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Accumulation of ^{241}Am in subcellular structures of the hepatopancreas of the lobster *Homarus gammarus*

F. Paquet[1], F. Goudard[2], J.-P. Durand[2], P. Germain[1], J. Pieri[2]

[1]Institut de Protection et de Sûreté Nucléaire, Département de Protection de l'Environnement et des Installations, Unité de Radioécologie Estuaires et Milieu Marin, IPSN/CEA, F-50444 Beaumont-Hague Cedex, France

[2]Laboratoire de Biochimie et de Radiobiochimie, Université de Nantes, 2, rue de la Houssinière, F-44072 Nantes, France

ABSTRACT: Some of the artificial radionuclides released by the nuclear industry are assimilated by marine organisms. In this study, an attempt is made to elucidate the detailed mechanisms of ^{241}Am accumulation within the intracellular constituents of lobster hepatopancreas; the experimental procedure involved contaminating lobsters with a radiolabelled meal. The monitoring of activity levels in the digestive gland shows that the intracellular incorporation of this radioisotope occurs through a 2-fold process of adsorption onto membranes coupled with endocytosis, taking place within 30 min of ingestion. The breakdown of exogenous ligands releases americium, which then appears to be distributed between subcellular structures via the mediation of a cytosolic vector. After 24 h, various cellular constituents are affected by autophagous processes which cause a reconcentration of ^{241}Am into residual bodies.

INTRODUCTION

A certain number of transuranic elements are released into the marine environment by the nuclear industry. Some of these radionuclides are incorporated into organisms and transferred to different levels of the food chain. These transfers have been analysed for a number of years by many different laboratories among the international scientific community. The main lines of study have focused on defining the kinetics of radionuclide accumulation or loss, as well as determining their preferential fixation onto various organs. However, the fundamental processes underlying radionuclide fixation at the subcellular level remain as yet unknown.

In the present study, a preliminary approach to this problem is described which makes use of the actinide ^{241}Am in lobster tissue. Americium is taken up by marine organisms at all trophic levels (Germain & Miramand 1984). In this context, the lobster, which is an edible species, represents a potential pathway to humans. Furthermore, some studies have already been undertaken on lobsters which allow the localization of this radionuclide in the digestive gland (Miramand et al. 1989) and its fixation onto 3 classes of cytosolic proteins (Goudard et al. 1991). An investigation of the dynamics of radionuclide accumulation on the subcellular scale should lead to a better understanding of transfer mechanisms within the organism concerned.

MATERIALS AND METHODS

Radiolabelling of lobster specimens. Seven male lobsters in the C_4 intermoult stage, with an average weight of 531 ± 72 g, were kept in individual 20 l tanks each containing 15 l of seawater at 14 °C. The specimens were then contaminated by ingestion of a mussel labelled with seawater containing ^{241}Am. The radioactive source was supplied by the Commissariat à l'Energie Atomique (C.E.A., France) and provided an ingested radioactivity equivalent to 51300 ± 6700 Bq for each specimen. The lobsters were then killed off at periods of 30 min and 1, 4, 16, 24 and 48 h after the americium meal and the hepatopancreas sampled for biochemical analysis.

Sampling and homogenization of the hepatopancreas. The procedure followed here is based on the method of Goudard et al. (1991). Some slight modifications were applied to the protocol:

The 2 lobes of the organ are placed on filter-paper and then briefly washed twice with the extraction solution [20 mM tris-HCl, 0.25 M sucrose, 5 mM ß-mercaptoethanol + 7 mM phenylmethyl-sulphonyl fluoride (PMSF), pH = 8]. The purpose of this step is to eliminate any americium, not incorporated into the cells, that is either adsorbed onto the external tubule walls of the hepatopancreas (in contact with the haemolymph, itself also contaminated) or caught up in the lumen of digestive tubules. This washing step diminishes the total activity of the organ by 5 to 10 %. The hepatopancreas is then drained on filter paper, weighed and homogenized at 4 °C in a Dounce apparatus using 20 volumes of extraction solution. The degree of homogenization is checked at the end of the procedure by observing an aliquot sample under the optical microscope. The homogenate is subsequently filtered through gauze and subjected to ultracentrifugation (Rotor SW41 Beckman L2) at successive accelerations of 900 × *g* (for 15 min); 12 000 × *g* (for 15 min); 45 000 × *g* (for 30 min); and 115 000 × *g* (for 70 min). In this way, 4 plug samples and a supernatant liquid are obtained from the homogenate. The 4 successive centrifuged fractions are thus composed of cell nuclei, mitochrondria + lysosomes, membranes, microsomes and the cytosol is represented by the supernatant.

To assist in the interpretation of ^{241}Am distribution, 3 different types of analysis were performed on each fraction: proteins (Lowry et al. 1951), specific activity of acid phosphatase (Andersch & Szczypinsky 1947) and specific activity of cytochrome oxidase (Cooperstein & Lazarow 1951).

RESULTS

Figs. 1 & 2 make it possible to evaluate the incorporation kinetics and degree of contamination of the organelles (in terms of Am%) as well as their ability to concentrate the radionuclide (Am%/Prot%) at various stages of the digestive cycle as defined by Barker & Gibson (1977).

Incorporation kinetics of extracted organelles (Fig. 1)

The rate of incorporation of the radionuclide into the cells is extremely high. Thirty minutes after ingestion of a labelled mussel, ^{241}Am can be detected in all the cellular organelles of the hepatopancreas (Fig. 1). Cytosol is the subcellular constituent with the highest

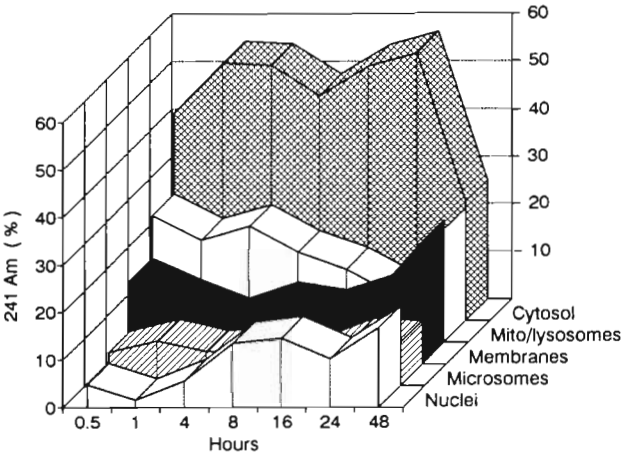

Fig. 1. *Homarus gammarus*. Evolution of the organelles' radioactivities during the digestive cycle. Subcellular fractions were obtained by differential centrifugation and, for each moment, ^{241}Am activity is expressed as the percentage of total radioactivity of the homogenate

americium level, containing more than 50 % of the total amount present in the hepatopancreas. The other types of organelle account for 5 to 20 % of the total cellular radioactivity. However, the partitioning of americium between the various subcellular structures is not static since this involves intra-cellular transfer mechanisms. Accumulation in the nuclei is progressive and regular, whereas the membranes are involved principally at the beginning and towards the end of the digestive cycle of the lobster. In other respects, the degree of contamination of the mitochrondria and lysosomes decreases regularly with time, and rises again 48 h after ingestion (T + 48 h). The cytosolic fraction exhibits a constant level of contamination up to T + 24 h, followed by a drop in activities after T + 48 h. In fact, microsomes are the only organelles measured here which show no marked change in contamination with time.

Americium concentration (Fig. 2)

When the plug sample activities are compared with the proteins from each fraction, it is evident that microsomes have the highest protein concentration for ^{241}Am, regardless of the time elapsed since ingestion. The pattern of accumulation appears to have either 2 or 3 phases within the nuclei, membranes and microsomes. In the cytosol, however, the specific activity remains almost constant throughout the digestive cycle.

The other salient feature of these data is the clear enrichment of americium observed in the membranes at T + 30 min. Radioactivity levels in this constituent are seen to fall sharply at T + 1 h, after which they increase again to form 2 distinct peaks.

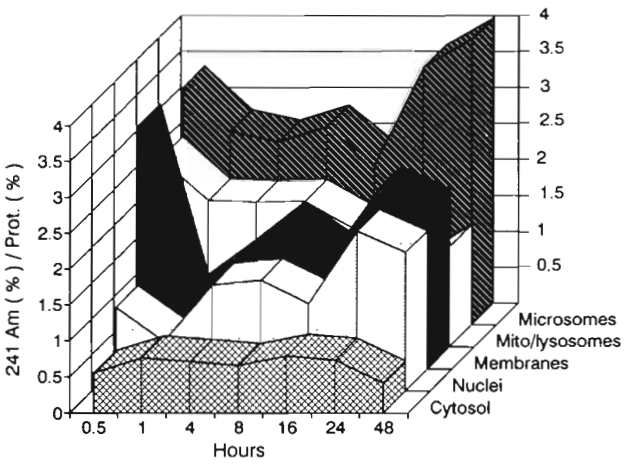

Fig. 2. *Homarus gammarus*. Evolution of the specific activity of subcellular fractions, during the digestive cycle. Specific activity is expressed as the quotient ^{241}Am (%)/protein (%)

DISCUSSION

The assimilation efficiency of the ^{241}Am ingested by lobster after a single meal was calculated and presented in a previous work (Paquet 1991). In that case, approximately 27 % of the initially ingested ^{241}Am was assimilated from food.

The results obtained in this study allow the investigator to define the mechanisms and kinetics of intracellular ^{241}Am accumulation, and the role of the organelles concerned. The use of 1 lobster at each time interval – in view of the extent of the experiment – led us to interpret our results cautiously. Nevertheless, numerous previous studies performed in our laboratory proved the reproducibility and the constancy of the digestive cellular cycle of the lobster, when used as described above.

An important question is whether, at the sampling time involved, mussel material remains in the lumen of the digestive tubules. The double buffer rinse (before homogenization) normally removes all the undigested food, but unfortunately it is possible that a part of the mollusc material remains in the tubule of the organ. The diameter of the aggregates which enter the gland is always less than 100 nm, corresponding to the soluble fraction (Hopkin & Nott 1980). For that reason this exogenous material should be co-distributed after centrifugation in the cytosolic (i.e. soluble) fraction of the hepato-pancreatic cells. Nevertheless recent studies in our laboratory tend to define the nature and the origin of the proteins obtained in this fraction, and lead us to believe that this phenomenon is negligible.

Mechanisms and kinetics of intracellular radionuclide incorporation

Adsorption/endocytosis

In the first place, accumulation of americium appears to take place by adsorption onto the cell membranes and then by endocytosis in the cytoplasm (Fig. 3). The high levels of membrane radioactivity at T + 30 min, followed by a very large decrease at T + 1 h, suggest that adsorption/endocytosis phenomena occur mainly during the first hour after ingestion. Americium would appear to be transferred towards small digestive vacuoles rich in acid hydrolases. In such an environment, the many different digestive enzymes in the vacuoles favour the chemical assimilation of absorbed compounds by maintaining the pH at a relatively low value (Fowler 1987). Under these pH conditions, americium

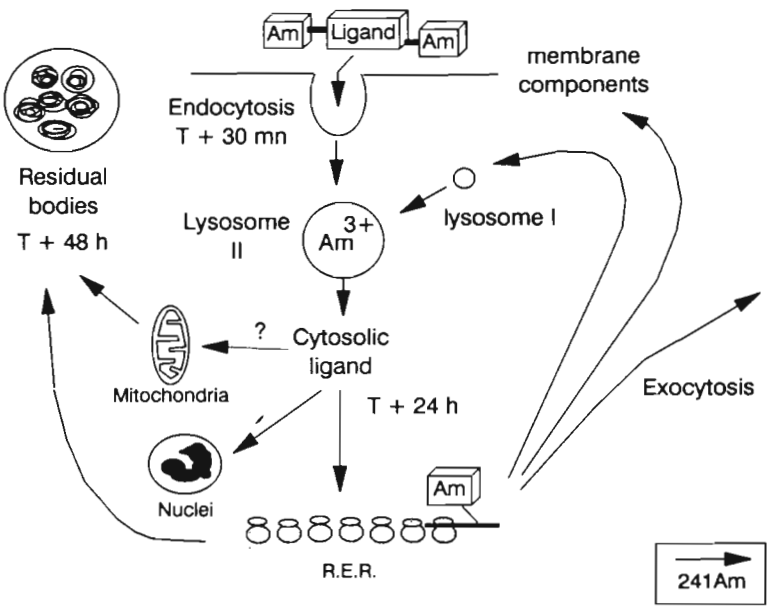

Fig. 3. *Homarus gammarus*. General scheme of ^{241}Am incorporation in the lobster hepatopancreatic cells. Americium is incorporated into pinocytic vesicles. The vacuoles coalesce with primary lysosomes, which contain numerous hydrolitic enzymes. Intravacuolar digestion transforms the metal into an ionic form which can pass through the lysosomal membrane and bind to cytosolic ligands. Some of the ligands can be proteinic components of mitochondria, nuclei or rough endoplasmic reticulum (R.E.R.) in the assembling stage in the cytosolic compartment. ^{241}Am binding on these structures leads to the organelle contamination. After this, proteinic synthesis in the R.E.R. can lead to the contamination of polypeptides which are destined for exocytosis, membrane components or primary lysosomes. Finally, autophagy of the organelles leads to the residual bodies' contamination

is probably present as Am^{3+} (Moulin et al. 1988), so it is free to diffuse across the membranes and spread throughout the cell (Viarengo 1985, Prescott 1989). Such mechanisms are likely to be extremely rapid, judging by the kinetics of incorporation into the other organelles.

Intracellular diffusion

Broadly speaking, the distribution pattern of ^{241}Am within subcellular stuctures may reflect 2 concomitant processes: simple adsorption onto nearby organelles or fixation onto cytosolic proteins that are destined for incorporation into cellular organelles. In the second case, the fixation of americium onto certain proteins destined for incorporation into the nucleus, mitochrondria or endoplasmic reticulum could bring about contamination of the organelles concerned. Subsequently, the contamination of newly synthesized proteins coming from the rough endoplasmic reticulum (R.E.R.) could cause a secondary labelling of proteins destined for the plasmic membranes, the primary lysosomes or for exocytosis (Fig. 3). Such an interpretation may account for the bimodal contamination of the membranes and the gradual contamination of the other organelles. During the first 24 h after ingestion, there is a regular transfer of activity from the secondary lysosomes, via the cytosol, towards the other organelles.

At T + 48 h, the autophagy of certain contaminated organelles can lead to the contamination of residual bodies. This secondary contamination occurs after a period of latency estimated here at about 1 or 2 d.

According to this model – in the case of chronic contamination – primary contaminated lysosomes become fused with the vacuoles formed by endocytosis, which are themselves contaminated. This leads to cyclic phenomena during intracellular incorporation of the radionuclide. In detail, this brings about an important enrichment of the radionuclide in the residual bodies of digestive cells.

Contribution of each subcellular fraction to the accumulation of ^{241}Am

Lysosomes

The role of lysosomes in the fixation of americium appears to be rather small since they absorb only 10 to 26 % of the total amount present. Nevertheless, some recent work (Goudard et al. 1991) has revealed a predominant fixation of americium onto the lysosomes of lobsters that were contaminated with radiolabelled food. Similar types of studies have been carried out on the crab (Guary 1980) and the crayfish (Bierkens et al. 1985, Vangenechten et al. 1985), as well as the starfish (Galey et al. 1983), the murex (Galey et al. 1984), the periwinkle (Galey et al. 1986) and a species of freshwater snail (Thiels 1982). All these results point towards a strong retention of the americium in the more massive constituents of the cell, here brought together in 2 of the centrifuged fractions (i.e. containing nuclei and mitochrondria + lysosomes).

Otherwise, various studies have been carried out on plutonium accumulation in the crab (Guary & Negrel 1981), the plaice (Leonard & Pentreath 1981) and in some species of mammal including the rat, dog and hamster (Boocock et al. 1970, Bruenger et al. 1971, Danpure & Taylor 1974, Gurney & Taylor 1975, Ragan 1975, Seidel et al. 1979). These investigations all show a preferential accumulation of radionuclides within the heaviest fractions, mainly the mitochondria and the lysosomes. By contrast, europium tracer studies carried out on *Carcinus maenas* (Bierkens & Simkiss 1988) have shown a preferential fixation of this element in the soluble fraction of the cellular material (59 %), in agreement with the experimental results presented here. In fact, Sullivan et al. (1988) have demonstrated that the subcellular distribution of metals in bivalve molluscs is a function of the specific element concerned; Cd, Cu, K and Mg appear to be concentrated in the cytosolic fractions, whereas Ba, Fe, Mn and Pb are associated with granular bodies.

The fixation of americium onto the lysosomes is entirely plausible, since these structures have always been considered as an important site for the elimination of cellular toxins (Fowler et al. 1975, Sternleib & Goldfisher 1976, Brown 1977, Jansen & Scholz 1979, Seidel et al. 1979, Viarengo et al. 1981, 1985, 1987, George 1983a, b, Moore 1985, Fowler 1987, Prasad et al. 1989) even though certain elements, such as silver, apparently show no concentration effect (Martoja et al. 1988). The fixation of metals onto the older lysosomes (residual bodies and tertiary lysosomes) takes place by fixation onto the products of lipid peroxidation (George 1983a, b) or onto polymerized metallothionein (Viarengo et al. 1985). These 2 alternative sites constitute, in fact, possible coexisting pathways for the elimination of metals which are incorporated into the lysosomes. From their studies on the rat and hamster, Seidel et al. (1979) consider that the fixation of plutonium by lysosomes is mediated by the glycoproteins. Goudard et al. (1991) have used chromatography in an attempt to characterize the lysosomal ligands responsible for the fixation of americium in the hepatopancreas of the lobster. It appears that the radionuclide is linked to very high molecular weight components in the lysosomes, but more precise information could not be obtained from these chromatographic analyses.

Table 1. *Homarus gammarus*. Distribution of ^{241}Am in subcellular fractions from digestive gland of lobsters. The radioactivity and the activity of marker enzymes for lysosomes and mitochondria, i.e. acid phosphatase and cytochrome oxidase, are expressed for each fraction. T: time of ingestion; prot.. protein; AP: acid phosphatase; CO: cytochrome oxidase; Lyso: lysosomes; Mito: mitochondria

	Nuclei	Mito/Lyso	Membrane	Microsome	Cytosol
T + 30 min					
241 Am (Bq)	275	1580	1048	414	2591
241 Am (%)	4.7	26.7	17.7	7	43.9
Prot. (%)	4	10.1	5.2	2.1	78.6
241 Am (%)/prot. (%)	1.2	2.6	3.4	3.3	0.6
AP (%)/prot. (%)	1.6	2.2	2.2	1.6	0.4
CO (%)/prot. (%)	0.8	6.1	0.7	0.0	0.0
T + 1 h					
241 Am (Bq)	100	1433	882	632	3612
241 Am (%)	1.5	21.5	13.2	9.5	54.2
Prot. (%)	2.2	10.5	13	3.5	70.8
241 Am (%)/prot. (%)	0.7	2.0	1.0	2.7	0.8
AP (%)/prot. (%)	2.0	2.6	2.9	1.1	0.2
CO (%)/prot. (%)	0.8	6.5	2.1	0.5	0.0
T + 4 h					
241 Am (Bq)	502	2186	831	646	4808
241 Am (%)	5.6	24.4	9.3	7.2	53.6
Prot. (%)	3.8	12.1	6.2	2.9	75.1
241 Am (%)/prot. (%)	1.5	2.0	1.5	2.5	0.7
AP (%)/prot. (%)	2.9	3.1	1.9	0.4	1.0
CO (%)/prot. (%)	0.9	2.8	1.1	0.2	0.0
T + 8 h					
241 Am (Bq)	1508	2117	1421	838	5277
241 Am (%)	13.5	19	12.7	7.5	47.3
Prot. (%)	8.8	9.3	6.2	2.7	73
241 Am (%)/prot. (%)	1.5	2.0	2.0	2.8	0.6
AP (%)/prot. (%)	1.5	1.7	2.9	1.0	0.6
CO (%)/prot. (%)	1.2	5.6	2.3	0.8	0.0
T + 16 h					
241 Am (Bq)	795	839	606	316	2943
241 Am (%)	14.5	15.3	11	5.7	53.5
Prot. (%)	12	9.2	7	2.6	69.2
241 Am (%)/prot. (%)	1.2	1.7	1.6	2.2	0.8
AP (%)/prot. (%)	1.4	2.3	1.9	0.9	1.3
CO (%)/prot. (%)	0.6	3.1	1.9	0.5	0.0
T + 24 h					
241 Am (Bq)	1255	1238	1765	1197	7005
241 Am (%)	10.1	9.9	14.2	9.6	56.2
Prot. (%)	4.5	10.1	5.7	2.7	77
241 Am (%)/prot. (%)	2.2	1.0	2.5	3.6	0.7
AP (%)/prot. (%)	2.0	3.2	3.0	1.9	1.3
CO (%)/prot. (%)	0.4	7.2	2.5	0.6	0.0
T + 48 h					
241 Am (Bq)	384	546	591	209	576
241 Am (%)	16.7	23.7	25.6	9.0	25.0
Prot. (%)	8.6	16.8	11.9	2.3	60.4
241 Am (%)/prot. (%)	1.9	1.4	2.2	4.0	0.4
AP (%)/prot. (%)	2.3	2.0	1.4	0.2	0.8
CO (%)/prot. (%)	0.9	2.2	1.9	0.0	0.0

In order to explain the differences observed between our results and the studies cited above, it is possible to assume that the high degree of homogenization during preparation of the fractions might have led to rupture of most of the lysosomes and a transfer of their contents towards the soluble fraction. However, if this had occurred, the activity of acid phosphatase would have been higher in the soluble fraction than actually observed (see Table 1). Furthermore, the constant nature of results obtained from all the lobster specimens seems to rule out any variation due to random degrees of homogenization.

In fact, there is another reason behind this apparent disparity. The mitochrondria + lysosomes fraction is highly heterogeneous in nature, containing many different constituents which have the same density as these organelles. As a consequence, the centrifugation may bring together numerous particles of different types. Guary & Negrel (1981) have shown that the strong enrichment of plutonium in the heavy fraction is correlated with the presence of calcium phosphate granules. These granules, which are widely found in the animal kingdom, exhibit a strong affinity for a large number of metals (George 1982). Although lobsters lack such granules, the cytoplasm of aged resorptive cells is seen to contain numerous residual bodies which result from auto- and heterophagous processes. Depending on their density, these residual bodies may be concentrated into the mitochrondria + lysosome or into the nuclei fraction. It is interesting to note that in the experiments on lobsters carried out by Goudard et al. (1991) specimens were used that had been contaminated for 18 d. Most of the other studies mentioned above either made use of chronically contaminated specimens or specimens that had been radio-labelled at a point in time and killed a few days later. Thus, we may conclude that, in these cases, the enhanced activities in the heavy fractions (nuclei, mitochrondria + lysosomes) could be due mainly to a contamination of residual bodies or diverse granules which concentrate radionuclide with the passage of time. The time lapses used in our experiments (0.5 to 48 h) are not sufficiently long to allow contamination of this type of organelle. Consequently, in the present study, the americium spike stays mostly in solution within the cytoplasm.

Mitochrondria

There is no evidence that americium can be fixed onto the mitochrondria. The radioactivity recorded in the heaviest fractions may be attributed entirely to the presence of tertiary lysosomes or residual bodies as described previously. Nevertheless, Bruenger et al. (1972) detected some ^{249}Cf in the mitochrondria of dog's liver. Similarly, Galey et al. (1983) have found ^{252}Cf and ^{241}Am in mitochrondria extracted from the pyloric coecum of starfish. Taylor (1969) and then Bruenger et al. (1970) have shown the fixation of Pu onto liver mitochrondria in the rabbit and dog, respectively. However, Seidel et al. (1979) were unable to detect the presence of Pu in the mitochrondria of the rat or hamster. None of the other studies cited in this section have shown any notable concentration of metals in mitochrondria. As a general rule, it would seem that these organelles have only a minor role in the biokinetics of transuranic elements.

Membranes

The strong affinity of americium for the membranous fractions can be explained, in the first instance, by adsorption of the ingested radionuclide onto the external walls of the plasmic membranes of digestive cells. Histo-autoradiography has shown that americium is located in the microvilli of the brush border of the cells (Miramand et al. 1989). Plasmic membranes are the interfaces through which all adsorbed elements must pass, so it is entirely reasonable to assume that americium could be fixed on such a site. The adsorption of americium, or ligand complexes containing the radionuclide, could take place on the surface coatings' plasmic membranes, which are composed of glycoproteins. The incorporation of americium by endocytosis brings about its transfer to another constituent of the cell, while the membrane is freed of the radioactive material. After a few hours, it is very likely that the fixation of americium onto cytosolic proteins, which are later integrated into the plasmic membrane, would cause a gradual recontamination of the membrane, but this time proceeding from inside the cell. Such a process of membrane contamination should thus display a biphase distribution in time.

Nuclei

Cell nuclei are seen to concentrate americium relatively well. Due to the complexity of nuclear structures, it is difficult to analyse the types of molecule (proteins or nucleic acids) which are involved in the radionuclide fixation. Nevertheless, some recent studies (Paquet 1991) have enabled conclusions to be drawn about the preferential fixation of this radionuclide onto the proteins of the nuclear matrix.

Microsomes

The strong preference of americium for the microsome-bearing fraction is to be noted with interest (see Fig. 2). This fraction contains the lowest absolute concentrations of proteinic material (Table 1), but the proteins that are present have an enhanced affinity for the radionuclide. However, the high americium activities associated with these structures remain to be elucidated. Maruyama & Nonomura (1984) have presented evidence for the existence of a protein exhibiting a strong affinity for calcium in the microsomes of *Pecten* sp. muscle tissue, but none of the studies listed above have shown any affinity with transuranic elements. Nevertheless, these microsomes are sites for the synthesis of numerous proteins, some of which are able to

interact with the americium. In other respects, certain proteins located in the R.E.R. undergo glycosylation processes which transform them to glycoproteins with various different functions (Prescott 1989). It is conceivable that americium is fixed onto the glycoproteins; the affinity of certain transuranic elements (Pu and Am) for these proteins has been demonstrated by *in vitro* experiments (Chipperfield & Taylor 1970).

Cytosol

The cytosolic fractions contain the highest measured radionuclide activities (Fig. 1), but the nature of the bonding is not yet determined. Work carried out by Goudard et al. (1991) has shown that most of the cytosolic americium is fixed onto 2 protein groups having molecular weights of 10 and 20 kDa and that the remaining amount may be fixed onto ferritine. Although the authors cited above have dismissed the possibility of fixation onto metallothioneins, the nature of the cytosolic proteins remains unidentified. Several hypotheses can thus be advanced to account for the observed phenomena.

The studies listed above draw attention to the high concentrations of glycoproteins in the radioisotope-contaminated structures. These glycoproteins occur within the microsomes, in the cellular mantle and also in the nuclear structures involved in radionuclide fixation. Glycoproteins are all synthesized in the cytosol, migrating towards their target organelles where they are almost immediately incorporated after their formation. They cover a range of molecular weights and appear to have a strong affinity for americium (Chipperfield & Taylor 1970). Thus, it is quite reasonable to assume that americium is fixed onto cytosolic glycoproteins.

CONCLUSIONS

This study draws a first assessment of intracellular transfers of ^{241}Am from a food source to an edible species. The results provide new data in radioprotection research and explain the biokinetics which were observed for whole organisms. The findings show regular accumulation of the residual bodies which result from various autophagic processes. The time taken to eliminate the radionuclide thus depends directly on the fate of these structures; in the case of the hepatopancreas of the lobster, residual bodies are only eliminated after the death of the contaminated cells. In that context, future studies will try to define the fine structure of the molecular compounds involved in the ^{241}Am fixation and will allow the role of the glycoproteins in the fixation of the actinide to be confirmed or invalidated.

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