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POLYAMINES EFFECT ON SUBCELLULAR FRACTIONATION OF RAT LIVER HOMOGENATE

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SUMMARY: 1) Spermidine and spermine added to the homogenizing medium are able to increase the sedimentation velocity of mitochondria, smooth microsomes, lysosomes, Golgi apparatus and plasma membranes. Spermine at 0.5 mM enhances the sedimentation and at 3 mM is able to sediment, at 600 g for 10 min, almost the totality of membranous components of the cell.
2) Smooth microsomes were used as a model to study the nature of spermine effect. The amount of spermine bound to 1 g of smooth microsomes would increase their density of about 0.02%. In the presence of 1 mM spermine the great majority of smooth microsomes are unable to pass through a 10 μ filter indicating an extensive aggregation of the particles.
3) Spermine induced aggregation of smooth microsomes can be reversed by either heparin or poly-D-glutamic acid.

It is known that inorganic cations present in the homogenizing medium exert a very pronounced effect on cellular organelles (1,2). They cause extensive aggregation enhancing the sedimentation velocity of the particles. In spite of the rather wide interest in this field not much attention has been given to the effect that organic cations, and among them polyamines, can exert on subfractionation of cellular organelles. A part from early reports (3-5) this practical and operative aspect of polyamines has been neglected. Polyamines are widely distributed in nature and their concentration is not always negligible (6). The highest contents are found in pancreas and ventral prostate of rat (9.6 and 13.4 μmol/g wet wt. respectively) (6). The content of these amines in the same tissue is also variable depending on its functional and hormonal status (7). Polyamines have been shown to have stabilizing effects in vitro on various cellular and subcellular components (3,5,8,9). Many of the results obtained can be explained by considering the polyamines as polyvalent cations. Because of their polybasic nature however, polyamines have a particularly high affinity for cellular polyanions, and thus have pronounced effects at rather low concentrations. These considerations even more emphasize the usefulness of a study aiming at determining the influence that polyamines have on subcellular fractionation. The purpose of this investigation was to find out: 1) the degree of interaction of polyamines with different cellular organelles; 2) the mechanism of their effect. We undertook also experiments to define media able to minimize the aggregation caused by spermine.

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MATERIALS AND METHODS

^{14}C spermine tetrahydrochloride (100 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, Buck., England). Spermidine trihydrochloride and spermine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis Mo., U.S.A.). All other chemicals were of analytical grade.

Since the aim of this study was to investigate the effect of certain ions on subcellular organelles, the presence of buffers would greatly interfere and consequently buffers were not added to the system. However due to the large buffering capacity of protein present during the experiment, the pH was held constant within the range of 7.05-7.15. Any compound whose addition would effect the pH of the medium was brought to pH 7.1 before being tested.

Male Wistar strain rats weighing 200-300 g were fasted overnight before being sacrified for the experiment. The livers were excised and homogenized in 0.5 M-sucrose in a Potter-Elvehjem homogenizer to give a 10% homogenate. The crude homogenate was centrifuged at 600 g for 10 min to obtain the 'nuclear' fraction. Smooth microsomes were prepared according to the modification of Dallner et al. (10) of the original procedure of Rothschild (11).

Linear sucrose gradients ranging from 20 to 50% were prepared with a mixing chamber. 2 mg of microsomal protein in 0.25 M-sucrose were layered on the gradient after the addition of the cation under investigation. In a first series of experiments the cation under investigation was added in equal amounts to the gradient media. After centrifugation at 55,000 g for 30 min fractions were collected with a fraction collector while a continuous measurement of A_{280} was performed.

When the binding of ^{14}C spermine to smooth microsomes was investigated ^{14}C spermine was added to smooth microsomes at a final concentration of 2 mM- and with a specific activity of 2 10^5 d.p.m./µmol. After centrifugation fractions were collected and ^{14}C spermine determined.

To measure increase in size, smooth microsomes with or without cation were sujected to Millipore filtration according to Dallner et al. (12).

Protein was determined according to Lowry et al. (13); RNA was estimated by the method of Munro and Fleck (14); DNA was estimated by the diphenylamine method of Burton (17). All enzymes were determined after dialysis of the homogenate and the subcellular fractions against four changes of 50 mM-Tris HCl pH 7.2 containing 0.2 M NaCl. This procedure was shown to be effective in removing most of the polyamines absorbed to the particles (16). All assays were performed under optimum conditions. The enzymic activities were determined according to the procedures referenced: cytochrome oxidase (17); NADPH-cytochrome c reductase (18); acid phosphatase (19); 5'-nucleotidase (20); galactosyltransferase (21).

RESULTS AND DISCUSSION

Effect of polyamines on differential centrifugation of rat liver homogenate.

Rat liver homogenate was centrifuged at 600 g for 10 min to obtain the 'nuclear' fraction which was assayed for enzyme functions characteristic of various organelles. To permit comparison between animals and from one marker enzyme to another, the distribution of enzyme activities or concentrations of protein, RNA and DNA are expressed as percentage of the total activities in the homogenate. As shown in Table I the 'nuclear' fraction contains the bulk of DNA and also some mitochondria (cytochrome oxidase) and large frag-

TABLE I

Sedimentation of protein, RNA, DNA and enzyme markers in the 'nuclear' fraction in the presence and absence of spermidine, spermine and $MgCl_2$.

Biochemical component	Per cent distribution in the 'nuclear' pellet					
	ADDITION					
	none	+3 mM-Spd	+0.5 mM-Sp	+1 mM-Sp	+3 mM-Sp	+10 mM-Mg^{++}
Protein	33.7	56.6	44.8	50.7	57.4	47.9
RNA	47.5	-	-	-	-	-
DNA	89	-	-	-	-	-
Cytochrome oxidase	34.5	92.1	71	84.8	97.4	63
5'-Nucleotidase	47	83	61.9	75	93.9	61
Acid phosphatase	16.6	70	33.2	56.3	71.7	39
Galactosyltransferase	25.5	84.5	59.2	73.1	93.8	59

Rat liver homogenates prepared in a medium containing the cation indicated were centrifuged at 6.4 10^3 g-min and protein, RNA, DNA and enzymic activities were determined. Recoveries represent the concentration of a marker relative to the concentration of this component in the crude homogenate. The absolute values/g of tissue in the homogenate are: protein 198.3 mg; RNA 11.6 mg; DNA 2.11 mg; cytochrome oxidase μmol cytochrome c oxidized/min; 5'-nucleotidase μmol adenosine deaminated/min; acid phosphatase μmol p-nitrophenol formed/min; galactosyltransferase μmol galactose transferred/20 min.

ments of plasma membranes (5'-nucleotidase) and Golgi apparatus (galactosyltransferase). When the liver homogenate is fractionated in the presence of either buffered 3 mM-spermidine or 3 mM-spermine (3 mM is the theoretical concentration of polyamines in a 25% w/v homogenate of rat ventral prostate) the subcellular distribution of the organelles in the aforementioned differential centrifugation is dramatically effected as shown in Table I. For comparison the homogenate was fractionated also in the presence of 10 mM-$MgCl_2$. The addition of either polyamine clearly results in an enhanced sedimentation of every organelle studied and, in the so called 'nuclear' pellet, to the bulk of DNA are associated at an almost equal extent mitochondria, lysosomes, plasma membranes and Golgi apparatus. Also illustrated in Table I is the effect of spermine concentration in the homogenizing medium. Concentrations lower than 0.5 mM-spermine were not tested as the theoretical concentration of endogenous polyamines in our 10% w/v homogenate would be already about 0.3 mM according to published values (7). Polyamines effect on cellular organelles may be attributed to:

a) binding of substantial amount of cation that increases the density of the particles;

b) aggregation of the particles.

Smooth microsomes were used as a model to study the nature of this effect.

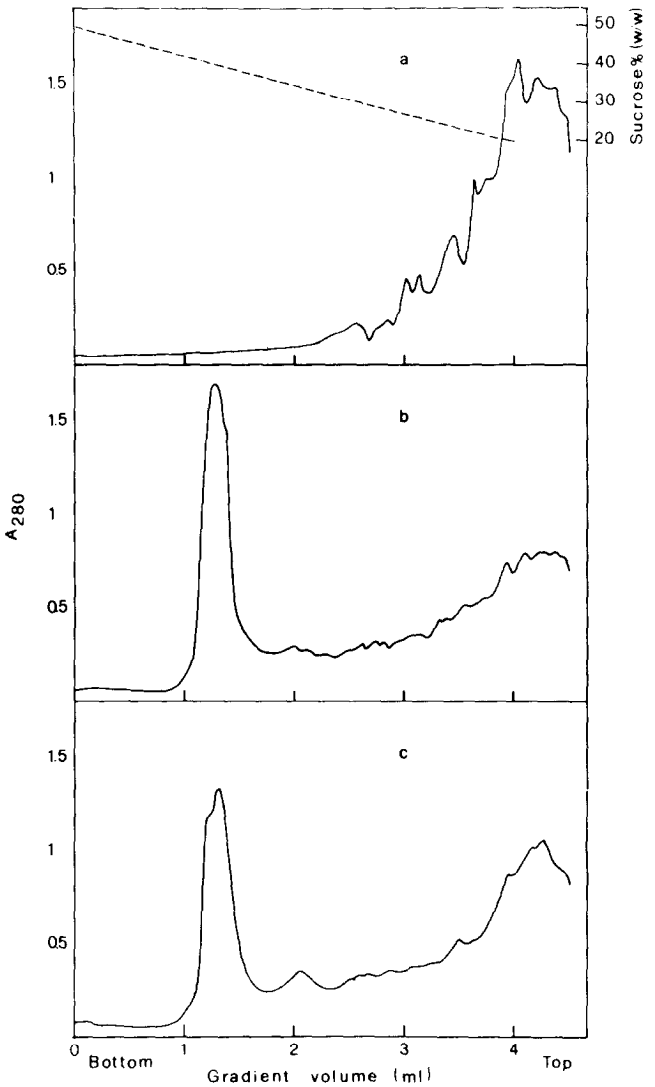

Fig. 1 : Sedimentation of liver smooth microsomes in a continuous sucrose gradient in the presence of spermine or $MgCl_2$

Smooth microsomes in 0.25 M-sucrose were layered over a continuous sucrose gradient ranging from 20 to 50% (w/w). The various additions to the media of the mixing chambers were: a) none; b) 2 mM-spermine; c) 10 mM-$MgCl_2$. The microsomal suspensions on the top of the gradient contained the same cation concentration as the gradient medium.

Effect of spermine on sedimentation of smooth microsomes on continuous sucrose gradient.

Smooth microsomes on a continuous gradient ranging from 20 to 50% fail to sediment after 30 min of centrifugation at 55,000 g. The addition of 2 mM-spermine to the microsomal suspension and to the gradient media results in sedimentation excepting a small fraction which remains at the top of the gradient (Fig.1). The addition of 10 mM-$MgCl_2$ also brings about sedimentation of the

majority of microsomes to the bottom of the tube after similar centrifugation. When microsomal suspensions containing 2 mM-spermine were centrifuged on the gradient described previously but in the absence of polycation in the media the protein sedimented. Evidently the aggregation of microsomes caused by spermine is essentially an irreversible process in this condition.

Association of spermine with smooth microsomes.

In order to estimate quantitatively the association of spermine with smooth microsomes, sedimentation in the presence of ^{14}C spermine was performed. ^{14}C spermine was added to the microsomal suspension and the aggregated microsomes were centrifuged on a continuous sucrose gradient with no spermine addition in the media. After centrifugation the protein content and the total activity of the various fractions were determined. Fig.2 presents the total activity of the fractions recovered from the gradient. Only a small fraction of the total activity was found to be associated with microsomes. The specific activity calculated per gram of protein was 3.3 µmoles and corresponds to 0.66 µmoles per gram (wet wt.) of smooth microsomes (0.23 mg of the polycation). Thus the maximum increase in density of the microsomal particles due to the binding of spermine would produce an increase in the sedimentation velocity of the smooth

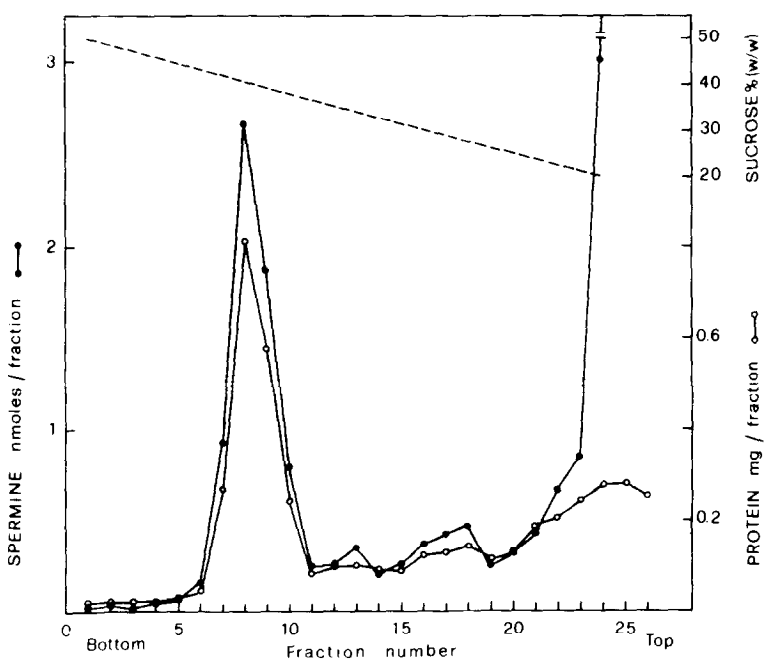

Fig. 2 : Association of ^{14}C spermine with liver smooth microsomes.
Smooth microsomes were aggregated in the presence of 1 µmole of ^{14}C spermine ($2\ 10^5$ d.p.m.). The microsomal suspension was layered on a continuous sucrose gradient ranging from 20 to 50% (w/w). After centrifugation fractions were collected and measurements of protein o—o, and ^{14}C spermine •—• present in each fraction were performed.

TABLE II

Spermine induced aggregation of liver smooth microsomal vesicles

Filter pore (μ)	Protein ($\underline{A}_{280}$) ADDITION none	+1 mM-Sp	+10 mM-Mg^{++}
None	2.100	-	-
0.3	1.035	2	2
0.45	1.814	2	8
0.8	2.004	3	7
1.2	2.052	2	8
5	2.080	5	10
10	2.060	8	2.075

Smooth microsomes prepared as indicated in the Materials and Methods section were diluted with 0.25 M-sucrose to 5 mg of protein per ml and preincubated in the presence of the cation indicated for 20 min at 0°C. 2 ml of the suspension were filtered through a Millipore filter of stated pore size, and the filter was washed by an additional volume of sucrose. The amount of microsomal material passed through the filter was estimated by measuring the absorbancy of the filtered suspension at 280 nm.

microsomal particles absolutely irrelevant and certainly not comparable with the obserbed one.

Spermine induced changes in size of smooth microsomes.

The degree of aggregation of smooth microsomal vesicles induced by spermine has been determined filtering microsomes through commercially available filters of uniform pore size (12). Table II demonstrates that about 90% of the untreated microsomes passed through the 0.3 μ filter and about 100% through filters of pore size of 0.45 μ or more. In the presence of 1 mM-spermine practicaly no protein passed through any filter tested and this aggregation is still more pronounced than the aggregation caused by 10 mM-$MgCl_2$. The apparent mean radius of spermine aggregated smooth vesicles could not be calculated but large aggregates were discernible. The increased sedimentation of microsomes induced by spermine may then be satisfactorely explained by the observed aggregation of the vesicles. Concerning the mechanism of aggregation it appears probable that these polycations act by binding to the lipid surface. Polyamines binding to membrane phospholipids has been suggested (22,23) and the bound polyamines might decrease the net negative surface charge density with consequent abolishement of repelling forces. From these data it appears that polyamines exerting aggregating effect on most of the cellular organelles might

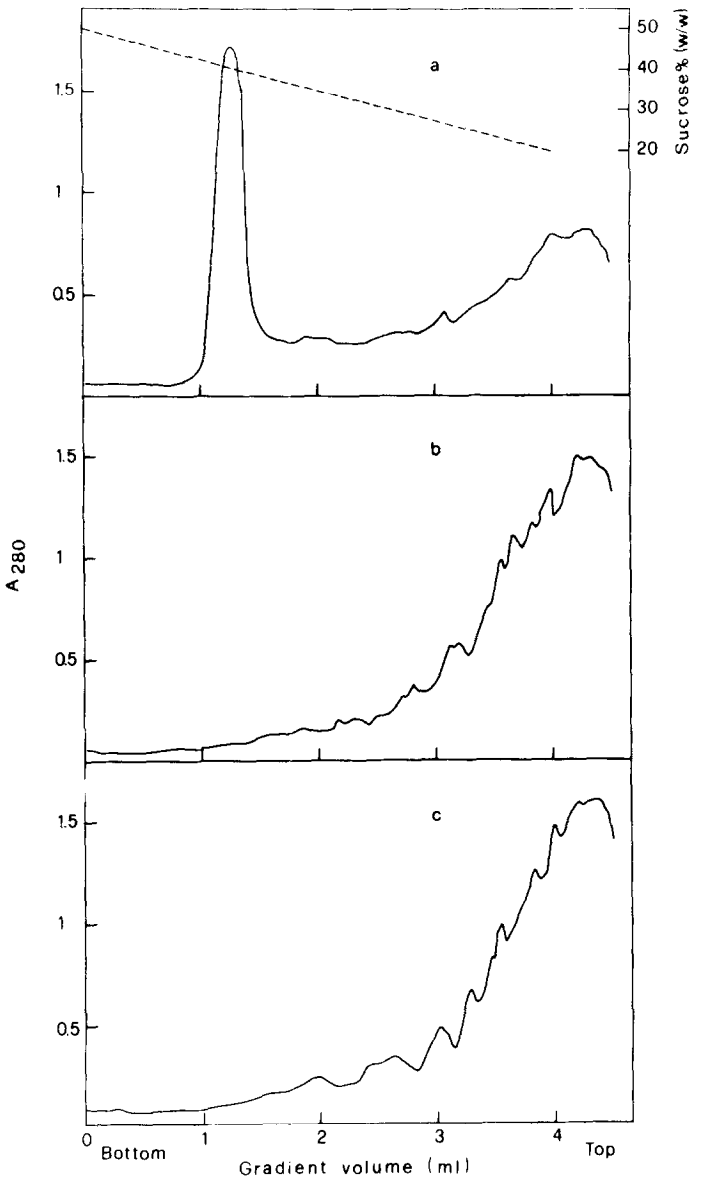

Fig. 3 : Effect of heparin and poly-D-glutamic acid on the sedimentation of spermine aggregated liver smooth microsomes.

Smooth microsomes in 0.25 M-sucrose were aggregated in the presence of 2 mM-spermine. After the additions of: a) none; b) 800 U/ml of heparin and c) 3 mg/ml of poly-D-glutamic acid, microsomal suspensions were incubated at 0°C for 5 min and then layered on a continuous sucrose gradient ranging from 20 to 50% (w/w).

jeopardize subfractionation studies of tissues rich in these biogenic amines. Particularly hazardous are also subcellular localization studies involving either differential rate centrifugation or differential density gradient sedimentation, in experimental models which induce variation in the content of polyamines.

Effect of various compounds on the enhanced sedimentation of smooth microsomes caused by spermine.

Compounds having a high affinity for polyamines and able to compete for them with cellular polyanions as phospholipids might be useful in minimizing artefacts. There is some evidence that polyamines can form complexes with the acidic heparin (8,24-26) and with poly-D-glutamic acid (27). Either heparin or poly-D-glutamic acid were added to microsomal suspensions aggregated in the presence of 2 mM-spermine to give a final concentration of 800 U/ml and 3 mg/ml respectively. As shown in Fig.3 these polyanions were able to prevent the aggregated microsomes from sedimenting in the sucrose density gradient. In case that the release of polyamines aggregation of other cellular organelles by these polyanions could be demonstrated, their addition to the homogenizing medium could be a useful method to successfully sequester polyamines so as to improve separation in the purification of cellular organelles.

ACKNOWLEDGEMENTS

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