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A. Taya
Institut für Genetik und für Toxikologie von Spaltstoffen

Kernforschungszentrum Karlsruhe

KERNFORSCHUNGSZENTRUM KARLSRUHE

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STUDIES ON THE BINDING AND TRANSPORT PROCESSES OF AMERICIUM-241 HYDROXIDE POLYMERS IN RAT LUNG AND BOVINE ALVEOLAR MACROPHAGES

Ahmed Taya

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Kernforschungszentrum Karlsruhe GmbH, Karlsruhe

Kernforschungszentrum Karlsruhe GmbH
Postfach 3640, 7500 Karlsruhe 1

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Abstract

The binding of Am-241 hydroxide polymers to the cell components of rat lung was investigated using differential centrifugation, density gradient centrifugation with different media, gel chromatography, free flow electrophoresis and electron microscopic autoradiography with Pu-241. The bovine alveolar macrophage cultures were introduced as an in vitro test system for Am-241 uptake.
From the biochemical and electron microscopic studies it can be concluded that Am-241 is taken up by pulmonary macrophages, where its first storage site is probably the lysosome. Then the Am-241 seems to be solubilized in the lysosomes and to be bound to the cytosolic ferritin of macrophages. Am-241 might be released from the cells and crosses the alveolar membranes as bound to transferrin or as low molecular weight form.

Americium-241-Hydroxid-Polymere: Untersuchungen von Bindungs- und Transportprozessen in der Rattenlunge und in Rinderalveolarmakrophagen

Zusammenfassung

Die Bindung von Am-241-Hydroxid-Polymeren an Zellkomponenten der Rattenlunge wurde mit Hilfe von differentieller Zentrifugation, Dichtegradienten-Zentrifugation mit verschiedenen Medien, Gel-Chromatographie, Trägerfreier Elektrophorese und elektronenmikroskopischer Autoradiographie unter Verwendung von Pu-241 untersucht. Zellkulturen mit Rinderalveolarmakrophagen dienten als in vitro-Testsystem für die Aufnahme von Am-241.
Anhand von biochemischen und elektronenmikroskopischen Untersuchungsergebnissen kann als sicher angenommen werden, daß Am-241 von Lungenmakrophagen aufgenommen wird; erste Ablagerungsorte in der Zelle sind wahrscheinlich die Lysosomen. In den Lysosomen scheint das Am-241 solubilisiert zu werden und danach an cytosolisches Makrophagen-Ferritin zu binden. Aus der Zelle freigesetztes Am-241 wird wahrscheinlich in niedermolekularer Form oder an Transferrin gebunden durch das Alveolarepithel ins Blut übertreten.

Abbreviation list

Enzymes

ALPD	Alkaline phosphodiesterase
AP	Acid phosphatase
AS	Arylsulfatase
GDH	Glutamatedehydrogenase
NAC	N-acetyl-ß-glocosaminidase
S'N	5'-nucleotidase

Subcellular fractions

CT	Connective tissues of lungs
Hom	Lung homogenate
N	The nuclear fraction which contains mainly cell nuclei, debris and unbroken cells
E	The postnuclear fraction
ML	A sediment which contains the majority of the lysosomes, mitochondria and the plasma membranes
P	The microsomal fraction which contains part of the plasma membranes and the endoplasmic reticulum
S	The soluble fraction, cytosol
P_1, P_2, P_3	The subcellular fractions prepared according to Nijjar method (section 2.1.5. and Table 5)
S_3	The plasma membrane-enriched fraction

Marker proteins

Ferr	Ferritin
RB	Acid ribonuclease
Tf	Transferrin

Miscellaneous

BAMs	Bovine alveolar macrophages
BSA	Bovine serum albumin
Ch	Chamber
d	Day
Ext	Extinction
FFE	Free flow electrophoresis
HM	Homogenate medium
hr	Hour
Lav	Lavaged
min	Minute
Perf	Perfused
PLS-^{51}Cr	Polystyrene particles labeled ^{51}Cr
S.E.	Standard error
TX 100	Triton X 100
Unperf	Unperfused

Contents

1. INTRODUCTION

Large quantities of transuranium elements are produced following the increased use of nuclear energy technology and accidental intakes of such elements by the personnel, mainly via inhalation, might happen. Therefore, the retenion, clearance and the biological effects of the transuranium elements, particularly ^{239}Pu, were extensively studied using different animal species.

The clearance from lung after inhalation of plutonium or other actinides depends largely on the physico-chemical properties of the inhaled material. For example, inhaled oxides could remain for a long time in the lungs, while moderately soluble materials, such as nitrate, are cleared rapidly from lungs (see Bair et al., 1973; Durbin, 1973; ICRP, 1986; Mewhinney and Craig, 1981).

Following inhalation, the insoluble material, $^{239}PuO_2$, was observed by electron microscopy or autoradiography of the ^{239}Pu (alpha emitter) to be taken up by alveolar macrophages (Bair et al., 1973; Sanders and Adee, 1968; Sanders et al., 1977) and in alveolar epithelial cells (Bair et al., 1973). Within the alveolar macrophages, these particles were deposited in phagolysosomes (Bair et al., 1973; Sanders et al., 1977). In a study with U/Pu mixed oxide, which has been recently done in this institute, was observed by electron microscope that these particles could be found in phagolysosomes of rat alveolar macrophages (Müller, unpublished results). Dust of CeO_2 was also found in rat alveolar macrophages, following intratracheal instillation (Pritchard et al., 1985). The phagocytosis of such particles by alveolar macrophages is not surprising, because

macrophages in different organs are responsible for taking up foreign material including particulate substances, such as colloids (see also discussion). The phagocytized particles are cleared mechanically via the removal of the loaded macrophages by mucociliary processes. Such a process is an important one in clearing particulate material from lungs (Rühle et al., 1983) see also discussion.

The picture is different with moderately soluble actinide compounds, such as $^{241}AmO_2$ and $^{244}CmO_2$, and with the soluble forms such as nitrate and chlorides salts of the actinides. The main clearance route of such compounds from lungs is via translocation to the blood stream whereby the transported elements are deposited, mainly in liver and skeleton (ICRP, 1986). In contact with lung fluid, the moderately soluble compounds, such as $^{241}AmO_2$, seem to be solubilized to produce hydroxide salts of low molecular weight which can cross the alveolar wall to the blood (Stradling et al., 1978a). $^{244}CmO_2$ is also solubilized in the lung fluid to produce negatively charged hydroxides of low molecular weight which do not react with surfactants and quickly cross the alveolar wall to the blood. Unlike $^{244}CmO_2$, the solubilization products of $^{239}PuO_2$ are positively charged, hence the binding to the lung fluid phospholipids which are taken up by alveolar macrophages (Cooper et al., 1980). Also small particles of plutonium dioxide (1nm) could cross the alveolar wall to the blood (Smith et al., 1977; Stradling et al., 1978b). In vitro uptake of $^{241}AmO_2$, which might be ingested via non-specific binding to the cell surface, by alveolar macrophages has increased the solubility of $^{241}AmO_2$ (Robinson and Schneider, 1980a and b). Within the macrophages

^{239}Pu was associated with iron deposits (Priest and Haines, 1982).

Thus, the alveolar macrophages, as well as lung surfactant may play a role in the solubilization of inhaled actinides leading to the release of soluble complex which can cross the alveolar wall.

While the retention of various actinide compounds has extensively been investigated (ICRP, 1986), mainly for radiation protection, there is an almost complete lack of mechanistic studies on their transport in lung, except the works of Stradling, Cooper and coworkers mentioned above. Such studies, however, are not only helpful for a better understanding of transport and solubilization processes, for which there is still need, (Bailey et al., in press), but also for certain effects of inhaled actinides in lung.

In the liver, the subcellular binding of transuranium elements has been extensively studied using differential and density gradient centrifugations. The association of different radionuclides such as ^{241}Am, ^{239}Pu or ^{59}Fe to liver lysosomes and ferritin has been reported by several workers (see discussion). The lysosomal association of ^{241}Am, ^{241}Pu, ^{237}Np and ^{144}Ce in liver of different animal species has been recently shown in this laboratory by using density gradient centrifugation and free flow electrophoresis (Wiener, 1984; Seidel et al., 1986).

In fact, little is known about the binding and storage of transuranium elements, particularly, the moderately soluble compounds in lung cells. Except the work of Glück (1982), no study has been reported concerning the intracellular binding of transuranium elements with lung cells using biochemical

techniques.

Therefore, to contribute for better understanding of the binding and transport of the moderately soluble compounds in lungs, the ^{241}Am hydroxide polymers which are instilled intratracheally, are used. The bovine alveolar macrophages will be used as a simpler model than the whole lung and as a kinetic model for solubilization and transport processes of the studied radionuclide. These purposes will be tried to be achieved by using the biochemical techniques that have been applied for liver (Sütterlin, 1982) accompanied by morphological studies using electron microscope autoradiography with ^{241}Pu.

Because of the lack of facilities for inhalation experiments, in the course of this work, the intratracheal instillation technique is used. In comparison with inhalation, the intratracheal technique is considered to be a non-physiological method, whereby the instilled material will be unevenly distributed within the lungs. The differences between inhalation and intratracheal instillation were shown in the quicker clearance of the inhaled material than the instilled ones, at the first phase. (Zalikin and Popov, 1977; Watson et al., 1969). This is attributed to the part of the inhaled material which deposits in the upper airways of the lungs, hence its rapid clearance by the cilia. However, the instillation method still has a lot of advantages, such as: it is a very simple technique and needs not any sophisticated equipments and minimizes hazards to laboratory personnel. Also this technique permits the introduction of large and therefore effective doses of material in short time (for more details see Brain et al., 1976).

Due to the complexity and the elastic properties of lungs,

relatively vigorous homogenization techniques (Gill and Reiss, 1973; Page-Roberts, 1972; Hoffman, 1972) were used. This would cause the disruption of the subcellular organelles, therefore homogenization techniques or modified ones for liver were applied to lungs (Jobe et al., 1981; de-Lumen et al., 1972). In this study the modified homogenization and fractionation techniques of de Duve (de Duve et al., 1955) used for liver (Sütterlin, 1982) will be applied to the whole lung.

For subcellular fractionation, the sucrose was often used as density gradient media. Because of its high osmolality, the lysosomes were not well separated from mitochondria. The separation of lysosomes in sucrose was possible, only if their density has been selectively changed by treatment of the animal, for example with Triton WR 1339 (Gruner et al., 1981; Sütterlin, 1982; Winter and Seidel, 1982). To avoid such changes in lysosomes, the iodinated non-ionic medium, metrizamide, was successfully used to separate lysosomes and other organelles from mitochondria (Wattiaux et al., 1978). This medium in contrast to sucrose, has lower osmolality and it is completely non-ionic, hence its suitability for the separation of many different types of biological material (Rickwood, 1983). Recently, this medium was introduced at our institute and applied to liver and lung fractionations (Sütterlin, 1982; Glück, 1982). Nycodenz, which will be used in this study, like metrizamide belongs to the non-ionic iodinated media and has similar properties (Rickwood, 1983).

Another density gradient medium is the colloidal silica-sol percoll, which has been used for cell organelles analysis (Pertoft et al., 1978a). Because of its low osmolality and

viscosity, it is possible to prepare from percoll an isotonic gradient medium with a mild density range. By such a gradient, it was not possible to separate mitochondria from lysosomes but the lysosomes and pericellular membranes which occur at similar density in the iodinated non-ionic media were differentiated (Sütterlin, 1982).

By the mean of the above-mentioned techniques, we will try to fractionate the whole lung. In addition, the cytosol will be analyzed on the basis of the different sizes (molecular weight) and the electric charges of the proteins using gel filtration and free flow electrophoresis, respectively.

It is always better to compare the biochemical studies with morphological ones. Therefore, electron microscope autoradiography with ^{241}Pu will be used. ^{241}Pu is a soft ß-emitter (21 KeV) and is, thus, suitable for high-resolution electron microscope autoradiography. Up to now, the method was only applied to liver (James and Rowden, 1969).

It should be kept in mind the differences in the biological behaviour of plutonium and americium (Durbin, 1973), when a comparison of the morphological and biochemical results is done. On the other hand, the use of ^{241}Pu as a tracer for ^{241}Am is justified since their subcellular behaviour is identical (Gruner et al., 1981; Sütterlin, 1982; Seidel et al., 1986).

The aims of the present study can be summarized as follows:

1. Comparison of the organ retention of ^{241}Am hydroxide polymers with that of other hydroxide colloids and insoluble particles

after intratracheal injection into rats.

2. Analytical subcellular fractionation of whole rat lung and bovine alveolar macrophages by conventional biochemical methods.
3. Analysis of binding of ^{241}Am to subcellular organelles of whole rat lung and of bovine alveolar macrophages by density gradient centrifugation in different media.
4. Analysis of intracellular distribution of intratracheally injected ^{241}Pu hydroxide polymers in parallel to the biochemical binding studies with ^{241}Am.
5. Studies on the binding of ^{241}Am in the cytosol by gel chromatography and free flow electrophoresis.
6. Development of a tentative model for the binding of ^{241}Am in lung and its transport to the blood.

2. MATERIAL AND METHODS

During this work both rat and bovine lungs were used. Accordingly, this section will be divided into two parts:

A) Experiments with rats and
B) Experiments with bovine lungs

2.1. Experiments with rats

2.1.1. Animals, radionuclides, application techniques

Sex:	Female rats
Breed:	Sprague Dawley
Weight:	180-220 g
Age:	6-8 weeks
Source of supply:	Charles River Wiga, Sulzfeld
Food:	Standard diet Altromin

The rats were allowed free access to food and water before and after the radionuclide instillation.

All radionuclides were purchased from the Radiochemical Centre, Amersham, U.K..
^{241}Am was supplied as $^{241}Am(NO_3)_3$ in 3n HNO_3. 10-20 µl of the ^{241}Am stock solution were added to 10 ml of 0.01n HNO_3. The pH of the solution was slowly adjusted to 8.5 with 0.1n NaOH. More than 40% of the ^{241}Am solution was filtered through 0.025 µm pore size filter.

The ^{59}Fe was supplied as ferric chloride in 0.1n HCl. 10 µl of this solution were added to 4 ml of 0.1n HCl. The pH was adjusted to 8 with 0.1n NaOH.

^{237}Np is stored as a solution in 3n HNO_3. 90 µl from the ^{237}Np stock solution were added to 4 ml of distilled water.

0.05 ml of $^{88}YCl_3$ were added to 6 ml of stable 0.025 M YCl_3 (Merck, Darmstadt) dissolved in 0.01n HCl. The pH of the solution was adjusted to 7 with NaOH.

0.01 ml of ^{241}Pu nitrate stock solution were added to 1.2 ml of 0.01n HNO_3. The pH was adjusted to 8.5 with 0.1n NaOH. The solution contained 1% of the daughter isotope ^{241}Am with respect to the ^{241}Pu beta activity.

The labelling of the polystyrene particles of 1.1 µm initial particle size (Serva, Heidelberg) with ^{51}Cr was performed according to the method of Hinrichs et al. (1978). The labelling was by emulsion polymerisation of ^{51}Cr-Acetylacetonate dissolved in styrene monomer using commercial microspheres. This was accomplished with two successive polymerization steps: radiation excitation ($13x10^6$ rads; ^{60}Co-γ-ray source) and radical polymerization initiated with potassium persulfate (Sigma, USA). 40% of the ^{51}Cr initial activity was attached to the particles. To remove the non-attached ^{51}Cr the particles were washed three times with distilled water and centrifuged at 1500 g for 10 minutes (in rotor type 60 Ti). The particles were resuspended by ultrasonic agitation. The ^{51}Cr-labelled polystyrene suspension was stored at final concentration of 0.25% (w/w) in H_2O. To

stabilize the suspension, 3 drops of 5% sodium lauryl sulfate were added (Sigma, USA). To determine the particle size distribution, a sample of polystyrene suspension was filtrated through a series of polycarbonate filters (Nuclepore Corp., Pleasanton, CA) of different pore sizes: 0.1, 1, 2, and 5 μm. An electron microscopic photograph of the final particles used for the experiments is shown in Fig. 35 (a,b). The suspension was stored at 0°C until use.

The doses of radioactivity and the masses of the different radionuclides are listed:

Radionuclide	Radioactivity	Mass
	Bq kg^{-1}	mol kg^{-1}
^{241}Am	$1-2x10^{4}$	$3-7x10^{-10}$
^{59}Fe	$1.5x10^{4}$	$0.20-3x10^{-10}$
^{237}Np	$0.4x10^{4}$	$6x10^{-7}$
*^{88}Y	$1.0x10^{4}$	$4x10^{-5}$
^{241}Pu	$1.5x10^{7}$	
PLS-^{51}Cr	$0.05x10^{4}$	

*The mass of the stable Y was considered.

In almost all cases, the animals were intratracheally injected and sacrificed under ether anesthesia. In some experiments, there was a need to keep the animals under anesthesia for a long time (for instance, during the perfusion of lungs), in this case the animals were intraperitoneally injected with 0.4 ml of 30 mg/1 ml H_2O nembutal solution (Nembutal: 5-ethyl-5-(1 methylbutal)barbituric acid -Na salt, Serva, Heidelberg).

The radionuclides ^{59}Fe, ^{88}Y, ^{237}Np and ^{51}Cr were measured for γ-activity with γ-scintillation spectrometer (Gamma 8000, Beckman) which is equipped with NaI crystal. ^{241}Am radioactivity can also be measured in γ-scintillation spectrometer (40% of the ^{241}Am decay is emitted as γ-rays). In the case of low γ-activity, the ^{241}Am was assayed by liquid scintillation counting for alpha activity (Seidel, Volf, 1972). The different organs of the animal and the differential fractions were first digested over night in a mixture of 70% $HClO_4$ + 30% H_2O_2 (1:1) then heated at 60°C for 2 hours. 15 ml of insta gel, which was used as a scintillator, were added. The fractions of the gradients, Free Flow Electrophoresis (FFE) and the column were directly filled with insta gel and measured in the Packard liquid scintillation spectrometer. ^{241}Pu is considered as a beta emitter, and was determined as the alpha activity of ^{241}Am. Besides, the ^{241}Pu contained 1% of the daughter isotope ^{241}Am, thus its activity could be also monitored by γ-activity determination.
In retention studies the radioactivity of the whole body of the animal was measured using a small animal whole body counter (Berthold), which comprised of two NaI crystals.

The rats were anesthetized in an ether desiccator until eye-lid movement ceased. The rat was removed from the desiccator and suspended on an animal support stand by a rubber band passed under the upper incisor teeth. The tounge was pulled to one side with tweezers, and a bent metal needle of 0.8x120 mm attached to a 1 ml syringe was inserted into the trachea. The dose (0.4 ml of injection solution used within 2 hours of preparation) was strongly injected with some air in order to ensure a good spray of the sample in the lungs. The needle was injected deeply enough

into the lungs to assure that the dose was injected in the alveolar region of the lungs. The end of the metal needle was coated with a plastic layer to avoid injuring the lungs. The needle could be felt rubbing down the cartilage of the trachea, which ensured that the needle was indeed in the trachea and not in the oesophagous.
In one experiment the rats were injected with ^{241}Am and ^{59}Fe, successively, within an interval of 1 hour.

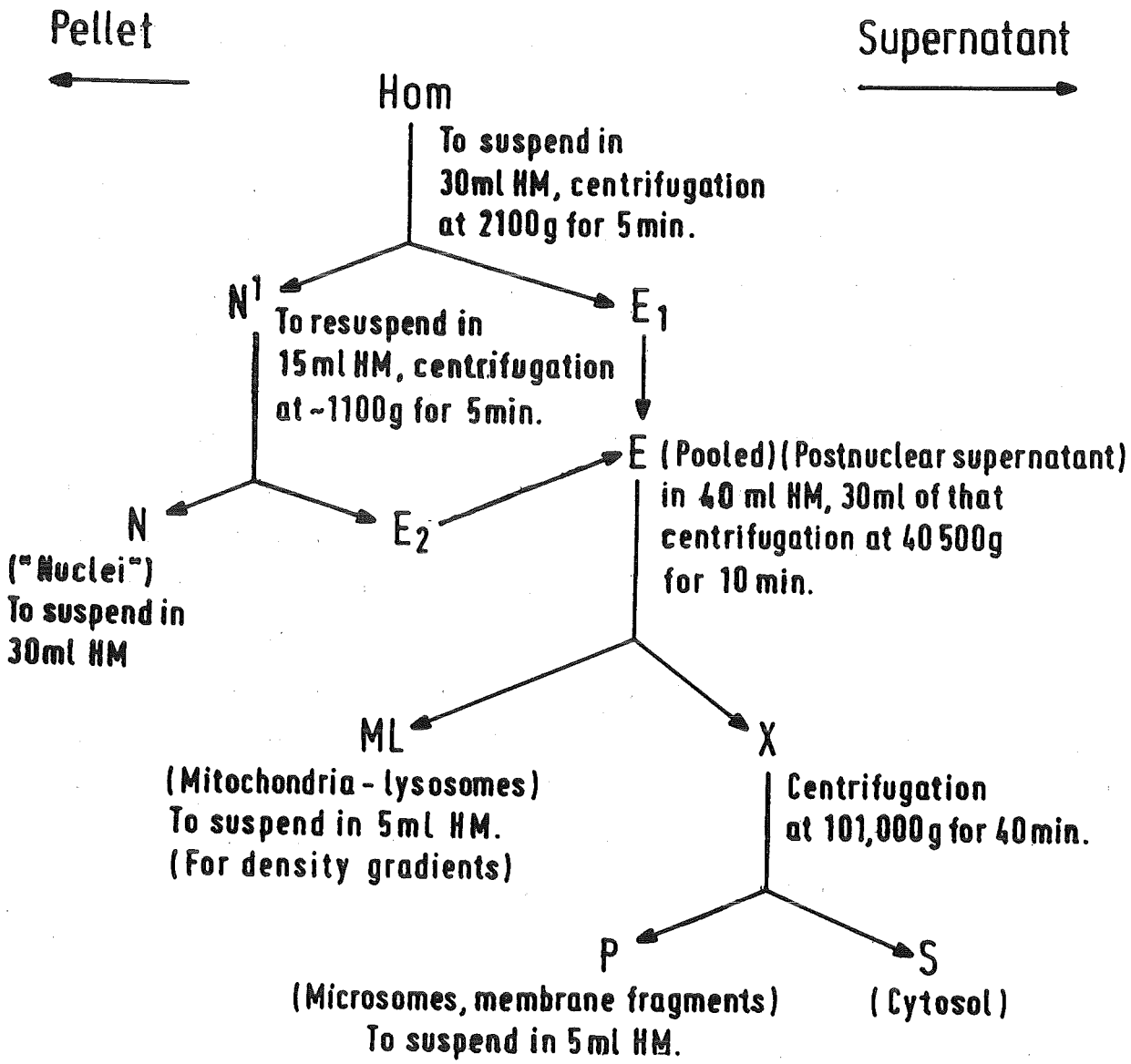
2.1.2. Perfusion of lungs and lung lavage
The rats were anesthetized by intraperitoneal injection of nembutal solution. 0.2 ml of Heparin (200 units/2ml, Promonta,Hamburg) were intravenously injected. The lungs were perfused through the pulmonary vessels with 80 ml of 0.9% NaCl.

The lungs were lavaged through a metal needle (the end of it was coated with a layer of plastic) which was tightly connected to the trachea. A 20 ml syringe was used to insert and to withdraw the lavage fluid. The lungs were lavaged 8 times with ice-cold 0.9% NaCl (5 ml each time).
The lavage fluid fractions were pooled and centrifuged at 250 g for 5 minutes in order to separate the cells from the fluid. The cells were resuspended in 2 ml 0.9% NaCl. Rat lungs were lavaged at different times after ^{241}Am injection.

2.1.3. Fractionation of lungs by differential centrifugation
The rats were sacrificed under ether or nembutal anesthesia by cutting the abdominal aorta, at different time intervals, 3-5 perfused or unperfused lungs were used in each experiment. All the preparations were performed on 0°C. The radioactivity of the

whole lungs was determined before homogenization. The centrifugation steps were accomplished in rotor type 60 Ti using Beckman centrifuge, Spinco L2 50B.

The homogenization of lungs was performed in ice-cold homogenate medium (HM, 0.25M Sucrose, buffered with 10 mM triethanolamine HCl, pH 7.2-7.4). The lungs were first pressed through a metal sieve with pore size of 2 mm in order to remove the connective tissues of the lungs (CT-fraction). The lung slices were suspended in HM and homogenized by three up and down strokes in 30 ml Potter Elvejhem homogenizer with teflon pestle rotating at 500 rpm. The homogenate was then transferred to a 15 ml Dounce homogenizer (S-Dounce) and homogenized with 3 hand-driven strokes. The resulting tissue suspension is designated as hom. The homogenate was centrifuged at 5.500 rpm for 5 minutes. The nucelar fraction (N) was resuspended in HM and homogenized again with 3 hand-driven strokes in S-Dounce homogenizer. All the homogenization and centrifugation steps are described in the following scheme (Gruner et al., 1981):



The resulting fractions were measured for radioactivity or kept at -20°C for marker enzyme determinations. The ML-fraction was further analyzed by density gradient centrifugation.

2.1.4. Metrizamide, Nycodenz and Percoll gradients

2.1.4.1. Metrizamide gradients

Metrizamide (2-(3-acetamide-5-N-methylacetamido-2,4,6-triiodobenzamdo)-2-deoxy-D-glucose) was supplied from Serva, Heidelberg. The range of osmolalities of metrizamide solutions that were used were 120-420 mOs/kg H_2O (Rickwood and Birnie, 1975). The desired density of metrizamide was prepared by dissolving metrizamide in H_2O. The linear metrizamide gradient was performed in dual-chamber gradient-making machine by mixing 21 ml of 9.2% metrizamide with 17 ml of 50.6% metrizamide. 2 ml of ML fraction were layered onto the gradient and subjected to centrifugation in vertical rotor VTi 50 at 26.000 rpm (60.000 g) for 2 hours using Beckman centrifuge, L2 50B at 4°C. The gradients were fractionated by pumping the solution out from the bottom of the tube, using paraffin oil. 22 fractions were collected. The gradient covered a density range of 1.05-1.25 g/ml. The fractions were measured for radioactivity, for enzyme determination they were kept at -20°C.

2.1.4.2. Nycodenz gradients

Nycodenz (N,N -Bis(2,3-dihydroxypropyl)-5-(N-(2,3-dihydroxypropyl)acetamido)-2,4,6-triiodo-isophatalatamine) was supplied from Serva, Heidelberg. The linear nycodenz gradient was prepared, centrifuged and fractionated in the same manner as the metrizamide gradient.

2.1.4.3. Percoll gradients

Percoll (Pharmacia, Freiburg) is a colloidal preparation of silica particles (average particle size about 17 nm) coated with polyvinylpyrralidone. This coating prevents reaction of the silica with the biological material. The colloidal particles form a solution of low osmolality (20 mOs/kg H_2O) (Pertoft et al.,

1978a).

The Percoll stock solution was diluted by adding 9 parts of Percoll solution to 1 part of 2.5 M sucrose to get iso-osmotic Percoll solution with density of 1.13 g/ml (320 mOs/kg H_2O). The desired density was obtained by diluting the iso-osmotic Percoll solution with 0.25 M sucrose (Percoll(R)-methodology and applications, Pharmacia, fine chemicals). By dilutions, the initial densities 1.05 and 1.06 g/ml were prepared. 2 ml of ML were mixed till homogeneous with 38 ml of Percoll medium and centrifuged at 19.000 rpm (33.000 g) for 5 minutes, 18°C, in Beckman vertical rotor VTi 50 (Sütterlin, 1982). The gradient was fractionated as the metrizamide gradient and 22 fractions were collected. The Percoll gradient was actually formed during the centrifugation and showed a sigmoidal shape. Three density regions were selected, regions I and III represent the parts from the bottom and the top with density increments and region II the very flat central portion of the gradient.

For further analysis the fractions from regions I and III were applied to linear nycodenz gradient (section 2.1.4.2.).

2.1.5. Pericellular membrane purification

The plasma membrane-enriched fraction was prepared according to the method described by Nijjar and Ho (1980). The rats were sacrificed 4 days after ^{241}Am injection. The lungs were homogenized in Potter Elvejhem homogenizer followed by 15 hand-driven strokes in tightly fitting Dounce homogenizer (S-Dounce). The preparation was performed in HM. The homogenate was filtrated through one layer of nylon mesh (the precipitate on

the mesh was called N'and the filtratable fraction E'). The E' fraction was brought to 20% (W/V). This fraction was subjected to a series of differential centrifugation to give the following fractions: P_1, P_2, P_3 and S_3 (see abbreviation list). A sample from S_3 (the plasma membrane-enriched fraction) was layered on a discontinuous sucrose gradient for further analysis.

The discontinuous sucrose gradient was prepared by layering from bottom to top with: 2.7 ml 45%, 2.7 ml 35%, 2.3 ml 30% and 2.3 ml 25% sucrose solutions (Nijjar and Ho, 1980). 2.3 ml of S_3 were layered on the top of the gradient and centrifuged in swinging 41 Ti rotor at 27.000 rpm (100.000 g) for 75 minutes, 4°C, using Beckman centrifuge L2 50B. Starting from the top of the gradient the following fractions were carefully withdrawn: 1.4 ml (F_1), 1.2 ml (F_2), 1.2 ml (F_3), 1.7 ml (F_4), 2.5 ml (F_5) and 4 ml (F_6). The fractions were diluted to 10% sucrose with H_2O, then centrifuged at 140.000 g for 60 min. The pellets were washed with HM, centrifuged as before, and suspended in a small volume of HM. The fractions were measured for radioactivity and for marker enzymes.

2.1.6. Analysis of cotysol by gel chromatography and free flow electrophoresis

The lungs were homogenized in free flow electrophoresis buffer ("chamber buffer"). The homogenization process was described in section 2.1.3. After filtration, the homogenate was suspended in 30 ml chamber buffer and centrifuged at 1100 g for 10 minutes. The postnuclear fraction (E) was centrifuged at 18.000 g for 20 minutes, followed by centrifugation of the supernatant at 101.000g for 40 minutes to get the cytosol fraction (Beckman et al., 1981) which was analyzed with gel chromatography and free

flow electrophoresis. The protein content of the perfused and unperfused lung cytosol ranged between 5-6 mg/1 ml cytosol.

2.1.6.1. Gel filtration: Sephacryl S-300

The Sephacryl S-300 selectivity range is: $1x10^4$-$1.5x10^6$ (Gel Filtration, Pharmacia).

Buffer

2.42 g Tris (hydroxymethyl)-aminoethane (0.02 M)

2.92 g NaCl (0.05 M)

0.2 g NaN_3 (0.02%)

in 1 l H_2O. The pH was adjusted to 8.6 with HCl.

Two columns were packed with Sephacryl S-300, one of 30 cm x 1.9 cm and the other of 60 cm (bed height) x 2.6 cm and calibrated with horse spleen ferritin (Pharmacia), human plasma transferrin (Sigma) and acid ribonuclease (Pharmacia) with molecular weights of: $4.5x10^5$, $7.8x10^3$ and 13.700, respectively.

The eluted fractions of the column were collected in fraction collector frac 100 (Pharmacia). The protein contents of the fractions were spectrophotometrically monitored at 280 nm.

Parameter	short column	long column
volume of cytosol applied	2 ml	4 ml
flow rate	0.6 ml/min	0.7 ml/min
fraction volume	2 ml	4 ml
number of fractions	50	90

After each run the column was washed with ferritin (1 mg/ml chamber buffer) and untreated lung cytosol, alternatively.
When the data from gel chromatography were processed, the void volumes of the short and long columns (20 ml and 100 ml, respectively) were subtracted from the total number of the fractions.

2.1.6.2. Free flow electrophoresis (FFE)
The free flow electrophoresis aparatus (Elphor VAP-11) was supplied from Bender λ Hobein, München.

Chamber buffer (in which the sample is suspended)
7.5 g triethanolamine (0.001 M)
2.85 g 100% acetic acid (0.001 M)
427 g sucrose (0.25 M)
in 5 l H_2O. The pH was adjusted to 7.4 with conc. NaOH.

Electrode buffer
75 g triethanolamine (0.01 M)
28.5 g 100% acetic acid (0.01 M)
in 5 l H_2O. The pH was adjusted to 7.4 with conc. NaOH.

Operation conditions
sample flow rate = 3 ml/hr (10-15 mg Prot/hr)
"chamber" flow rate = 3-4 ml/hr for each of the 90 fractions
temperature = 5.5°C.
current = 160-180 mA
voltage = 1400 V.
90 fractions were collected. 3 ml from each fraction were taken for radioactivity determination. The protein content in each

fraction was photometrically assayed.

The binding of ^{241}Am and ^{59}Fe to the lung cytosol and to ferritin, transferrin, and bovine serum albumin (BSA) were studied in vitro.
20-35 Bq ^{241}Am (at pH 1 and pH 8) were added to 1 ml cytosol of perfused and unperfused lungs. The samples stood for 30 min at room temperature.
In another experiment, 1.85×10^2 and 0.37×10^2 Bq. ^{59}Fe (pH 8) were added to 1 ml cytosol of unperfused lungs.
Another 20-30 Bq of ^{241}Am (pH 1, pH 8) were added to 1-2 mg/1 ml of ferritin, transferrin and BSA dissolved in chamber buffer. In one experiment 20 Bq of ^{241}Am (pH 1) were added to 1-2 mg transferrin dissolved in 1 ml of physiological bicarbonate solution (2 g $NaHCO_3$, 0.075 g Na_2HPO_4, 0.6 NaCl, pH 7.4 adjusted with 0.1 n HCl). The ^{59}Fe, ^{241}Am labelled cytosol and proteins were analyzed by FFE and S-300 column (see sections 2.1.1. and 2.1.6.).

2.1.7. Preparation of ferritin rich fraction
The ferritin rich fraction was prepared either from a whole lung or from the cytosol.
From total lung homogenate:
3 rats were sacrificed 3 days after ^{241}Am injection. The lungs were perfused as described in section 2.1.2.. Then they were sliced and homogenized in a volume of H_2O equivalent to 1.5 times the weight of the tissue. The total lung homogenate was heated at 75°C for 10 minutes, then filtered through a blue band filter. The filtrate was further treated in a series of centrifugations as described by Gonyea et al. (1976). The final pellet, which

contained the ferritin, was resuspended in a minimal volume of water.

From cytosol:

4 rats were sacrificed 3 days after ^{241}Am and ^{59}Fe injection. The cytosol fraction was prepared from the perfused lungs as described in section 2.1.6., also heated at 75°C for 10 minutes and subjected to further centrifugation as just described. The "ferritin fraction" was resuspended in 5 ml chamber buffer (see section 2.1.6.2.).

2ml of the "ferritin fraction" were applied to the Sephacryl S-300 column.

2.2. Experiment with Bovine Alveolar Macrophages (BAMs)

2.2.1. Cell preparation and general culture techniques

2.2.1.1. Bovine lungs lavage

The bovine lungs of healthy animals from both sexes and different ages were supplied from the slaughter house, Karlsruhe. The lungs were lavaged within 1-2 hours after slaughter, with 10-15 liters of 0.9% NaCl. The cells were sedimented at 200 g for 10 minutes in rotor JA10 using Beckman centrifuge J2-21 and washed once with saline solution. The cells were resuspended in 50 ml incubation medium and kept on ice until use. Samples contaminated with blood were discarded. The cells were counted in a Neubauer counting chamber (Bender λ Hobein, München). The yield was $8x10^8$-$1x10^9$ cells per lung. Over 85% of the cells excluded trypan blue.

Smears of the cells were routinely stained (using the combined May-Grünwald-Giemsa staining method) and inspected for differential cytology. They were also controlled in the laboratory of Broncheoalveolar Cytology, Department of Pulmology of the Robert-Koch-Klinik, Freiburg. More than 90% of the cells

were alveolar macrophages. The differential cytology corresponded to that of healthy humans and that described for bovine lung in the literature (for electron microscope micrographs of BAM see Figs. 36a and b).

2.2.1.2. General culture techniques

The BAMs were incubated with ^{241}Am in different media in order to find the optimum medium for BAM incubation. The following media were used:

HEPES medium (Castranova et al., 1980).

8.18 g NaCl (0.14 M)

0.372 g KCl (0.005 M)

0.9 g Glucose (0.005 M)

2.38 g HEPES (0.001 M) (N-2-hydroxyethylpiperazine N -2-ethane-sulfonic acid, Sigma)

in 1 l H_2O. The pH was adjusted to 7.4 with 1n NaOH.

Waymouth medium

Waymouth medium (MB 752/1) was supplied from Gibco Europe, Scotland, as a powder. It was dissolved in 10 l H_2O. 22 g of $NaHCO_3$ were added.

There was no need to work under sterile conditions, because the cells were cultured for short times.

"Lungs surfactants":

The first wash of the lung was used as a sample of "surfactant". The cells were incubated in: HEPES medium, HEPES + 4% BSA, HEPES + "surfactants" (1:1), Waymouth medium, Waymouth + 4% BSA, Waymouth + surfactants (1:1), and "surfactants".

$10-15x10^6$ cells were incubated in 5 ml of medium (in 20 ml urine

beakers). After 30 min of incubation at 37°C, 2.9×10^3 Bq ^{241}Am solution (pH 8) were added to each culture. The cells were incubated for 1.5 hr at 37°C (in shaking water bath). Samples were incubated in triplicate. At the end of incubation the cells were sedimented at 200 g for 5 minutes using lab centrifuge (Martin Christ, Osterode/Harz). The cells were washed once with the incubation medium and then resuspended in 2 ml of medium. The ^{241}Am radioactivity of the incubated cells was determined.

For most incubation experiments, HEPES medium was used because of its low hydrolysis at pH 7.4 (Good et al., 1966) and thought to have minimum interaction with the ^{241}Am hydroxide polymers (see also discussion).

2.2.2. Special culture techniques

To test the influence of incubation temperature, viable cells were incubated in 5 ml HEPES medium (2.8×10^6 cells/1 ml medium) at 0°C, 10°C, 22°C, and 37°C for one hour. 2.5×10^3 Bq of ^{241}Am were added to each culture (the samples were incubated in duplicate). The incubated cells were centrifuged, washed with HEPES medium, centrifuged again and resuspended in 2 ml HEPES medium. The ^{241}Am content in the incubated cells was determined.

To test the influence of incubation time, samples of cells in 5 ml HEPES medium (2.5×10^6 cells/1 ml medium) were incubated with or without BSA at 0°C and 37°C with 2.6×10^3 Bq ^{241}Am for different times: 10 min, 20 min, 30 min, 1 hr, 2 hr, 3 hr and 4 hr.

The incubated cells were sedimented and washed with medium then resuspended in 2 ml HEPES medium. The cells were measured for

radioactivity. The cells which were incubated at 0°C and 37°C (in HEPES medium without 4% BSA) were treated with 0.05% trypsin (dissolved in HEPES medium) for 0.5-1 min at room temperature (2 ml of 0.05% trypsin for each culture). To stop the trypsin, 2 ml of 0.035% (in HEPES) trypsin inhibitor were added. The trypsin-treated cells were sedimented and resuspended in 2 ml HEPES. The trypsin supernatants and the trypsin-treated cells were measured for ^{241}Am radioactivity.

The effect of the ^{241}Am concentration was systematically tested in a special experiment. To duplicates of cell samples (in 5 ml HEPES medium) the following ^{241}Am radioactivities were added: $5.9x10^2$, $1.2x10^3$, $2.5x10^3$, $4.8x10^3$ and $8.36x10^3$ Bq. The cell samples ($2.6x10^6$ cells/1 ml medium) were incubated at 0°C and 37°C for one hour. The incubated cells were centrifuged, washed and resuspended in 2 ml HEPES medium.

2.2.3. Fractionation of BAMs by differential and density gradient centrifugation

$200-400x10^6$ cells were suspended in 50 ml HEPES medium, pre-incubated at 37°C for 30 min and $1x10^4-1.6x10^4$ Bq of ^{241}Am were added. The cells were incubated at 37°C for 2 hours; sedimented and washed with 2% BSA (dissolved in HEPES) followed by washing 3 times with homogenate medium (HM). The cells were resuspended in 5ml HM. The washings and the incubated cells were measured for ^{241}Am radioactivity. A sample of cells was kept at -20°C for protein assay.

The cells were first homogenized in HM by 15 hand-driven strokes in a tightly fitting Dounce homogenizer (S-Dounce). The

homogenate was centrifuged at 2100 g for 5 min (in rotor type 60 Ti). The N-pellet was resuspended and homogenized again by 15 hand-driven strokes in S-Dounce homogenizer and centrifuged at 1100 g for 5 min. The pooled E fraction was subjected to the series of further centrifugation steps described in section 2.1.3.. The volumes of BAM differential fractions were as follows:
Homogenate 5 ml, N fraction 10 ml, pooled E fraction 10 ml, ML and P fractions 3 ml each, and S fraction 10 ml homogenate medium.
The ML fraction of the bovine alveolar macrophages was further analyzed by density gradient centrifugation on nycodenz and percoll gradients (as described in section 2.1.4.).

2 ml of the cytosol were layered on Sephacryl S-300 column (30x1.9 cm). The operation conditions were as described in section 2.1.6.1.

The linear nycodenz gradient was performed as described in section 2.1.4.

Linear percoll gradient
The nycodenz gradient was mainly used to separate the mitochondria from the other organelles. A linear percoll gradient was used in order to try to get better separation of the plasma membranes from the lysosomes. Nine volumes of the percoll stock solution were diluted with one volume of 2.5 M sucrose to get iso-osmotic percoll solution. The percoll gradient was preformed as metrizamide with 21 ml of 0.25 M sucrose + 17 ml of the iso-osmotic percoll solution (section 2.1.4.1.). 2 ml of ML

fraction were layered on the preformed percoll gradient and centrifuged in vertical rotor type VTi 50 at 19,000 rpm (33,000 g) for 1 hour using Beckman centrifuge L2 50B, at 4°C. The linear percoll gradient was fractionated as metrizamide gradient, and 40 fractions of 1 ml volume were collected.

The density of the percoll gradient ranged from 1.025-1.19 g/cm^3. The gradient remained linear from fraction 3 to fraction 30. The last fractions formed a flat density region.

The conditions of the marker enzymes were similar to that described in section 2.3.2., except that the volumes of the samples added, were half of that given in table 1 (percoll fractions had 1 ml volume).

2.3. Determination of densities, enzymes activities and protein concentration

2.3.1. Determination of densities

Metrizamide

The densities of the metrizamide gradient fractions were determined by measuring the refractive index (η) of the fraction in an Abbé refractometer. From the following relationship between the refractive index (η) and the density (ρ), the densities of the gradient fractions can be calculated (RICKWOOD, 1983):

$$\rho = 3.35\ \eta - 3.462 \quad \text{at } 20°C.$$

Nycodenz

In a similar way, the densities of the fractions of nycodenz gradients were determined. The relationship between the refractive index η and density ρ g/ml (RICKWOOD, 1983) as follows:

$$\rho = 3.242\ \eta - 3.323 \quad \text{at } 20°C.$$

Percoll

The refractive index (x) of percoll gradient fractions were measured in an Abbé refractometer. From the following relationship the densities can be calculated

$$Y = -8.448 + 7.042x$$

where Y is the density of the fraction.

Sucrose

The sucrose concentrations were measured in an Abbe refractometer. The densities were determined from the Int. Critical Tables of Numerical Data, Physical Chemistry and Technology (Vol. II, London, 1927).

2.3.2. Determination of enzyme activities

The following marker enzymes were used:

Organelle	Enzyme
Lysosomes	N-Acetyl-ß-glucosaminadase
	Acid phosphatase
	Aryl sulfatase
Pericellular membranes	Alkaline phosphodiesterase
	5'-Nucleotidase
Mitochondria	Glutamate dehydrogenase

The conditions for the determination of the enzyme activities in the differential and gradient fractions are given in table 1. The enzyme reaction was started by adding the substrate to the sample. The samples were then incubated at 37°C (except GDH which was kinetically assayed at 25°C. For abbreviation see the list). After stopping the enzymes, the samples were kept at 0°C for 15 minutes, then centrifuged at 3000 rpm for 15 min in Hettich Lab.

centrifuge.

The extinctions of the enzyme products were measured in spectrophotometer M25 (Beckman). Samples of differential and gradient fractions in which the enzymes were stopped before adding the substrates, were used as blanks (all methods of enzyme determination have already been described in detail by Sütterlin, 1982 and Gruner, 1978).

2.3.3. Bio-Rad protein assay

The protein of FFE, gel filtration fractions and of the differential fractions were assayed (as well as by the spectrophotometrical method) by Bio-Rad protein assay (Bio-Rad Laboratories, München, FRG). This assay is based on the shift of the absorbance of an acidic solution of Coomassie Brillant Blue G-250 from 465 nm to 595 nm when binding to proteins occurs.

The Bio-Rad dye was diluted 1:5 with H_2O before application (Bio-Rad Laboratories, München). The solution was filtered to remove aggregates. 2.5 ml of the diluted Bio-Rad solution were added to 50 µl of sample (the FFE and S-300 column fractions do not need any dilution). The extinction was measured at 595 nm 5-30 min after preparation. The differential centrifugation fractions were first diluted 1:10 with H_2O and centrifuged to remove the aggregates. As blanks, 2.5 ml of Bio-Rad solution were used. Different concentrations of Bovine Serum Albumin (Serva) were used as a protein standard (Bio-Rad protein assay, 1981). The assay was performed in triplicate.

2.4. Light and electron microscope autoradiography

2.4.1. Light microscope autoradiography

Rat alveolar macrophages were obtained by lavaging the rat lungs

2 weeks after ^{241}Am injection (section 2.1.2.). The macrophages were smeared on slides which had been previously treated with alcohol. The smears were fixed in methanol. Before the stripping film AR 10 (Kodak, London) was applied, the slides were dipped in chromalaun-gelatine solution. The slides were exposed for 2 weeks at 4°C. After that the slides were developed using Kodak D19, followed by fixation in Kodak Unifix powder dissolved in H_2O. The autoradiograms were stained using the combined May Grünwald - Giemsa staining method.

2.4.2. Electron microscope autoradiography

Two rats were intratracheally injected with $1.5x10^7$ Bq/kg ^{241}Pu solution (section 2.1.1.) and sacrificed after 24 hours and 7 days, respectively. Sections from the lungs with the highest radioactivity were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 followed by washing 3 times with 0.1 M cacodylate buffer. The lung sections were then postfixed with 1% OsO_4 in palade solution.

The dehydration process began in an alcohol series and finished in propyleneoxide. Epon-Araldit was used as an embedding medium. The samples were cut with Sorval MT2-B Ultramicrotome into sections of 90nm thickness. The sections were transferred onto grids without formvar coating. Ilford L4 emulsion was layered onto the sections by the wire loop method and exposed for 15 weeks at 4°C. The sections were developed in amidol followed by contrasting with uranyl acetate and lead citrate. The electron microscope Elmiscop IA, Siemens, was used.

For electron microscope autoradiography of BAMs, $20x10^6$ cells were incubated in 5 ml HEPES medium and $3x10^4$ Bq ^{241}Pu were added. The cells were incubated at 37°C for 2 hours.

For BAM electron microscope autoradiography the same procedure as for the lung sections was applied except that the cells were mixed with agar before the dehydration process.

2.5. Evaluation and presentation of the data

For whole body retention studies the rats which were injected at 0-day, were taken as 100%. For ^{51}Cr, ^{59}Fe and ^{88}Y, the radioactivities were corrected for physical decay. The activity of the daughter isotope ^{233}Pa of ^{237}Np was subtracted from the ^{237}Np radioactivity.

For calculating the retention in the organs the initially injected dose was taken as 100%. The ^{241}Am content in the skeleton was calculated by multiplication of the femur activity by factor 20 (Seidel, 1977). The same factor was taken for ^{237}Np, for ^{88}Y and ^{59}Fe only the activities of the femur were given because of the lack of information on the femur to skeleton activity ratio.

The radioactivity in CT, N, E was presented as a percentage of the lung burden (for BAM, from the radioactivity of the cells). The radioactivity, enzyme and protein content in ML, P and S or P_1, P_2, P_3 and S_3 was related to the activity of E fraction (as 100%). The protein content in N and E fractions was calculated relative to the protein content of the whole homogenate (for both rat lungs and BAM).

The distribution profiles of radionuclides, enzymes and proteins in metrizamide and nycodenz gradients were presented in the form of $\Delta Q/\Delta\rho$ (frequency on the ordinate) as a function of the density (abscissa) (as described by Beaufay et al., 1976). ΔQ represented

the percent of the activity in one fraction related to the total activity of the gradient, where $\Delta\rho$ was the density increment in the same fraction.

The data for sucrose gradients (Fig. 13), percoll gradients (Table 7 and Fig. 33), FFE and gel chromatography are expressed as a percentage of the total activity recovered, respectively.

The peaks of the distribution profiles of density gradients, FFE and gel filtration from repeated experiments are qualitatively identical but not quantitatively. Therefore, only representative experiments are shown. The quantitative changes can be attributed to different rat number which was used in each experiment. In addition to quantitative differences, the peaks of the FFE distribution profiles are shifted due to deviations in the electrical current. Also representative experiments are shown from BAM results. The quantitative differences of BAM repeated experiments are related to the use of different bovine lung in each experiment.

3. RESULTS

3.1. Studies with ^{241}Am and ^{59}Fe in whole rat lungs

3.1.1. Retention

Fig. 1 shows the retention of ^{241}Am in the whole body and lungs of a rat. About 50% of the ^{241}Am whole body radioactivity was cleared with a half life of 15 days, and the remainder was cleared slowly. Most of the ^{241}Am content was rapidly eliminated from the lungs with a half life of 4 days, and about 6% of the initial dose was retained in the lungs after 175 days. The ^{241}Am was mainly translocated from lungs to liver and skeleton (Fig. 2). After one week 22 % of the initial dose of ^{241}Am was deposited in the liver and then cleared in a matter of days. In the skeleton, ^{241}Am was accumulated and had already reached about 18% of the initial dose after one month.

The ^{241}Am radioactivity could not be detected in the blood two hours after ^{241}Am injection. 2.5% of ^{241}Am (from initial dose) could be found in the gastrointestinal system, minutes after ^{241}Am administration. After two weeks 1% of ^{241}Am could still be detected.

The results after lung lavage are summarized in Table 2. The ^{241}Am content of the cell free lavage fluid decreased from 65% of the total lung burden immediately after ^{241}Am instillation (which was 89% of the lung lavage activity) to 6% by the 14^{th} day or 35% of the lavage material, respectively.

Smears from the free cells of the lavage material showed that more than 95% of the cells were alveolar macrophages.

The ^{241}Am content of the cells increased from 5% of the lung burden immediately after ^{241}Am injection (7% of the lavage material) to 12% (67% of lavage material) by the 14th day.
Figs. 37 (a,b) show the light microscope autoradiograms of rat alveolar macrophages, 2 weeks after ^{241}Am injection. It can be seen in both autoradiograms, that most of the ^{241}Am tracks were found within the cells and some single tracks which were due to free ^{241}Am were also found outside the cells.
The perfusion process did not affect the ^{241}Am content in the lungs, because ^{241}Am disappeared rapidly from the blood The perfused lung burdens (% of initial dose) at 3 and 7 days were 46% and 24%, respectively (compare with Fig. 1).

After one week, 85% of ^{59}Fe (from injected dose) was still retained in the whole body of the rat (Table 3). As with ^{241}Am, ^{59}Fe was rapidly cleared from the lungs, and only 37% remained after one week. In the extrapulmonary tissues, 11% of the injected ^{59}Fe dose was deposited in the liver after one week and a small amount was found in the femur. However, a considerable amount 29%, was still in the blood after one week (Table 3).

3.1.2. Gross subcellular distribution in the lung
The subcellular distribution of ^{241}Am in the differential fractions is presented in Table 4. Irrespective of the time of sacrifice, 30-45% of ^{241}Am lung burden was found in both the nuclei (N) and the postnuclear fractions (E) between 2 and 35 days after ^{241}Am instillation, and only 5-12% of the ^{241}Am activity was associated with the connective tissues (CT). In the postnuclear fraction, 30-40% of ^{241}Am in the E fraction was sedimented with the mitochondria-lysosome rich fraction.

Approximately, 30-50% was recovered in the cytosol fraction (S). The recoveries of ^{241}Am in the differential fractions ranged between 80% and 87%. The subcellular distribution of ^{241}Am in the differential fractions of perfused lungs gave similar results to that of unperfused lungs.

The relative distribution of the marker enzymes in the differential fractions showed that more than 70% of the lysosomes (according to N-acetyl-ß-glucosaminidase, acid phosphatase and aryl sulfatase determinations) 65% of pericellular membranes (determined with alkaline phosphodiesterase and 5'-nucleotidase) and about 70% of the mitochondria (determined with glutamate dehydrogenase) in the E fraction were sedimented in the ML fraction.

The subcellular distribution of ^{59}Fe is presented in Table 6. More than 60% of the ^{59}Fe lung burden was associated with the E fraction and about 20% sedimented in the N fraction. Approximately 40% of the E ^{59}Fe content was retained in the cytosol, and about a third was sedimented with the P fraction. Only 13% sedimented with the ML fraction.

3.1.3. Density gradient studies with the mitochondria-lysosome fraction

Figs. 4 and 5 show the distribution profiles of ^{241}Am and the marker enzymes after centrifugation of the ML fraction from unperfused lungs on metrizamide gradients 2 and 7 days after ^{241}Am insillation, respectively. The glutamate dehydrogenase profiles (GDH, mitochondria marker) show that the mitochondria were well separated from the lysosomes and pericellular membranes, and that they sedimented within high density fractions (1.2-1.26 g/cm^3). The marker enzymes of lysosomes,

N-acetyl-ß-glucosaminidase (NAC), acid phosphatase (AP) and aryl sulfatase (AS) and that of pericellular membranes, alkaline phosphodiesterase (ALPD) and 5'-nucleotidase(5'N) appeared approximately in the same buoyant density range with maximum at 1.1 g/cm^3. The lysosomal (except NAC) and membrane enzymes had an activity peak at the top of the gradient, whereas the NAC had a low activity peak. The ^{241}Am profile had two major peaks: one at a density of 1.1 g/cm^3 and the other at 1.19 g/cm^3. ^{241}Am activity was also found in the top of the gradient, which this could be due to activity of free ^{241}Am. The ^{241}Am peak occurring at 1.1 g/cm^3 coincided with the marker enzymes for lysosomes and pericellular membranes.

Fig. 6 shows the distribution profiles of ^{59}Fe and marker enzymes after centrifugation of the ML fraction of unperfused rat lung on a metrizamide gradient 7 days after ^{59}Fe instillation. The mitochondria were well separated from the lysosomes and pericellular membranes and found within a high density range (1.1 -1.23 g/cm^3). The ^{59}Fe distribution profile was even more scattered than that produced by the ^{241}Am.

Fig. 7 presents the distribution profiles of ^{241}Am and the marker enzymes after centrifugation of the ML fraction on nycodenz gradients, 2 days after ^{241}Am instillation. As with metrizamide gradients, the mitochondria were well separated from the lysosomes and pericellular membranes and sedimented within a density range of 1.16 - 1.20 g/cm^3. The lysosomes and the pericellular membranes occurred at approximately the same isopycnic site, 1.13 g/cm^3, a higher density than that obtained using metrizamide gradients (Figs. 4 and 5). The ^{241}Am profile with median density of 1.14 g/cm^3, showed some correlation with

the lysosomes and pericellular membranes but not with the mitochondria. Lungs which 7 days previously had been instilled with ^{241}Am, were perfused and the ML fraction was layered on a nycodenz gradient (Fig. 8). The mitochondria (GDH) were well separated from the lysosomes and pericellular membranes within a density range 1.15 - 1.19 g/cm^3. The profiles of ^{241}Am and the lysosome and membrane markers coincided with each other, the major peaks occurring at 1.13 g/cm^3.

The ^{59}Fe profile of the ML fraction on a nycodenz gradient, 7 days after ^{59}Fe injection (Fig. 9) was correlated with the lysosome and membrane markers, the major peak occurring at a density of 1.125 g/cm^3. The mitochondria (GDH) however, sedimented at a higher density.

Fig. 10 shows the distribution profiles of cytosol protein from unperfused lungs 3 days after ^{241}Am instillation, and ^{241}Am-labelled ferritin on both nycodenz and metrizamide gradients. Most of the cytosol occurred in the lighter gradient fractions. The ^{241}Am profiles showed a major peak which coincided with the cytosol peaks at the top of the gradients. The ferritin and ^{241}Am profiles behaved alike in both mycodenz and metrizamide with two major fractions, one near the top of the gradient and the second at 1.14 g/cm^3.

3.1.4. Special studies with pericellular membranes

Table 5 shows the subcellular distribution of ^{241}Am in the differential fractions prepared according to the method of Nijjar and Ho (1981), 4 days after ^{241}Am instillation. The purpose of the preparation was to obtain a fraction enriched with

pericellular membranes (S3). The results presented in Table 5 cannot be directly compared with those in Table 4, because of the different centrifugation conditions. About 43% of the ^{241}Am activity in the E fraction was recovered in the S3 fraction. The S3 fraction being shown according to alkaline phosphodiesterase determination to contain more than 50% of the E fraction pericellular membranes. Most of the mitochondria and lysosomes sedimented in the P1 and P2 fractions, respectively. The recovery of ^{241}Am in the differential fractions was 71% of the ^{241}Am lung burden.

A sample from the fraction of purified pericellular membranes, S3, was layered on a discontinuous sucrose gradient (Fig. 13). It can be seen that there is no correlation between the ^{241}Am distribution profile, which has a clear maximum at 1.048 g/cm^3, and the alkaline phosphodiesterase one, which has a small peak at 1.095 g/cm^3.

As an another attempt to obtain a better separation of lysosomes from the pericellular membranes, the self-forming percoll gradient was introduced. Table 7 presents the distribution of ^{241}Am and of lysosome and pericellular membrane marker enzymes, 3 days after ^{241}Am instillation. Two initial percoll densities, 1.05 and 1.06 g/cm^3 were used. According to the table, lysosomes sedimented at the bottom of the gradients, whereas the membranes tended to remain in the top fractions. However, the behaviour of the two lysosomal markers is different: according to N-acetyl-ß-glucosaminidase, 70-75% of the lysosomes was found in the high density region (region I, in both gradients) while for the other lysosomal marker, acid phosphatase, only 40-45% was found in the high density region (I). About 40% of the ^{241}Am radioactivity sedimented in the high density region (I), and approximately 30%

in both the intermediate and light density regions (II and III, respectively).

The fractions of the density regions I (bottom) and III (top) of a percoll gradient of initial density 1.06 g/cm^3 were further analyzed on a nycodenz gradient. The radioactivity profiles are given in Fig. 12. The ^{241}Am radioactivity from the bottom occurred at high densities in nycodenz with the major peak at 1.15 g/cm^3, but the ^{241}Am distribution profile of the top (region III) was scattered over the light density fractions.

3.1.5. Gel chromatography of cytosol

The elution patterns of cytosol, marker proteins, ferritin, transferrin, ^{241}Am and ^{59}Fe are presented in Figs. 14-21.

After chromatography of cytosol from unperfused and perfused lungs, four peaks occurred (Fig. 14) of which the first and the second eluted in the high molecular weight region. The third peak overlapped with the descending edge of the marker protein ribonuclease (RB, molecular weight 13700), and the last peak eluted in the very low molecular weight region. After lavaging the perfused lungs, the intensity of the third peak decreased, indicating that this peak was contributed to by the lung fluid (compare with the elution pattern of lung fluid, Fig. 14).

The fractions of cytosol from perfused lavaged lungs were determined for protein content by Bio-Rad, and the elution pattern showed a major peak up to the 17th fraction, suggesting that the material eluted in the following higher fractions might not contain any proteins.

Figs. 15 and 16 show the elution patterns of cytosol of

unperfused and perfused lungs, respectively, as well as the ^{241}Am distribution profiles, 1, 3 and 7 days after ^{241}Am instillation. There is no essential differences between the protein profiles of perfused and unperfused lungs and no change with time. Also the distribution of ^{241}Am did not change with time. In both figures, the ^{241}Am eluted in a major peak which overlapped with the ferritin one (70% of ^{241}Am activity recovered eluted within this peak). A small percentage of ^{241}Am also eluted with low molecular weight components. The elution patterns of cytosol from perfused lungs, 3 days after ^{241}Am and ^{59}Fe instillation (Fig. 17) showed that most of the activities of ^{241}Am and ^{59}Fe were eluted in a major peak which coincided with the ferritin. Also a small activity peak could be observed in the low molecular weight region.

A ferritin rich fraction was prepared (see section 2.1.7.) from heated total lungs and from cytosol, 3 days after ^{241}Am and ^{59}Fe instillation. The elution profiles of ^{241}Am, ^{59}Fe and the proteins are presented in Fig. 18. The major peak of the protein profiles eluted very early in the high molecular region. This peak corresponded to the denatured proteins in the ferritin rich fraction. The ^{241}Am and ^{59}Fe radioactivities again recovered in a major peak which overlapped with the eluted ferritin peak (more than 50% of both the ^{59}Fe and the ^{241}Am activity in the cytosol was recovered in the ferritin rich fraction).

When ^{241}Am (pH 1-2) was added in vitro to the cytosol of unperfused and perfused lungs, the ^{241}Am eluted in a bimodal peak, where both marker proteins ferritin and transferrin or BSA eluted (Fig. 19). In the case of cytosol from unperfused lungs, a considerable amount of ^{241}Am activity eluted in the low molecular weight region, and this could be attributed to the interaction of

^{241}Am with the inorganic constituents of the cytosol and the blood serum. The ^{241}Am was more scattered in the low molecular weight region of the profile of the perfused lung cytosol. The addition of ^{241}Am (pH 8) to cytosol from unperfused, perfused and perfused lavaged lungs gave a major peak of ^{241}Am which eluted with ferritin (Fig. 19). Another ^{241}Am peak could be found in the low molecular weight region of the unperfused lung cytosol profile, and a smaller peak eluted in the case of ^{241}Am (pH 1). The recoveries of the ^{241}Am from the column were over 80%.

Most of the ^{241}Am activity eluted with the ferritin fraction when ^{241}Am of pH 8 was added to ferritin (Fig. 20). ^{241}Am samples of pH 2 and pH 8 were added to a sample of transferrin as well as to BSA. In both cases a very low ^{241}Am activity was recovered (20-30% of the applied ^{241}Am activity) and a considerable amount of ^{241}Am remained on the resin. A very small percentage of ^{241}Am eluted with the transferrin peak (Fig. 20). When ^{241}Am of pH 2 was added to transferrin dissolved in physiological bicarbonate solution, a major ^{241}Am peak eluted in the low molecular weight components. Similar results were obtained with citrate buffer. Traces of ^{241}Am eluted with the transferrin peak. Similar results were observed with BSA (results not shown).

In order to obtain better separation of the cytosol proteins, a long column of Sephacryl S-300 was used. Fig. 21 shows the elution profiles of cytosol (from perfused lungs) and ^{241}Am, 3 days after ^{241}Am administration. The ^{241}Am eluted in a major peak which overlapped with the ferritin one. Another small ^{241}Am peak coincided with the transferrin or BSA peak. 60% of the total ^{241}Am recovered eluted with the major peak. The recovery of ^{241}Am from the column was 73% of the applied ^{241}Am activity.

3.1.6. Analysis of cytosol by free flow electrophoresis

The distribution profiles of cytosol proteins, other proteins, ^{241}Am and ^{59}Fe are presented in Figs. 22-27. Fig. 22 shows the distribution patterns of the cytosol proteins of unperfused lungs, 3 days after treatment of the rats with ether, nembutal and ^{241}Am-free nitrate solution (pH 7.4) plus ether anesthesia. The protein distribution remained unchanged after the above-mentioned treatments. The sample inlet was located around fraction 75, and as can be noticed from the distribution profiles the proteins were negatively charged.

After perfusion of the lungs, the intensities of some peaks were reduced, mainly the peaks which eluted between fractions 21-37 and 57-81 where the blood serum proteins occurred (compare the profiles of cytosol from perfused and unperfused lungs measured at 280 nm, Fig. 23). The lavage of perfused lungs caused reduction of the peak occurring between fractions 14-24. This peak could be contributed to by the lung fluid (compare with the lung fluid in the same figure). The fractions were also measured for their protein content by the Bio-Rad method. In these profiles, the peaks around fractions 3 and 15-18, which are detectable by measuring the extinction at 280 nm, were lacking. When the extinction at 280 nm was monitored, pure chamber buffer gave a peak in the very first fractions (see the chamber buffer profile, Fig. 23). For perfused and unperfused lavaged lung profiles (Bio-Rad profiles), a very broad peak with maximum around fraction 33 occurred. A similar profile was obtained for unperfused lungs (Bio-Rad profile), except the peaks occurred between fractions 57-81, confirming that these peaks were contributed to by the blood serum proteins.

Independent of time, the ^{241}Am distribution patterns occurred

with two major peaks (with both unperfused and perfused lung cytosol, Figs. 24 and 25, respectively). The bigger peak eluted around fraction 45 and the smaller around fraction 33. In an experiment performed 3 days after intratracheal instillation of ^{59}Fe, the distribution pattern of ^{59}Fe (with perfused lung cytosol) coincided that of ^{241}Am.

The distribution patterns of ^{241}Am which was added as ^{241}Am of pH 1-2 to unperfused and perfused lungs cytosol (fig. 26) show that the ^{241}Am was scattered over a broad peak (fractions 33-55) indicating an unspecific binding of ^{241}Am (pH 1-2) to the cytosol proteins. The distribution patterns of ^{241}Am which was added as ^{241}Am of pH 8, show a major peak with maximum around fraction 45 (Fig. 26). 37 Bq and 185 Bq of ^{59}Fe (pH 8) were added to 1 ml of unperfused lung cytosol. The pattern of ^{59}Fe with low activity (Fig. 26, unperfused lung, $^{59}Fe(1)$) shows that the ^{59}Fe was scattered over the cytosol profile. With a higher activity dose (185 Bq/1 ml cytosol) the ^{59}Fe eluted with a major peak between fractions 50-65 (the same figure, unperfused lung $^{59}Fe(2)$) where the transferrin is expected to elute (see Fig. 27).

The proteins ferritin, transferrin and bovine serum albumin (BSA) were incubated in vitro with ^{241}Am (pH 2 and pH 8), (Fig. 27). Transferrin, in comparison with ferritin and BSA, migrated slowly and eluted at fractions 50-65. BSA and ferritin occurred in fractions 40-49 (with maximum around fraction 47) and 33-49 (with maximum around fraction 40) respectively. The ^{241}Am distribution pattern overlapped well with the ferritin one. Both ^{241}Am and BSA migrated with similar electrophoretic mobility rates, but the patterns were not well overlapped. As the other hand, ^{241}Am

migrated more quickly than the transferrin, and its radioactivity eluted around fraction 35, suggesting that the ^{241}Am hydroxide (pH 8) was negatively charged.

The recoveries of ^{241}Am and ^{59}Fe were regularly over 90% of the applied radioactivity.

3.2. Studies with ^{241}Am and Bovine Alveolar Macrophages

3.2.1. Uptake studies

The uptake of ^{241}Am bovine alveolar macrophages (BAM) in different media is presented in Table 8. The highest uptake of ^{241}Am was observed in HEPES and Waymouth media. The addition of BSA to these media inhibited considerably the uptake of ^{241}Am by BAMs. HEPES medium was chosen for the further incubation experiments (for more details see the discussion).

It can be seen, from Fig. 28, that the ^{241}Am uptake by the BAMs increased with temperature.

Fig. 29 show that the ^{241}Am uptake by BAMs increased linearly with ^{241}Am concentration, when the cells were incubated at 0°C and 37°C. At higher ^{241}Am concentrations the uptake curve became non-linear.

Figs. 30 and 31 show the uptake of ^{241}Am by BAMs depending on time. When the cells were incubated with ^{241}Am at 37°C, there was an initial rapid uptake for the first 15 minutes, then the uptake process increased slowly (Fig. 30). The cultures that were incubated on ice, showed also an intial rapid uptake, but after 1.5 hours the uptake process reached a plateau. After 4 hours,

the cells incubated on ice had taken up about 41% of the ^{241}Am activity taken by cells incubated at 37°C. The uptake process was strongly inhibited when the cells were incubated with 4% BSA at 37°C (Fig. 30). In order to differentiate between the ^{241}Am taken inside the cells and that adsorbed at the cell membranes, the cells incubated at 0°C and 37°C were treated with trypsin (Fig. 31). After trypsin digestion, 40% of the ^{241}Am taken by the cells incubated at 37°C was removed. In the case of the cells incubated on ice, 70% of the ^{241}Am was removed.

According to the viability test and protein determinations of the cells during the incubation time, most of the cells were still viable (the protein content of the cells being 0.095-0.12 mg/10^6 cells). The recoveries of ^{241}Am in the uptake experiments were over 90%.

For homogenization experiments, the cells were washed with 2% BSA (to remove the part of ^{241}Am adsorbed on the cell membranes) and not with trypsin because the cells tend to aggregate after such a treatment. About 45% of the ^{241}Am taken by the cells was removed following the washing with BSA.

The relative subcellular distribution of ^{241}Am and the protein content are presented in Table 9. The ^{241}Am was equally distributed in the N and E fractions (40% from the cells activity). Most of the ^{241}Am in E sedimented with the ML fraction (64% of E). 45% of the protein content of the cell homogenate was recovered in E and most of it was found in the cytosol (65% from E or 2-3 mg protein/1 ml cytosol). Only 15% were in the ML fraction.

3.2.2. Density gradient centrifugation

Fig. 32 shows the distribution profiles of ^{241}Am and the marker enzymes after centrifugation of the ML fraction of BAM on nycodenz gradients. The mitochondria (GDH) were well separated from the lysosomes and the pericellular membranes. There was a considerable activity of the lysosomal and membrane markers in the light fractions around density 1.07 g/cm^3. Another major peak occurred at 1.2 g/cm^3. The ^{241}Am profile coincided with the profiles of the lysosomal and membrane markers. A considerable ^{241}Am activity was found in the light fractions (1.07 g/cm^3). Also a major peak occurred around 1.125 g/cm^3.

To get better separation of lysosomes from pericellular membranes preformed linear percoll gradients were used (Fig. 33). The lysosomal markers gave rise to a major peak around fraction 16 (density 1.082 g/cm^3). The acid phosphatase (AP) showed another peak around fraction 22 (density 1.045 g/cm^3). The profiles of the pericellular membrane markers (ALPD and 5'N) had a maximum around fraction 22 (density 1.045 g/cm^3). The ^{241}Am distribution pattern showed a maximum at fraction 20 (density 1.054 g/cm^3). The ^{241}Am profile coincided with part of the acid phosphatase profile and with the leading edge of 5'-nucleotidase, but not with the other lysosomal and membrane markers.

Fig. 34 shows the elution profiles of ^{241}Am and the other components after gel chromatography of BAM cytosol. In contrast to the results after use of whole rat lung, the recovery of ^{241}Am from the column was very low (20% of the applied radioactivity), indicating that most of the ^{241}Am remained on the column. Nevertheless, most of the ^{241}Am recovered eluted at fractions where ferritin occurred. A smaller percentage of ^{241}Am eluted at

the low molecular weight region.

3.3. Electron microscopic autoradiography with ^{241}Pu

Figs. 38 (a,b,c, and d) and 39 (a,b,c, and d) present the electron microscopic autoradiograms from rat lungs, 24 hours and 7 days after intratracheal injection of ^{241}Pu, respectively.

After 24 hours, the beta tracks of ^{241}Pu were still found in the alveolar space of the rat lung and some tracks were seen at the basement membranes (Fig. 38a). Agglomerations of beta tracks were found with electron dense, globular inclusions in cells which were located at the lining surface of the alveolar space (Figs. 38 b and c). Some tracks could be also seen in the nuclei of such cells (Fig. 38c). Large agglomerations of beta tracks could be seen in a cell surrounded with blood vesicles. The cell contained electron dense and transparent inclusions (Fig. 38d). After 7 days, the agglomerations of beta tracks were no longer seen but were spead uniformly over the cell. In cells located at the lining surface of the alveolar space beta tracks could still be seen on the electron dense and transparent inclusions Figs. 39 a and b), and more beta tracks were found in the nuclei (Fig. 39 b). Other tracks were found with large electron transparent inclusions in cells containing a lot of vesicular structures (Figs. 39 c and d). Also the nucleus of such type of cells contained some beta tracks (Fig. 39 d).

Figs. 40 (a,b,c,and d) present the electron microscopic autoradiograms of bovine alveolar macrophages. Agglomerations of beta tracks of ^{241}Pu could be seen in the cytoplasm of the cells (Figs. 40 a and b), as well as in the nuclei (Figs. 40 c and d).

3.4. Complementary studies with other radionuclides

Retention and subcellular studies have been performed with ^{51}Cr-labelled polystyrene particles (PLS-^{51}Cr), ^{88}Y and ^{237}Np in rats.

Fig. 3 shows the retention of ^{51}Cr-labelled polystyrene particles (PLS-^{51}Cr) in the whole body and lungs of a rat. 32% and 27% of the initial PLS-^{51}Cr dose were cleared from the whole body and lungs, respectively, within 20 days. After that, the PLS-^{51}Cr content in the whole body was unchanged (53% at the 77^{th} day), whereas, in the lungs, the PLS-^{51}Cr radioactivity was slowly eliminated (40% of the 77^{th} day). Filtration of the PLS-^{51}Cr sample through a series of filters of different pore sizes gave the following particle size distribution: $>$ 5 μm (1.5%), 2-5 μm (21%), 1-2 μm (52%) and 0.1 μm (17%) (compare with the electron microscopic micrographs of PLS-^{51}Cr, Figs. 35a and b).

86% of the 1-2 μm fraction was retained in the lungs one week after its injection. 5 days after the instillation of $<$ 0.1 μm fraction, no PLS-^{51}Cr activity was detected in the lungs.

During the retention period, there was no translocation of PLS-^{51}Cr from the lungs to the other organs of the rat.

Table 3 summarized the retention of ^{241}Am and other radionuclides in rats. After 10 days, 72% of the injected ^{237}Np still remained in the whole body of the rat. ^{88}Y was slowly cleared and in the whole body was about 76% of the injected dose after 104 days. The ^{237}Np content in the lungs after 10 days was 61%. ^{88}Y was very slowly removed from the lungs and about 70% of the injected dose remained in the lungs, after 104 days.

It can be noticed that the amount of the radionuclide which remained in the lungs, increased with the mass of the injected radionuclide. In the extrapulmonary tissues, a small amount of ^{237}Np was found in the liver, and in the skeleton the ^{237}Np

content was as high as found with ^{241}Am (14% after 10 days). Traces of ^{88}Y radioactivity were found in the liver and femur.
The subcellular distribution of ^{88}Y and PLS-^{51}Cr are given in Table 6. Most of the ^{88}Y activity was found in the N fraction (80% of the lung burden) and only 5-8% was recovered in the postnuclear fraction (E). Of the ^{88}Y content in E, 75-79% was sedimented with the ML fraction, while in the cytosol ^{88}Y activity could not be detected.
Lung homogenate was incubated in vitro with ^{88}Y, and the N fraction was prepared. 17% of the added ^{88}Y was sedimented with the N fraction.

About half of the PLS-^{51}Cr lung burden was sedimented with the N fraction. 20-35% of PLS-^{51}Cr activity was found in E fraction. Most of the PLS-^{51}Cr in the E fraction was found in the ML fraction, and in the cytosol no activity could be detected. A sample of PLS-^{51}Cr was incubated in vitro with lung homogenate, and the N fraction was prepared. Another sample of PLS-^{51}Cr was added to homogenate medium alone and treated in the same manner as the N fraction. 26% and 17% of the PLS-^{51}Cr was sedimented in the N fraction and in the homogenate medium, respectively. More than 85% of the radionuclides (from the lung burden) were recovered in the subcellular fractions.

The distribution profiles of PLS-^{51}Cr (in the ML fraction) on a nycodenz gradient, 5 and 12 days after PLS-^{51}Cr instillation, behaved as the free PLS-^{51}Cr particles in nycodenz (Fig. 11) where most of the PLS-^{51}Cr radioactivity occurred at light density of 1.07 g/cm^3.

4. DISCUSSION

The main aim of this work is to investigate the storage and transport of ^{241}Am in lungs. However, we will first discuss the subcellular fractionation of whole rat lung and BAMs, then the subcellular distribution and binding of ^{241}Am in rat lung, accompained by morphological studies using electron microscope autoradiography with ^{241}Pu, followed by the ^{241}Am uptake with BAMs, and finishing with a proposed tentative model for binding and transport of ^{241}Am in lung.

As known, the lung is a very complex and heterogeneous organ which contains more than 40 cell types, as well as large amounts of fibrous tissue and aqueous media (Crapo et al., 1983; Gail and Lenfant, 1981). This makes lung homogenization and fractionation difficult in comparison, for example, with liver. In spite of this we succeeded in obtaining subcellular fractions from rat lung, as well as from BAMs using the same differential centrifugation conditions. The subcellular organelles lysosomes, mitochondria, and pericellular membranes were sedimented in the ML fraction. In the cytosol fraction, some lysosomal activity could be detected, which might be ascribed to lysosomal rupture during the fractionation procedure. In addition to that lysosomal enzymes which are present in the extracellular lining of the lung (Hook, 1978), could be found in the lung cytosol fraction. From the density gradient studies with metrizamide and nycodenz, it can be seen that, the mitochondria (and the endoplasmic reticulum which appeared with the mitochondria, see Glück, 1982) were well separated from the other ML components of both rat lung (Figs.

4-9, see also section 3.1.3.) and BAMs (Fig. 32, see also section 3.2.2.). However, the crucial problem when working with iodinated non-ionic density gradient media is that lysosomes and pericellular membranes band at very similar densities. Our attempts to overcome this problem by use of self-forming percoll or preformed percoll gradients were not fully successful, but a lysosome rich fraction from the whole lung was obtained (Table 7) and the majority of the BAM lysosomes were separated from the pericellular membranes (the behaviour of 5'-nucleotidase, the membrane marker, and acid phosphatase, the lysosome marker, will be discussed later).

In conclusion it is possible to separate lysosomes and pericellular membranes from mitochondria (and endoplasmic reticulum) by conventional cell fractionation techniques. The subcellular organelles of the whole lung and that of the BAMs are similarly distributed with regard to density. This is surprising when the complexity of the lung cell populations is considered.

The analysis of the cytosol from perfused and unperfused lungs by gel chromatography yielded four peaks (Fig. 14, extinction was measured at 280 nm). The first and the third peak could be contributed to by the lung fluid (see also section 3.1.5.), while the fourth peak, in the low molecular region, could be attributed to non-protein constituents (which were not detected when the cytosol was measured by "Bio-Rad" technique). Determination of the cytosol by "Bio-Rad" suggested that most of the lung proteins are of molecular weight higher than that of transferrin. The BAM cytosol was distributed in one major peak of high molecular weight which could be attributed to the cytosol proteins, while

the low molecular weight peak could represent the non-protein constituents of the cytosol (Fig. 34).

The analysis of the cytosol by FFE indicates that most of the cytosol constituents are negatively charged (Fig. 23). Monitoring the cytosol fractions at 280 nm or by the "Bio-Rad" method indicates that the bulk of the cytosol proteins eluted between fractions 25-50 (Fig. 23). After lavage, the second peak of the cytosol profile monitored at 280 nm (Fig. 23) was less pronounced. This peak was not monitored by "Bio-Rad" method. This suggests that the second peak could be attributed to the negatively charged phospholipids of lung fluid. After lung perfusion the peaks between fractions 55 and 85 were reduced indicating that they were contributed to by blood serum proteins. The first peak in the profiles can be attributed to the constituents of the chamber buffer (Fig. 23). It is worth noting that the cytosol protein distribution profiles might not be influenced by anesthesia (Fig. 22).

In conclusion, by using the electrophoretic properties of the cytosol and the differences in the molecular size it is possible to identify some of the cytosol constituents.

The trivalent ^{241}Am tends to be hydrolized at high pH in aqueous medium, and above pH 7 colloidal particles (hydroxide polymers) are formed (Starik and Ginzberg, 1960). Most of the ^{241}Am in the injected solution could not be filtered through a 0.025 μm pore size filter, indicating that ^{241}Am in the injected solution (pH 8) was in colloidal form, and according to its behaviour in the FFE, these particles might be negatively charged (Fig. 27,

distribution profile of transferrin and ^{241}Am). The other radionuclides, ^{59}Fe, ^{88}Y (trivalent state) and ^{237}Np at pH 7 also form colloidal particles. Thus, all the injected radionuclides have a similar chemical form.

In comparison with the retention results of ^{88}Y and ^{237}Np (Table 3) which might form bigger colloidal particles than ^{241}Am and with the insoluble PLS-^{51}Cr retention studies (Fig. 3, section 3.4.), the ^{241}Am hydroxide polymers were solubilized and cleared rapidly from the whole body and lungs (Fig. 1). This actually confirms that the ^{241}Am colloid is moderately soluble compound and that was expressed in higher deposition of ^{241}Am in the extrapulmonary tissues (Fig. 2) than the ^{88}Y and ^{237}Np (Table 3) while no PLS-^{51}Cr activity could be detected in the extrapulmonary tissues probably, because of the short half-life time of ^{51}Cr. The differences between ^{241}Am and ^{59}Fe (Table 3) are due to the fact that the former is a non-physiological metal while the second is a physiological one which is involved in the body's metabolism.

The rapid clearance of PLS-^{51}Cr from the whole body and lungs at the first phase (Fig. 3) could represent the clearance of polystyrene particles by macrophages which were removed by the ciliary escalator toward the esophagus (Schlesinger et al., 1982). The 0.1 μm fraction of the PLS-^{51}Cr injected dose might contain very small particles which could cross the alveolar wall to the blood, thus contributing to the rapid clearance of PLS-^{51}Cr particles. Clearance by macrophages could be one route for ^{241}Am clearance from lungs, but could not explain the very rapid clearance from lungs and the relatively high extrapulmonary

deposition which suggest that the main clearance route of ^{241}Am from lungs might be through the blood circulation (to be discussed later). The PLS-^{51}Cr content in the whole body is higher than that in the lung, at longer times. This could be attributed to measurement errors of the whole body counter. At comparable times, Glück (1982) reported that less ^{241}Am hydroxide polymers were retained in the rat lungs. This could be attributed to the application of different intratracheal technique.

Similar results were obtained for retention in lungs and for extrapulmonary deposition, when $^{241}AmCl_3$ was injected intratracheally into rat lungs (Zalikin and Popov, 1977; Zalikin et al., 1968) and when rats inhaled $^{241}Am(NO_3)_3$ (Ballou et al., 1980). However, ^{241}Am in a stable and soluble chemical form, such as citrate, was removed from the lungs more quickly than other ^{241}Am chemical forms and higher ^{241}Am amounts were translocated to the liver and skeleton (Stradling et al., 1978a; Crawley and Goddard, 1976).

Within the lungs, most of the ^{241}Am hydroxide polymers were found in the lung tissues and in the lavaged alveolar macrophages, but ^{241}Am activity was also found in the lung fluid (Table 2). Light microscopic autoradiography of alveolar macrophages show that the ^{241}Am α-tracks were found within these cells (Figs. 37a and b). It is probable, that the amount of ^{241}Am taken up by the alveolar macrophages is underestimated. This could be due either to the low yield of macrophages which could be lavaged or to uneven distribution of the injected ^{241}Am within the lungs. To obtain more information about the distribution of ^{241}Am within the lung cells, the electron microscopic autoradiography with ^{241}Pu was introduced. Most of the ^{241}Pu ß-tracks were found within cells

which have the appearance of macrophages (Figs. 38a-d and 39a-d). The ß-tracks were seen in both alveolar and ,probably, interstitial macrophages (Fig. 38c and d, respectively), indicating that those macrophages are responsible for the uptake of ^{241}Pu hydroxide polymers. Such a statement should be extended to ^{241}Am with caution because of the different chemistry of both radionuclides. However, it is not unexpected that ^{241}Am would be taken up by these macrophages.

Rahman and Lindenbaum (1964) reported that when polymeric plutonium was intravenously injected, it was found in the Kupffer cells (liver macrophages). When ferric hydroxide plutonium colloid was injected into rats, the colloid was initially concentrated in the Kupffer cells and spleen macrophages (Priest and Haines, 1982). In another study colloidal ferric hydroxide particles enveloped with potassium polyvinyl surface molecules were intravenously injected into rats. These particles were negatively charged (as ^{241}Am hydroxide polymers). It was found that such particles were taken up by Kupffer cells in the liver and macrophages in the spleen and no particles could be detected in other cell types (Ono and Awai, 1984). All these studies support the hypothesis that the lung macrophages might be the cell type in which the ^{241}Am hydroxide polymers are deposited. In fact this is consistent with the general function of the reticuloendothelial cells to which the macrophages belong, in taking up particulate material.

The subcellular distribution of ^{241}Am in rat lungs (Table 4) in comparison with that of ^{88}Y and PLS-^{51}Cr (Table 6) suggests that the ^{241}Am hydroxide polymers were solubilized intracellularly, while the others were not. Most of the ^{88}Y and PLS-^{51}Cr activity was found within the N fraction, where it might be contained

either in heavy lysosomes which could sediment at low speeds or within unbroken cells. One might think that because ^{88}Y and PLS-^{51}Cr are big particles, they could sediment as free particles at low speeds, but it is not the case because only few activity was detected in the N pellet when both nuclides were incubated with lung homogenate (see section 3.4.).
At the subcellular level also there are differences in the distribution of ^{241}Am and ^{59}Fe (Tables 4 and 6, respectively) which indicate that both elements have different intracellular transport systems. This is in agreement with the subcellular distribution of monomeric ^{59}Fe and ^{241}Am in rat liver (Sütterlin, 1982). Similar subcellular distribution of ^{241}Am in rat lung was obtained by Glück (1982).

The ^{241}Am in the N fraction (Table 4) could be found either within unbroken cells, which were not disrupted during the homogenization procedure or within heavy lysosomes. It is also possible that free ^{241}Am which could be released during the homogenization process, could bind nonspecially to the homogenate components. However, if to extend the observations of the electron microscope autoradiograms where a lot of ^{241}Pu ß-tracks were found with lung cell nuclei (Figs. 38c and 39b,c), a lot of ^{241}Am activity in the N fraction could be associated with lung cell nuclei.
^{241}Pu ß-tracks were also found in the liver cell nuclei of rats and Chinese hamsters (Seidel et al., 1985) and also in the nuclei of rat hepatic parenchymal cells (James and Rowden, 1969). Gurney and Taylor (1975) observed that ^{239}Pu was associated with the nuclei of liver cells. Other metals, such as beryllium, could be deposited in the cell nuclei (Williams and Skilleter, 1983).

However, little is known about the interaction of transuranium elements with the cell nuclei, therefore such findings need to be investigated further.

From the studies with the iodinated non-ionic density media (metrizamide and nycodenz), the mitochondria can be excluded as the binding site of ^{241}Am hydroxide polymers at the intracellular level (Figs. 4,5,7 and 8, see also section 3.1.3.). Similar results were obtained for ^{59}Fe (Fig. 6 and 9), but iron could be found in the mitochondria. The self-forming percoll gradient results indicate that a considerable amount of ^{241}Am could be associated with lysosomes of high density (Table 7), while the association of ^{241}Am with pericellular membranes which occurred with a lot of ^{241}Am in the light density percoll regions (Table 7), could be excluded in view of the lack of correlation between the ^{241}Am activity and the pericellular membranes in discontinuous sucrose gradient (Fig. 13, see also section 3.1.4.). These biochemical studies indicate that intracellularly, ^{241}Am is deposited, most probably in lysosomes of low and high densities (Table 7 and Fig. 12). This is supported by morphological studies with electron microscopic autoradiography of ^{241}Pu. The ^{241}Pu ß-tracks are associated with lysosome-like structures (Figs. 38c-d and 39a,b). ^{241}Pu ß-tracks were observed over electron lucid vesicles which might represent the surfactant bound ^{241}Pu taken up by the alveolar macrophages (Figs. 39c and d, see also Cooper et al., 1980) (the role of the lung surfactants will be discussed later). It is worth noting that there was no ^{241}Pu association with the pericellular membranes. If the electron microscope autoradiography technique were to be extended to ^{241}Am the chemical differences between ^{241}Pu and ^{241}Am should be taken in consideration.

Intracellularly, the insoluble particles might behave differently to the moderately soluble compounds. The behaviour of PLS-^{51}Cr in a nycodenz gradient (Fig. 11) suggests that these particles could be free in the cytoplasm or found in digestive vacuols as a result of PLS-^{51}Cr phagocytosis by lung cells, particularly, alveolar macrophages (Wetzel and Korn, 1969).

Except the work of Glück (1982) who obtained similar distribution profiles for ^{241}Am hydroxide polymers in metrizamide, there are not any biochemical studies of the transuranium elements in lungs. The majority such studies have been performed with liver. Therefore the literature concerning the liver studies will be cited. ^{239}Pu, as well as ^{241}Am, were found in the lysosomes of rat liver cells (Rahman and Lindenbaum, 1964; Taylor, 1972; Gurney and Taylor, 1975; Boocock et al., 1970). Studies at this institute with monomeric ^{239}Pu and ^{241}Am have shown that both radionuclides were associated with liver cell lysosomes of different animals (Gruner et al., 1981; Winter and Seidel, 1982; Sütterlin et al., 1984). ^{241}Pu ß-tracks have been observed within "dense granules" (James and Rowden, 1969). Following inhalation, ^{239}Pu particles were localized in macrophage phagolysosomes (Sanders et al., 1977).

There are still some aspects concerning the density gradient results that should be discussed. The behaviour of the marker enzymes in the density gradients, as well as in the subcellular fractions have already been dicussed (Sütterlin, 1982; Glück, 1982). The differences in the distribution profiles of ^{241}Am and the marker enzymes in metrizamide and nycodenz are not fully understood. However, this could be due to the heterogeneity of lung and not due to the effect of the gradient media on the

biological material (Rickwood, 1983). The other aspect concerning the ^{241}Am activity which sedimented at high densities in both metrizamide and nycodenz gradients (Figs. 4,5,7). This activity could be attributed either to ferritin or hemosiderin bound ^{241}Am which attached with lysosomal membrane fragments as a result of lysosome disruption during the homogenization process or to ferritin-bound ^{241}Am which is adsorbed on the endoplasmic reticulum (Gruner, 1978). Free ferritin which is thought to be the ^{241}Am binding protein in the cytosol (to be discussed later) is excluded here because the cytosol and horse spleen ferritin sedimented at light densities in both metrizamide and nycodenz gradients (Fig. 10). Ferritin and hemosiderin which are found in the lysosomes of cells (Bradford et al., 1969; Essner and Novikoff, 1960; Peters and Seymour, 1976) are thought to be the binding proteins of ^{239}Pu in the lysosomes (Stover et al., 1970; Neu-Müller, unpublished results) and probably ^{241}Am.

In percoll gradients, the lysosomes were found not only in high density regions, but in the low ones too. This heterogeneity of the lysosomes could originate from the different cell types of rat lung. However, it cannot be excluded that lysosomes from the same cell type are heterogeneous (Pertoft et al., 1978b; Rome et al., 1979). It should also kept in mind that change in the density of lysosomes could happen during the fractionation procedure. The different behaviour of the lysosomal enzymes acid phosphatase and N-acteyl-ß-glucosaminidase in self-forming percoll gradient (Table 7) could be due either to that acid phosphatase unlike N-acetyl-ß-glucosaminidase, is thought to be bound weakly to lysosomal membranes (Baccino et al., 1971), thus it will be easily dissociate and release following lysosome disruption during gradient centrifugation, or to the presence of

heterogeneous lysosomes from the lung cell types with different concentration levels of hydrolases (see also below).

The results of the lung cytosol analysis by gel filtration suggest that most of the ^{241}Am in the cytosol (70%) might be bound to high molecular weight protein (>400,000) which eluted where marker ferritin fractions occurred (Figs. 15,16 and 21). The elution of ^{241}Am together with ^{59}Fe after analysis of the cytosol (Fig. 17) and "ferritin" rich fraction (Fig. 18) which has a reddish colour and was prepared by heating the cytosol or the whole lung homogenate, suggest strongly that the high molecular weight protein might be ferritin which is considered to be the storage site of the intracellular iron. A small amount of ^{241}Am in the cytosol could be found as low molecular weight complexes, such as citrate (compare with Fig. 20). It is also likely that ^{241}Am bound to transferrin or albumin (Fig. 21) which are available in the extracellular lining of lung (George and Hook, 1984).

The behaviour of ^{241}Am in the high electric field of FFE (Figs. 24 and 25) indicates that ^{241}Am is indeed bound to the cytosol proteins (compare to Fig. 23). The binding of ^{241}Am to lung fluid (mainly lung surfactants) might be excluded because no ^{241}Am has migrated with lung fluid (second peak in Figs. 24 and 25) which has higher negative electric charge than the protein-bound ^{241}Am. This is not unexpected because both ^{241}Am hydroxide polymers and the lung surfactants are negatively charged. That is actually consistent with the findings of Cooper et al. (1980) who reported that the negatively charged $^{244}CmO_2$ was not bound with lung fluid unlike the positively charged $^{238}PuO_2$.

If the ferritin is the protein most likely to bind ^{241}Am in the cytosol, the in vitro incubation results of ^{241}Am (Fig.

19,20,26,27 and sections 3.1.5. and 3.1.6.) indicate that ^{241}Am might bind to ferritin as hydroxide polymer (^{241}Am at pH 8), while it might bind to transferrin or albumin in monomeric form (pH1-2). ^{241}Am could also form low molecular weight complexes which could be citrate. The in vitro incubation of ^{241}Am with transferrin and BSA (Figs. 20 and 27) did not succeed to show any specific binding of Am to both proteins, therefore more investigations are required.

It might be thought that the presence of ferritin which might bind ^{241}Am in the cytosol is due to lysosomes which have been disrupted during the homogenization procedure. However this possibly is excluded if the following points are considered: de Duve and Beaufay reported that about 10% of the cell ferritin is bound to lysosomes (de Duve and Beaufay, 1957). Boocock has mentioned that most of the ferritin sedimented with the microsomal fraction P (Boocock et al., 1970). In addition, it was found that 10-20% of the lysosomes could be disrupted during the fractionation procedure. All the above-mentioned points are inconsistent with large amounts of ^{241}Am which might be bound to ferritin in the cytosol.

The binding of transuranium elements to cell proteins was investigated by several workers. ^{239}Pu was found to be associated with protein of molecular weight of more than 300,000 following the analysis of rat lung cytosol on Sephacryl S-200 (Glück, 1982). In FFE similar distribution patterns were obtained for ^{239}Pu. In liver cells, most of ^{241}Am, ^{239}Pu and ^{59}Fe were associated with ferritin (Bruenger et al., 1976; Boocock et al., 1970; Stover et al., 1970). In blood serum plutonium forms stable complexes with transferrin (Stover et al., 1968; Turner and

Taylor, 1968a, 1968b), while the trivalent actinides, such as ^{241}Am, seems to form less stable complexes with blood serum proteins which could dissociate during the separation procedure. However, several workers have reported that some of the ^{241}Am was bound to both albumin and transferrin (Boocock and Popplewell, 1966; Bruenger et al., 1969; Turner and Taylor, 1968b). Using affinity chromatography, it has been found that ^{241}Am is bound rather to transferrin and not to albumin (Cooper and Gowing, 1981).

We may conclude that with respect to the structural cell elements, ^{241}Am which was intratracheally instilled as hydroxide polymers, is bound to lysosomes and in the cytosol the great majority of ^{241}Am might be bound to ferritin. The binding of ^{241}Am to lung fluid can be excluded.

As mentioned before, it is likely that ^{241}Am hydroxide polymers are deposited in the alveolar macrophages of rat lungs. Therefore, short-term cultures of bovine alveolar macrophages as a simpler model than the whole lung were introduced. The medium in which the BAMs and ^{241}Am were incubated, was chosen so that it would have minimum interaction with ^{241}Am. This facilates the work by having defined system which excludes the interference of unknown factors which could affect the ^{241}Am uptake process by the cells. The HEPES medium was chosen because it binds no metals and has a very low dissociation constant, so even the possibility of binding with ^{241}Am hydroxide polymers is excluded (Castranova et al., 1980; Good et al., 1966). Waymouth medium was not used because it contains negatively and positively charged amino acids which could bind to ^{241}Am, thus affecting the uptake process. The BSA, as well as the lung "surfactants" have an inhibitory effect

on the nuclide uptake (Table 8).

The observations of the kinetic studies (Figs. 29-31 and section 3.2.1.) suggest that ^{241}Am hydroxide polymers were taken up by BAMs in a two-fold process: first a rapid adsorption of ^{241}Am hydroxide on the cell surface followed by the ingestion or engulfment of the surface-bound ^{241}Am. Such an uptake process is known as "adsorptive pinocytosis" (Silverstein et al., 1977). From these studies it is not clear whether ^{241}Am uptake by BAMs takes place via cell receptors or via nonspecific binding of ^{241}Am to the cell membranes. Therefore, more investigations in this direction are required. However, the uptake of $^{241}AmO_2$ by rabbit alveolar macrophages was described as taking place via nonspecific binding of the $^{241}AmO_2$ particles to the cell surface followed by subsequent phagocytosis and/or pinocytosis (Robinson and Schneider, 1980a). Similar systems were described for the uptake of colloidal gold by rabbit peritoneal macrophages (Gosselin, 1967) and for colloidal beryllium by alveolar macrophages (Hart and Pittman, 1980). The inhibitory effect of BSA on the ^{241}Am (Fig. 30) and its capability to remove the ^{241}Am bound to the cell surface could be due to nonspecific interaction of BSA with the ^{241}Am hydroxide polymers.

The ^{241}Am subcellular distribution results in BAM (Table 9), in comparison with that in rat lung (Table 4), suggests that the ^{241}Am is deposited first within ML components at the intracellular level, therefore a small amount of ^{241}Am was found in the cytosol. In N fraction ^{241}Am could be found within BAM nuclei where ^{241}Pu ß-tracks have been observed (Figs. 40c and d). Using the nycodenz gradient, the mitochondria were excluded as the binding site of the intracellular ^{241}Am in BAMs (Fig. 32). The preformed linear percoll gradient results (Fig. 33) suggest

that ^{241}Am might be found within phagolysosomes which are formed from the fusion of pinocytic vesicles-containing ^{241}Am and primary lysosomes. As a result, the phagolysosomes become lighter than the primary lysosomes (Seglen and Solheim, 1985). The difference between 5'- nucleotidase and alkaline phosphodiesterase could be due either to the heterogeneity of the plasma membranes (Toda et al. 1975) or to the irreversible inactivation of alkaline phosphodiesterase in the phagolysosomes by changing the pH or as a result from the action of lysosome hydrolases. 5'-nucleotidase activity has been observed in phagolysosomes of macrophages following phagocytosis of polystyrene particles (Werb and Cohn, 1972). The correlation of the ^{241}Am profile to the acid phosphatase and not to N-acetyl-ß-glucosaminidase and aryl sulfatase could be attributed to either uneven hydrolase representation within secondary lysosomes of macrophages or to differences in the stability of the various enzymes within the secondary lysosomes (Wiener and Curelaru, 1975). It could be also attributed to the presence of several phosphatases in the macrophage lysosomes and/or adsorption of solubilized enzyme onto various cellular components (Canonico et al., 1978). Acid phosphatase was found in digestive vacuoles (Lowrie et al., 1979).

These biochemical findings could be supported by the electron microscope autoradiography. ^{241}Pu ß-tracts can be seen within structures which might represent phagolysosomes (Figs 40 a,b and d).

In spite of the low recovery of ^{241}Am (20%) from gel filtration, most of the ^{241}Am recovered eluted with a high molecular weight protein (>400.000). Such a protein might represent ferritin which has been found in human alveolar macrophages (Costabel et al.,

1984). A small amount of ^{241}Am could be found as a low molecular weight complex in the BAM cytosol.

In the light of the previous discussion, we will try to build up a tentative model for binding and transport of ^{241}Am in lungs.

The ^{241}Am hydroxide polymers were cleared rather quickly from the lungs. One possible route of clearance could be via migration of the alveolar macrophages containing ^{241}Am to the lung airways where they are swept up by the mucociliary action and carried out into the esophagus. Such a mechanism is thought to be an important one in clearance of particulate material, such as PLS-^{51}Cr, from lungs (Rühle et al., 1983; Spritzer et al., 1968; TGLD, 1966). In fact this might explain the presence of ^{241}Am activity in the stomach, even two weeks after ^{241}Am instillation.

As mentioned before, the translocation of considerable amounts of ^{241}Am in the liver and skeleton indicates that the main clearance route of ^{241}Am from the lungs is through the blood circulation. This suggests that ^{241}Am in the blood is found in two chemical forms: the first is as colloidal ^{241}Am hydroxide polymers, which, as a general feature of colloidal substances, tend to deposit in the liver and the second, as soluble form of ^{241}Am which, deposits in skeleton (Durbin, 1973). In fact, ^{241}Am hydroxide polymers could be solubilized rapidly in the lung shortly after ^{241}Am injection, to form hydroxides of low molecular weight which can cross the alveolar epithelium and the glomerular membranes to the blood (Stradling et al., 1978a). The soluble chemical form of ^{241}Am which deposited in the skeleton might be formed either in the lung or in the blood following the solubility of the ^{241}Am hydroxides which cross the alveolar wall to the blood.

As noted before, the ^{241}Am hydroxide polymers might not bind to

the lung surfactant, leading to enhancement of ^{241}Am clearance from the lungs. Also $^{244}CmO_2$ did not bind to lung surfactant unlike the positively charged $^{238}PuO_2$ (Cooper et al., 1980). Such difference between americium and plutonium was shown in more rapid clearance of ^{241}Am from rat lungs than ^{239}Pu (Glück, 1982). In addition, ^{241}Pu was seen within electron transparent structures in alveolar macrophages (Fig.40). This could be attributed to surfactants-binding ^{241}Pu which were taken up by alveolar macrophages. Also, the inhibitory effect of surfactants on the ^{241}Am uptake by BAMs could be ascribed to the electrostatic repulsion between ^{241}Am and surfactants which are both negatively charged. In parallel to the translocation mechanism, ^{241}Am could be taken up by alveolar macrophages in the lungs, and it might cross the epithelial wall to the interstitium where it could be taken up by the interstitial macrophages. Gold was also found in interstitial and alveolar macrophages (Pääkkö et al., 1984). According to BAM's results, the ^{241}Am colloid is deposited first in the lysosomes of the macrophages through pinocytic vesicles which fuse later with the lysosomes, in contrast to monomeric radionuclides, such as ^{239}Pu and ^{241}Am which occur first in the cytoplasm and later it would be found within the lysosomes (Taylor, 1972; Bruenger et al., 1976). The electron microscope autoradiography of rat lungs with ^{241}Pu shows that after one week the agglomerations of ß-tracks were no longer seen (Figs. 38b,d and 39a-d) indicating that the ^{241}Pu was solubilized within the macrophages. Robinson and Schneider reported that the solubility of $^{241}AmO_2$ was increased after its phagocytosis by alveolar macrophages (Robinson and Scheider, 1980b). Thus the ^{241}Am colloids might first be solubilized in the lysosomes then bind to soluble ferritin in the cytoplasm. The

mechanism of such a pathway is still unknown. However, from our in vitro experiments it seems that the ^{241}Am is bound to ferritin as hydroxides. It was shown autoradiographically that aggregation of ^{241}Am particles occurred with "brownish pigment" which was thought to be ferritin (Rudnitskaya and Moskalev, 1975). George and Hook (1984) reported that the proteins of molecular weight less than 150,000 can cross the alveolar wall of the lungs from the blood stream. Therefore ferritin (molecular weight of 450,000) could be excluded as the transport protein of ^{241}Am. The alveolar macrophages have transferrin receptors (Costabel et al., 1984). Transferrin, as well as the other plasma proteins, is found in the extracellular lining of the lung, and it can cross the epithelial wall of the lungs to and from the blood stream (George and Hook, 1984). As noted before, transferrin could be the transport protein of ^{241}Am in the blood. All these facts together support the assumption that ^{241}Am could be released from the macrophages and is transported to the blood by transferrin which can cross endothelial membranes (Soda and Tavassoli, 1984). It is possible that ^{241}Am is released from macrophages to lung fluid (Table 2) in another soluble form such as low molecular weight hydroxides or citrate complex which is found in blood and urine (Stradling et al., 1978a) and then transported to the blood stream via the alveolar wall. It is also possible that ^{241}Am is released from macrophages by an exocytosis process.

5. SUMMARY

1. a) The retention of ^{241}Am hydroxide polymers was studied in the whole body and lungs of rat. In comparison with the insoluble ^{51}Cr-polystyrene and the big colloidal particles of ^{237}Np and ^{88}Y, the ^{241}Am was cleared rapidly from the whole body and lung and translocated, mainly to liver and skeleton.

b) A system for short-term culture of bovine alveolar macrophages (BAMs) was developed. The conditions, kinetics of ^{241}Am hydroxide polymers' uptake and their intracellular fate were analyzed.

c) Within the lungs ^{241}Am was found with the lavaged alveolar macrophages and (during the first days) in the cell-free lavage fluid.

2. By using conventional homogenization and fractionation techniques, it was possible to separate mitochondria from lysosomes and pericellular membranes of whole rat lung and BAMs (using iodinated non-ionic gradient media) while lysosomes and pericellular membranes could partially be separated by percoll gradients. Using gel chromatography and free flow electrophoresis it was possible to separate some of the cytosol constituents.

3. After differential centrifugation, ^{241}Am is found in "nuclei", mitochondria-lysosome and cytosol fractions. In analogy to the findings after electron microscope autoradiography with ^{241}Pu it is concluded that ^{241}Am is associated with the nuclei of

rat lung cells and BAMs. In the ML fraction, a substantial part of ^{241}Am is associated with lysosomes, the remainder could not be correlated with a cell•organelle. This conclusion was supported by the observations with electron microscope autoradiography, where ^{241}Pu ß-tracks have predominantly been seen in lysosome-like structures.

4. In cytosol, ^{241}Am is associated with proteins of the molecular weight of ferritin (>400.000) and (to about 5-10%) also with low molecular weight components. Electrophoretically, ^{241}Am migrates together with the bulk of the cytosol proteins and not with the highly negatively charged surfactant components.

5. A tentative model for binding and transport of ^{241}Am in lungs is proposed:

   Initially, one fraction of ^{241}Am is already solubilized in the lung fluid leading to the formation of low molecular hydroxides which can cross the alveolar wall to blood stream. In parallel, ^{241}Am is taken up by pulmonary macrophages of which a part can by cleared by the mucociliary processes. Within the macrophages most of the ^{241}Am hydroxide polymers become associated with lysosomes (where they are solubilized) and with the cytosolic ferritin.

   It can be speculated that the ^{241}Am is released from the cells and crosses the alvèolar membranes as bound to transferrin.

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7. TABLES AND FIGURES

Table 1: Conditions of Enzyme Determinations for Gradient Fractions

Enzyme	Sample	Buffer	Substrate	Incubation	Inhibitor	Manipulation	λ(nm)	Reference
N-Acetyl-β-glucosaminidase	0.2 ml	0.5 ml (Citrate)	0.10 ml	20 minutes at 37°C	0.5 ml Ethanol	0.5 ml+2.5 ml $NaHCO_3$ solution wait 10 min.	400	Beaufay (1974)
Acid phosphatase	0.2 ml	1 ml+10µl TX 100 (Acetate)	0.1 ml	1 hour at 37°C	0.2 ml $HClO_4$ solution	0.5 ml+0.5 ml solution B+100µl TX 100+2.5 ml Solution A.Wait30min.	660	Appelmans (1955)
Arylsulfatase	0.2 ml	10µl TX 100 (Acetate)	0.2 ml Substrate dissolved in buffer	1 - 2 hour at 37°C	2 ml PTA	1 ml+2 ml quinone solution wait 30 min.	515	Roy (1954)
Alkaline phosphodiesterase	20 - 50 µl	0.5 ml (Glycine-Acetate)	0.1 ml	20 minutes at 37°C	0.5 ml Ethanol	measure directly	400	Beaufay (1974)
5'-Nucleotidase	0.2 ml	1 ml+10µl TX 100 (Tris buffer)	0.1 ml	1 hour at 37°C	0.2 ml $HClO_4$ solution	As for acid phosphatase	660	Michell (1965)
Glutamate dehydrogenase	0.2 ml	1 ml (Triethanolamine)	0.1 ml α-keto glutarate 30 µl ADP 50 µl NADH	Room Temperature		Measured Kinetically for 5 minutes	365	Williamson (1967)

- The volumes of samples for differential fractions were half of that for gradient fractions (for glutamate dehydrogenase - 50 µl). The other conditions remained unchanged.

- All details of the assays are taken from Sütterlin (1982) and Gruner (1978).

Table 2: Removal of ^{241}Am from Rat Lung by Lavage

Days after ^{241}Am injection	% from lung burden[a]			% from lavage material[b]	
	Lavaged Lungs	Cells	Fluid	Cells	Fluid
0 +	25 ± 3	5 ± 0.4	65 ± 1	7 ± 2	89 ± 0.5
1	56 ± 4	15 ± 3	28 ± 3	35 ± 1	64 ± 1
3	66 ± 1	14 ± 3	19 ± 1	42 ± 1	56 ± 1
7	77 ± 1	12 ± 2	10 ± 1	55 ± 1	46 ± 0.4
14	82 ± 2	12 ± 2	6 ± 1	69 ± 0.5	35 ± 1

The values are the arithmetic mean of 3 experiments ± standard error.

Lavage = cells + lung fluid

\+ minutes after injection

a Total ^{241}Am lung content at sacrifice= 100

b Total ^{241}Am removed by lavage = 100

Table 3: Retention of Different Intratracheally Injected Radionuclides in Rat Organs.

radionuclide	^{241}Am		^{59}Fe		^{237}Np		^{88}y		
organ	2d.	7d.	2d.	7d.	3d.	10d.	4d.	11d.	104d.
Whole body	86 ± 1	76 ± 1	91 ± 1	85 ± 5	80 ± 4	72 ± 3	88 ± 2	79 ± 2	76 ± 2
Lungs	45 ± 3	30 ± 3	48 ± 1	37 ± 4	70 ± 2	61 ± 3	86 ± 1	82 ± 4	69 ± 3
Liver	12 ± 1	22 ± 3	10 ± 1	11 ± 1	2 ±0.1	1 ±0.2	0.7±0.1	0.8±0.3	
Skeleton	8 ± 1	14 ± 1			9 ± 1	14 ± 1			
Femur			1 ±0.1	0.2 ±0.1			0.03±0.01	0.04±0.01	0.1±0.03
Blood			18 ± 1	29 ± 1					
Injected mass mol/kg	$3\text{-}7\times10^{-10}$		$0.2\text{-}3\times10^{-10}$		6×10^{-7}		4×10^{-5}		

The values are the arithmetic of 3-6 rats ± S.E.

Table 4: Relative Subcellular Distribution of ^{241}Am in the Unperfused Rat Lung

Days after ^{241}Am injection	% from lung burden			% from E		
	CT	N	E	ML	P	S
2	8	36	38	28	14	48
3	6	20	44	38	12	37
4	5	38	44	34	16	43
5	5	35	47	38	13	39
7	8 ± 0.8	39 ± 2	38 ± 3	33 ± 0.7	16 ± 1	43 ± 0.8
10	5	34	42	40	19	36
22	12	45	30	39	13	43
35	10	41	32	58	16	27

One experiment per time period, 5 experiments for 7^{th} day (arithmetic means ± S.E.)

For abbreviations see the list.

Table 5: Relative sebcellular Distribution of ^{241}Am and Marker Enzymes in Unperfused Rat Lung, 4 Days after ^{241}Am Injection (The Fractions were prepared according to Nijjar, 1981, section 2.1.5).

	% from lung burden			% from E'			
	CT	N' [a]	E' [b]	P_1	P_2	P_3	S_3
^{241}Am	5 ± 0.8	28 ± 1	38 ± 3	32 ± 6	17 ± 1	5 ± 1	43 ± 6
NAC				16 ± 3	57 ± 2	10 ± 0.8	21 ± 1
ALPD				26 ± 3	20 ± 2	11 ± 1	52 ± 4
GDH				65 ± 6	18 ± 5	3 ± 0.4	13 ± 0.6

The values were the mean of 3 experiments ± standard error (S.E.).

a N' fraction - the fraction which remained on the nylon mesh after filtration of the homogenate.

b E' is designated to the filtrable fraction

For abbreviations see the list.

Table 6: Relative Subcellular Distribution of the Different Radionuclides in the Unperfused Rat Lung

Days after ^{59}Fe injection	% from lung burden			% from E		
	CT	N	E	ML	P	S
2	7	22	60	14	27	42
7	4	19	75	12	32	39

Days after ^{88}y injection	% from lung burden			% from E		
	CT	N	E	ML	P	S
4	2	83	9	79	8	-
11	9	79	5	75	7	-

Days after PLS-^{51}Cr injection	% from lung burden			% from E		
	CT	N	E	ML	P	S
12	10	49	35	∿100	5	-
19	7	51	21	∿100	6	-

One experiment

For abbrevations see the list

Table 7: Relative Distribution of ^{241}Am and Marker Enzymes after Centrifugation of ML Fraction (from Unperfused Rat Lung) in Self-Forming Percoll Gradient, 7 Days after ^{241}Am injection.

Initial percoll densities and density regions	% of total ^{241}Am activity*	% of total enzyme activity*		
1.05 g/cm^3		NAC	AP	5'N
I. 1.073 - 1.053	39	75	46	27
II. 1.053 - 1.05	29	4	3	9
III. 1.05 - 1.04	32	21	51	64
1.06 g/cm^3				
I. 1.078 - 1.06	39	70	40	19
II. 1.06 - 1.057	31	5	5	20
III. 1.057 - 1.048	30	25	25	62

Two experiments per value.

* Percentage of activity in each density region relative to the total activity of the gradient recovered.

For abbreviations see the list.

Table 8: Incubation[a] of Bovine Alveolar Macrophages with ^{241}Am in Different Media

Medium	HEPES	HEPES + "Surfactants" (1:1)	HEPES + 4% BSA	Waymouth	Waymouth + "Surfactants" (1:1)	Waymouth + 4% BSA	"Surfactants"[b]
^{241}Am activity (cpm/10^6 cells)	200 ± 10	153 ± 15	26 ± 1	240 ± 20	204 ± 9	65 ± 3	168 ± 11

a The cells were incubated at 37°C for 90 minutes.
(For further experimental details see section 2.2.1).

b "Surfactants" = cell free lavage fluid from the same lung.
Representative for two experiments. Each point is the arithmetic mean of three determinations ± S.E.

Table 9: Relative Subcellular Distribution of ^{241}Am and Protein Content in Bovine Alveolar Macrophages[a].

^{241}Am Distribution

% from cells' activity		% from E			n
N	E	ML	P	S	
44 ± 3	40 ± 3	64 ± 3	14 ± 2	21 ± 3	8

Protein Content[b]

% from the protein content of the homogenate		% from E			n
N	E	ML	P	S	
27 ± 1	45 ± 5	15 ± 0.5	8 ± 0.1	66 ± 1	2

Arithmetic mean ± S.E.

a The cells were incubated at 37°C for 2 hours in HEPES medium. For more experimental details see section 2.2.3.

b For protein assay see section 2.3.

n Number of experiments. For abbreviations see the list.

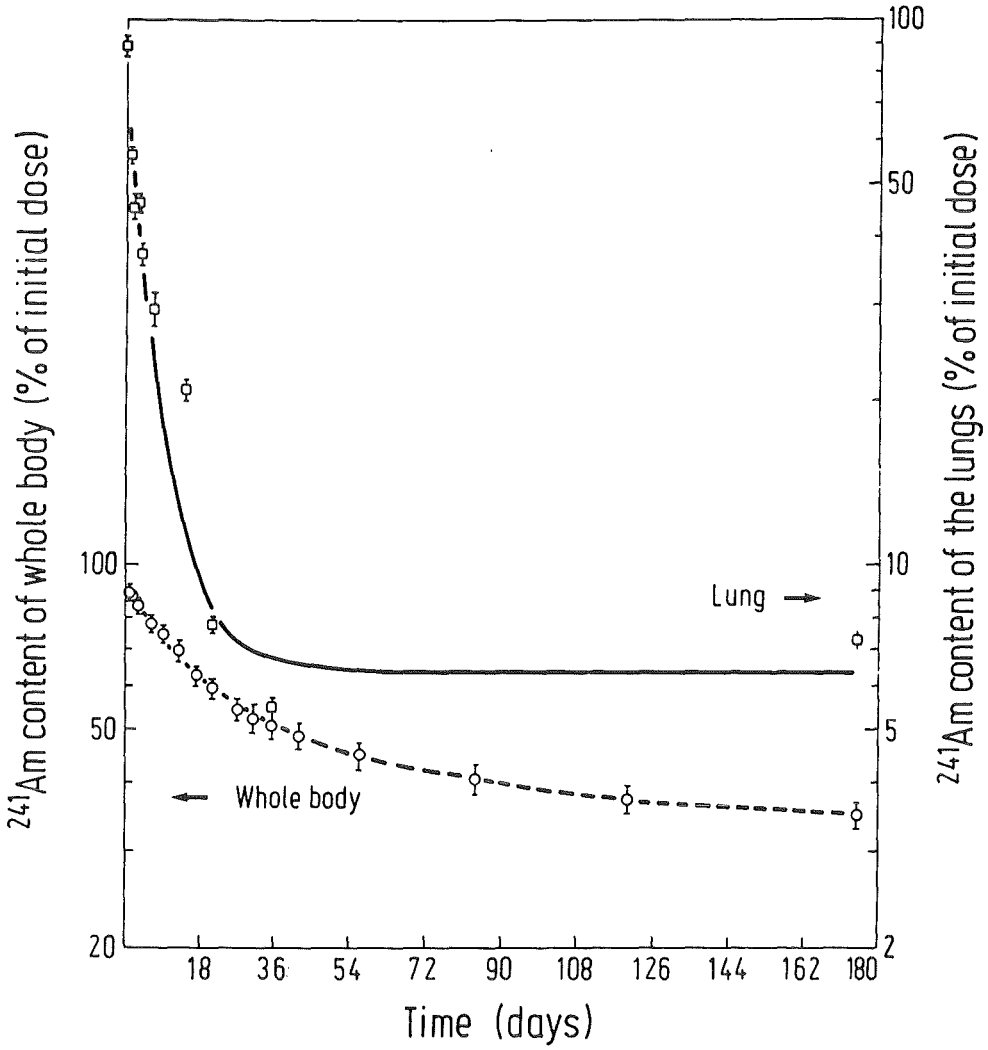


Fig. 1: Retention of intratracheally injected ^{241}Am colloid in whole body and lungs of rats. Arithmetic means of 5 values ± S.E. For presentation form see section 2.5.

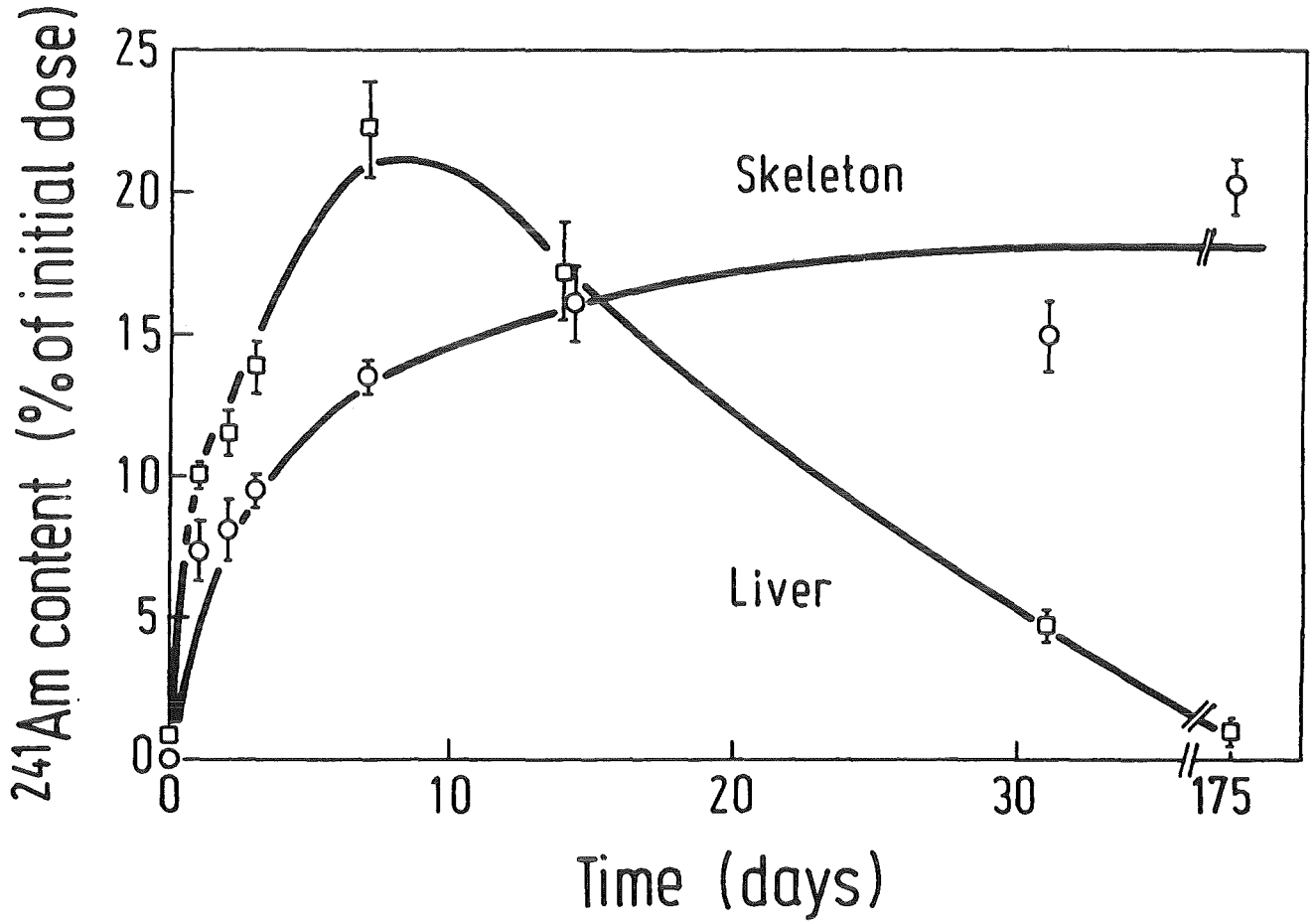


Fig. 2: Deposition of ^{241}Am colloid in skeleton and liver after intratracheal injection of ^{241}Am colloid. Arithmetic means of 3-6 rats ± S.E.

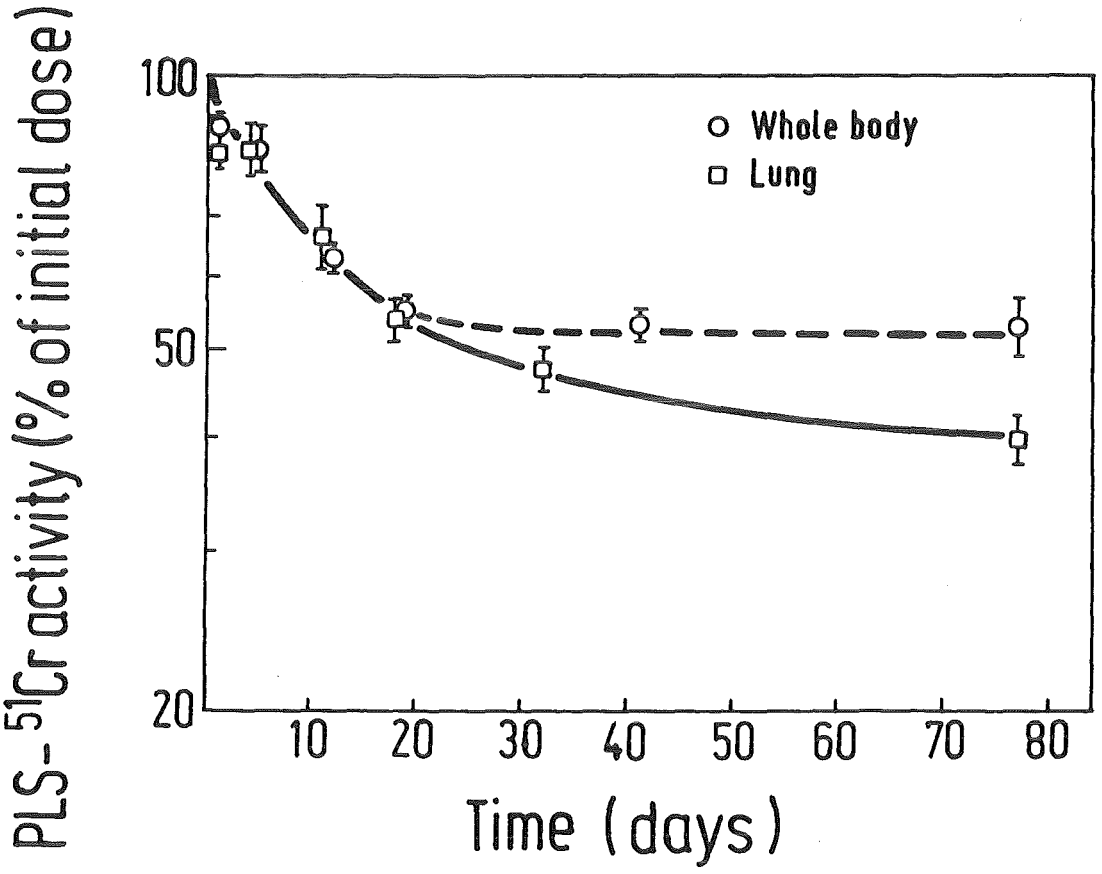


Fig. 3: Retention of intratracheally injected ^{51}Cr labelled polystyrene particles in the whole body and lungs of rat. Arithmetic means of 3-6 rats ± S.E.

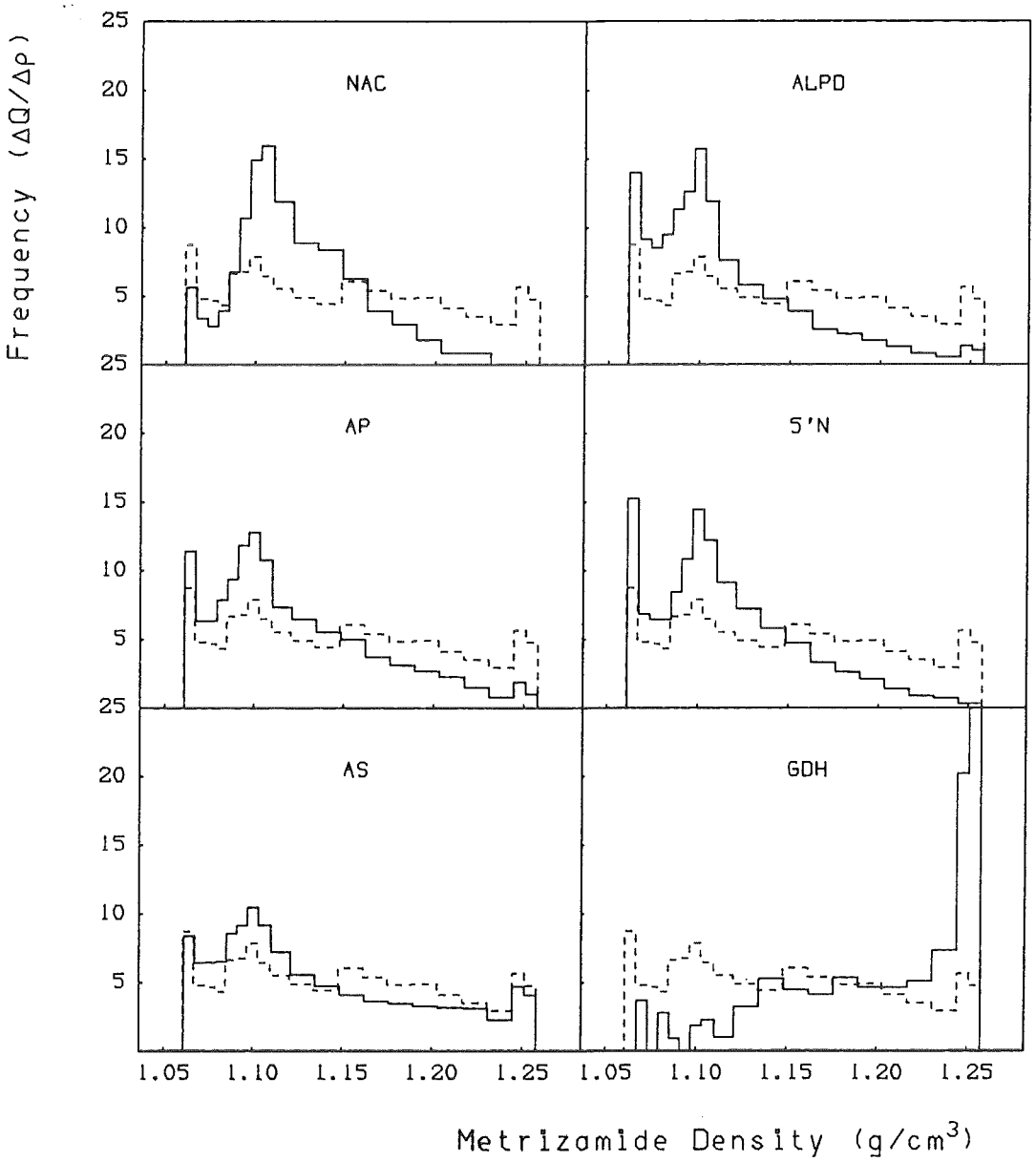


Fig. 4: Distribution profiles of the activity of ^{241}Am and of marker enzymes after centrifugation of an ML fraction (from unperfused rat lung) in a linear metrizamide gradient (2 hr, 60,000 g_{av}), 2 days after ^{241}Am instillation. Representative for 2 experiments. Dotted line = ^{241}Am profile. For abbreviations see the list and form of presentation section 2.5.

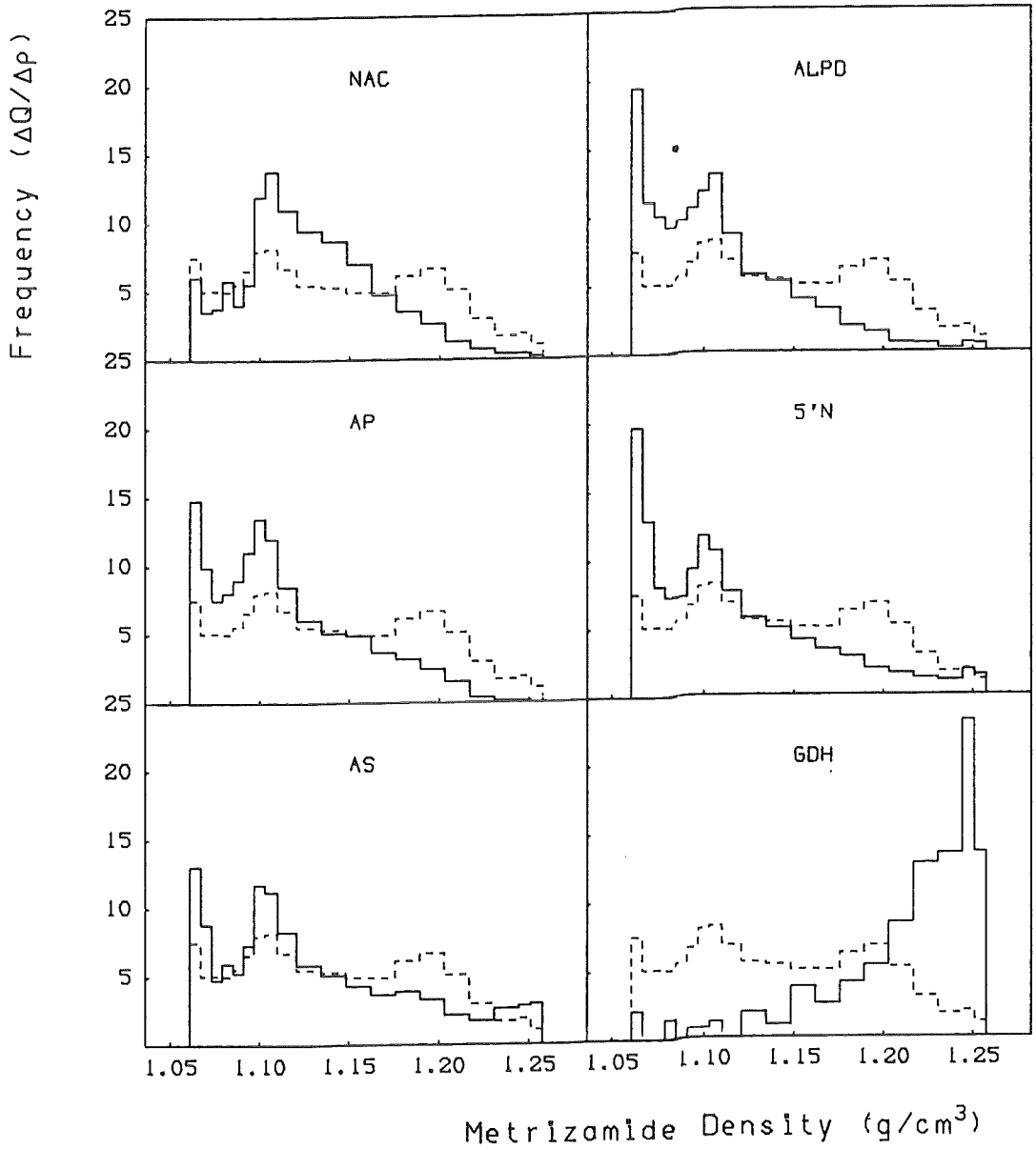


Fig. 5: Distribution profiles of the activity of ^{241}Am and of marker enzymes after centrifugation of an ML fraction (from unperfused rat lung) in a linear metrizamide gradient (2 hr, 60,000 g_{av}), 7 days after ^{241}Am instillation. Representative for 2 experiments. Dotted line = ^{241}Am profile. For abbreviations see the list.

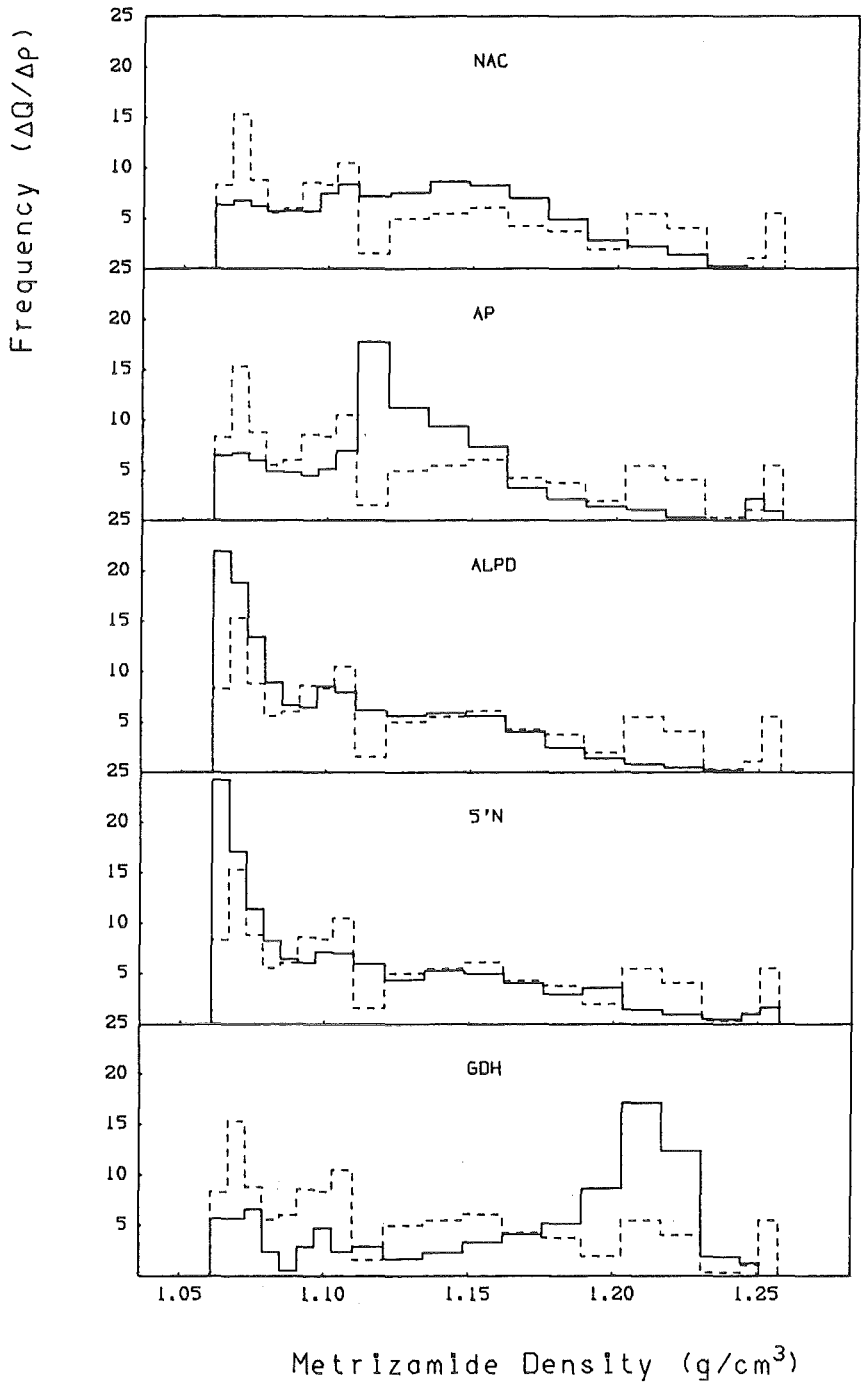


Fig. 6: Distribution of activity of ^{59}Fe and marker enzymes after centrifugation of an ML fraction (from unperfused rat lung) in linear metrizamide gradient (2 hr, 60,000 g_{av}) 7 days after ^{59}Fe instillation. One experiment. Dotted line = ^{59}Fe profile. For abbreviations see the list.

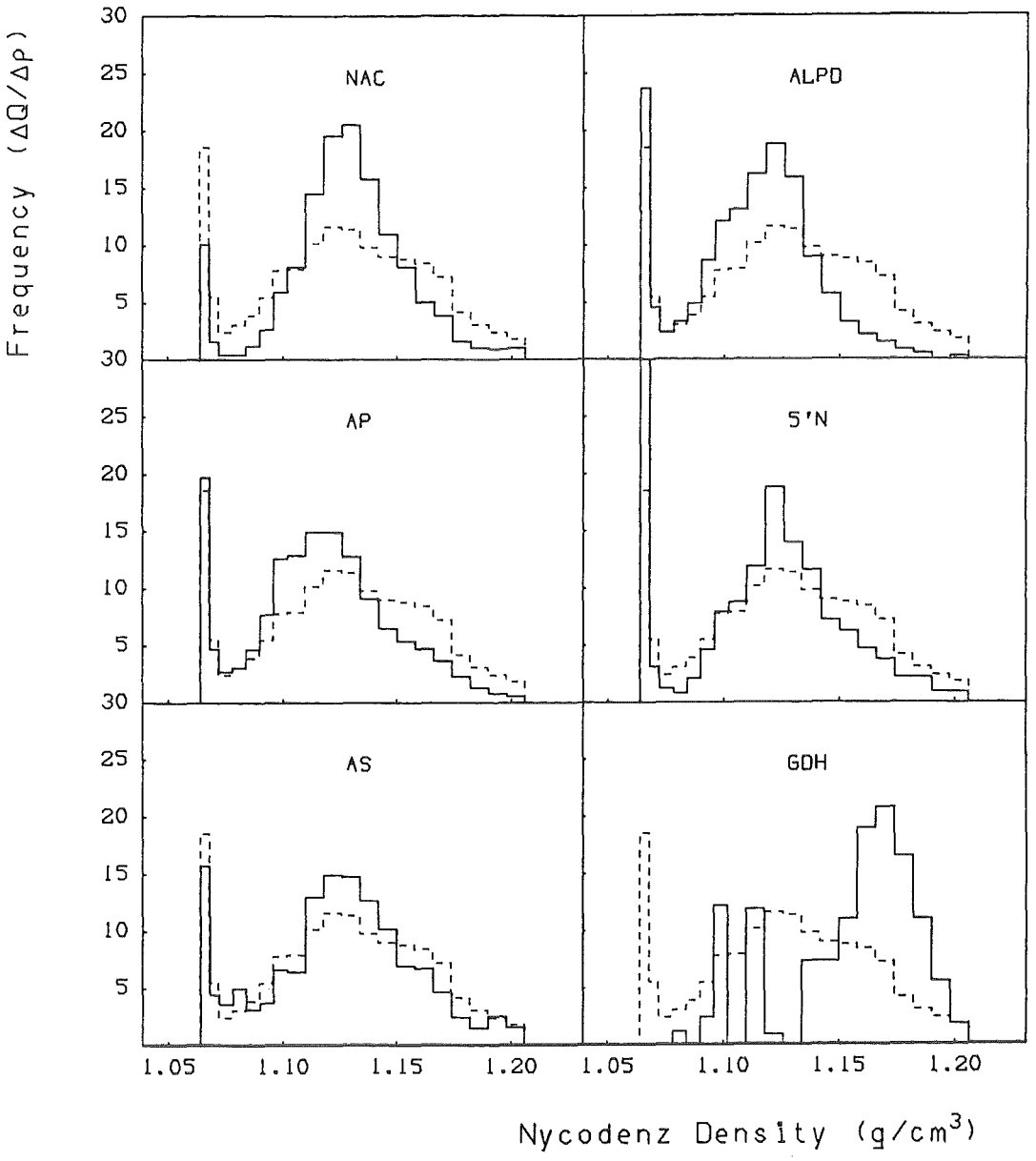


Fig. 7: Distribution of activity of ^{241}Am and marker enzymes after centrifugation of an ML fraction (from unperfused rat lung) in linear nycodenz gradients (2 h, 60,000 g_{av}) 2 days after ^{241}Am instillation. Representative for 2 experiments. Dotted line = ^{241}Am profile. For abbreviations see the list.

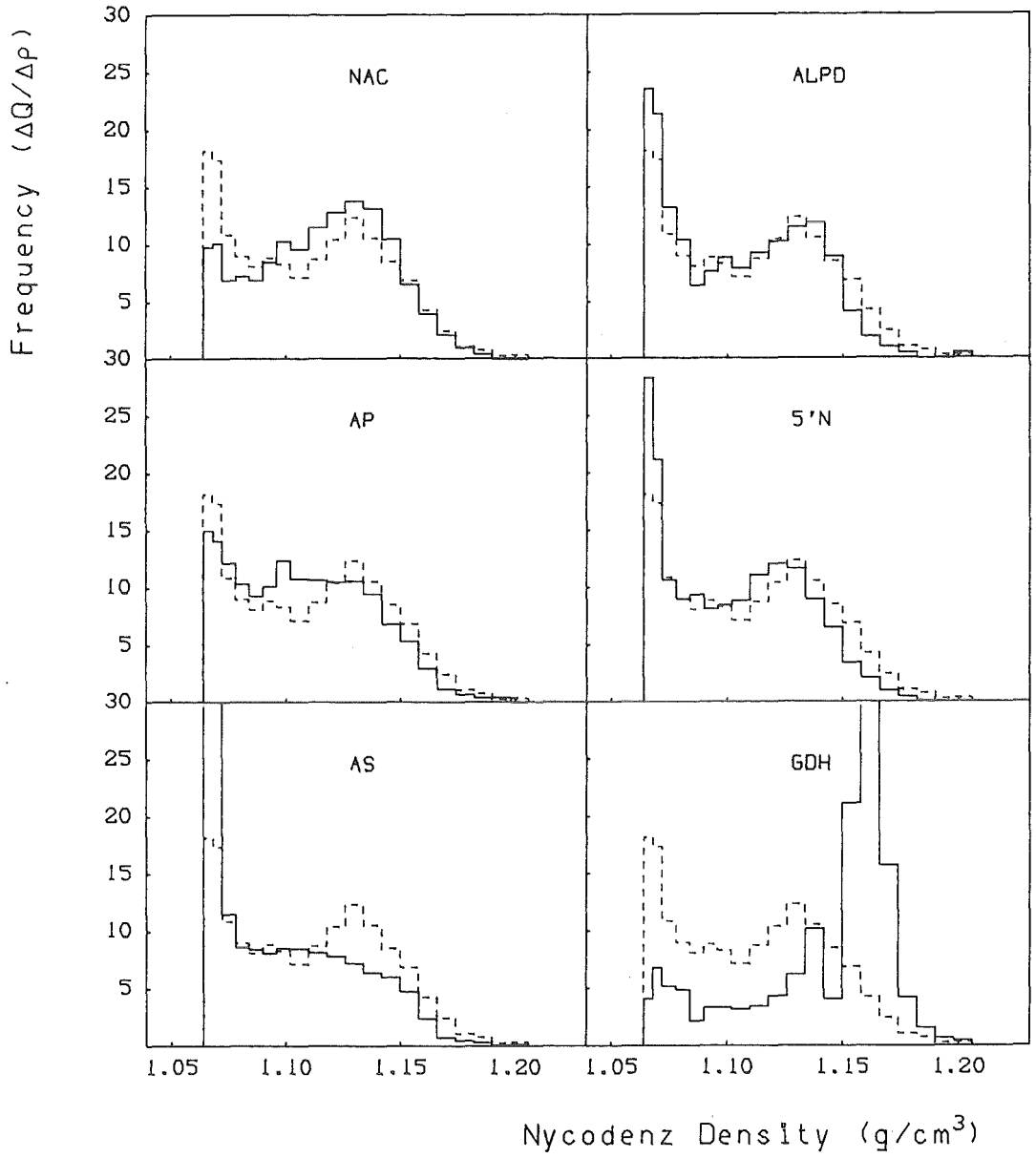


Fig. 8: Distribution of activity of ^{241}Am and marker enzymes after centrifugation of an ML fraction (from perfused rat lung) in linear nycodenz gradient (2 hr, 60,000 g_{av}) 7 days after ^{241}Am instillation. Representative for two experiments. Dotted line = ^{241}Am profile. For abbreviation see the list.

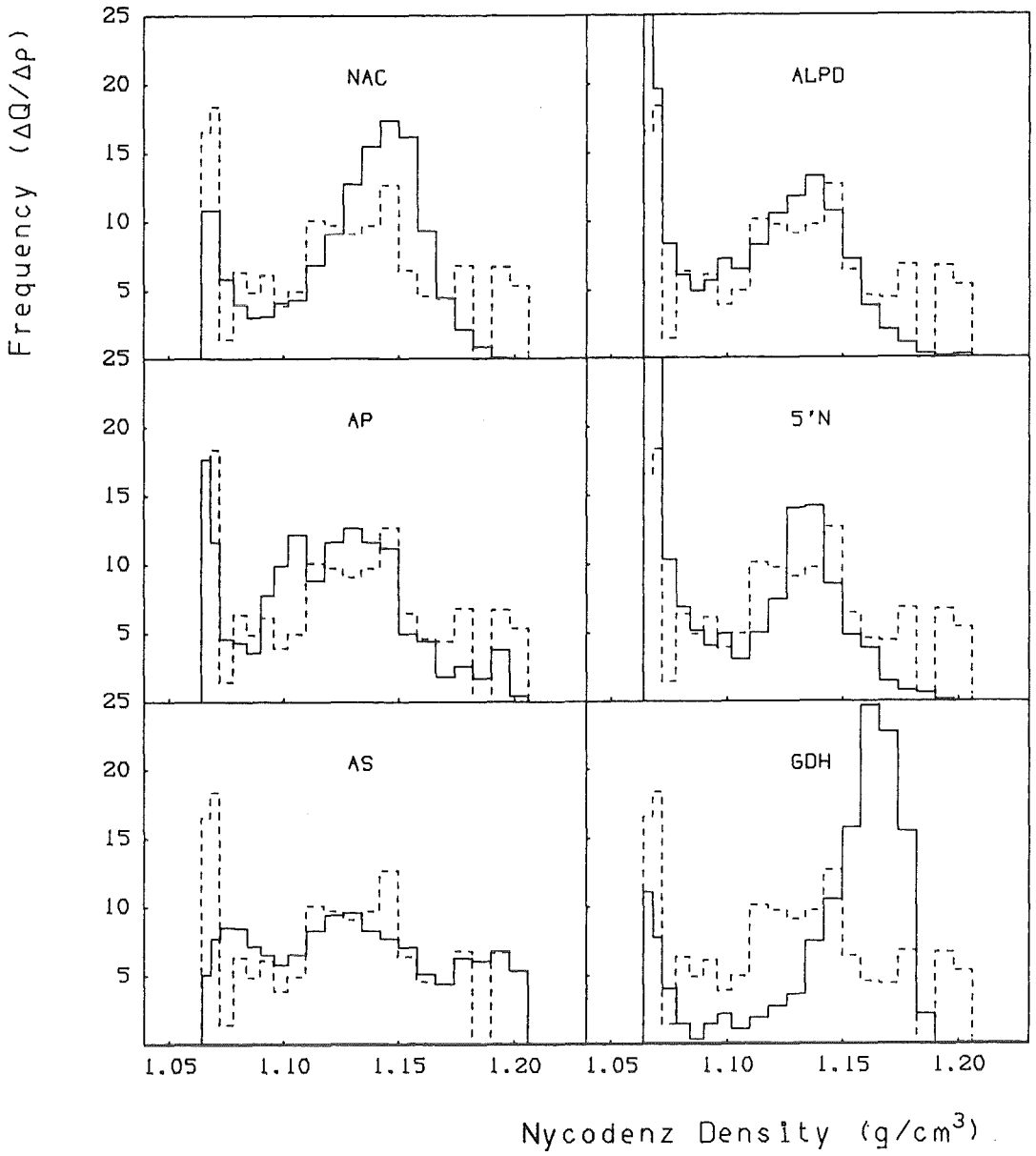


Fig. 9: Distribution of activity of ^{59}Fe and marker enzymes after centrifugation of an ML fraction (from unperfused rat lung) in linear nycodenz gradient (2 hr, 60,000 g_{av}) 7 days after ^{59}Fe instillation. One experiment. Dotted line = ^{59}Fe profile. For abbreviations see the list.

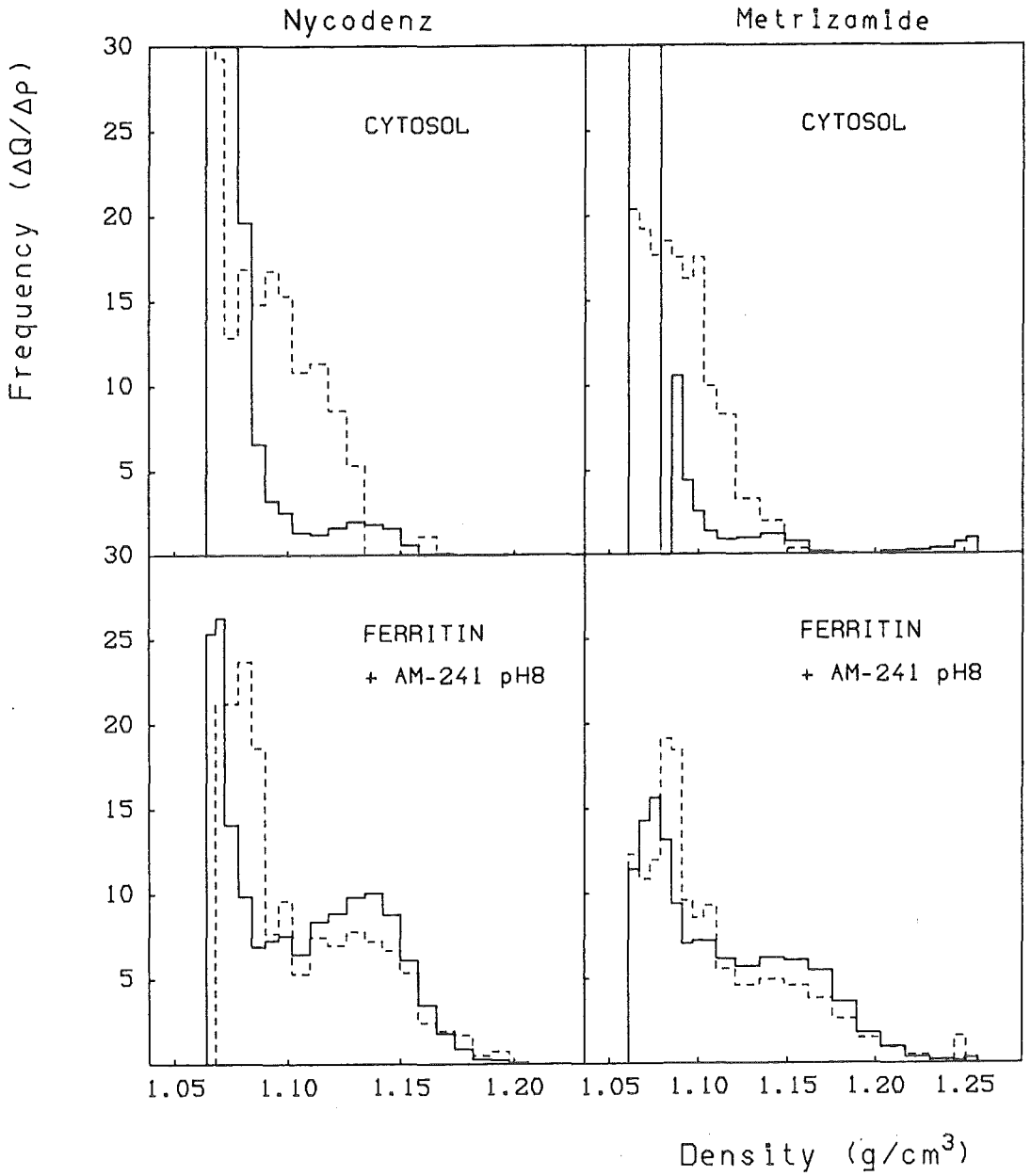


Fig. 10: Distribution of ^{241}Am, cytosol (from unperfused rat lungs, 3 days after ^{241}Am instillation) and ferritin (labelled with ^{241}Am of pH 8) after centrifugation in linear nycodenz and metrizamide gradients (2 hr, 60,000 g_{av}). Dotted lines = ^{241}Am profiles. One experiment each.

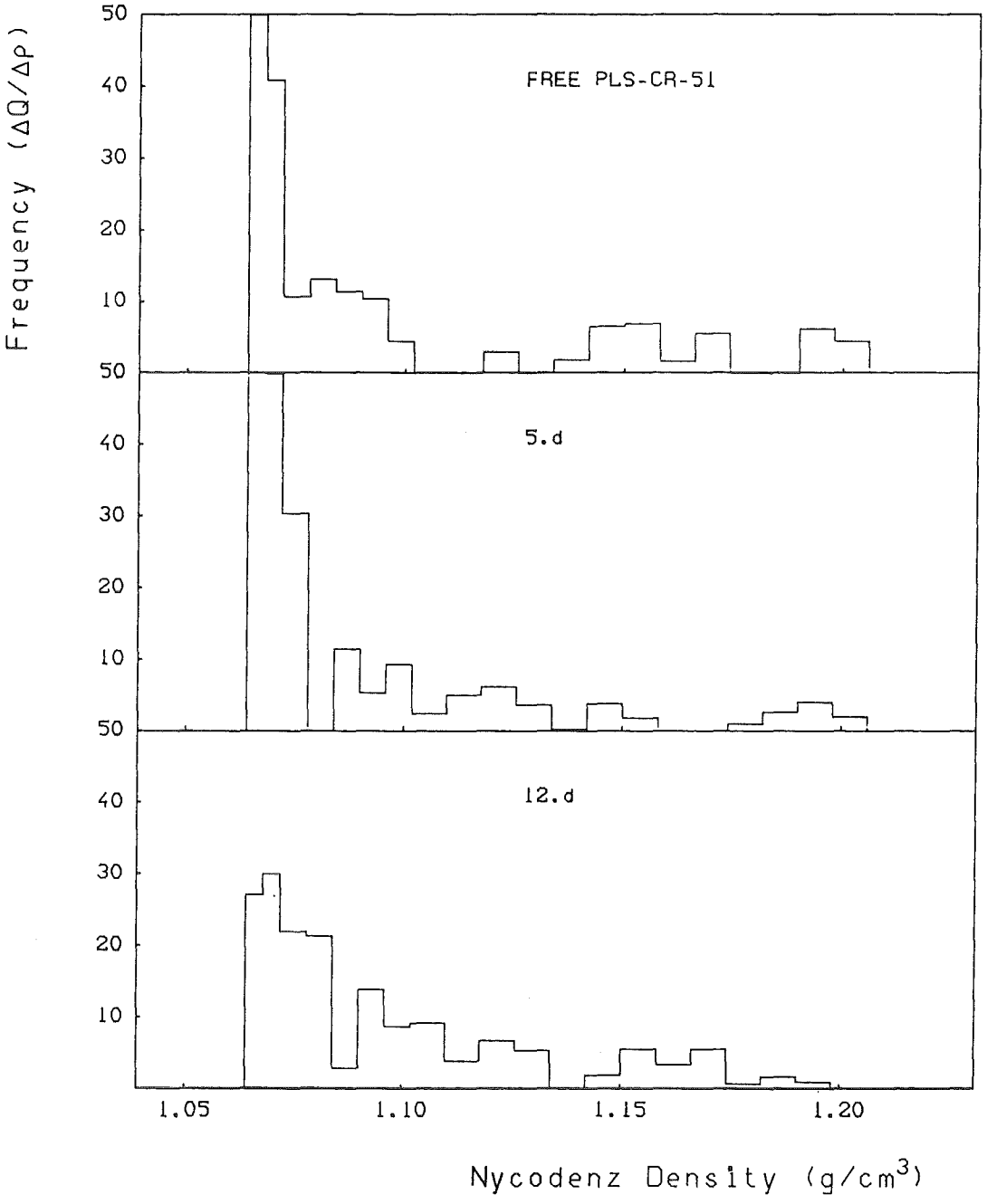


Fig. 11: Distribution of activity of ^{51}Cr labelled polystyrene (as free particles and with ML fraction, 5 and 12 days after PLS-^{51}Cr instillation) after centrifugation in linear nycodenz gradient (2 hr, 60,000 g_{av}).

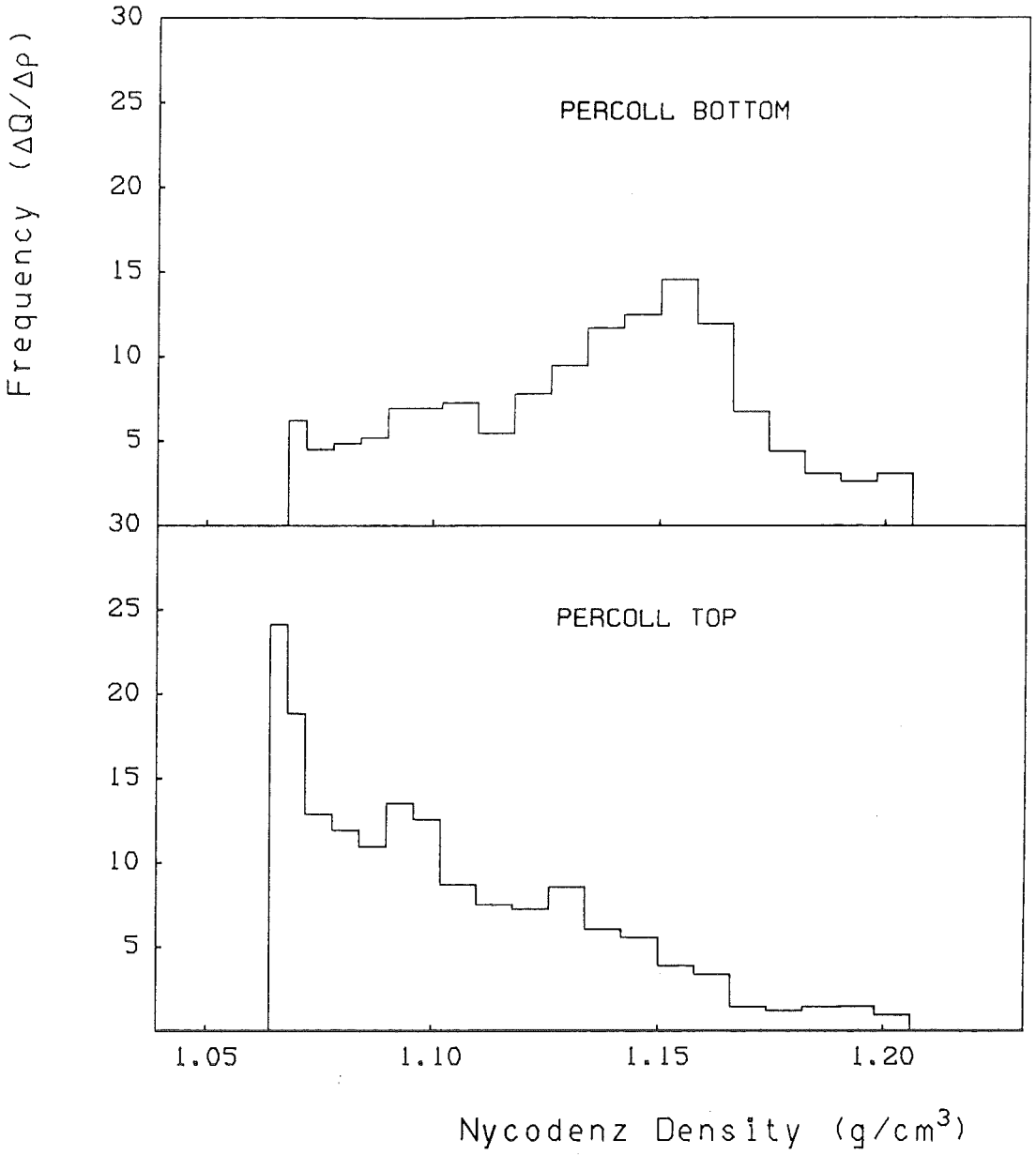


Fig. 12: Distribution of activity of ^{241}Am (the fractions of percoll bottom (region I) and percoll top (region III), see Table 7) after centrifugation in linear nycodenz gradient (2 h, 60,000 g_{av}) 2 days after ^{241}Am instillation.

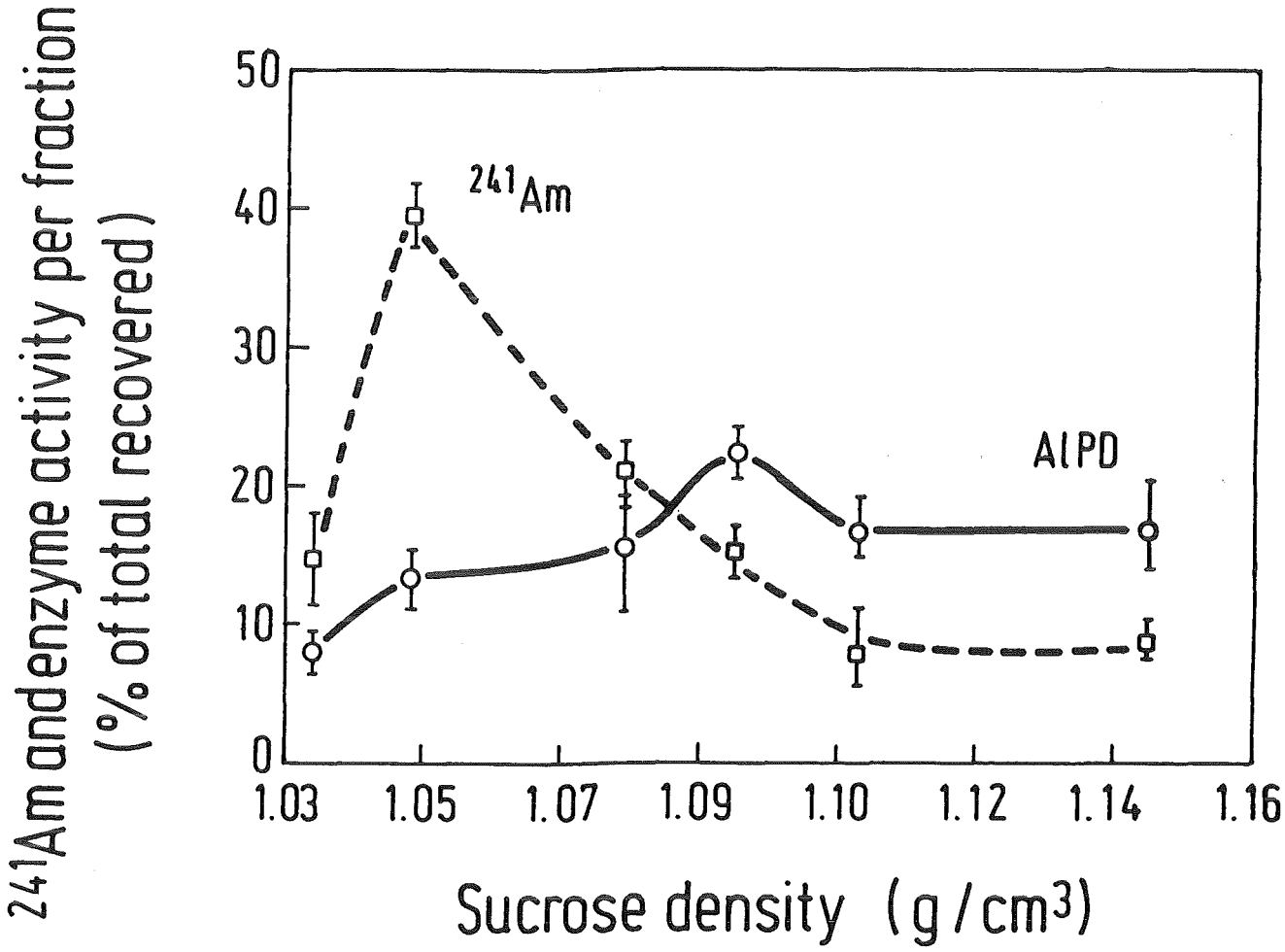


Fig. 13: Distribution of activity of ^{241}Am and alkaline phosphodiesterase (ALPD) after centrifugation of a fraction (S_3, see Table 5) enriched in pericellular membranes in a discontinuous sucrose gradient (75 min, 100,000 g_{av}). 4 days after ^{241}Am injection. Each point is the mean of 3 values ± S.E.

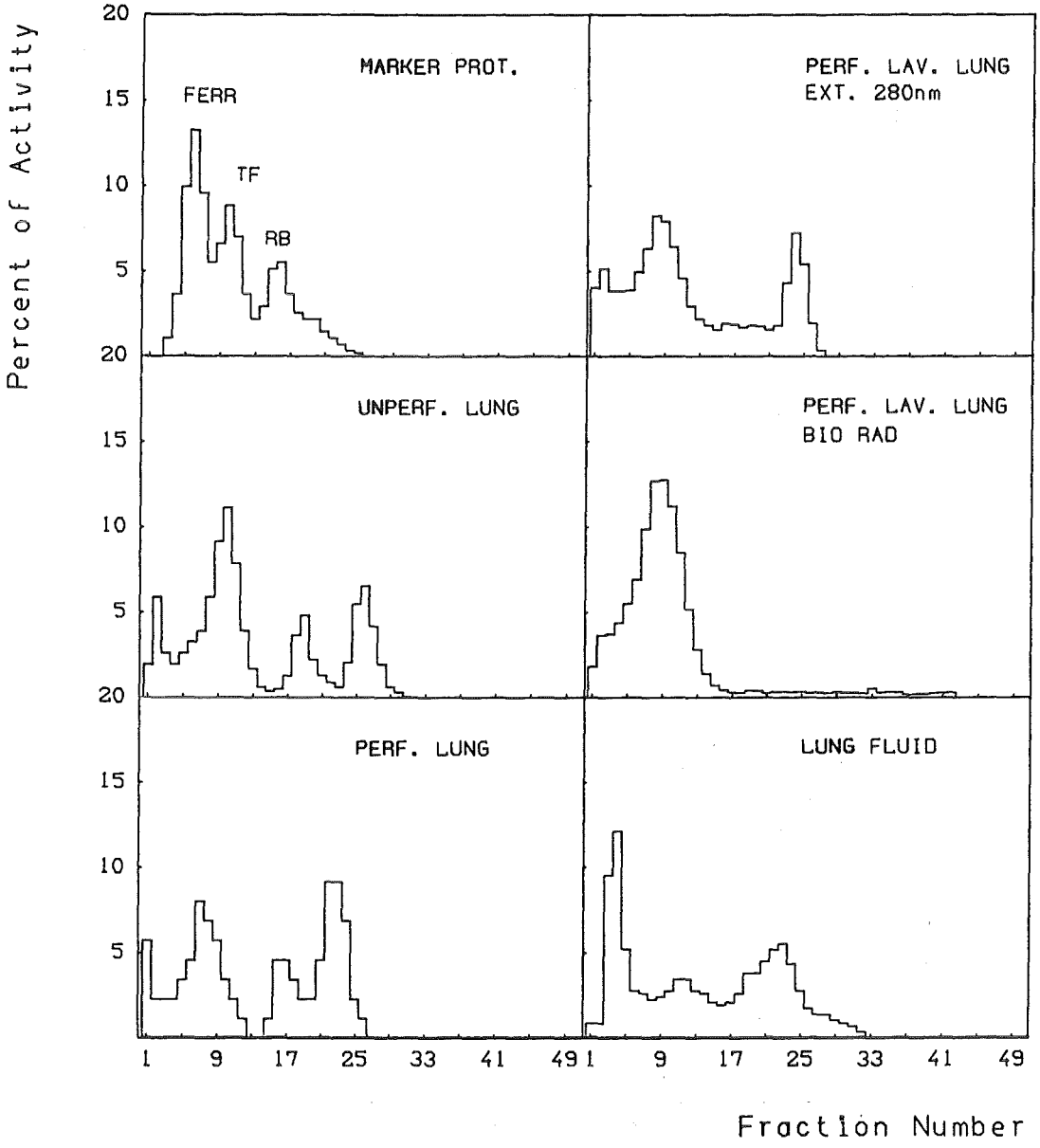


Fig. 14: Elution profiles of cytosol proteins of rat lung after chromatography on sephacryl S-300 (for experimental details see sections 2.1.6.1. and 2.5.). For abbreviations see the list.

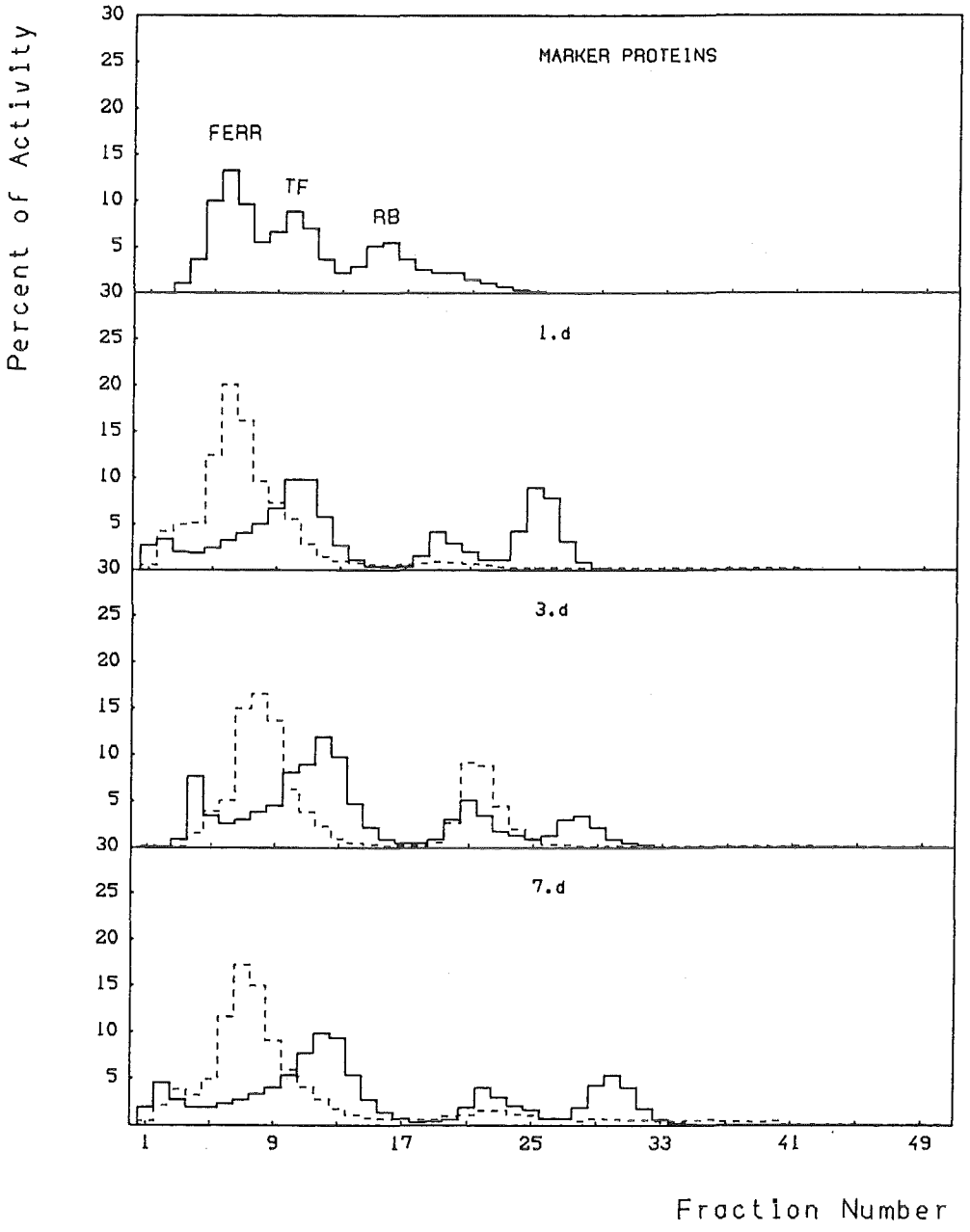


Fig. 15: Elution profiles of marker proteins, ^{241}Am activity and cytosol proteins (of unperfused rat lung) after chromatography on sephacryl S-300, 1, 3 and 7 days after ^{241}Am instillation (experimental details in section 2.1.6.1. and 2.5.). Representative for 2 experiments. Dotted lines = ^{241}Am profiles. For abbreviations see the list.

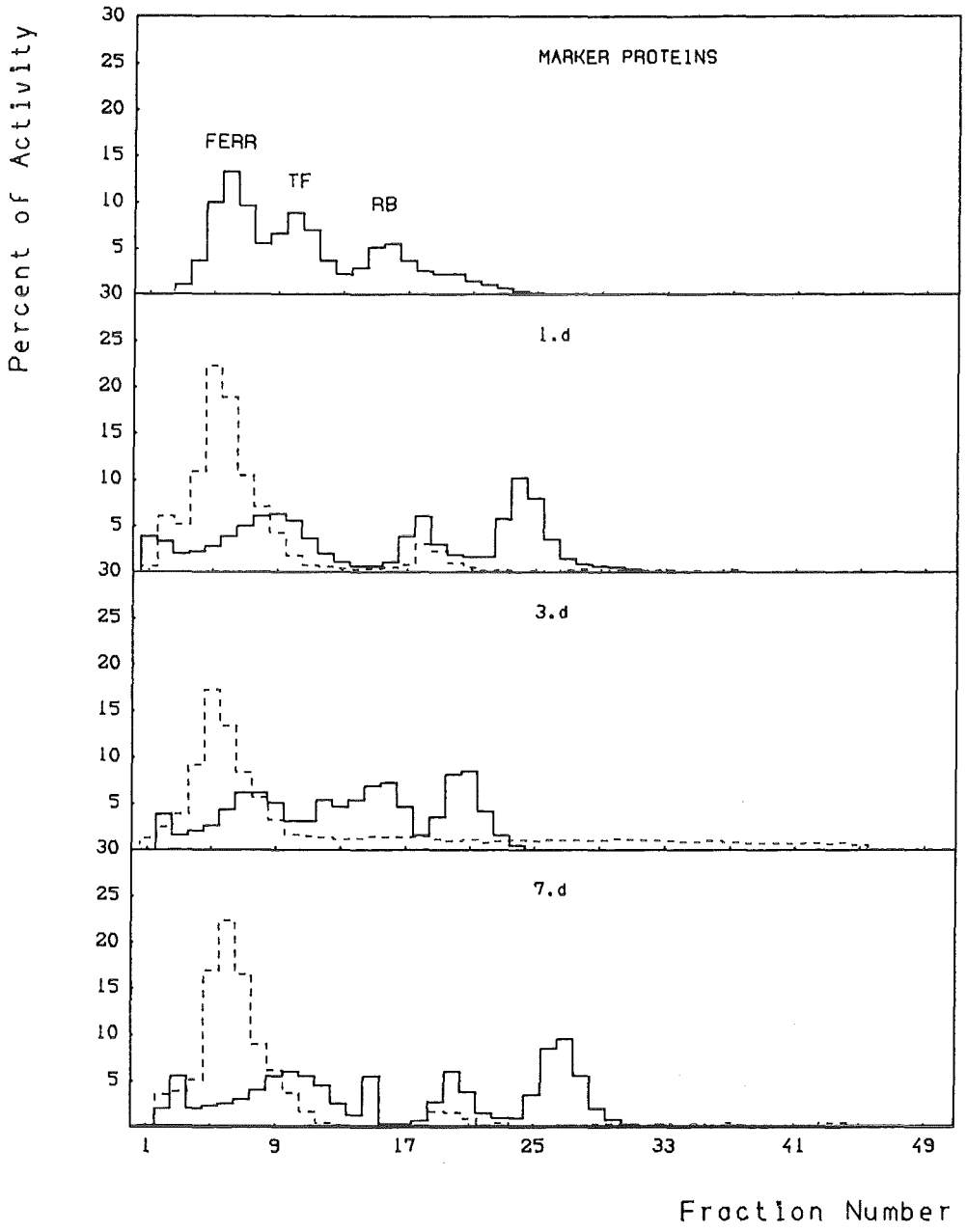


Fig. 16: Elution profiles of marker proteins, ^{241}Am activity and cytosol proteins (of perfused rat lung) after chromatography on sephacryl S-300, 1, 3 and 7 days after ^{241}Am instillation. Representative for 2 experiments. Dotted lines = ^{241}Am profiles (more details in Fig. 15).

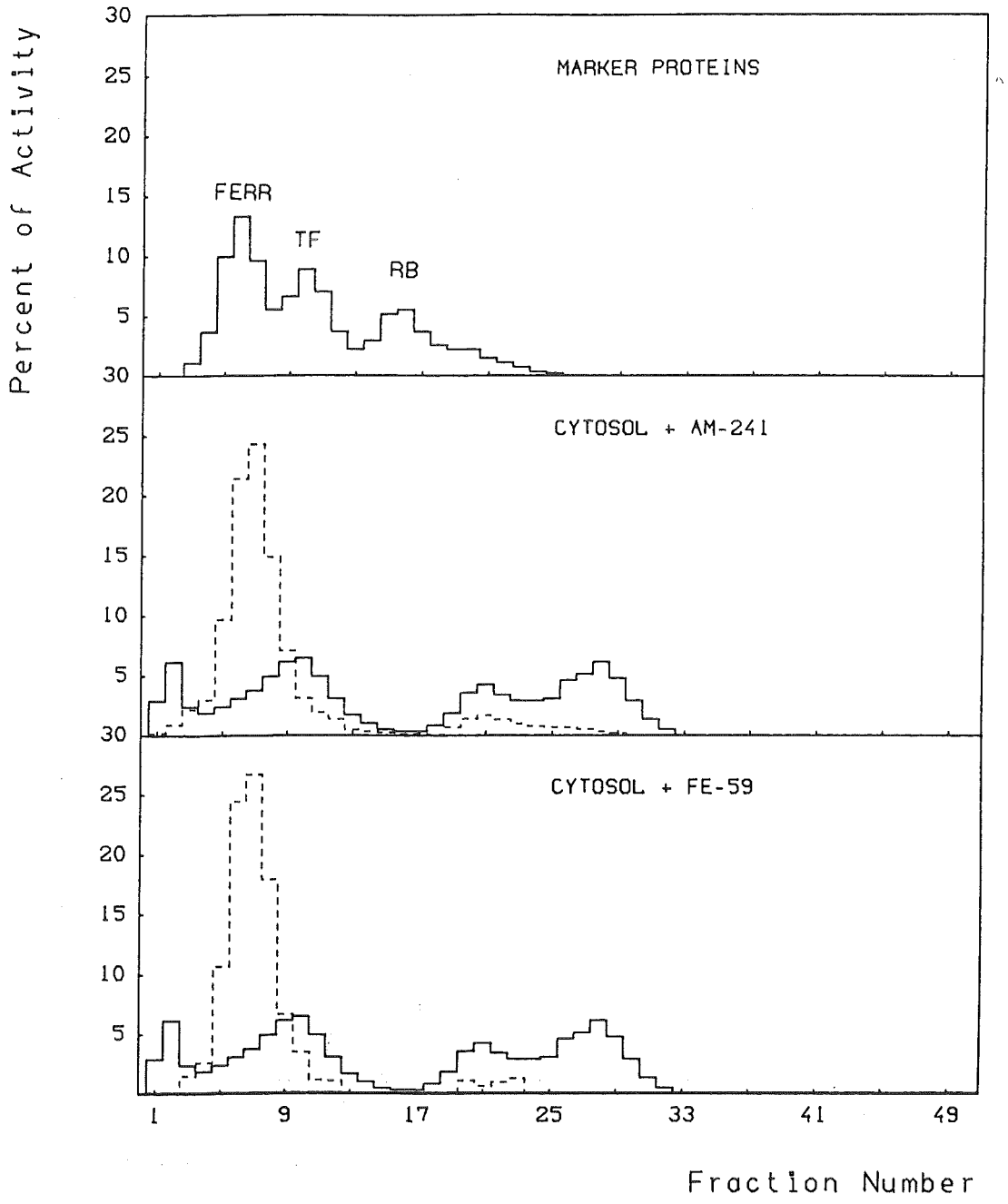


Fig. 17: Elution profiles of marker proteins, ^{241}Am and ^{59}Fe activities and cytosol proteins (of perfused rat lung) after chromatography on sephacryl S-300, 3 days after ^{241}Am and ^{59}Fe instillation. One experiment. Dotted lines = ^{241}Am or ^{59}Fe profiles (experimental details in section 2.1.6.1.). For abbreviations see the list.

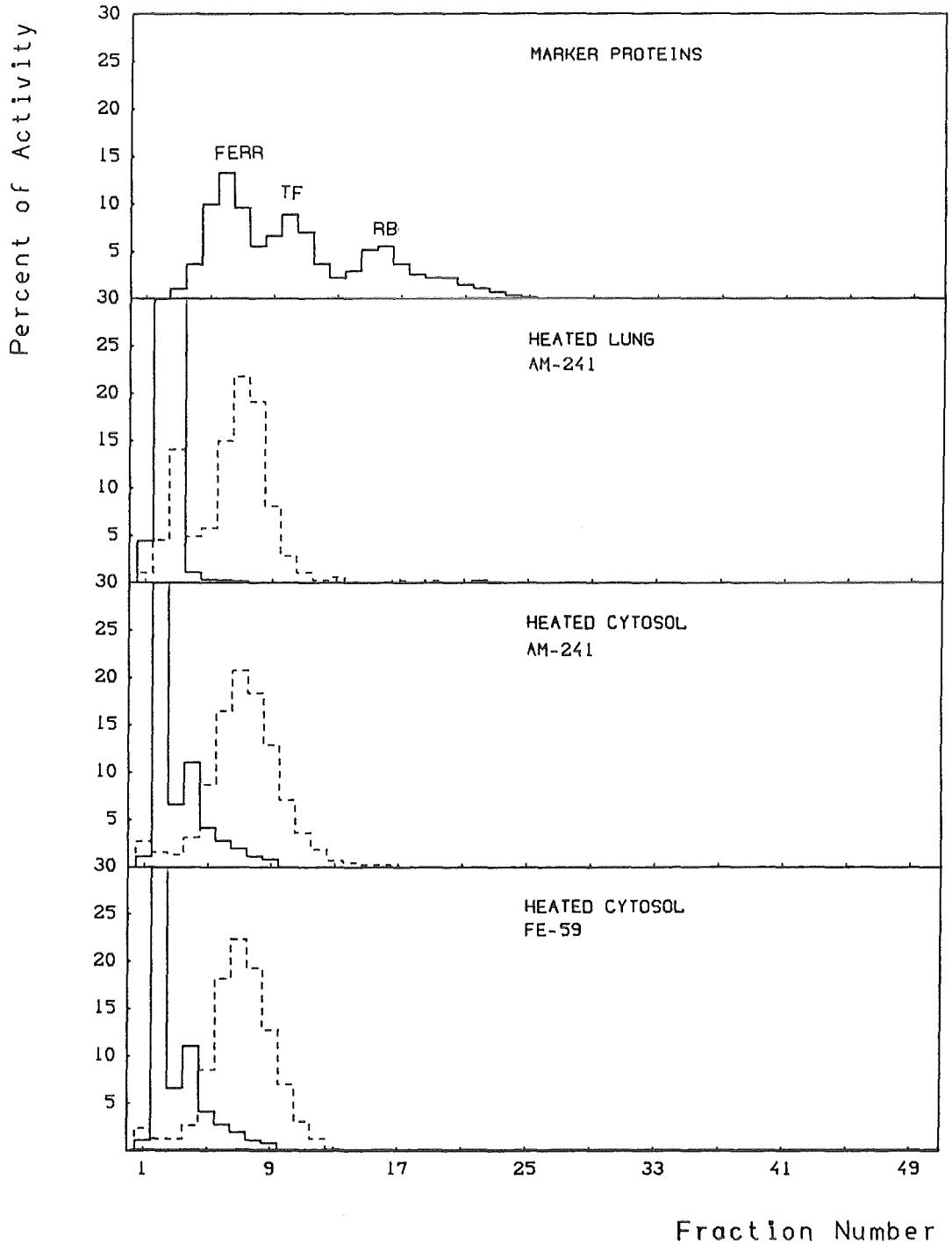


Fig. 18: Elution profiles of marker proteins, ^{241}Am and ^{59}Fe activities, and proteins (of the ferritin rich fraction which was prepared from heated total lungs and heated cytosol. more details in section 2.1.7.), chromatography on sephacryl S-300, 3 days after ^{241}Am or ^{241}Am and ^{59}Fe instillation. One experiment. Dotted lines = ^{241}Am or ^{59}Fe profiles. For abbreviations see the list.

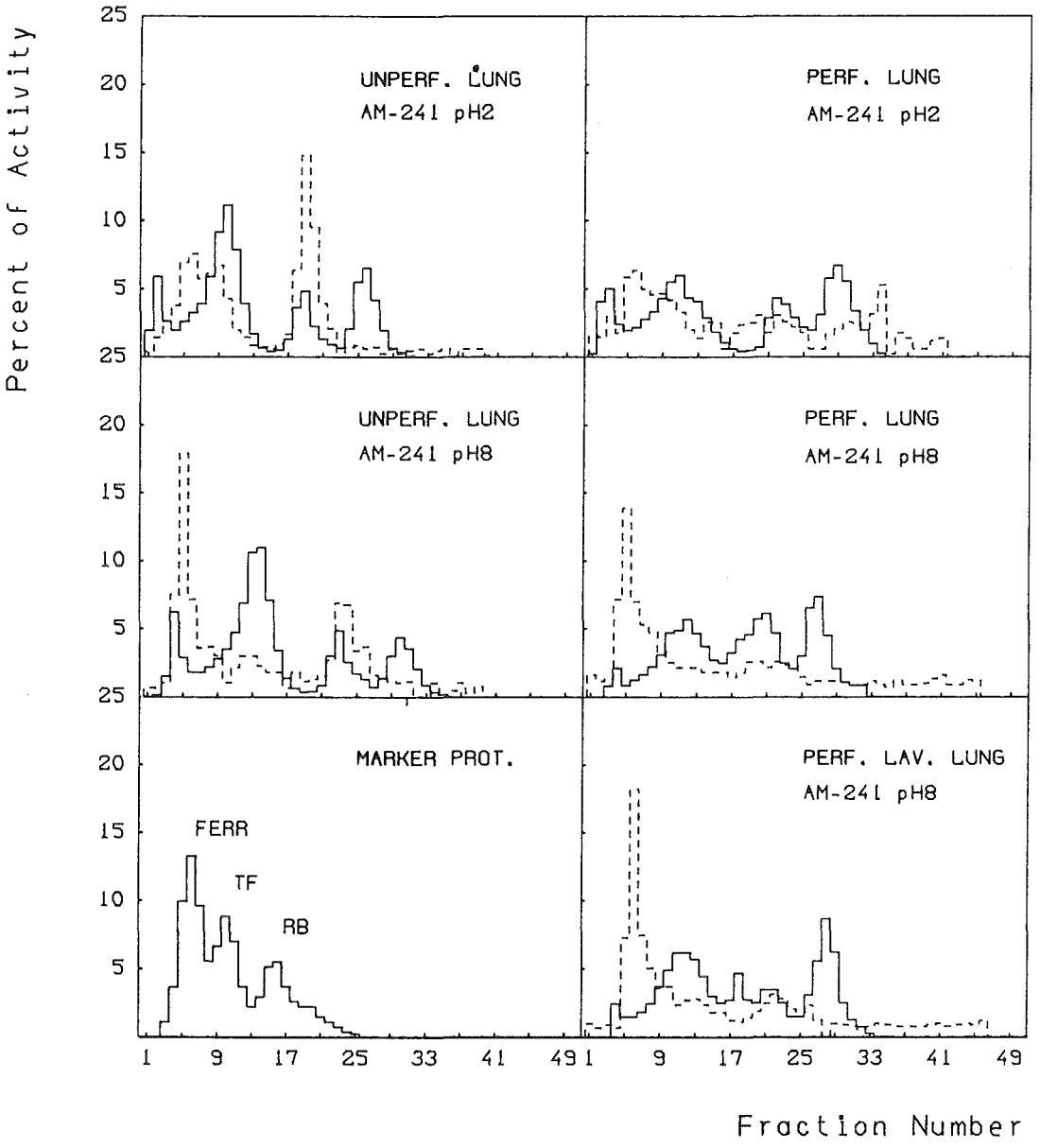


Fig. 19: Elution profiles of ^{241}Am activity, cytosol (from unperfused, perfused and perfused lavaged rat lung) and marker proteins. Chromatography on sephacryl S-300 after in vitro labelling with ^{241}Am (pH 2 and pH 8). Representative for 2 experiments. Dotted lines = ^{241}Am profiles (more experimental details in section 2.1.7.). For abbreviation see the list.

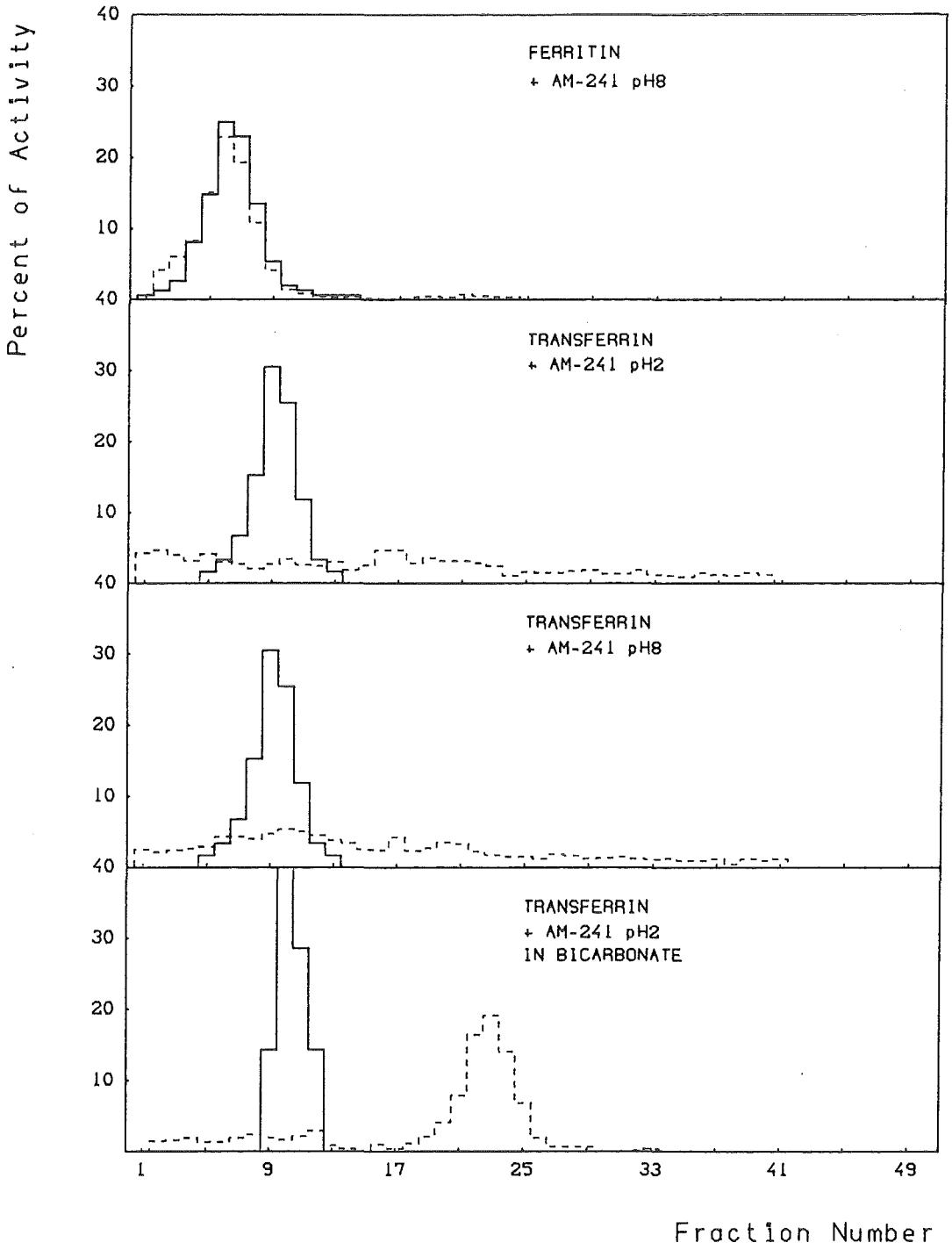


Fig. 20: Elution profiles of activity of ^{241}Am, ferritin and transferrin. Chromatography on sephacryl S-300, after in vitro labelling with ^{241}Am (pH 2 and pH 8). Representative for 2 experiments. Dotted lines = ^{241}Am profiles (experimental details in section 2.1.7.). For abbreviations see the list.

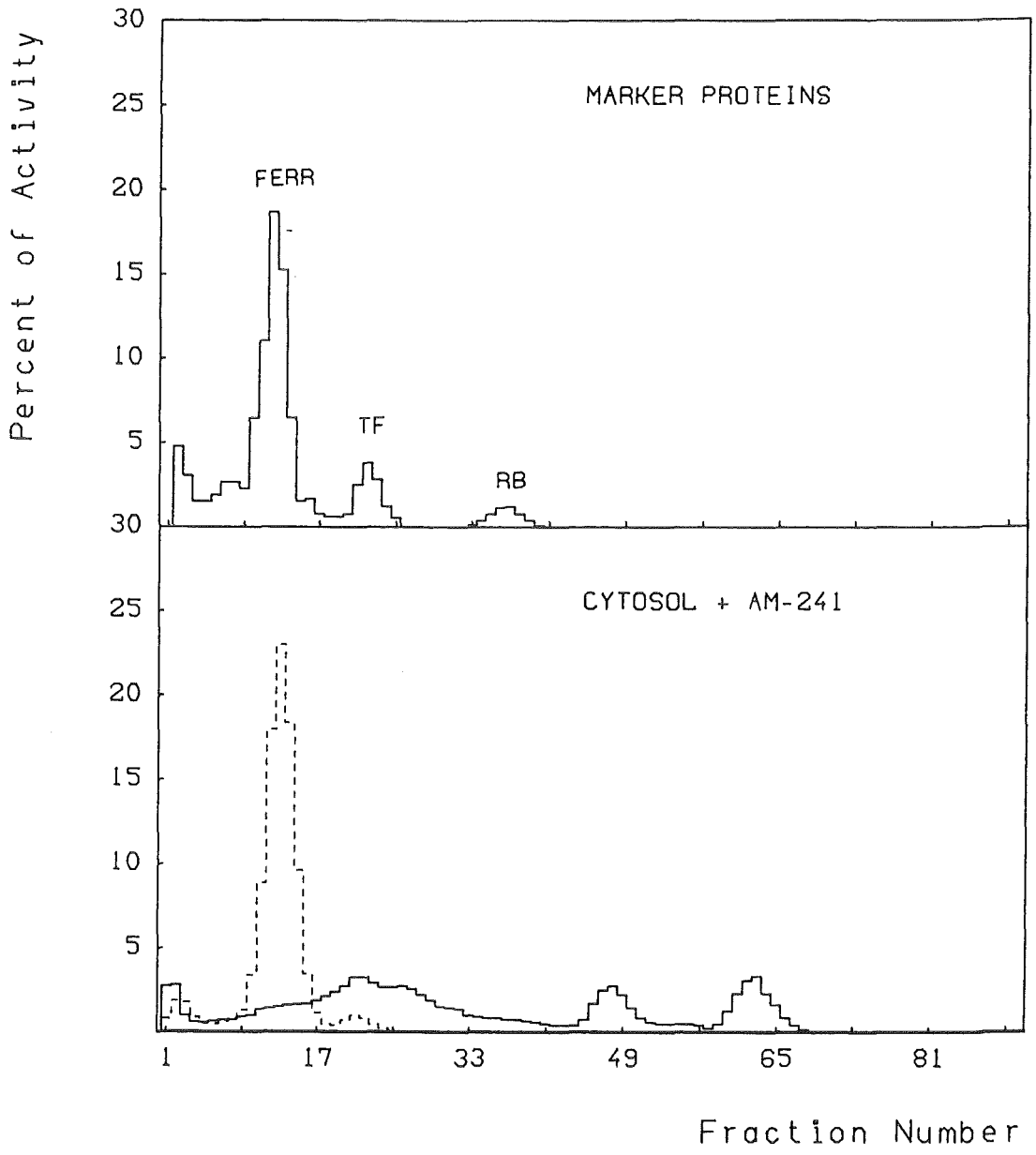


Fig. 21: Elution profiles of marker proteins, activity of ^{241}Am and cytosol (of perfused rat lung). Chromatography on a "long" sephacryl S-300 column, 3 days after ^{241}Am instillation. One experiment. Dotted line = ^{241}Am profile. (experimental details in section 2.1.6.1.). For abbreviations see the list.

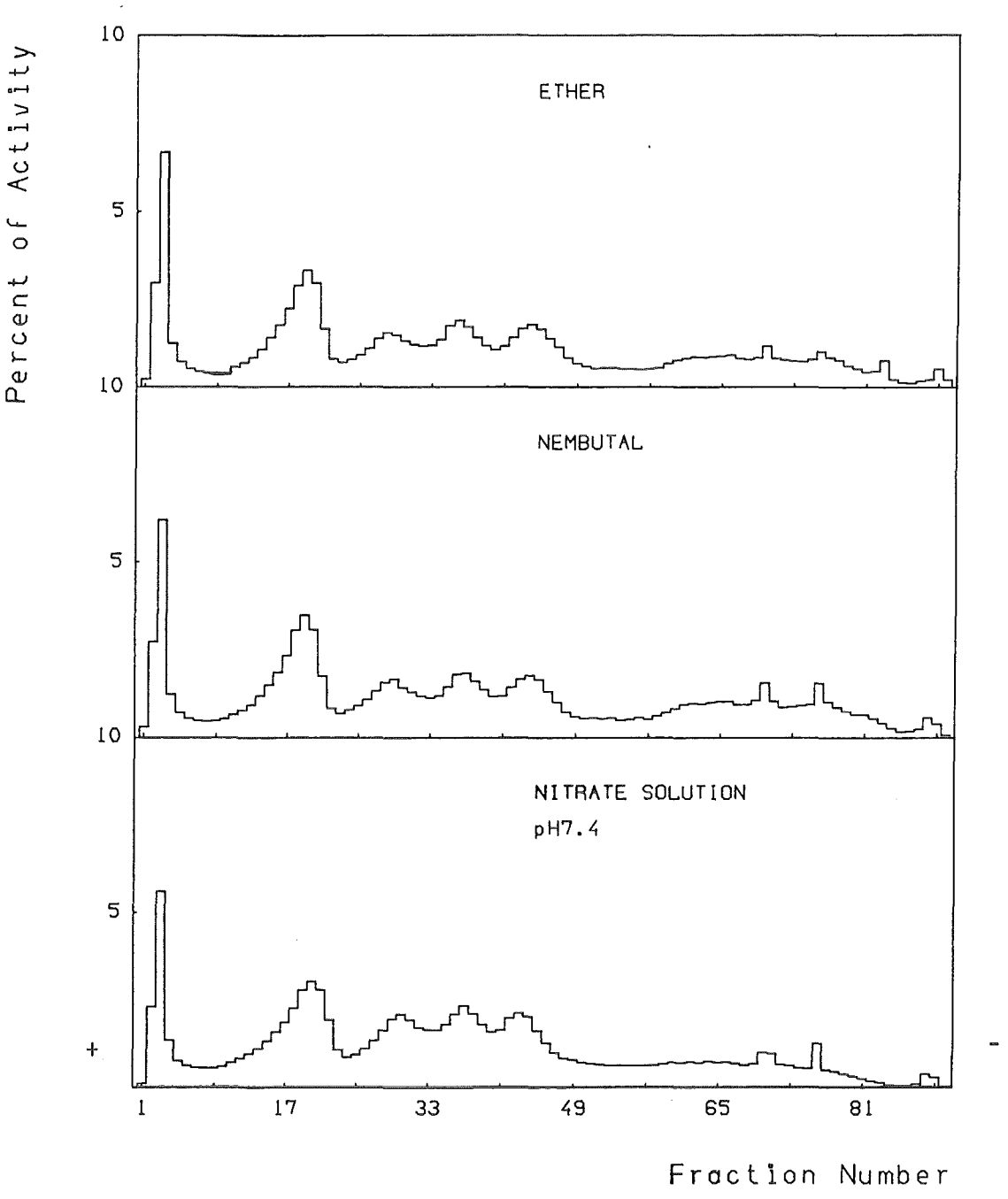


Fig. 22: Analysis of cytosol (from unperfused rat lung) by free flow electrophoresis (FFE), 3 days after anesthesia of rats with ether, nembutal or intratracheal injection of nitrate solution (pH 7.4) (with ether anesthesia). Representative for two experiments.

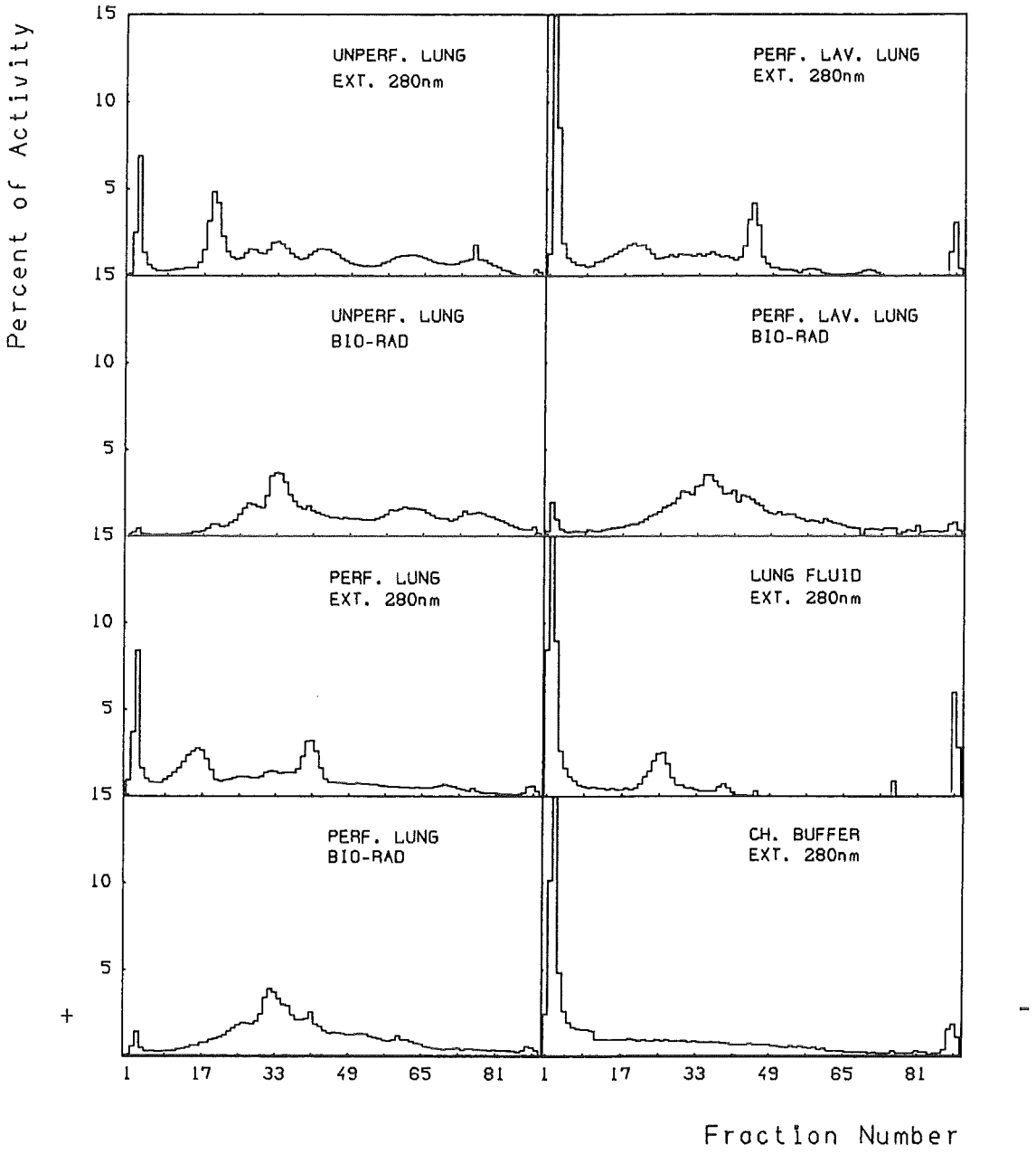


Fig. 23: Analysis of cytosol of unperfused, perfused and perfused lavaged rat lung, of lung fluid and of chamber buffer by FFE. The proteins in the fractions were measured at 280 nm or by the Bio-Rad method (experimental details in sections 2.1.6.2. and 2.3.). Representative for two experiments. For abbreviations see the list.

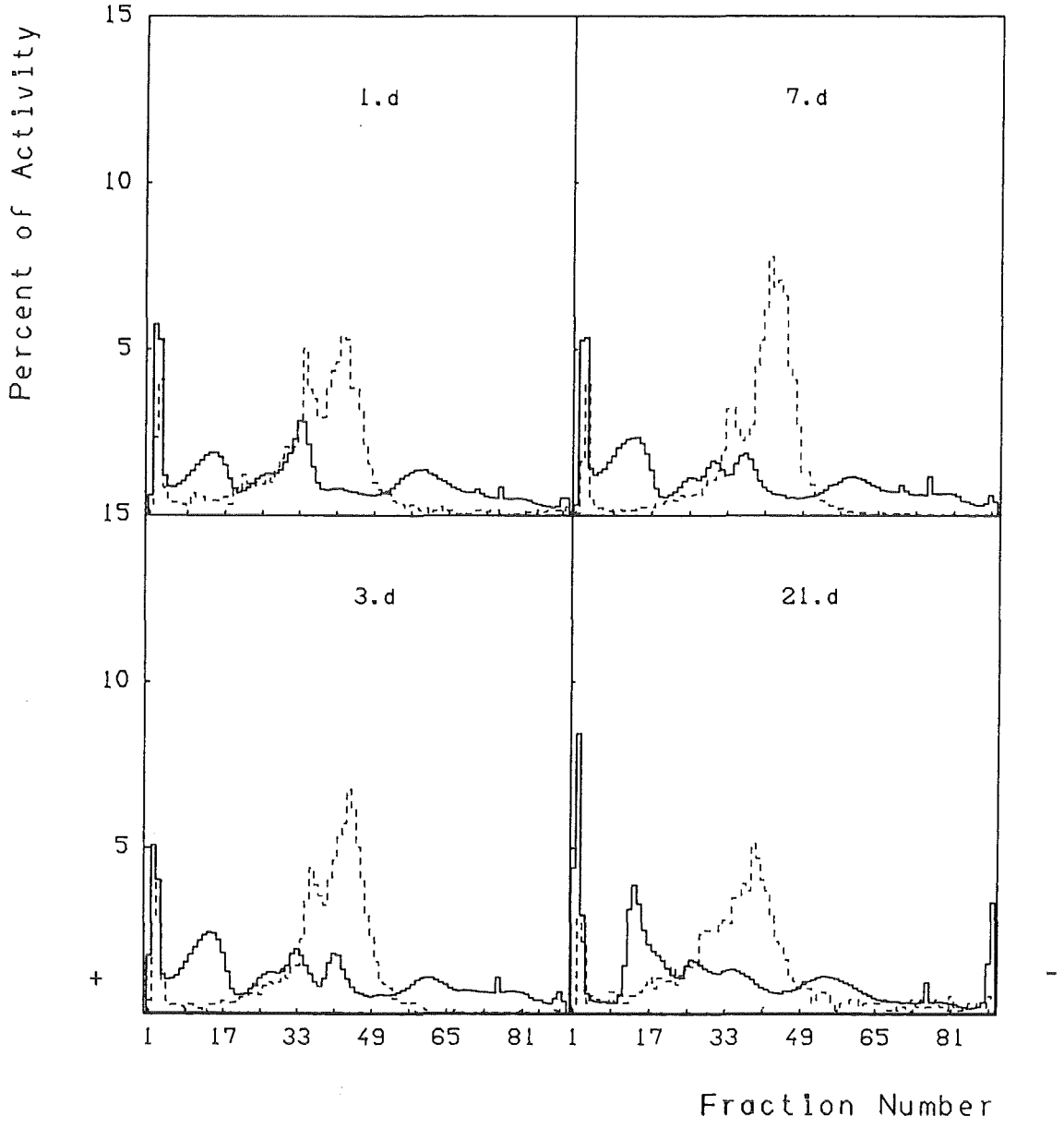


Fig. 24: Analysis of cytosol (from unperfused rat lung) by FFE, after 1, 3, 7 and 21 days of ^{241}Am instillation, Representative for 2 experiments. Dotted lines = ^{241}Am profiles (experimental details in section 2.6.1.2.).

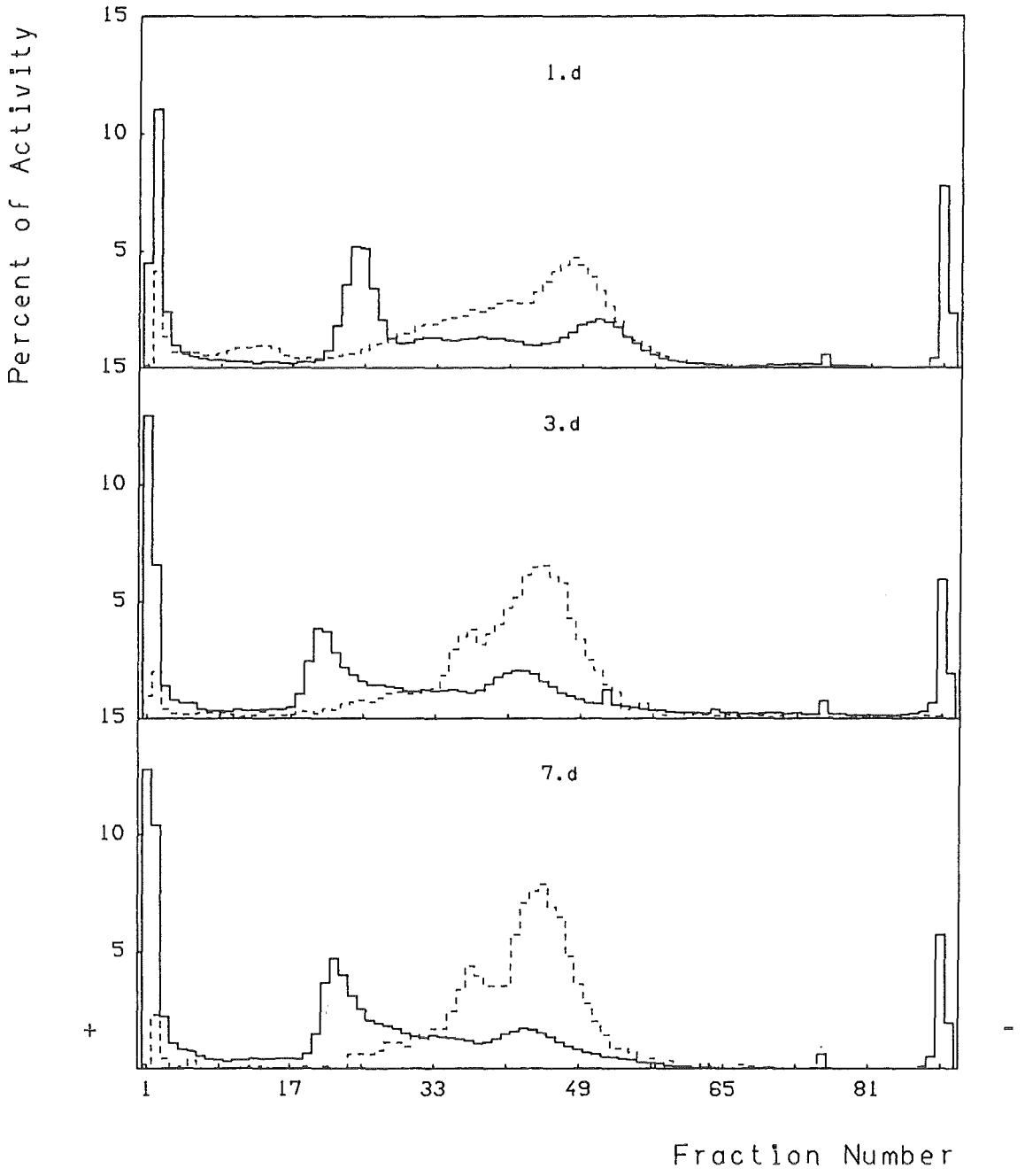


Fig. 25: Analysis of cytosol (from perfused rat lung) by FFE, 1, 3 and 7 days after ^{241}Am instillation. Representative for 2 experiments. Dotted lines = ^{241}Am profiles (experimental details in section 2.6.1.2.).

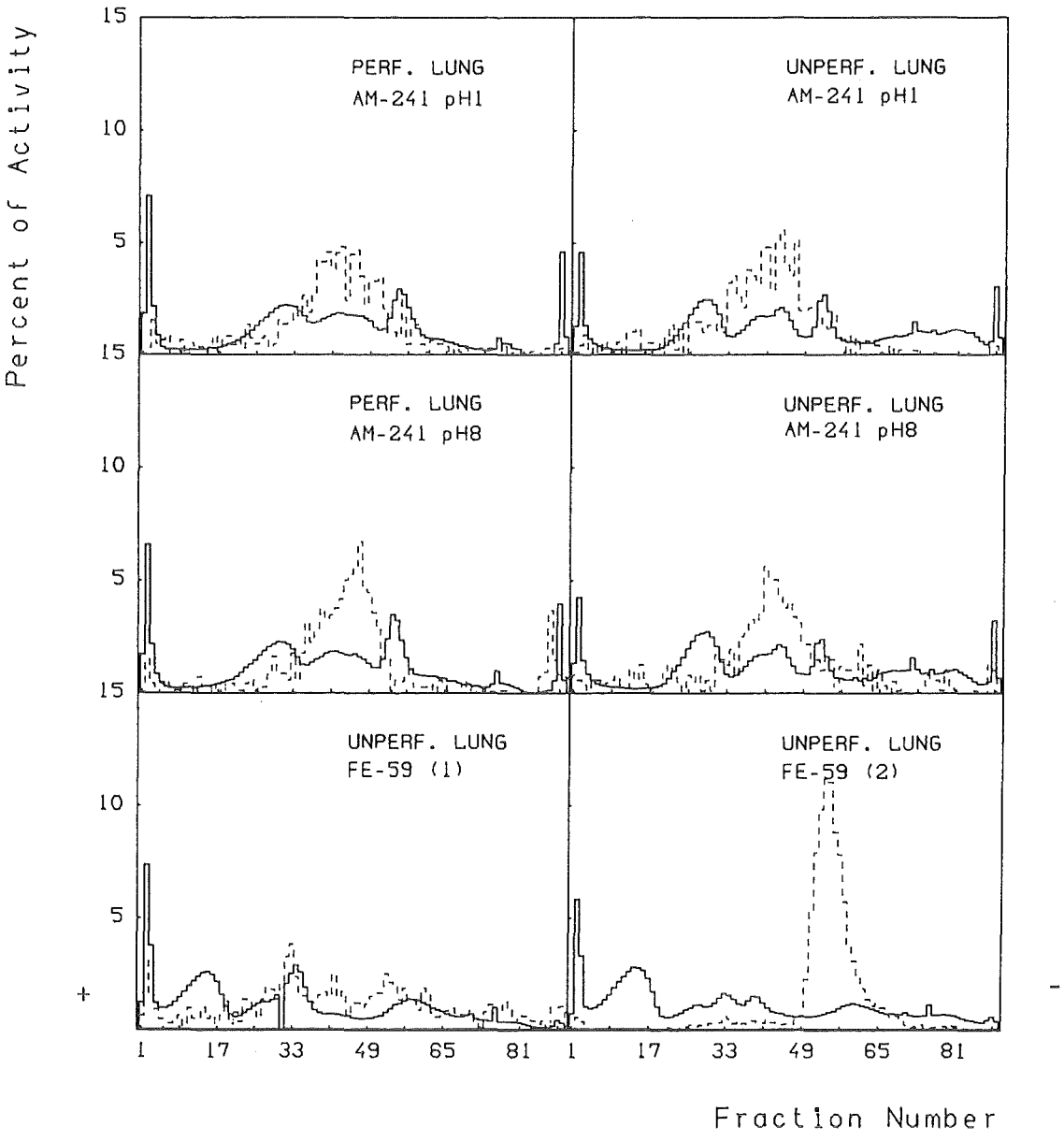


Fig. 26: Analysis of cytosol (from unperfused and perfused rat lung) by FFE, after in vitro labelling of the cytosol with ^{241}Am (pH 1 and pH 8) and ^{59}Fe (pH 8) ($^{59}Fe(1)$ - 37 Bq/1ml cytosol, $^{59}Fe(2)$ - 185 Bq/1ml cytosol). Representative for 2 experiments. Dotted lines = ^{241}Am or ^{59}Fe profiles. For abbreviations see the list (experimental details in section 2.1.7.).

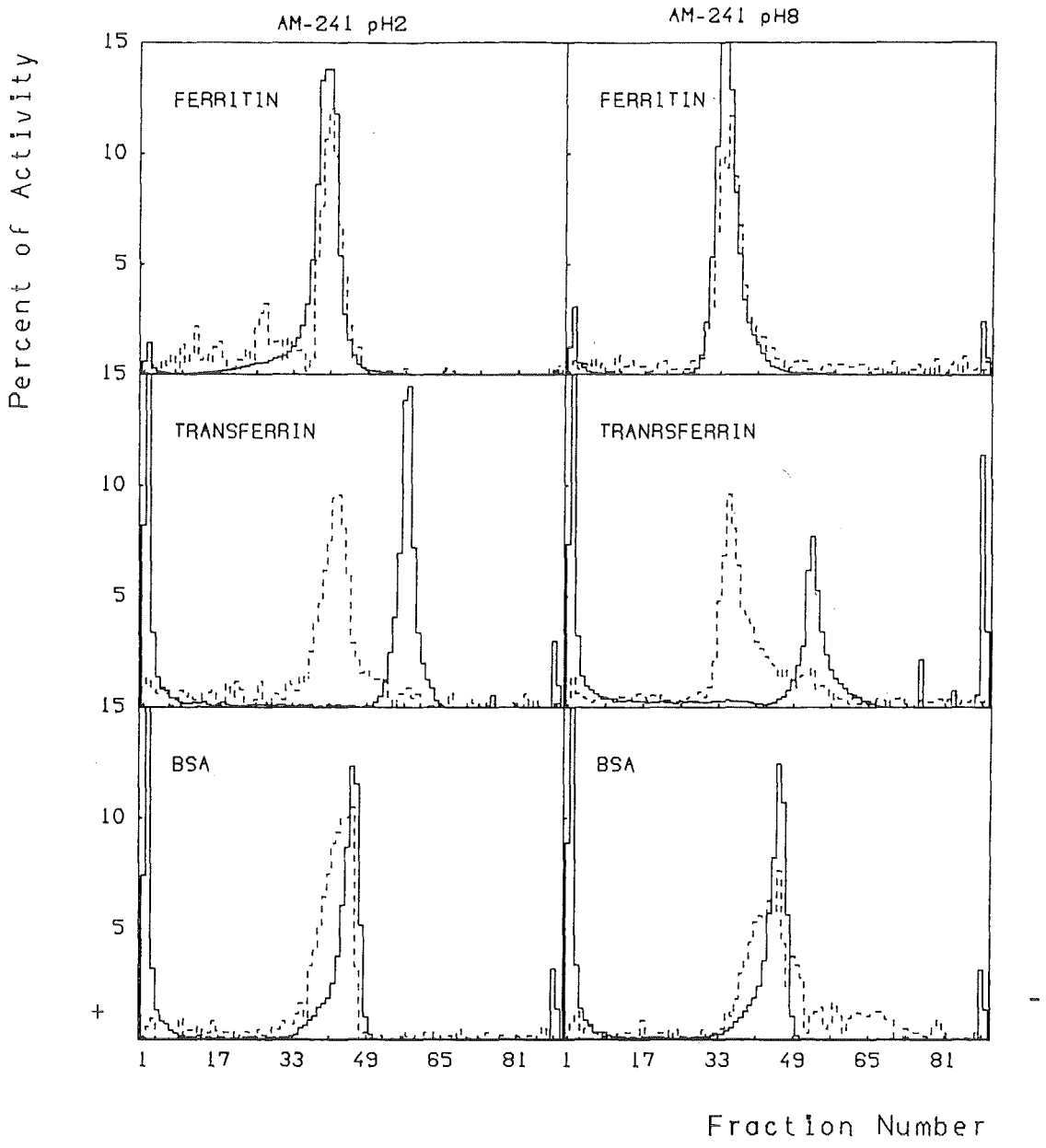


Fig. 27: Analysis of ^{241}Am and proteins by FFE, after in vitro labelling of ferritin, transferrin and bovine serum albumin (BSA) with ^{241}Am (pH 2 and pH 8). Representative for two experiments. Dotted lines = ^{241}Am profiles (experimental details in section 2.1.7.).

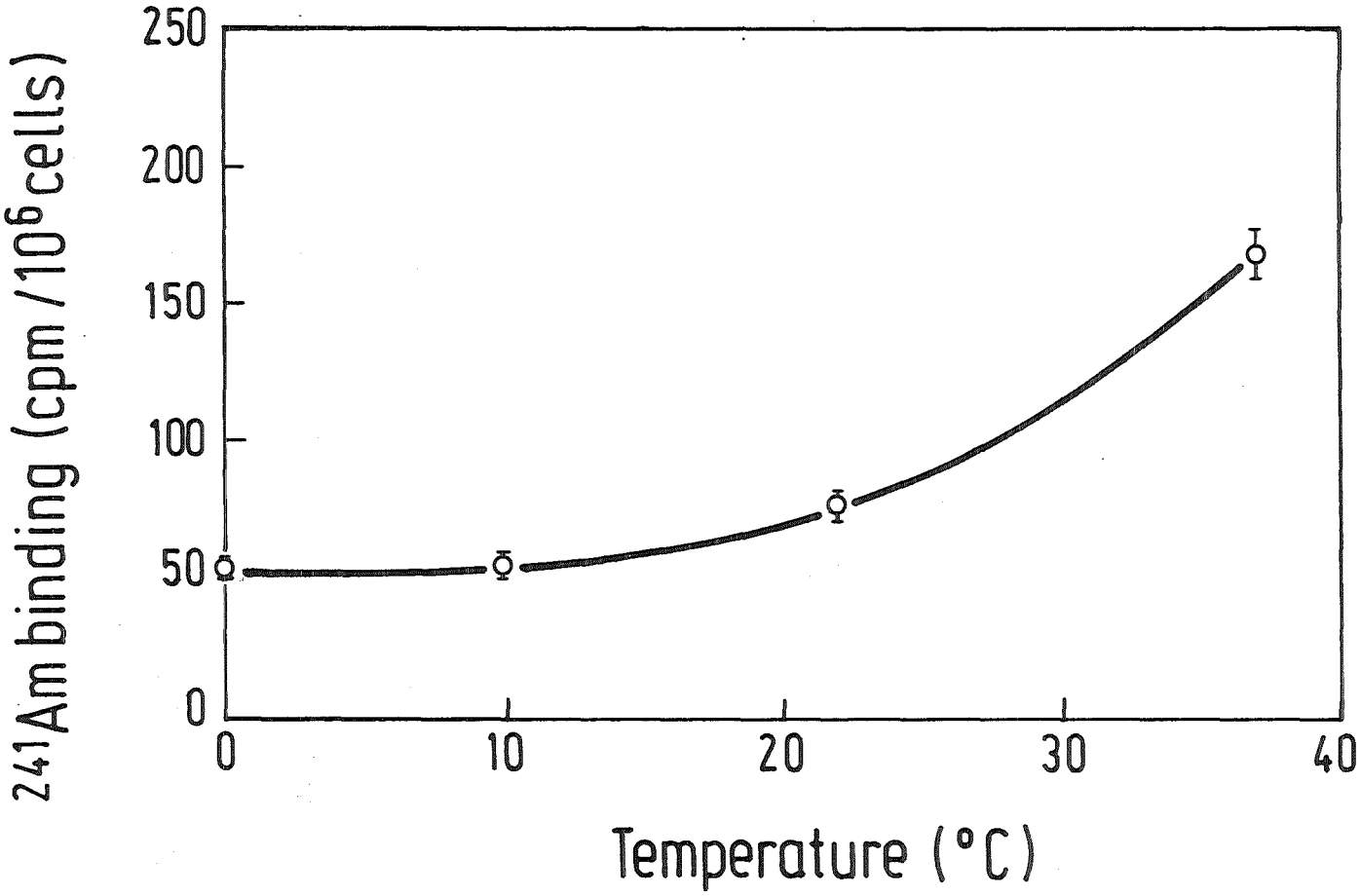


Fig. 28: Temperature dependence of the uptake of ^{241}Am colloid by bovine alveolar macrophages (for experimental details see section 2.2.2.). Representative for two experiments. Each value is the arithmetic mean of three determinations ± S.E.

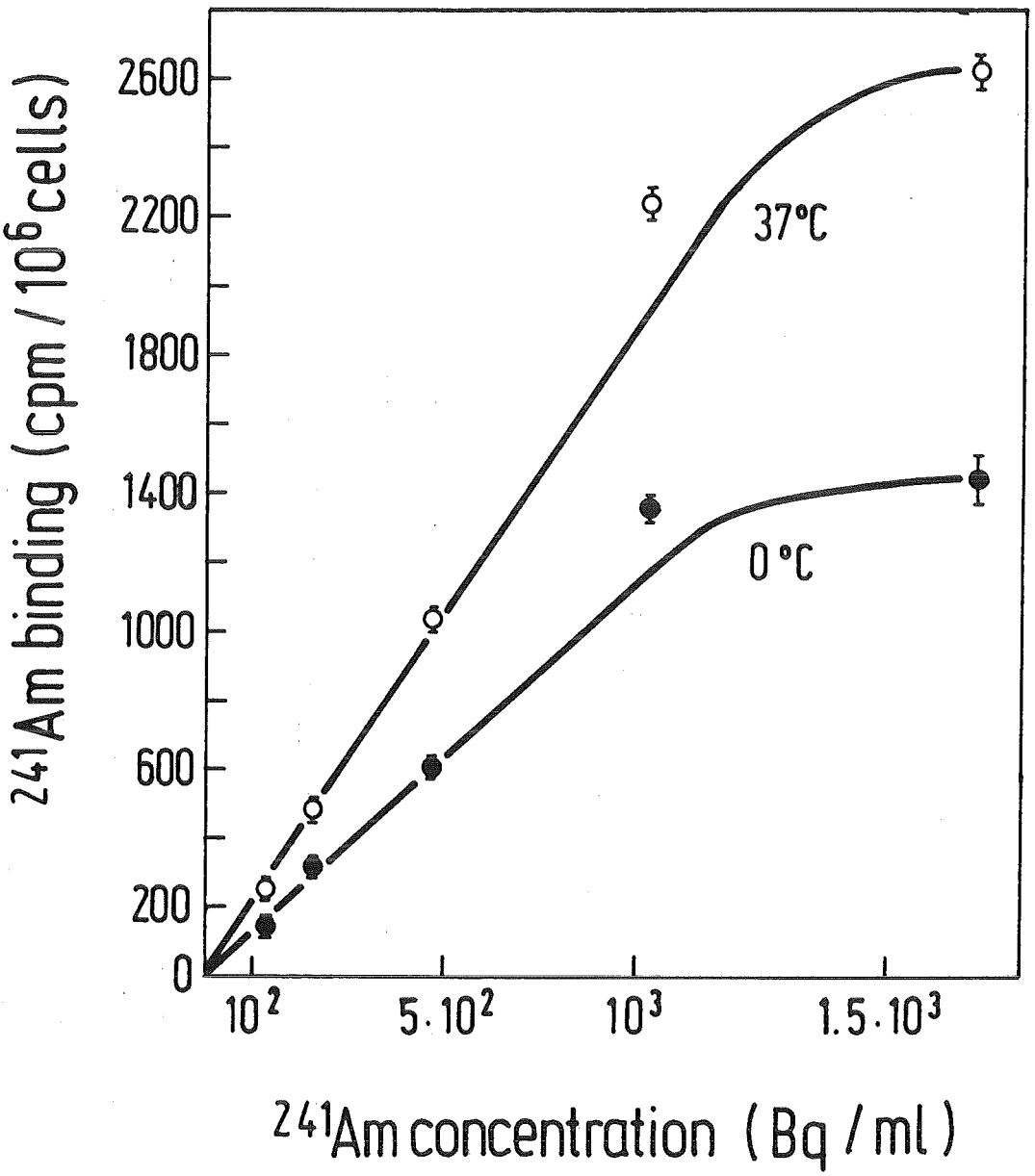


Fig. 29: ^{241}Am concentration dependence of the uptake of ^{241}Am colloid by BAMs. The cells were incubated in HEPES at both 37°C and 0°C for one hour (experimental details in section 2.2.2.). Representative for two experiments. Each value is the arithmetic mean of two determinations ± S.E.

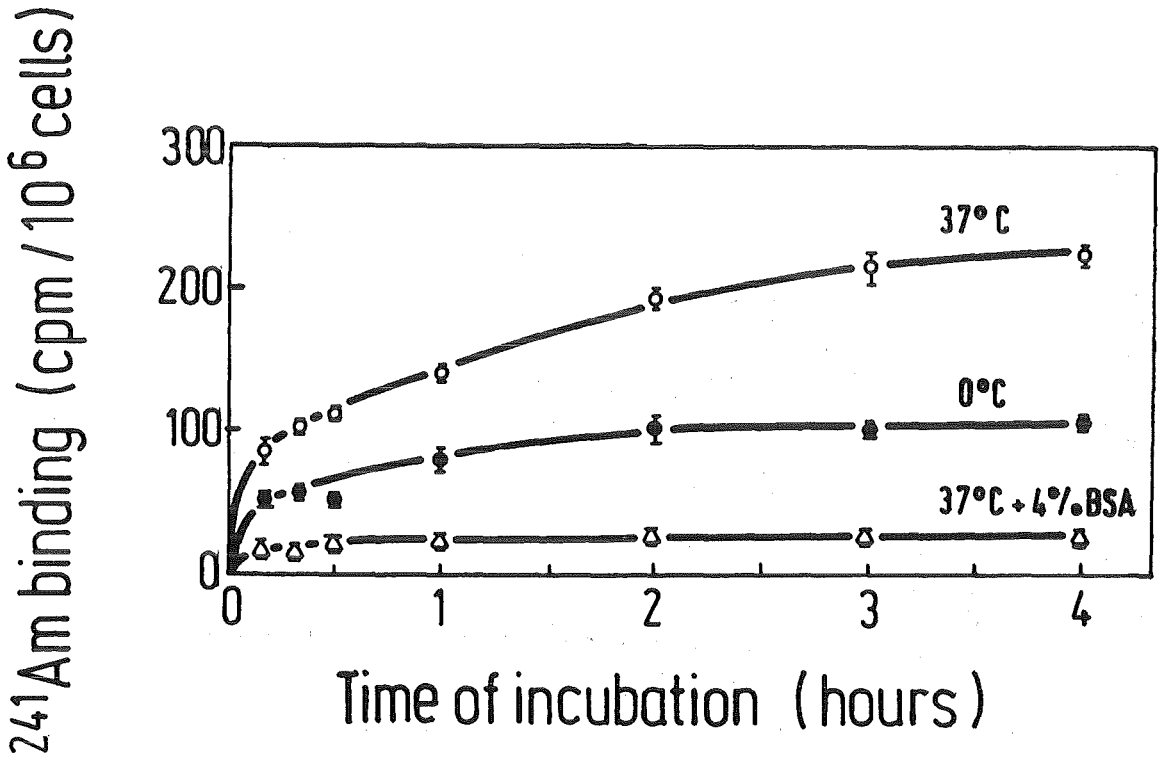


Fig. 30: The uptake of ^{241}Am colloid by BAMs as a function of time. The cells were incubated in HEPES (also in presence of 4% BSA) at 0°C and 37°C (experimental details in section 2.2.). Representative for two experiments. Each value is the arithmetic mean of two determinations ± S.E.

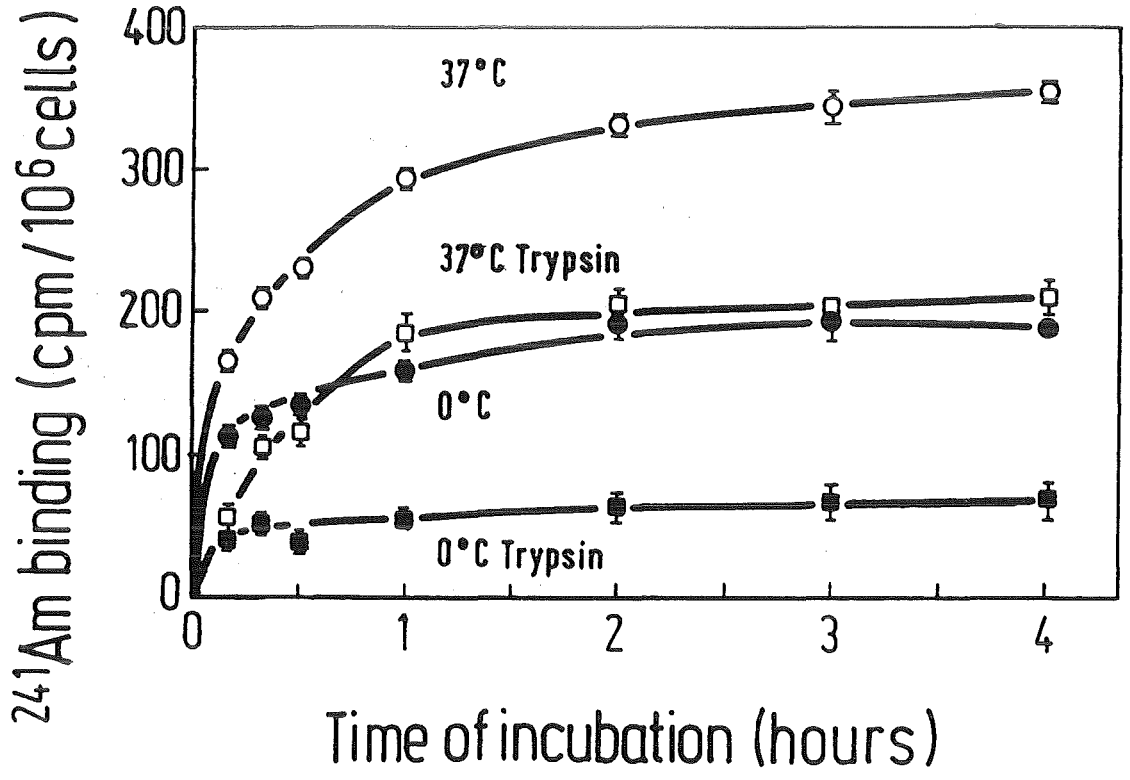


Fig. 31: The uptake of ^{241}Am colloid by BAMs as a function of time. The cells were incubated as described in Fig. 30 followed by trypsin digestion of cells incubated with ^{241}Am at 0°C and 37°C (for more experimental details see section 2.2.2.). Representative for 2 experiments. Each value is the arithmetic mean of two determinations ± S.E.

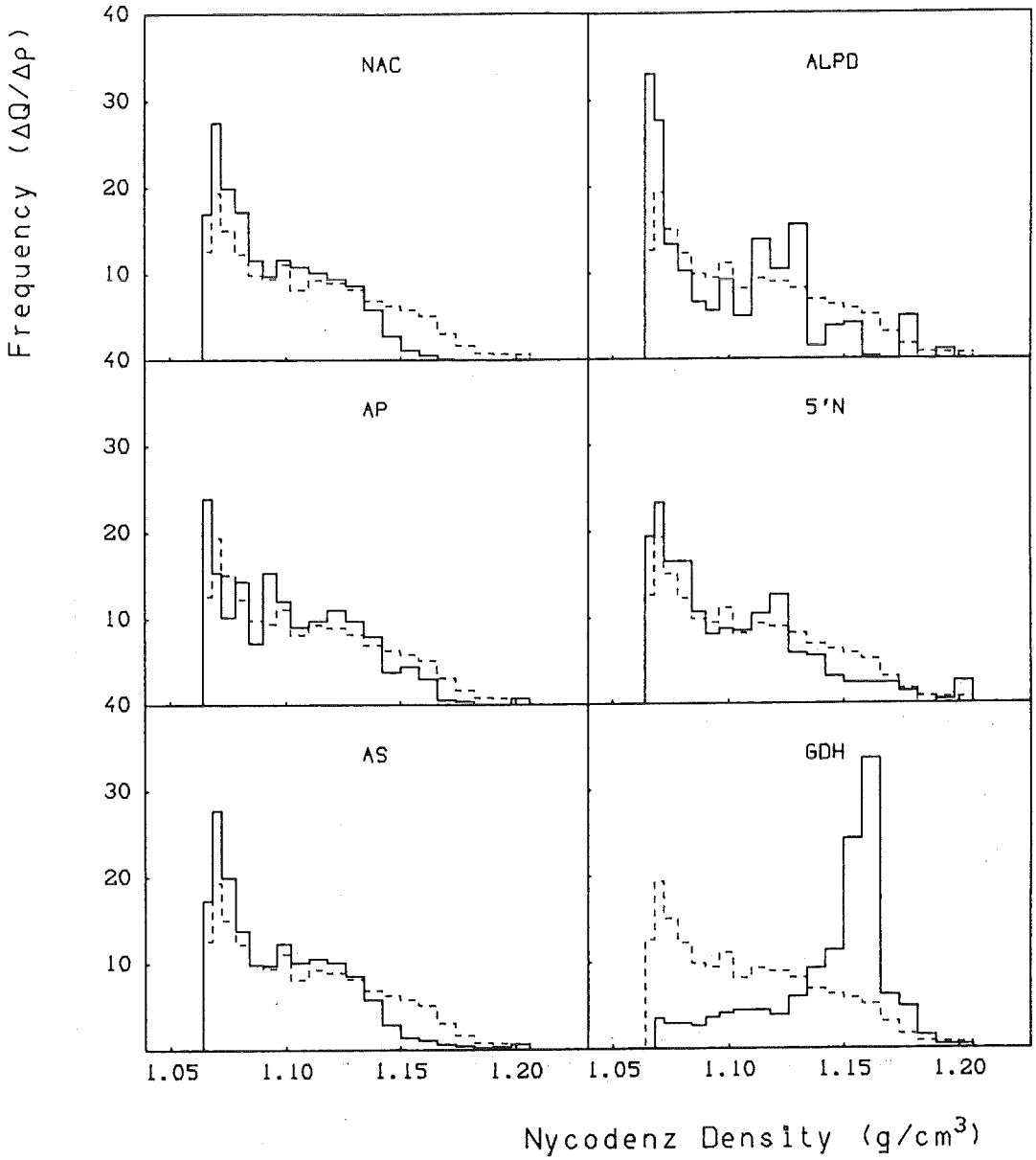


Fig. 32: Distribution of ^{241}Am and marker enzymes after centrifugation of the ML fraction of BAMs in linear nycodenz gradient (2 h, 60,000 g_{av}). Representative for two experiments. Dotted line = ^{241}Am profile (experimental details in section 2.2.3. and form of presentation in section 2.5.).

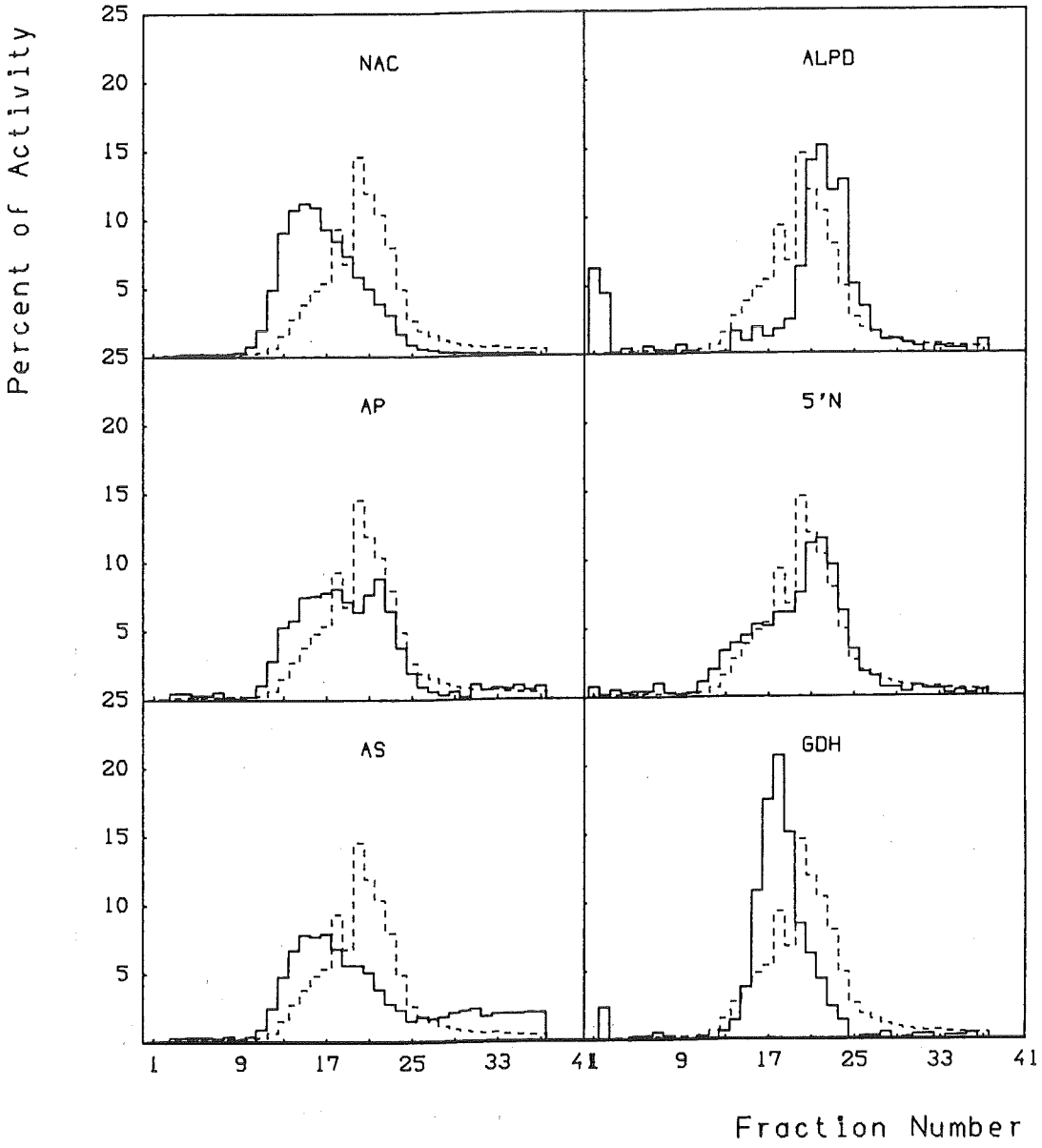


Fig. 33: Distribution of ^{241}Am and marker enzymes after centrifugation of ML fraction of BAMs in preformed linear percoll gradient (1 hour, 33,000 g_{av}), 2 hours after BAMs were incubated with ^{241}Am (for more details see Fig. 32 and section 2.2.3.). Representative for 3 experiments. Dotted line = ^{241}Am profiles. For abbreviations see the list.

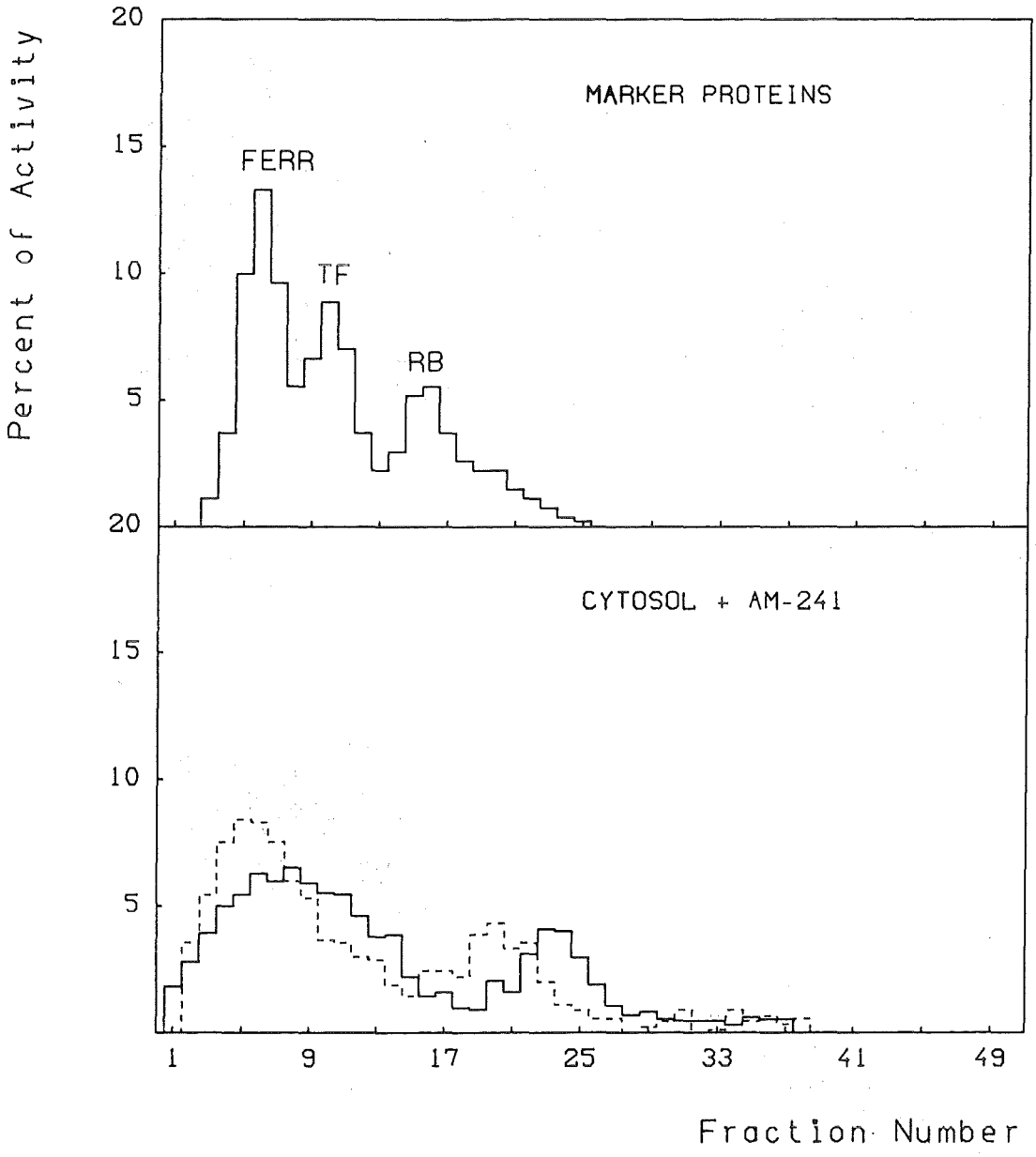


Fig. 34: Elution profiles of marker proteins, ^{241}Am and cytosol proteins of BAM (after 2 hr incubation, see section 2.2.3.) from the short sephacryl S-300 column. One experiment. For abbreviation see the list.

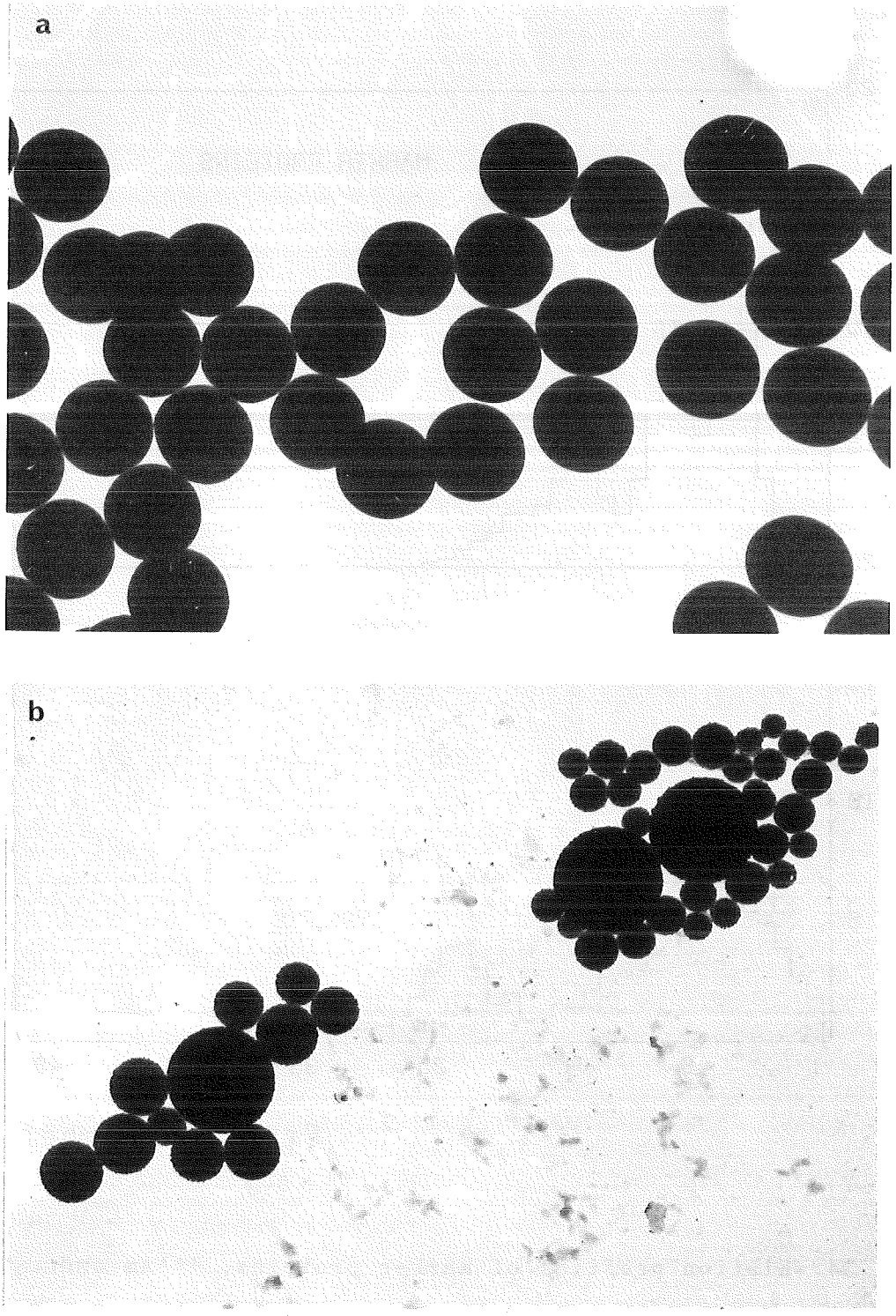
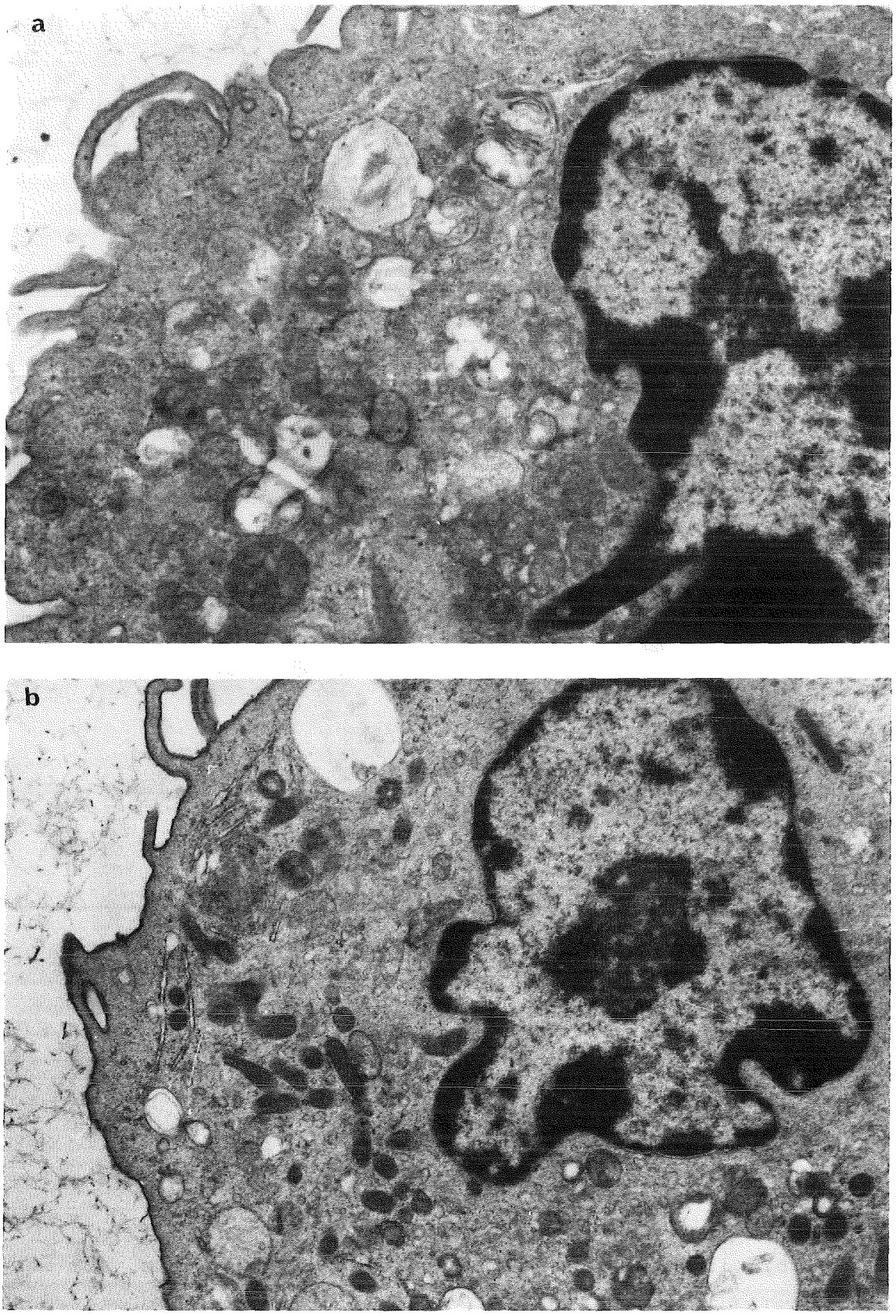
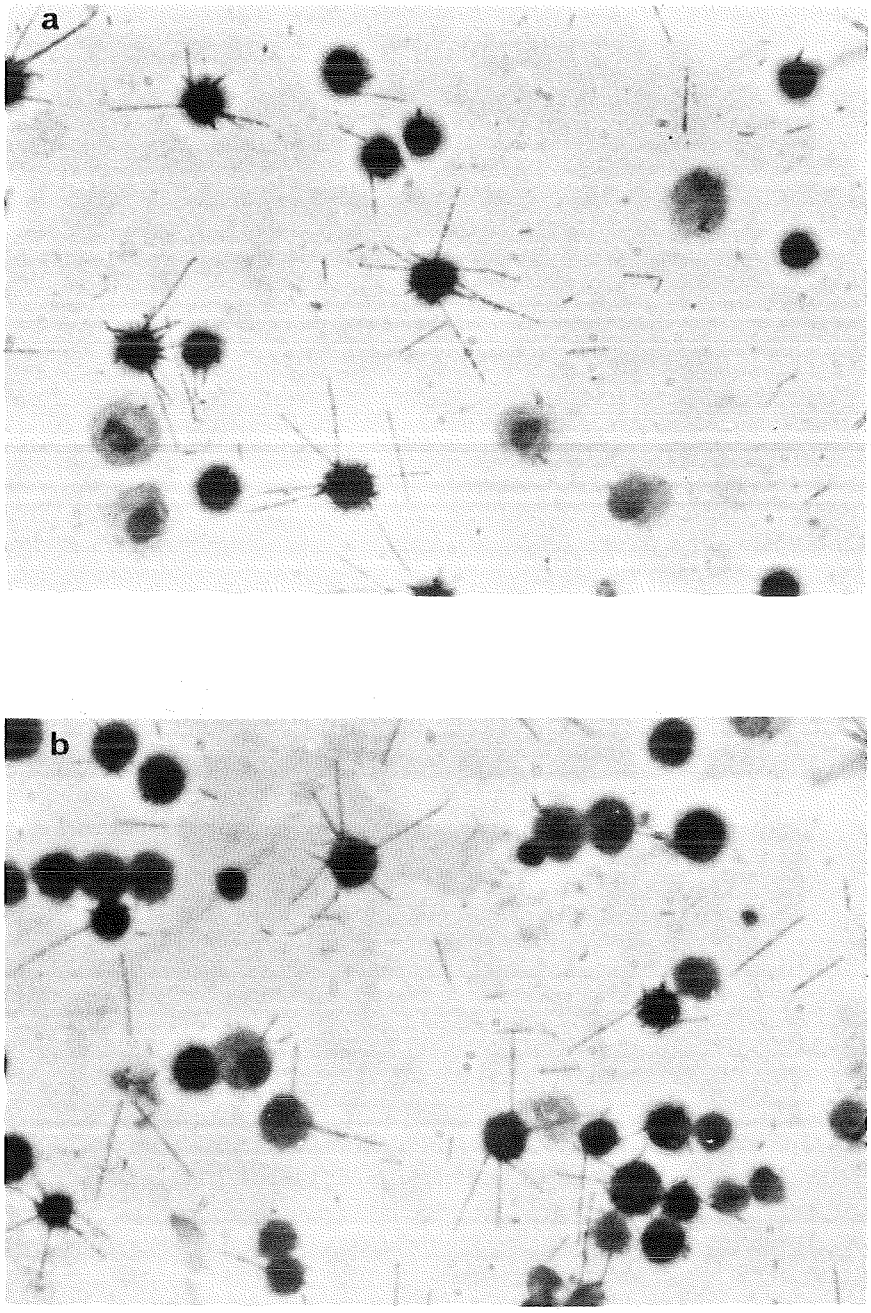


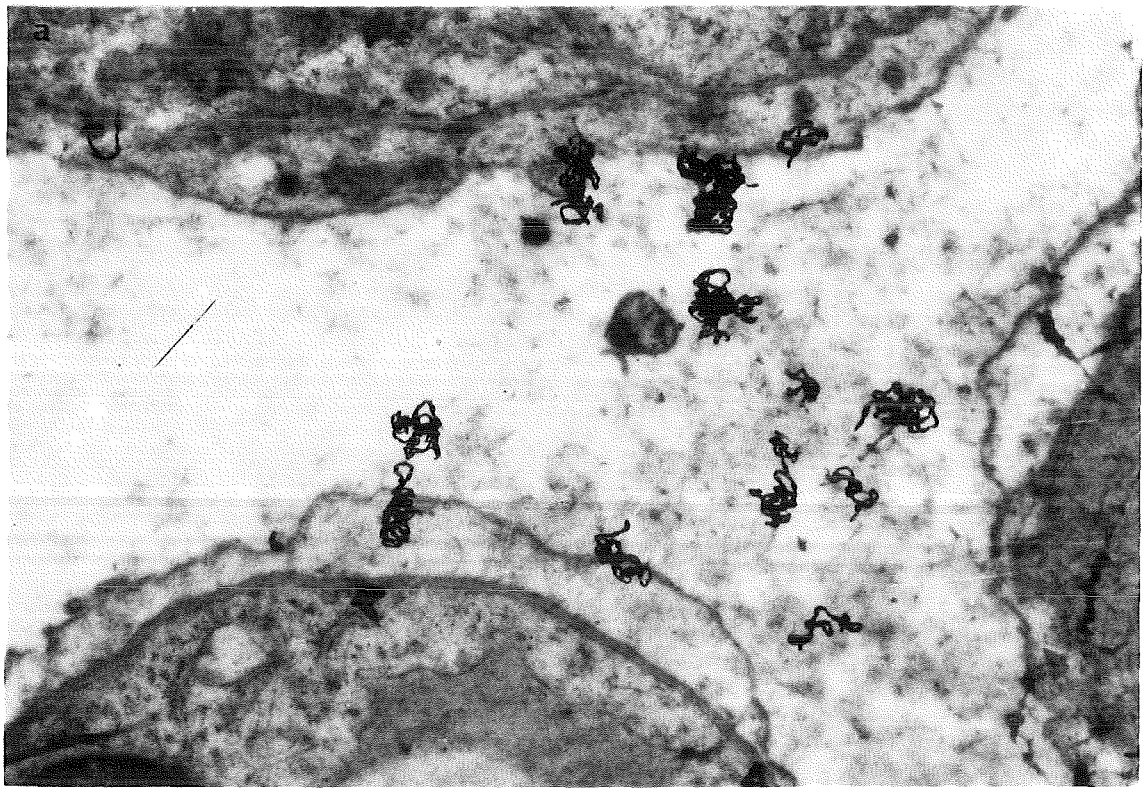
Fig. 35: Electron microscopic micrographs of polystyrene particles x 12000.

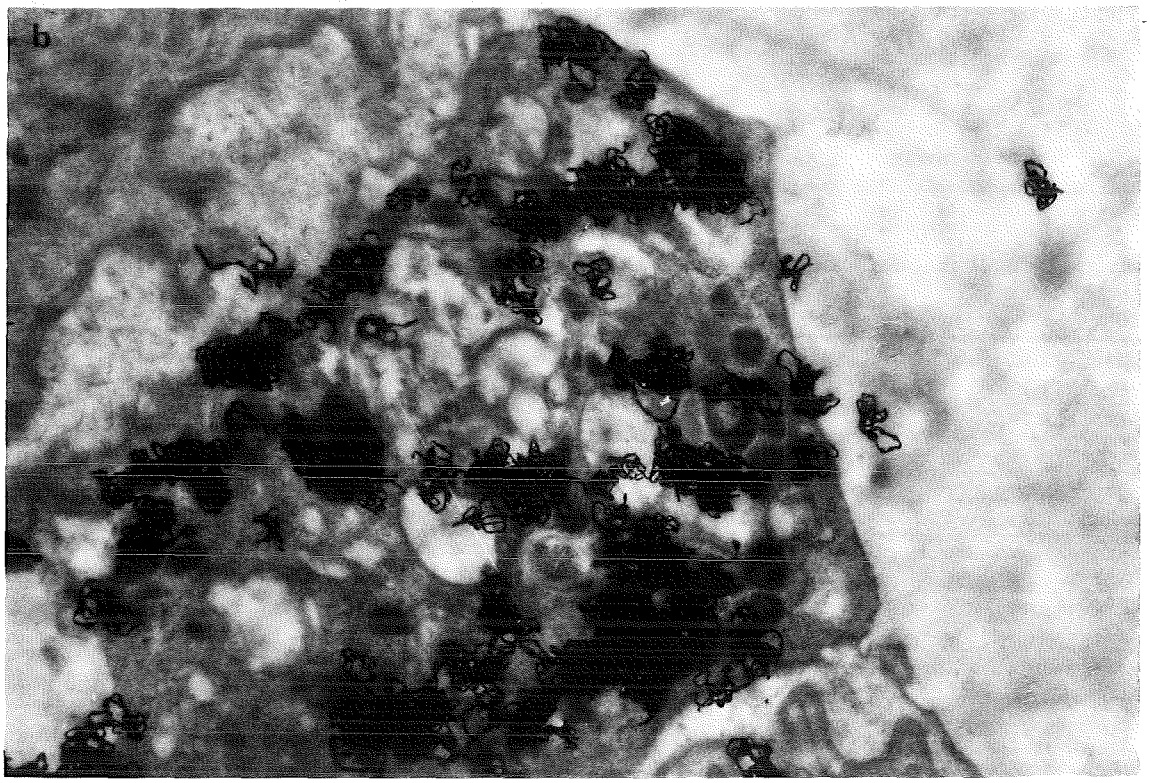


Figs. 36 (a,b): Electron microscopic micrographs of bovine alveolar macrophages after two hours culture in HEPES medium (37°C) x 12000.

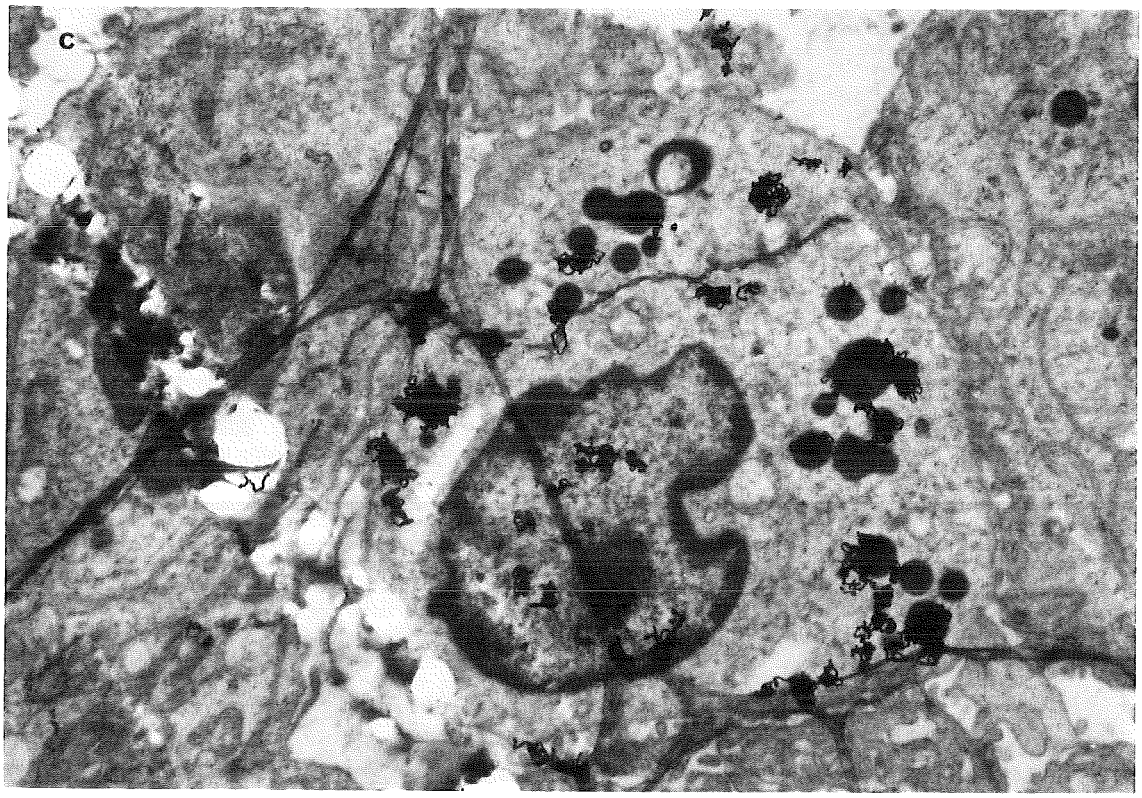


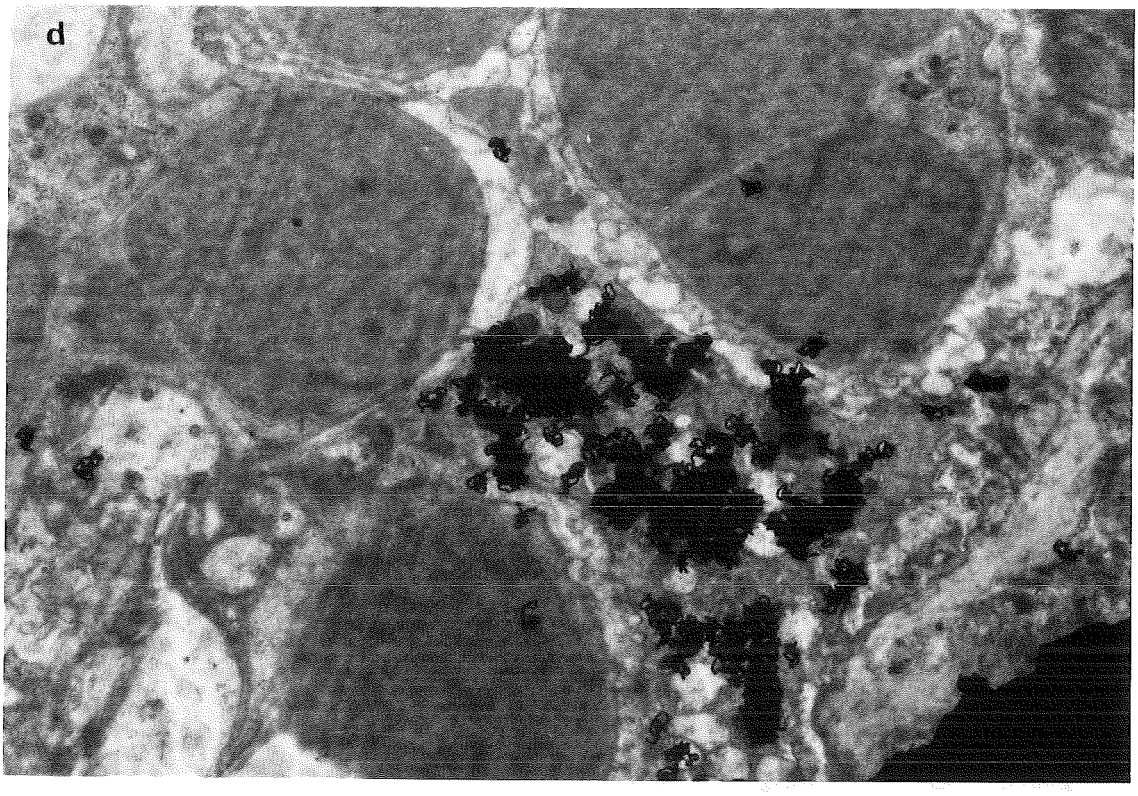
Figs. 37 (a,b): Light microscopic autoradiograms of the lavage cell pellet, two weeks after intratracheal injection of ^{241}Am colloid into rats. The exposure time was two weeks (for more details see section 2.4.1.) x 1200.



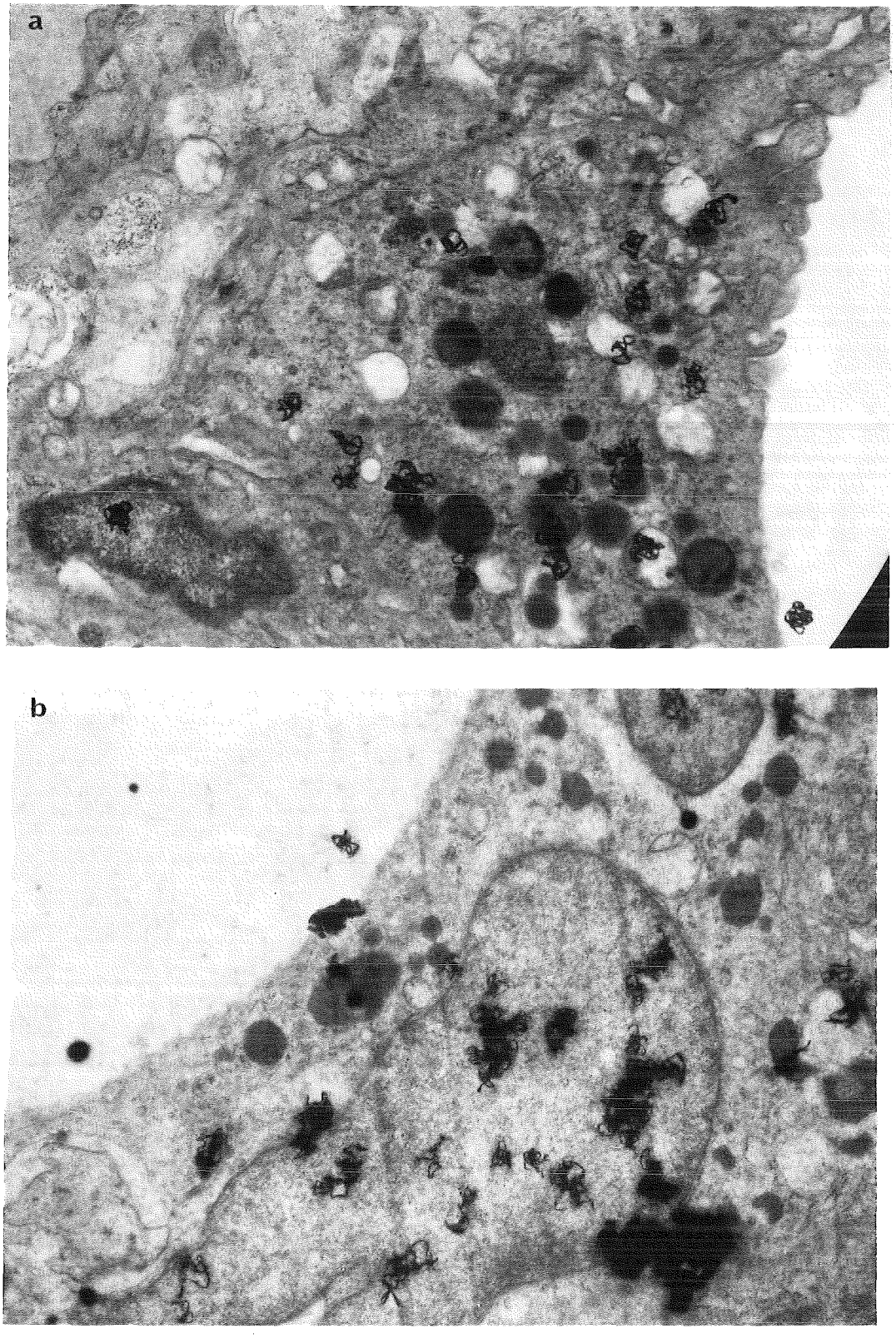


Figs. 38 (a,b): Electron microscopic autoradiograms of rat lungs, 24 hours after intratracheal instillation of ^{241}Pu colloid (more details in section 2.4.2.) x 12000.

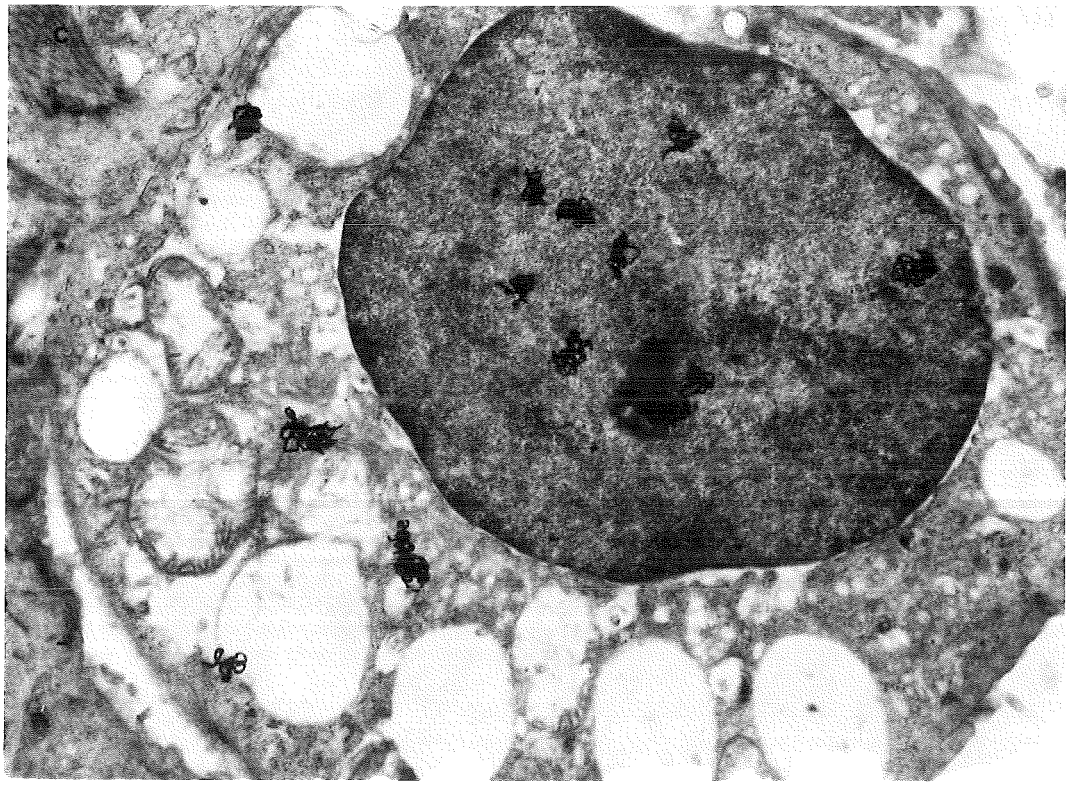


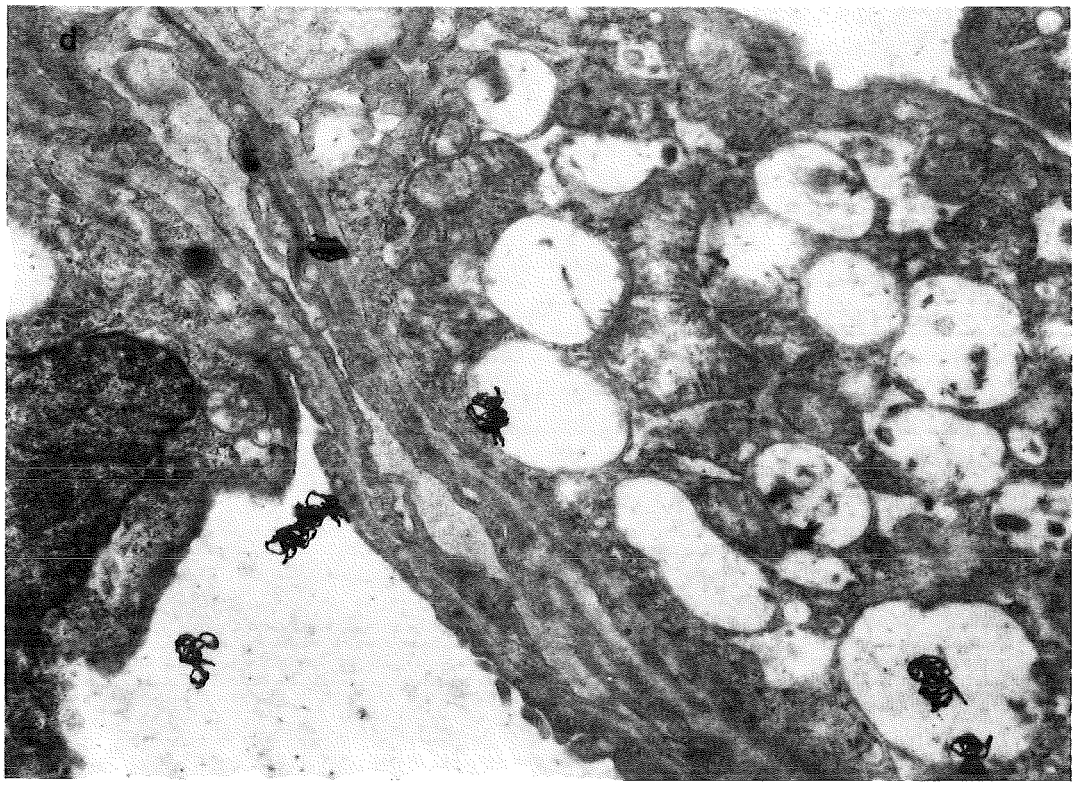


Figs. 38 (c,d): Electron microscopic autoradiograms of rat lungs, 24 hours after intratracheal instillation of ^{241}Pu colloid (more details in section 2.4.2.) x 12000.

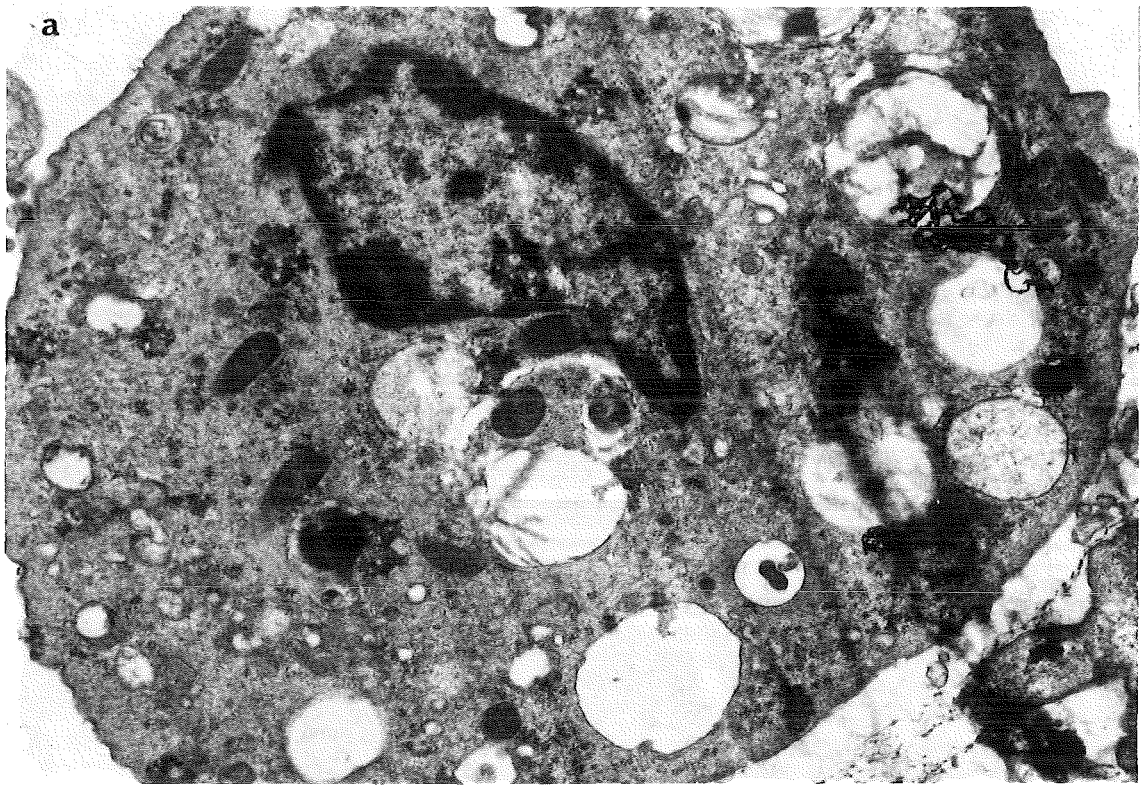


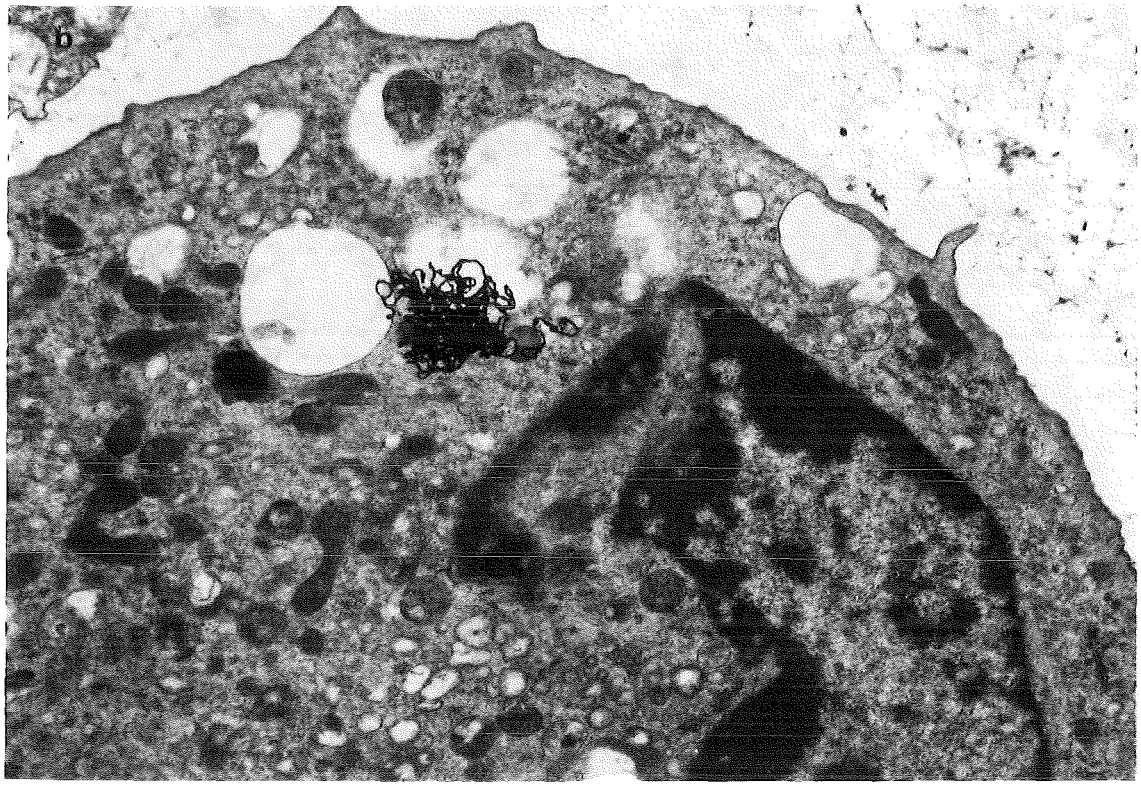
Figs. 39 (a,b): Electron microscopic autoradiograms of rat lungs, 7 days after intratracheal instillation of ^{241}Pu colloid (more details in section 2.4.2.) x 12000.



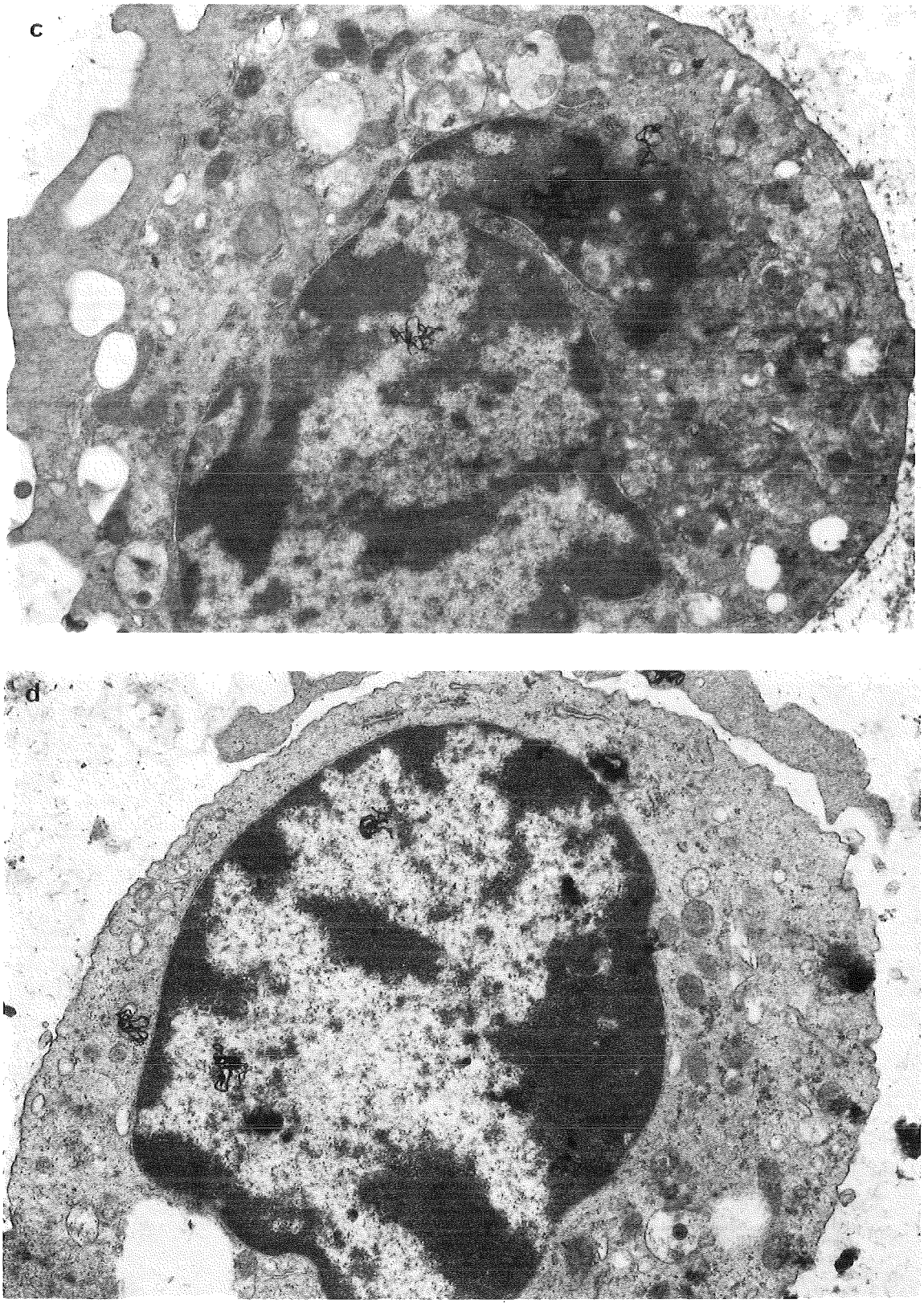


Figs. 39 (c,d): Electron microscopic autoradiograms of rat lungs, 7 days after intratracheal instillation of ^{241}Pu colloid (more details in section 2.4.2.) x 12000.





Figs. 40 (a,b): Electron microscopic autoradiograms of bovine alveolar macrophages, 2 hours after incubation with ^{241}Pu colloid (details in section 2.4.2.) x 12000.



Figs. 40 (c,d): Electron microscopic autoradiograms of bovine alveolar macrophages, 2 hours after incubation with ^{241}Pu colloid (details in section 2.4.2.) x 12000.