	3.7
Gly	8.8	7.9

* Determined as cysteic acid. Tryptophan was not determined.

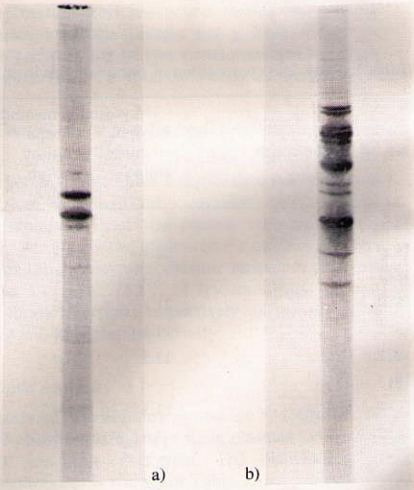

Fig. 1. Polyacrylamide gel electrophoresis: Plasma (a) and lysosomal membrane (b).

4. Carbohydrate analysis

The neutral carbohydrate contents (hexoses, deoxyhexoses, pentoses and heptoses) were estimated by the α-naphthol reaction[9]. They are listed together with hexosamines and *N*-acetylneuraminic acid in Table 6.

Table 6. Carbohydrate constituents in μg per mg of protein.

	Plasma membrane	Lysosomal membrane
Neutral carbohydrates	28	45.6
Glucosamine*	8.6	25.4
Galactosamine*	traces	5.5
N-Acetylneuraminic acid**	10.4	16.1

* Values obtained with a Beckman Spinco Automatic Amino Acid Analyzer.
** Determined by the WARREN method[10].

The values for the carbohydrates in lysosomal membranes clearly exceed those in the plasma membranes.

Table 7 summarizes the gross composition of the various membrane constituents.

Table 7. Composition of plasma and lysosomal membranes as % of dry weight.

	Plasma membrane	Lysosomal membrane
Protein	67.8	73.0
Lipids	29.1	20.2
Phospholipids	15.1	14.0
Neutral lipids	5.6	1.8
Cholesterol	5.9	2.8
Cholesterol (esterif.)	2.5	1.6
Carbohydrates	3.1	6.8
Neutral sugars	1.8	3.3
Hexosamines	0.6	2.3
Sialic acid	0.7	1.2

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Discussion

The fractionation procedures used yielded highly purified membrane material from both sources suitable for direct comparison. Microsomal (16%) and mitochondrial (0.1%) contaminations of the plasma membranes were in the range reported by other authors[11–14]. The preparation of plasma membranes free of microsomal contamination has never been successful as judged by the glucose-6-phosphatase assay. This might be explained by the electron microscopic observations which report multiple points of continuity between the plasma membranes and the endoplasmic reticulum[15]. Lysosomal membranes were prepared from triton filled lysosomes which are essentially free of mitochondria and microsomes. In order to isolate the organelles in an active phase of pinocytosis the lysosomes were isolated two days after Triton WR-1339 application and not after the usual four days interval[7,16]. This lowers the yield of lysosomal membranes by about 30—40%. In comparison with the homogenate the specific activity of the acid phosphatases in the lysosomal fraction is 20 times higher, and in the lysosomal membranes 35 times higher (Table 1). Lysosomal membranes obtained by either sonication or dialysis against a hypotonic solution are identical with respect to the specific activity of their acid phosphatase and their phospholipid composition, thus indicating that the properties of the membrane fraction are independent of the preparation method applied.

Since all biological membranes consist of the same main constituents, proteins, lipids and carbohydrates, but accomplish quite different functions, their diversities must be based on the detailed arrangements of the various compounds. The best studied substances of membranes are the phospholipids. Their presence and composition in the

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plasma membranes, as described in this paper agree well with the findings of several other authors [17–19]. A high amount of sphingomyelin (20%) appears to be characteristic for cellular surface membranes of various cell types[20,21]. Our data show that the lysosomal membrane contains an even higher content of sphingomyelin (32%). This distinguishes the lysosomal membrane clearly from other intracellular membranes[22,23]. With the exception of some preliminary results[24,25] concerning the lysosomal membrane, sphingomyelin has been described as a major lipid constituent of intracellular membranes only in the GOLGI apparatus[26].

Among the cellular membranes there seem to exist two types as judged from the phospholipid composition, one type containing only phosphatidylcholine, the other an appreciable amount of sphingomyelin in addition to phosphatidylcholine. The sum of choline phospholipids constantly accounts for 50–60% of the total phospholipids. A similar mutual exchange of the choline containing phospholipids has been described for the erythrocyte ghost of ruminants and nonruminants[27]. Unfortunately the role of sphingomyelin in membranes so far is unknown and the high content in plasma and lysosomal membranes cannot be interpreted. The fatty acid composition of the glycerophospholipids shows some differences between both types of membranes, the plasma membrane containing in general more polyunsaturated fatty acids. In both membranes the degree of unsaturation is lower compared with other cell fractions (mitochondria, microsomes[22]). Polyunsaturated fatty acids cover a great surface area whereas saturated ones are more tightly packed.

In view of this fact the high content of cholesterol in plasma and lysosomal membranes should be mentioned. Cholesterol produces a condensing effect as observed in lipid monolayers[28] thus stabilizing the molecular arrangement in the membrane structure. This fact may be of biological importance since the plasma membrane is the external barrier of the cell. The pinocytotic process produces the situation that exogenous material in high concentration has to be sequestered inside the cell. This function of the lysosome suggests that the lysosomal membrane should be provided with similar properties.

The high percentage of saturated fatty acids in the phospholipids, the high cholesterol and sphingomyelin content lend the lysosomal membrane stabilizing physico-chemical properties. Not only cholesterol but also sphingomyelin exert a condensing effect on lipid monolayers which might find expression in tightening the packing of the lipid phase of membranes[29].

The total amino acid composition of the plasma membranes reported here is in good agreement with data published by other authors[30,31]. It is worthwhile to mention the absence of hydroxyproline indicating that the plasma-membrane fraction is not contaminated by collagen. The plasma membranes contain a significant excess of acidic amino acids (23.3%) as opposed to the basic ones (10.4%). They may contribute to the negative charge of the surface of the cell[32,33]. The lysosomal membrane shows the same distribution and therefore it may be concluded that their surface also carries a negative charge. Neutral

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sugars and hexosamine concentrations correspond well to the sialic acid content in both membranes as one may expect since they are the main constituents of glycoproteins.

Our analytical data underline certain points of similarity, namely the high content of sphingomyelin, cholesterol and sialic acid and the excess of acidic amino acids. Furthermore with regard to these characteristics plasma and lysosomal membranes differ from other membranes of mammalian cells. The composition of the secondary lysosomal membrane allows the interpretation that, during the pinocytotic process, areas of the plasma membrane bud into the cell and form parts of the membrane of the secondary lysosome.

On the other hand the similarity between the sphingomyelin and cholesterol contents of both membranes may merely reflect similar functions. These two main lipid constituents common to both membranes are known to stabilize membranes. The strikingly different protein patterns and carbohydrate contents of the plasma and lysosomal membranes prompted further comparative studies of the enzymic properties of the two membranes. The results of these investigations are reported in a subsequent paper.

Experimental

Preparation of the subcellular fractions

For the simultaneous isolation of the membrane fractions 1.7 mg per g of body weight of Triton WR-1339 in 0.9% saline was injected into the tail vein of inbred rats weighing about 200 g. After 36 h the animals were starved for 12 h before sacrifice. The livers were quickly removed and chilled in ice. The organs were minced with scissors and homogenized in 9 volumes of 0.25M sucrose by means of 25 strokes in a Dounce homogenizer with a loose fitting rubber pestle. All operations were carried out at 4°C. The flow sheet (Fig. 2) summarizes the fractionation steps.

Materials and reagents

AMP, glucose-6-phosphate and cytochrome *c* were obtained from Boehringer Mannheim GmbH and *p*-nitrophenyl phosphate and FOLIN-CIOCALTEU's phenol reagent from Merck, Darmstadt. Triton WR-1339 was purchased from Serva, Heidelberg. All the other chemicals were of analytical grade.

Analytical procedures

Protein was determined by the LOWRY method[34].

5'-Nucleotidase was measured by the release of inorganic phosphate from AMP and was carried out in 0.5 ml of 0.1M Tris-HCl buffer (pH 8.5) containing 10mM AMP and 10mM $MgCl_2$. After incubation at 37°C for 15 min the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid.

Glucose-6-phosphatase was determined under the same conditions in 0.5 ml of citrate buffer (0.1M, pH 6.5) containing 40mM glucose 6-phosphate.

Inorganic phosphate was assayed by the method of CHEN *et al.*[35].

The assay for *acid phosphatase* was carried out according to LINHARDT and WALTER[36]. *Cytochrome c oxidase* was determined by the method of FREEMAN[37] using the molar extinction coefficient of MASSEY[38].

All enzyme activities were expressed as μmol of substrate utilized per mg of protein per min.

Total lipids were extracted with chloroform/methanol 2:1. The extracts were washed with water, concentrated under N_2 and dissolved in chloroform/methanol 2:1. Portions were taken for the determination of total lipid phosphorus by a modified BARTLETT[39,40] procedure.

Phospholipids were separated by thin-layer chromatography (silica gel H, solvent system: chloroform/methanol/acetic acid/water 56:30:8:4). The spots were visualized with iodine vapour and assayed for phosphorus. The fatty acid methyl esters of phospholipids separated by thin-layer chromatography were obtained by transmethylation with 5% HCl in methanol. The analysis was carried out by quantitative gas liquid chromatography on a 2.5% diethylene glycol succinate polyester column (2 m, 180°C) using a Perkin-Elmer Gas Chromatograph equipped with a flame ionization detector. Identification was performed by the use of pure fatty acid standards. The percentage of unsaturated fatty acids was calculated from the original fatty acid methyl ester analysis and that after catalytic hydrogenation (PtO_2).

Separation of *neutral lipids* was achieved by thin-layer chromatography (silica gel H, solvent system: petroleum ether/ether/acetic acid 70:30:1) Quantitation was

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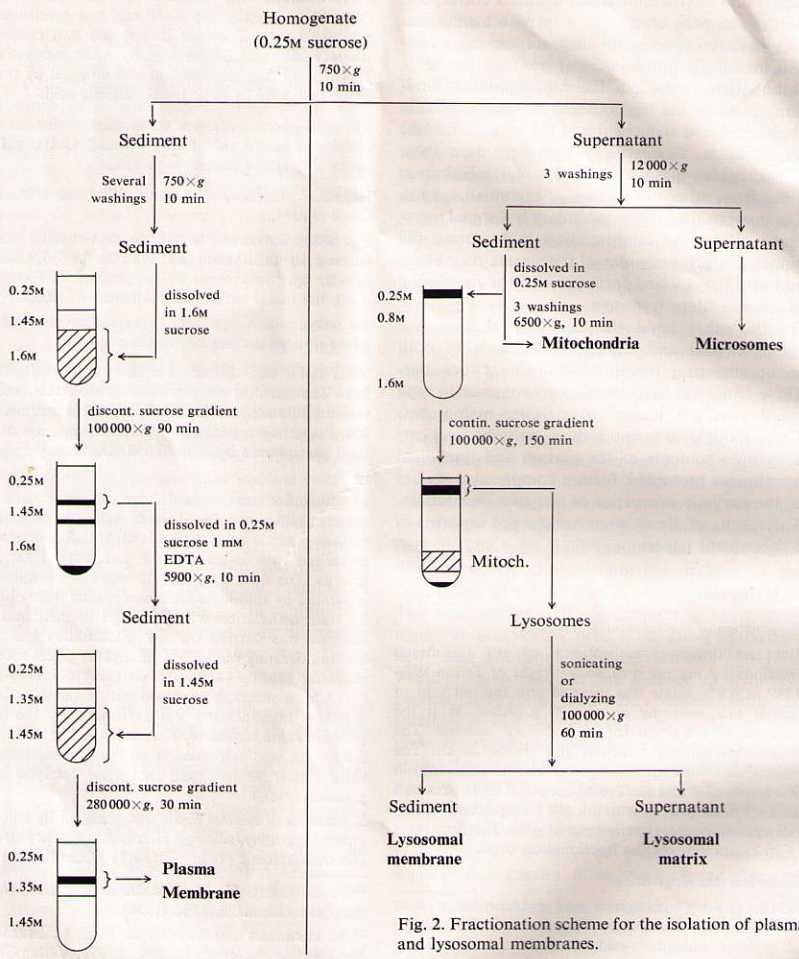

Fig. 2. Fractionation scheme for the isolation of plasma and lysosomal membranes.

carried out by the determination of ester groups according to STEPHENS and SNYDER[41].

Total cholesterol was measured by the method of ZAK[42].

Amino acid analysis was performed after hydrolysis of whole membranes in 6N HCl (110°C) on a Beckman Unichrom Amino Acid Analyzer. Cysteine was determined as cysteic acid after performic acid oxidation[43].

Polyacrylamide disc electrophoresis was carried out after solubilisation of the membranes with 0.05M K_2CO_3.

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The alkaline extract of membranes was adjusted to pH 3.5 with acetic acid and separated with a gel system according to NEVILLE[44]. Furthermore polyacrylamide gel electrophoresis was performed with the system of TAKAYAMA *et al.*[45] as modified by FLEISCHER *et al.*[8]. The gels were stained with Coomassie Blue (0.25% in 7% acetic acid).

Neutral sugars (hexoses, deoxyhexoses, pentoses, and heptoses) were estimated by the quantitative α-naphthol reaction[9] at 560 nm using a mixture of galactose, mannose and ribose (1:1:1) as standard.

Glucosamine and *galactosamine* were analyzed after 19 h hydrolysis in 3N HCl at 110°C on a Beckman Unichrom Amino Acid Analyzer.

Sialic acid was determined by the WARREN method[10].

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