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Heparin–Sepharose as a Tool in the Subcellular Fractionation of a Polyamine-Rich Organ (Rat Ventral Prostate)

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Abstract

Heparin–sepharose forms complexes with putrescine, spermidine, and spermine and indirect measurements of its affinity for polyamines gives values similar to those obtained with free heparin. A direct measurement of the binding of heparin–sepharose to spermine gives an apparent dissociation constant (K_d) of $1.5 \times 10^{-6} M$ spermine.

Unlike free heparin, heparin–sepharose does not cause either disruption of the nuclei or more sutble modifications able to modify their sedimentation behavior.

The heparin–sepharose polyamine complex formed by the addition of heparin–sepharose to the homogenate can easily be removed and the homogenate can be processed according to normal schedules.

Heparin–sepharose is able to sequester 85% of the exchangeable spermine present in the homogenate of rat ventral prostate.

The distribution of the marker enzyme galactosyltransferase (Golgi apparatus) on a sucrose density gradient was followed to assess the usefulness of heparin–sepharose in minimizing the aggregation of cellular organelles brought about by polyamines.

Index Entries: Heparin–sepharose, in subcellular fractionation; sepharose–heparin, in subcellular fractionation; polyamine-rich organ, subcellular fractionation; rat ventral prostate, subcellular fractionation; fractionation, subcellular, by heparin–sepharose.

Introduction

It was previously found that heparin added either to rat liver microsomes aggregated by spermine or to rat ventral prostate homogenate is able to sequester polyamines, minimizing the aggregation of subcellular organelles caused by these

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polycations (*1, 2*). These results allowed the development of reproducible and reliable methods to improve separation in the purification of cellular organelles from rat ventral prostate and other polyamine-rich organs (*2, 3*). Heparin however, inducing physical and functional changes (*4–6*), causes disruption of nuclei. The formation of a nucleoprotein gel highly effects the fractionation of membranous material and nuclei must then be removed before the addition of heparin (*2*). This difficulty could be overcome by the use of heparin rendered unable to enter the nuclei and then unable to displace DNA from complexes with histones. Heparin bound to agarose is commercially available and might be able to fulfill this requirement.

The purpose of this investigation was to evaluate: (1) the parameters of binding of heparin–sepharose to polyamines in vitro; (2) its lack of interactions with chromatin; (3) its ability to sequester polyamines present in vivo; (4) its effectiveness, when added to a rat ventral prostate homogenate, in releasing polyamines caused aggregation of subcellular organelles.

Materials and Methods

Heparin–sepharose Cl-6B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); heparin, sodium salt from Sigma Chemical Co. (St. Louis, MO, USA) and ^{14}C-spermine tetrahydrochloride (100 mCi/mmol) from the Radiochemical Centre (Amersham, Buck., England). All other chemicals were of analytical grade.

The binding of polyamines to heparin and heparin–sepharose was inferred from competitive binding studies performed with toluidine blue in 5 m*M* 3-(*N*-morpholino) propane sulfonic acid (MOPS) buffer at pH 7 and room temperature by the method described by Gianazza and Righetti (*7*).

The measurement of binding of ^{14}C-spermine to heparin–sepharose was performed by incubation of various concentrations of ^{14}C-spermine in 5 m*M* MOPS buffer, pH 7, at room temperature for 1 h with constant shaking. The reaction mixture was then layered on 3 mL of 1.5*M* sucrose and centrifuged for 5 min at 100*g*. Heparin–sepharose spermine complex collected at the bottom of the tubes and was dissolved in 0.5 mL Soluene-350 and added with 10 mL of Instagel.

The binding of spermine present in rat ventral prostate homogenate to heparin–sepharose was determined by incubation of various amounts of heparin–sepharose with 1 mL of homogenate added with ^{14}C-spermine (1×10^5 dpm). The 10% (w/v) homogenate was prepared by dissecting and homogenizing the ventral prostates obtained from male Wistar strain rats, in 0.25*M* sucrose with a Potter-Elvehjen homogenizer. After 1 h incubation the reaction mixtures were layered on 3 mL of 1.5*M* sucrose and centrifuged for 2 min at 100*g*. The nuclei did not sediment under these centrifugation conditions. The radioactivity in the pellets was determined as described above.

To study the subfractionation of Golgi apparatus, the homogenate that did not enter the 1.5*M* sucrose was collected with the aid of a Pasteur pipet. Some 1.5*M*

sucrose was also sucked and the homogenate was brought again to 0.25*M* sucrose with the aid of a Bausch & Lomb refractometer. A 1 mL volume of homogenate was layered on a discontinuous sucrose density gradient. The tubes were centrifuged using a SW 50 rotor at 142,000*g* for 1.5 h. After centrifugation, fractions were collected and the enzyme galactosyltransferase was assayed under optimum conditions, as previously described (*8*).

Protein was determined according to Lowry et al. (*9*); polyamines were extracted as described by Seiler (*10*) and determined according to Seiler and Wiechmann (*11*); DNA was estimated by the diphenylamine method of Burton (*12*).

Results and Discussion

Binding of Polyamines to Free and Sepharose-Bound Heparin In Vitro

Heparin has been used to sequester polyamines because of its ability to form complexes with these polycations (*13, 14*). Although the studies mentioned demonstrated the binding of various polyamines to heparin, no extensive study of the dissociation constants of the complexes has been reported. On the other hand, the use of heparin–sepharose to substitute free heparin will be more successful the more similar are their affinities for polyamines. We have attempted to get such data by competitive binding studies performed with toluidine blue according to the method described by Gianazza and Righetti (*7*). The curves in these plots represent binding isotherms since the values in the ordinate are proportional to the fraction of occupied sites, f (expressed as the ratio of number of bound molecules to number of binding sites in the chain), and the value in the abscissa is proportional to the term $\ln (X) + \ln K_0$ (*15*). Figure 1 shows the binding isotherms of spermine, spermidine, and putrescine to free heparin. The isotherms differ about one order of magnitude in terms of concentration and the doses required to achieve 50% of maximal inhibition ($f = 0.5$) of the binding of toluidine blue to the polyanion are $2 \times 10^{-5}M$ spermine, $2 \times 10^{-4}M$ spermidine, and $4 \times 10^{-3}M$ putrescine.

Figure 2 shows a similar experiment conducted in the presence of heparin–sepharose. In this case the increase of OD of the α-band of toluidine blue measured did not result from the reversal of the metachromatic effect, but from the increase in the concentration of the dye released from the complex with heparin–sepharose. It can be seen that the isotherms do not significantly differ from those obtained with free heparin and also that the $f = 0.5$ values are almost identical. A direct measurement of the binding parameters of the spermine/heparin–sepharose interaction was also performed. Figure 3a shows the binding curve of ^{14}C-spermine to heparin–sepharose; the Hill plot of the data (Fig. 3b) gives a value of 1.6 ± 0.3 for the Hill coefficient and a $K_d = 1.5 \pm 0.4 \times 10^{-6}M$ for spermine. From these data it is evident that free and sepharose-bound heparin have similar high affinities for polyamines.

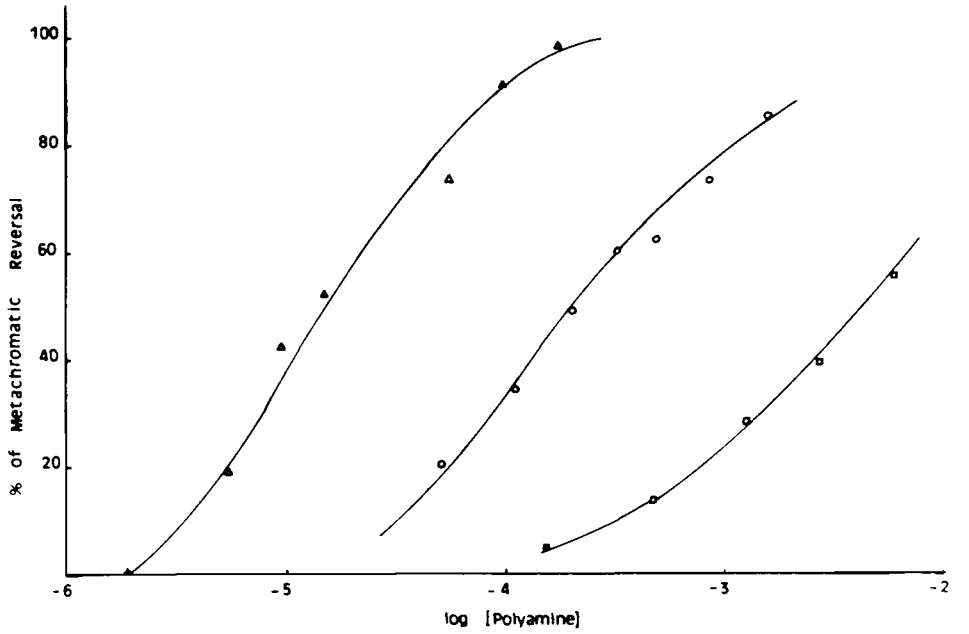

Fig. 1. Binding isotherms of putrescine, spermidine, and spermine to free heparin. To 1 μ*M* toluidine blue in a 3 mL cuvet, enough heparin (usually about 1–2 μEq.) was added to obtain the fully metachromatic complex. To the preformed complex, increasing amounts of putrescine □, spermidine ○, or spermine △ were added to bring about the metachromatic reversal.

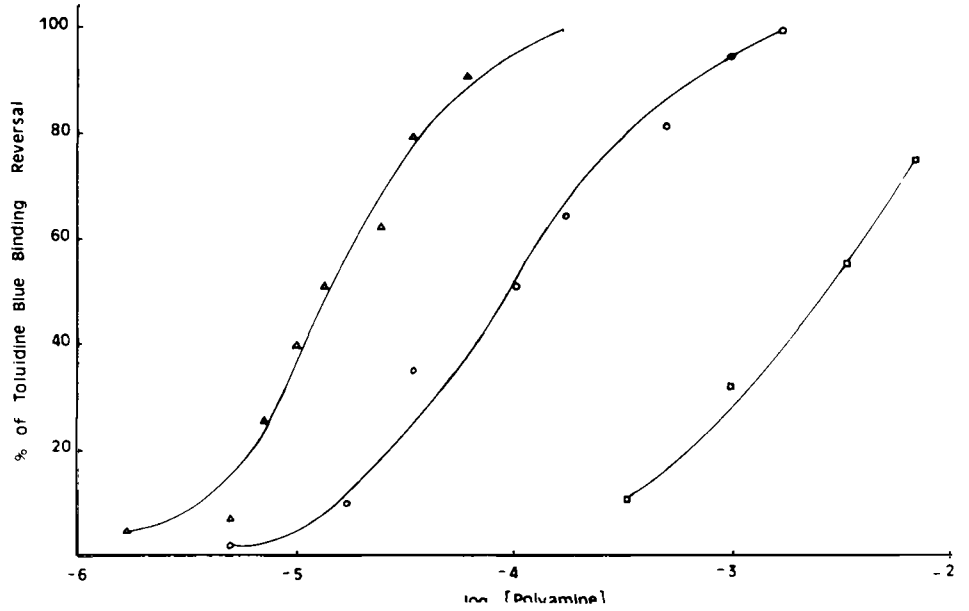

Fig. 2. Binding isotherms of putrescine, spermidine, and spermine to heparin–sepharose CL-6B. To 1 μ*M* toluidine blue in a 3 mL cuvet enough packed heparin–sepharose (usually about 10–20 μL) was added to obtain the total binding of the dye. To the preformed complex increasing amounts of putrescine □, spermidine ○, and spermine △ were added to release the dye from the complex.

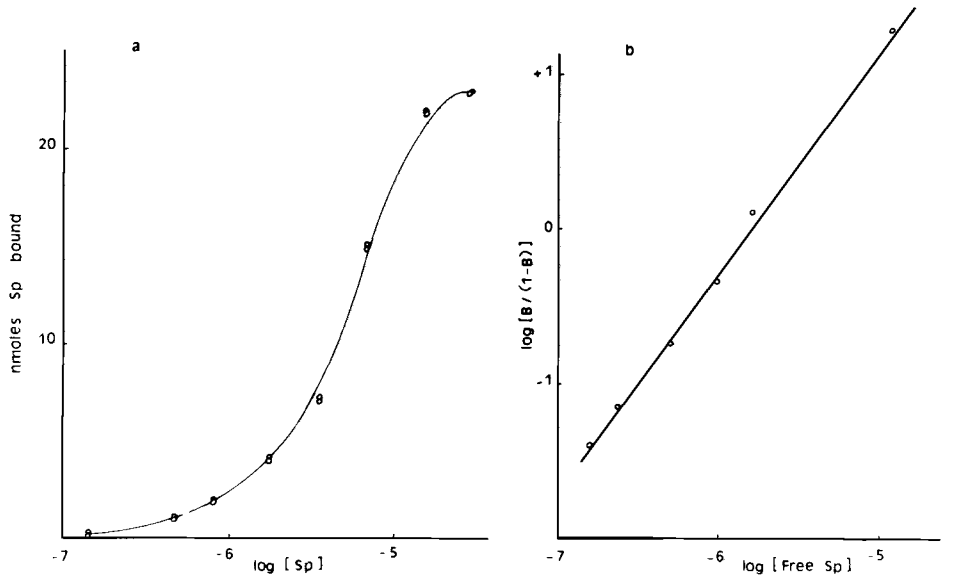

Fig. 3. Binding of spermine to heparin–sepharose. (a) Various concentrations of ^{14}C-spermine were added to 10 μL of packed heparin–sepharose and after a 1-h incubation the spermine/heparin–sepharose complex was recovered and the ^{14}C-spermine bound was determined as indicated in the Materials and Methods section. (b) A Hill plot of the same data from Fig. 3a.

Effect of Heparin–Sepharose on the Sedimentation of Nuclei

The major drawback in the use of free heparin to sequester polyamines from homogenates is its penetration into the nuclei, where it can exchange histones from the DNA–histone complex in a macromolecular ion exchange type of mechanism. This produces marked chromatin dispersion, marked or complete nucleolar dispersion, and lysis of nuclear membranes (*4–6*). Heparin–sepharose CL-6B is an adsorbent in which heparin is coupled to large (40–210 μm) beads of sepharose. As shown, heparin bound to this matrix is still able to interact with polyamines, but it is not expected to enter the nuclear pores, being coupled to beads 4–40 times larger than nuclei. When rat ventral prostate homogenate was mixed with heparin–sepharose, no apparent nucleoprotein gel was observed. To exclude a less evident and more subtle modification of the physical characteristics of the nuclei, we evaluated their ability to sediment through a 2.1*M* sucrose solution. The complex of polyamines and heparin–sepharose was easily removed from the homogenate because of the high density of the resin. The homogenates were layered on 1.5*M* sucrose and centrifuged for 2 min at 100*g*. The polyamines/heparin–sepharose complex is recovered in the pellet and the homogenate can be processed as usual. Sedimentation of treated and untreated homogenates on 2.1*M* sucrose in the presence of 1 m*M* $MgCl_2$ gave similar yields of nuclei, as percentage of total tissue DNA, with a small but reproducibly better recovery from heparin–sepharose-treated homogenate (10%). This phenomenon could be explained by considering the aggregating effect caused by polyamines on cellular components. Nuclei may aggregate with subcellular particles of low density and

the resulting complex may then exhibit intermediate sedimentation behavior. This explanation could also account for the different yields of rat ventral prostate nuclei obtained from normal, castrated, and testosterone-treated rats (*16*). The Triton X-100 wash employed by other authors to normalize the yield (*16*) is known to remove both the inner and outer membranes of the nuclear envelope (*17*), and the envelope-denuded nuclei may be less liable to aggregation with other organelles.

Binding of Spermine Present In Vivo to Heparin–Sepharose

From the data so far obtained it is evident that heparin–sepharose is able to bind polyamines with high affinity and does not cause disruption of nuclei. To evaluate its ability to sequester the polyamines present in a rat ventral prostate homogenate, different amounts of this adsorbent were incubated with 1 mL of homogenate. ^{14}C-spermine (1×10^5 dpm) was added to the homogenate to give a theoretical specific activity of 3.7×10^5 dpm/μmol. This presupposes the possibility that added radioactive spermine may mix uniformly with endogenous polyamine. This almost certainly is not the case since the spermine trapped in closed compartments does not mix with exogenous spermine. Figure 4 shows that the binding is proportional to the amount of heparin–sepharose added and that 0.3 mL of packed

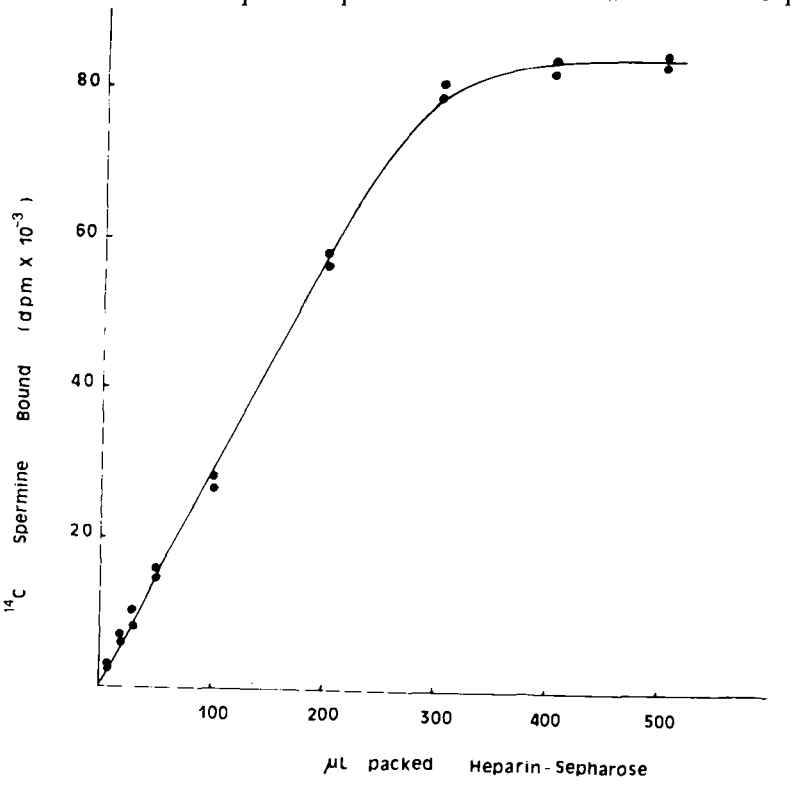

Fig. 4. Binding of ^{14}C-spermine, added to rat ventral prostate homogenate, to heparin–sepharose. ^{14}C-spermine (1×10^5 dpm) was added to 1 mL of rat ventral prostate homogenate (10% w/v). Increasing amounts of packed heparin–sepharose were added and after a 1-h incubation the spermine/heparin–sepharose complex was recovered and the ^{14}C-spermine bound was determined as indicated in the Materials and Methods section.

heparin–sepharose binds 80% of the radioactive spermine. This does not correspond to the total spermine present, as already pointed out, but to the exchangeable pool that is responsible for aggregation. Further addition of heparin–sepharose does not further sequester considerable amount of ^{14}C-spermine since the amine probably entered the nucleus and is no longer accessible to heparin–sepharose. The amount of spermine present in the homogenate bound per milliliter of heparin–sepharose is rather low compared to the amount bound to heparin–sepharose in a two-component binding system (Fig. 3). This may result from many not mutually exclusive mechanisms: (1) occupancy of the binding sites on heparin by other compounds, as well as spermidine and putrescine; (2) strong competition for spermine by heparin–sepharose and other structures with high affinity for this amine: (3) very low concentration of exchangeable spermine compared to its average concentration in this homogenate (0.29 m*M*); (4) ionic conditions in the homogenate.

Effect of Heparin–Sepharose Added to the Homogenate of Rat Ventral Prostate on the Sedimentation of Golgi Apparatus

Heparin–sepharose added to a 10% w/v rat ventral prostate homogenate apparently is not able to sequester the totality of exchangeable spermine. The spermine left either may be trapped in close and/or unaccessible compartments and thus not be involved in aggregation phenomena, or may be firmly held on cellular membranes by strong ionic- and/or covalent-specific bindings. In this latter case it may still exert some aggregating effect. Preliminary experiments were set to determine whether the residual amount of polyamines was still causing aggregation despite the heparin–sepharose treatment. We studied the distribution of Golgi apparatus or a sucrose density gradient following the distribution of one of its marker enzymes: UDP-galactose:glycoprotein galatosyltransferase. The choice of this cellular organelle was made because of the availability of data on the distribution of these particles in a homogenate deprived of nuclei and supplemented with free heparin (*2*). After removal of the polyamines/heparin–sepharose complex, the homogenate was brought to 0.25*M* sucrose and layered on a discontinuous sucrose density gradient: 1.5*M*, 1.25*M*, and 0.6*M* in 50 m*M* Tris HCl, pH 7, and 1 m*M* EDTA. A portion of homogenate was subjected to the same operation with the addition of sepharose CL instead of heparin–sepharose. Figure 5 shows the distribution of the enzyme in the gradient. The total recovery of the enzymes from the gradients plus the pellets was 95 and 93% for those that were heparin-treated or not homogenates, respectively. The distribution on the gradient of galactosyltransferase from the homogenate depleted of polyamines is very similar to the result obtained when free heparin is used to sequester polyamines. Heparin–sepharose probably does not completely abolish the aggregation as the centrifugation conditions (1.5 h and 142,000*g*) and also the specific activity of the enzyme (15 nmol galactose transferred/min/mg of protein) are neither so extreme nor so good as in the case of free heparin (5 h and 142,000*g* and 32 nmol galactose transferred/min/mg of protein, respectively). However, the high reproducibility of the distribution of the enzyme gives reason to believe that our aim has been reached and that rat ventral prostate may be subfractionated without undue difficulty.

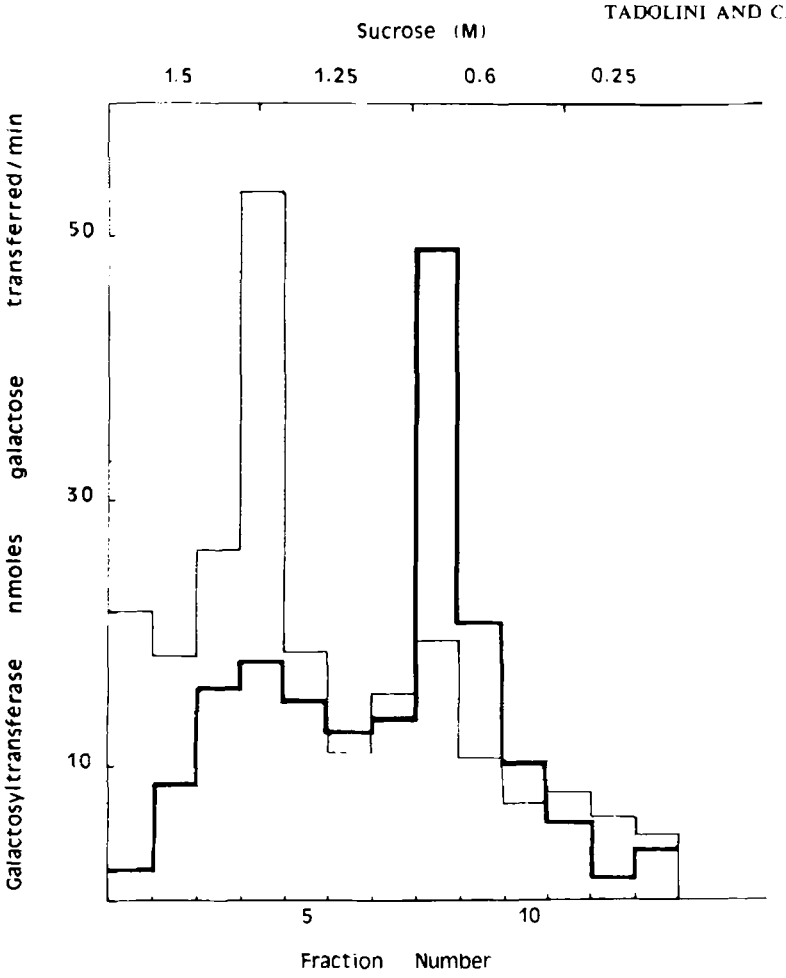

Fig. 5. Distribution of galactosyltransferase after centrifugation of rat ventral prostate homogenate deprived of polyamines by heparin–sepharose treatment on a discontinuous sucrose density gradient. The rat ventral prostate homogenates either deprived of polyamines by heparin–sepharose treatment (heavy line) or treated with Sepharose CL (thin line) were layered on a discontinuous sucrose density gradient and centrifuged at 142,000*g* for 1.5 h. Galactosyltransferase present in the fractions and in the pellets was assayed as indicated in the Materials and Methods section.

Acknowledgments

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