APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 9, 153–159 (1984)

# Representative Applications of Heparin–Sepharose in the Removal of Polyamines from Biological Materials

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Received September 27, 1983; Accepted November 1, 1983

## ABSTRACT

Many experimental systems would greatly benefit from the availability of a simple and effective technique to remove polyamines from biological materials. We have examined the possibility of utilizing heparin–sepharose in the removal of polyamines from rat heart mitochondria, DNA–spermine complex, and fetal calf serum.

(1) Heparin–sepharose removes 90% of spermine adsorbed to the cytoplasmic surface of rat heart mitochondria.

(2) Heparin–sepharose almost totally removes spermine from DNA–spermine complex, leaving less than 0.003 mol spermine/mol DNA phosphorus.

(3) Heparin–sepharose is highly effective in removing spermine and spermidine (99.5 and 95% adsorbed, respectively) from fetal calf serum. Under the same experimental conditions only 50% of putrescine is adsorbed. A higher amount of resin corresponding to an increased capacity for putrescine must be used to achieve a satisfactory removal of putrescine.

**Index Entries:** Heparin–sepharose, polyamine removal by; mitochondria, adsorbed spermine removal from; DNA, removal of spermine complexed to; serum, removal of polyamines from; polyamines, removal from biological materials; sepharose–heparin, for polyamine removal.

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0273–2289/84/0400–0153$02.00

## INTRODUCTION

Putrescine, spermidine, and spermine are found in a wide variety of animals, bacteria, yeasts, and plants and may be considered ubiquitous in biological materials (*1*). Because of their polybasic nature, they have a particularly high affinity for biological polyanions. Polyamines are known to bind nucleic acids (*2–5*) phospholipid membranes (*6–8*), lipopolysaccharides (*9,10*), heparin (*11,13*), and proteins (*14,15*). The presence of polyamines bound to different biological polyanions very often gives rise to technical problems. Polyamines cause aggregation of cellular organelles in the homogenate of tissues rich in these amines, jeopardizing subcellular fractionation studies and organelle purification (*12*). Adsorption of polyamines to the outer bilayer of the membrane of cellular organelles does not allow the evaluation of polyamine content of the organelle. This problem prevents the study of the subcellular distribution of polyamines and the study of polyamine transport from cytoplasm, where these amines are synthesized, to other cellular compartments. Polyamines strongly bind to biologically active peptide fractions from tissues or cultured cells (*14*). This binding has proved extremely harmful since the addition of the peptide–polyamine complex to cultures containing polyamine oxidase activity results in the formation of toxic aldehydes that are inhibitory to a variety of cells (*16*). Sometimes the strength of the binding of polyamines to polyanions has been exploited. Spermine has been utilized to fractionate the soluble ribonucleic acid of rabbit liver (*2*) and synthetic polynucleotides (*3*). Selective precipitation of DNA by spermine is obtained even in the presence of protein and triphosphates (*16*). However, the removal of polyamines from nucleic acid–spermine complex is time consuming. Sometimes the presence of polyamines in biological material may be responsible for observable phenomena. Polyamines present in serum thus may mediate some of the effects of serum on the cyclic nucleotides content of cultured cells (*18*), and the raised polyamines levels in same patients may mediate the onset of some clinical disorders (*19*).

These and many other experimental systems would greatly benefit from the availability of a simple and effective technique to remove polyamines from biological materials. For some of the examples referred to, namely, subcellular distribution and compartmentation of polyamines, such a technique would probably allow a real breakthrough in the field of polyamine biochemistry. Recently, we have shown that the resin heparin–sepharose has a high affinity and capacity for polyamines (*20*) and that it may be utilized to minimize subcellular aggregation brought about by polyamines (*21*). We have further investigated the suitability of this resin to remove polyamines and in the present report we examine the possibility of utilizing heparin–sepharose in the removal of polyamines from rat heart mitochondria, DNA–spermine complex, and fetal calf serum.

## MATERIALS AND METHODS

Spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, and calf thymus DNA II were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). $^{14}$C-spermine tetrahydrochloride (115 mCi/mmol) $^{14}$C-spermidine trihydrochloride (122 mCi/mmol), $^{3}$H-putrescine dihydrochloride (19 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Buck., England).

Heparin–sepharose CL-6B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), fetal calf serum from GIBCO, Europe (Paisley, Scotland UK), and BTS-450 from Beckman Inc. (Irvine, CA, USA).

Mitochondria were isolated from heart of adult male Wistar rats by the method described by Sordahl et al. (*22*). Isolated mitochondria were resuspended in 0.25*M* sucrose, 20 m*M* MOPS (morpholinopropanesulfonic acid), pH 7.2, without addition of bovine serum albumin, which often contains one amine oxidase (*23*). All assays were made using known polarographic methods (*24*).

In each test, 2–3 mg of mitochondrial protein were added with $5 \times 10^5$ dpm $^{14}$C-spermine for a tracer in 1 mL of 5 m*M* MOPS buffer, pH 7. After 5 min incubation at room temperature, the suspension was added to 0.5 mL of swollen heparin–sepharose and the resulting slurry was kept stirred for 10 min. The suspension was then poured onto a column that was eluted and washed with buffer.

Calf thymus DNA was dissolved in 10 m*M* MOPS buffer, pH 7.2. DNA concentrations were determined by absorbance at 260 nm, based on $A_{260} = 1$ for 50 μg/mL. To 1 mL of 75 μg/mL DNA, 0.5 μmol $^{14}$C-spermine ($5 \times 10^6$ dpm) were added and, after stirring, the solution was left at room temperature for 15 min. The suspension was then added to 0.5 mL of swollen heparin–sepharose and the slurry was treated as described for the mitochondria, except for a 45 min incubation.

Fetal calf serum that contains very low levels of polyamines (17) was supplemented with a final concentration of $1 \times 10^{-5}M$ $^{14}$C-spermine ($1 \times 10^5$ dpm), $1 \times 10^{-5}M$ $^{14}$C-spermidine ($1 \times 10^5$ dpm), and $1 \times 10^{-6}M$ $^{3}$H-putrescine ($1 \times 10^6$ dpm) in three different sets of experiments. After addition of labeled polyamine, 1 mL serum was applied to column of 0.5 mL swollen heparin–sepharose gel. The column was eluted and washed with buffer.

Eluted labeled polyamines were determined by scintillation counting. Adsorbed labeled polyamines were determined diluting 5–10 times the washed, nonregenerated column with buffer and drawing a fixed amount of resuspended resin. The radioactivity content of the resin was determined, after digestion with BTS-450, by scintillation counting.

The heparin–sepharose resin was regenerated for reuse as suggested by the manufacturer.

Protein concentration was determined spectrophotometrically by the method of Lowry et al. (*25*).

## RESULTS AND DISCUSSION

### *Removal of Spermine from the Cytoplasmic Surface of Mitochondrial Membranes*

One of the most important unresolved question in the study of the biological functions of polyamines is their subcellular distribution. None of the data obtained so far answers this question adequately since adsorption of polyamines on the cytoplasmic surfaces of organelle membranes does not allow the determination of the polyamine contents of the organelles. The use of heparin–sepharose to remove adsorbed polyamines may be a novel approach.

The possibility of evaluating the ability of the resin heparin–sepharose to remove spermine from the cytoplasmic surface of the mitochondria is based on two assumptions: that added labeled polyamine equilibrates with adsorbed spermine during the preincubation time, and that added labeled polyamine does not enter the mitochondria because of alteration of the mitochondrial membrane caused by the isolation procedure.

The former is confirmed by previously published experiments (*12*). Addition of polyamines to mitochondrial suspension causes an almost instantaneous aggregation of the organelles since the polycation becomes attached to the particle surface, decreasing the high net negative surface charge density that they usually possess with consequent abolition of repulsive forces. Furthermore, a direct measurement of the second-order rate constant of aggregate formation of spermine–phospholipid vesicle complex is about 43 $s^{-1}M^{-1} \times 10^3$ spermine (unpublished observation). The binding process is then very rapid.

The latter assumption is confirmed by the following experiments. The inability of mitochondria to oxidize externally added NADH is attributable to the impermeability of the mitochondrial membrane to this substrate and is, in fact, a most sensitive criterion for the "intactness" of isolated mitochondria (*26*). Alteration of the mitochondrial membrane as a result of the isolation procedure would permit oxidation of external NADH by mitochondria. Addition of NADH to the mitochondrial suspension did not stimulate oxygen consumption, as determined by the conventional polarographic method (*24*). Also, the values for glutamate plus malate oxidation of the P:O ratio (2.98) and of the respiratory control ratio (5.8) indicate the good quality of the rat heart mitochondria utilized in the removal experiments of adsorbed spermine. Intact and coupled rat heart mitochondria were added with labeled $^{14}C$-spermine to label the adsorbed pool of this polyamine.

After 5-min incubation, heparin–sepharose was added and the suspension was stirred by a rotating unit in order to achieve uniformity. After the affinity reaction was completed, the suspension was poured onto a chromatographic column. The column was washed with the buffer and the radioactivity present in the eluate was determined. The experiment was repeated in triplicate with two different mitochondrial preparations and the results were highly reproducible. The heparin–sepharose column is able to remove 90 ± 2% of the labeled spermine. Under similar experimental conditions and in the absence of mitochondria, the heparin–sepharose column is able to completely remove spermine from the solution. Then either the spermine present in the eluate is strongly bound to some component of the cytoplasmic membrane of the mitochondrion or it has been transported into the mitochondrial compartment and is inaccessible to the resin.

### *Removal of Spermine from the DNA–Spermine Complex*

It has been found that spermine can complex with DNA, apparently with neutralization of the negatively charged phosphate groups of the latter (*4–5*). At sufficiently high spermine concentrations, the complexes with DNA form precipitates (*17*). The precipitation of spermine–DNA complex has been exploited to purify DNA from impurities and buffer components. The removal of spermine from the complex, however, is time consuming and is achieved either by exchange with other cations or by high ionic strength buffers (*17*). We investigated the effectiveness of heparin coupled to sepharose beads to achieve this result without the reported limitations of the previously published methods.

Addition of labeled spermine to a solution containing DNA resulted in a aggregation or condensation reaction. Aggregation could be followed by measuring the turbidity at 300 nm. The reaction was completed after 10 min at room temperature. An amount of heparin–sepharose, corresponding to a spermine adsorption capacity exceeding twice the amount of spermine present, was added to the test tube and the suspension was kept stirred to insure a good degree of interaction. The determination of the adsorption kinetic could be followed measuring the decrease in turbidity of sample aliquots withdrawn at different time intervals. Under our experimental conditions, 30 min was a sufficient time lapse to insure the completion of the affinity reaction. The suspension was then poured onto a column and washed with the buffer. Labeled spermine was almost totally absent from the eluate, leaving less than 0.003 mol spermine/mol DNA phosphorus.

### *Removal of Polyamines from Serum*

One of the first-shown effects of polyamines in animals was a marked renal toxicity. Parenteral administration of spermine to a variety of animals produces proteinuria, serum nonprotein nitrogen retention

followed by death from renal failure within a week (*27*). In humans, vomiting, albuminuria, hematuria, acetonuria, azotemia, and hyperglycemia are observed after intramuscular injection of spermine (28). The polyamine content of human serum is rather low, but rises to high levels in many situations mostly pathological: during pregnancy (*29*), in patients with cancer (*30*), in chronic hemodialysis patients (*19*), and in patients with extensive cellular necrosis (scalds, etc.). The presence of high levels of polyamines in the serum of patients may grow synergistically worse in some pathological conditions, and may itself cause the onset of clinical disorders.

This hypothesis may be experimentally studied only by comparing the pathophysiologies of experimental animals whose serum polyamines have been removed or left unchanged.

The procedure developed to remove polyamines from other biological material was then utilized for the selective removal of polyamines from serum. Fetal calf serum was used after addition of labeled polyamines: 1 mL of serum was applied to a 0.5-mL column of swollen heparin–sepharose and was eluted with buffer. The eluate was collected and the concentration of protein and polyamines was determined. The resin was highly effective in removing spermine and spermidine (99.5 and 95% adsorbed, respectively), probably because of the tight binding that contracts in the presence of polyamine and resists the ionic strength of serum. On the contrary, only 50% putrescine is adsorbed. Putrescine interacts with heparin–sepharose with either two or one charge at two different classes of binding sites (*20*). The latter electrostatic linkage may not be considered tight binding and an increase in salt concentration elevates the probability that dissociation of the bond occurs. Half of the putrescine in a serum ionic environment appears to be in a finite adsorption equilibrium with the adsorbent and to move down the column. The movement is slower than the flow rate of the eluting liquid as the putrescine peak is retarded with respect to the protein peak. To completely adsorb putrescine, a higher amount of resin, corresponding to an adsorption capacity of the first binding class higher than the putrescine loaded on the column, must be used (*20*).

The protein adsorbed by the column is only 1.3% of the total and its chemical nature is well-defined. The major plasma proteins with affinity for immobilized heparin have been identified (*31,32*) and are principally coagulation proteins, complement protein, and lipoproteins. Depauperation of these factors in serum may be a major drawback also if the use of heparin–agarose for extracorporal removal of plasma components has already been successfully performed (*33*).

## ACKNOWLEDGMENTS

This work was supported by grants from Italian CNR. We are grateful to Professor C. A. Rossi for his interest in this research and for providing all facilities.

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