Mobilisation of Plutonium and Iron from Transferrin and Ferritin by Hydroxypyridone Chelators

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Plutonium is a metal of major importance in the nuclear industry and it has a high radiotoxicity which expresses itself mainly by the appearance of osteosarcoma long after its deposition in the body [1]. Because of its high radiotoxicity there is considerable interest in the development of chelating drugs which will enhance the, normally slow, elimination of plutonium from the bodies of persons who may become accidentally contaminated [2].

For the past 25 years the agent of choice for the chelation therapy of plutonium, or americium, contamination has been the polyaminopolycarboxylic acid diethylenetriaminepentaacetic acid (DTPA) administered intravenously as the tri-sodium-calcium-, or as the less toxic tri-sodium-zinc salt [2]. Although it is very effective in removing plutonium from its complexes in plasma and body fluids it is much less effective in mobilising the metal from the skeleton or the liver, the two principal organs of deposition [3]. In blood plasma plutonium is bound specifically to the iron-transport protein, transferrin [4] and in cells the metal is often found in association with the iron-storage protein ferritin [5]. The affinity of plutonium for iron-binding sites has led Raymond *et al.* to design a series of 'plutonium-specific' chelators based on natural 'siderophores' [6], several of these, especially LICAM(C), has shown a high effectiveness for the removal of plutonium from experimental animals [7, 8], comparable to that of DTPA.

Both DTPA and LICAM(C) are most effective when injected intravenously and from a practical point of view it would be very convenient to have an agent which could be administered orally over a long period of time. Kontoghiorghes [9] has recently synthesized a series of hydroxypyridone chelators with a view to their use orally for the treatment of the iron overload in thalassaemia.

In this paper, the ability of six pyridine chelators to release plutonium from its complexes with transferrin and ferritin *in vitro* is described and compared with their ability to release iron from these proteins.

Experimental

Materials and Methods

Chelators

1,2-Dimethyl-3-hydroxy-pyrid-4-one (**L1**), 2,4-dihydroxy-pyridine-1-oxide (**L3**) and 2-hydroxy-4-methoxy-pyridine-1-oxide (**L6**) were prepared as previously described [10]. 2-Hydroxy-pyridine-1-oxide (**L4**) was obtained from the Aldrich Chemical Company, Gillingham, U.K., 2-mercapto-pyridine-1-oxide (OM) and mimosine (1-aminopropionic acid-3-hydroxy-pyrid-4-one) from the Sigma Chemical Co, U.K. Human apo-transferrin was purchased from Merck, Darmstadt and purified as previously described [11]; horse spleen ferritin (iron content > 12%, Serva, Heidelberg, F.R.G.) was used without further purification. Plutonium-238 as nitrate and $^{59}FeCl_3$ were obtained from Amersham-Buchler, Braunschweig, F.R.G. Sephadex G-50 was purchased from Deutsche Pharmacia, Freiburg, F.R.G.

Preparation of Labelled Proteins

Transferrin or ferritin were dissolved in 0.14 M NaCl–0.02 M tris-HCl buffer (pH 7.4) to give solutions containing 14 micromol protein/dm^3 (assuming a molecular weight of 70 000 for transferrin and 400 000 for ferritin). Sufficient $^{59}FeCl_3$, or $^{238}Pu(NO_3)_4$, was added to 2 cm^3 of the ferritin solution to give protein:metal ratios of 2:1 and 250:1 respectively, and the pH was adjusted to 7.4 with solid $NaHCO_3$, the solutions were then allowed to stand at 4 °C for at least 24 h. For the preparation of labelled transferrin sufficient NTA was added to the $^{59}FeCl_3$ or $^{238}Pu(NO_3)_4$ solutions to give an NTA:metal ratio of 4:1, the pH was adjusted to 7.4 and the solutions allowed to stand at room temperature for 2 h. The metal–NTA complexes were added to the transferrin solution, to yield the same protein metal ratios as for ferritin, and allowed to stand for at least 24 h at 4 °C.

Metal-release Studies

Equal volumes (0.15 ml) of the 14 μM protein–metal solution were mixed with the same volume of a 7 mM solution of the appropriate chelating agent in the NaCl–tris-HCl buffer and allowed to stand for 60 min, or for a shorter or longer time.

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TABLE I. Release of Plutonium-238 from Transferrin or Ferritin by Chelating Agents *In Vitro* Following 1 h Incubation[a]

Chelating agent	Plutonium-238 recovered (%)			
	Transferrin		Ferritin	
	Protein	LMW[b]	Protein	LMW[b]
Metal–protein complex only	91 ± 6	8 ± 5	89 ± 2	7 ± 2
Diethylenetriaminepentaacetic acid (DTPA)	9 ± 3	90 ± 4	19 ± 8	79 ± 9
1,2-Dimethyl-3-hydroxy-pyrid-4-one (L1, A)	36 ± 9	64 ± 10	26 ± 10	71 ± 12
2,4-Dihydroxy-pyridine-1-oxide (**L3**)	8 ± 2	88 ± 7	7 ± 1	91 ± 9
2-Hydroxy-pyridine-1-oxide (**L4**)	26 ± 2	71 ± 3	39 ± 3	57 ± 2
2-Hydroxy-4-methoxy-pyridine-1-oxide (**L6**)	50 ± 4	46 ± 6	28 ± 3	68 ± 5
2-Mercapto-pyridine-1-oxide (OM)	63 ± 12	33 ± 11	86 ± 9	11 ± 7
Mimosine	17 ± 4	81 ± 8	28 ± 6	71 ± 7
N^1,N^5,N^{10},N^{14}-Tetrakis(2,3-dihydroxy-4-carboxybenzoyl)-tetra-azatetradecane (LICAM(C))	7 ± 1	93 ± 3	9 ± 2	89 ± 7

[a]Chelating agent:protein ratio 500:1; protein:plutonium ratio 250:1. [b]LMW = recovery in the low molecular weight fraction from the Sephadex G-50 columns, 3 to 7 measurements per substance ±1 standard deviation.

TABLE II. Release of Iron-59 from Transferrin or Ferritin by Chelating Agents *In Vitro* Following 1 h Incubation[a]

Chelating agent	Iron-59 recovered (%)			
	Transferrin		Ferritin	
	Protein	LMW[b]	Protein	LMW[b]
Iron–protein complex only	95	5	95	4
2,4-Dihydroxy-pyridine-1-oxide (**L3**)	27	73	56	44
2-Mercapto-pyridine-1-oxide (OM)	91	6	83	16
N^1,N^5,N^{10},N^{14}-Tetrakis(2,3-dihydroxy-4-carboxybenzoyl)-tetra-azatetradecane (LICAM(C))	48	51	77	21

[a]Chelating agent:protein ratio 500:1; protein:iron ratio 2:1. [b]LMW = recovery in low molecular weight fraction from the Sephadex G-50 columns.

Chromatographic Analysis

To separate protein-bound from non-protein-bound metal 0.1 ml aliquots of the metal–protein, or metal–protein–chelator, solutions were placed on the top of a column (0.6 cm diameter × 16 cm) of Sephadex G-50 and eluted with 0.14 M NaCl–0.02 M tris-HCl buffer at pH 7.4; 40, 1 ml, fractions being collected. The radioactivity in each fraction was assayed by gamma scintillation counting for ^{59}Fe or by liquid scintillation counting for ^{238}Pu.

Results

The results of the chromatographic analyses of the ^{238}Pu–transferrin and ^{238}Pu–ferritin complexes, and of the mixtures of the metal–protein complexes with six pyridine derivatives are listed in Table I. This Table also shows the data from experiments with two other chelators, DTPA and LICAM(C), which were chosen as reference substances for this study. The recovery of the ^{238}Pu from the Sephadex G-50 columns averaged 100 ± 6(SD)% in 64 experiments.

To provide a direct comparison of ^{59}Fe release under these experimental conditions a limited study was performed with three chelators, LICAM(C), **L3** and OM; the results of this study are listed in Table II. In this series of experiments the recovery of ^{59}Fe from the G-50 columns averaged 90 ± 9(SD)% in 8 runs.

A standard time of 1 h was selected for reaction between the metal–protein complexes and the chelators. In order to check that this was a reasonable choice a ^{238}Pu–ferritin–mimosine mixture was analysed at 5, 60 and 120 min after mixing, the results are shown in Table III.

Discussion

The analyses of the ^{238}Pu–transferrin and ferritin complexes showed that about 90% of the radioactivity

TABLE III. Time Course of Release of Plutonium-238 from Ferritin by Mimosine *In Vitro*[a]

Time after addition of chelator (min)	Plutonium-238 recovered (%)	
	Protein	LMW[b]
5	39 ± 5[c]	60 ± 5
60	31 ± 3	68 ± 3
120	26 ± 2	73 ± 2

[a]Chelating agent:protein ratio 500:1; protein:metal ratio 250:1. [b]LMW = recovery in the low molecular weight fraction from the Sephadex G-50 column. [c]Standard deviation, mean of 3 estimations.

eluted with the protein at the void volume with only about 8% 'tailing' over into the region where the ^{238}Pu–chelator complexes eluted. With the reference chelator, LICAM(C), the situation was reversed for both proteins, about 90% of the ^{238}Pu being recovered in the low molecular weight fraction and only 7 to 9% with the protein, an essentially similar picture was obtained with DTPA. Of the six keto-hydroxypyridine compounds tested only one, 2,4-dihydroxy-pyridine-1-oxide (**L3**), showed an ability to release ^{238}Pu from both proteins which was comparable to that of LICAM(C) or DTPA, although the amino acid, mimosine which was shown previously to be effective in mobilising iron from transferrin [12] showed only a slightly reduced ability to release the radionuclide from its transferrin complex. With the other four substances tested between 26% (**L4**) and 63% (OM) of the ^{238}Pu remained bound to transferrin while the corresponding figures for ferritin were 26% (**L1**) and 86% (OM).

The rather weak ability of 1,2-dimethyl-3-hydroxy-pyrid-4-one to release ^{238}Pu from transferrin or ferritin may be compared with the results of Kontoghiorghes [13] who found this compound to cause the release of about 80% of the iron from diferic transferrin at longer incubation periods. In contrast 2,4-dihydroxy-pyridine-1-oxide was much more effective in removing ^{238}Pu than ^{59}Fe from either ferritin or transferrin in the present studies, Tables I and II. In discussing the difference between the chelators it must be recognised that in the case of ferritin the plutonium is being mobilised together with, and in competition with iron. The differences perhaps reflect the differences in the sizes of the Fe^{3+} and Pu^{4+} ions, 0.65 and 0.96 nm, respectively [6], the molecular structure of the binding sites of the chelators [12], the length of the incubation period and the site of chelator action.

The results of the limited study of the kinetics of ^{238}Pu release from ferritin, Table III, using mimosine indicate a much faster mobilisation curve than those obtained by Kontoghiorghes for iron and mimosine (unpublished observation) and similar chelators [14] probably because the plutonium is deposited on ferritin, at least under these *in vitro* conditions, in a more labile form than iron.

In comparing the abilities of the various compounds tested it should be emphasised that their metal binding capacities differ in that the pyridine chelators are bidentate whereas LICAM(C) and DTPA are octadentate. At pH 7.4 the former chelators bind iron in the ratio of 3 mol chelators to 1 mol iron [10] and the latter two with a minimum ratio of 1 to 1. Similarly, more mol of the pyridine chelators would be needed to form a complex with plutonium than the octadentate chelators under the same conditions. It is also important to remember that both the protein:metal and chelator:metal ratios differ widely for the two metals. The transferrin:iron ratio of 2:1 is about the same as that normally expected in blood plasma and the transferrin:plutonium ratio was selected to be representative of that which might be cxpected in the blood after an accidental contamination severe enough to necessitate treatment with a chelating agent. The chelator:protein ratio of 500:1 was selected on the basis of other studies *in vitro* which had shown that for DTPA and LICAM(C) this ratio promoted maximal mobilisation of ^{238}Pu from transferrin [15]. Further, at this chelator:transferrin ratio, the mobilisation of ^{238}Pu from an apo-transferrin complex and from rat serum, in which the transferrin was approximately half saturated with iron, was similar [15].

Competitive binding studies [16] have shown that iron and plutonium compete for the iron binding sites on the transferrin molecule and that, as for iron, two atoms of plutonium may bind to each transferrin molecule. It is not yet known whether plutonium binds equally to the N- and C-terminal binding sites on the transferrin molecule, or whether it shows a preference for one of the two sites. No spectrophotometric studies of the effects of chelators on the binding of plutonium to transferrin are available thus it is not yet possible to discuss the mechanisms of plutonium release from transferrin and to compare them with those for iron.

On the basis of the *in vitro* studies reported here, only two of the six pyridine compounds namely 2,4-dihydroxy-pyridine-1-oxide, mimosine and 1,2-dimethyl-3-hydroxy-pyrid-4-one show an ability to mobilize ^{238}Pu from transferrin or ferritin sufficient to justify studies in experimental animals.

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