PLASMA CLEARANCE, TISSUE DISTRIBUTION AND CATABOLISM OF CATIONIZED ALBUMINS WITH INCREASING ISOELECTRIC POINTS IN THE RAT

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SUMMARY

1. The intravenous injection of polycations produces acute renal failure and proteinuria, and an experimental disease very similar to disseminated coagulopathy. The purpose of this work was to investigate further, in the rat, the plasma disappearance rate, the tissue distribution and the catabolism of albumins with modified isoelectric points.
2. Human serumalbumin was cationized with hexanediamine and labelled with radioiodine.
3. During 10 to 180 minutes after their intravenous injection to the rat, these modified ^{125}I labelled albumins were cleared from the plasma at a rate which increased with their isoelectric point.
4. 1 and 3 hours after the injection of highly cationic proteins (isoelectric point higher than 9.5), the tissular protein bound ^{125}I concentration was the highest ($\sim$ 3.5 % of the injected activity / g) in the spleen and liver. A significant amount of the basic proteins was found in the kidney and in the lung (0.75 to 1 % /g). Their concentration was much lower in other tissues.
5. The whole body radioactivity was significantly lower 24 hours after the injection of ^{131}I labelled cationized albumins than of native albumin, independently of their isoelectric point. However, expressed as a percentage of the 24 h retention, the body radioactivity at later times was higher for cationic than for native albumin.
6. We conclude that cationized albumins are cleared from the plasma, mainly by the reticuloendothelial system, at a rate directly related to their isoelectric point. The cationized albumins are catabolized very rapidly initially, but a fraction of the injected protein remains in the body for a longer time than native albumin.

INTRODUCTION

Cationized albumins have been infused intravenously to the rat (1) or into the renal artery of the dog (Lambert P.P., Doriaux M., Sennesael J., Vanholder R., Lammens-Verslijpe M. : unpublished work) to test the hypothesis that the negative charge barrier of the glomerular wall plays a major role in the restriction to albumin filtration. Two conclusions could be drawn from these experiments : First, albumins with a basic isoelectric point (pI) have a higher urinary clearance than native albumin (1). Second, urinary excretion of native albumin was increased in animals who received cationized albumin in short term experiments. This increase of native albumin urinary clearance has also been observed when other polycations, with different structure and molecular weight such as protamine (2) or hexadimethrine (3) were infused. The animals developed a variable degree of renal failure. Lambert et al. infusing highly cationized serumalbumin into the renal artery of the dog, observed that the perfused kidney was the site of capillary obstruction by agglutinated red cells and aggregated platelets. Amorphous deposits were seen in the subendothelial space, and clusters similar to those described by Kanwar and Farquhar (4) after cationized ferritin infusion were seen in both laminae rarae of the glomerular basement membrane (GBM). A hemorragic syndrome developed, with a fall of the platelet count and plasma fibrinogen concentration, and the appearance of fibrinogen degradation products. The experimental disease had thus some common features with an intravascular disseminated coagulation or a hemolytic-uremic syndrome.

In long term experiments, it was shown that cationized antigens injected intravenously were deposited in the GBM and induced the development of an immune complex glomerulonephritis (5, 6).

Because of the increasing interest for the pathogenic role of polycationic substances, we felt useful to investigate in more details the fate of cationized albumins injected into the circulation, as a function of their pI. Using radiolabelled cationized albumins, we studied in the rat the short term (1 h to 3 h)

plasma clearance and organ distribution of cationized human serumalbumins with increasing pI. The long term disappearance rate of these proteins was assessed by whole body counting up to 20 days after their intravenous injection.

MATERIAL AND METHODS

Preparation of the cationized albumins

Human serumalbumin (Sigma, Fraction V) was cationized according to the technique described by Danon et al. (7). The degree of cationization was controlled by varying the pH at which the reaction was performed. Briefly, 3 g of serumalbumin were dissolved in 30 ml of distilled water and slowly added to 200 ml of a 2 M hexanediamine solution (Fluka AG) adjusted to the desired pH with 6 N HCl. 3 g of carbodiimide hydrochloride (Fluka AG) were added in 30 minutes; the solution was kept on ice to avoid heating. The addition of the activator was repeated after 1 h. The pH was controlled continuously and readjusted with 0.2 N HCl. After an overnight incubation at room temperature, the solution was dialyzed against distilled water for 72 h, with 6 bath changes. The retentate was lyophilized.

Labelling of the albumins

The native and cationized albumins were labelled with ^{125}I (short term studies) or ^{131}I (long term studies) using chloramide T and ICl (8, 9) (C. Leroy, I.R.E., Fleurus, Belgium). 3 to 4 mg of albumin were incubated during 3 minutes with chloramine T (15 µg/ml). ICl (1 iodine atom per protein molecule) and the radioiodine were added at a concentration of 10^{-4} M in a borate buffer (pH 7.2). The labelled protein was separated from free radioiodine on a Sephadex G 25 column and lyophilized in 0.15 M NaCl / 0.1 M phosphate buffer (pH 7.2). Each vial contained 0.25 mg of albumin with a specific activity of 0.5 to 1 mCi/mg (18.5 to 37 MBq/mg).

Characterization of the labelled and unlabelled albumins

The pI of the cationized samples was measured by isoelectric focusing on polyacrylamide gel (LKB Multiphor 2117), using ampholites PAG plates 1804-101 (pH

range 3.5 - 9.5). Four samples: A_1 (pI : 7.2 - 8.7), A_2 (pI : 7.9 - 9.5), A_3 (pI > 9.5) and A_4 (pI > 9.5) were obtained for a cationization pH of 8.2, 7.8 and 6.5 respectively. Samples A_3 and A_4 were further characterized by their precipitation pH in distilled water, which was found to be 10.8 and 11.0 respectively.

When chromatographed on Sephadex G 200, the cationized albumins eluted in two main peaks considered to represent monomers and dimers respectively. The percentage of monomers, estimated from surface measurements of the peaks, was 68, 77, 77 and 64 % for A_1 to A_4. After labelling, the percentage of monomer was unchanged for A_1 and A_2; it decreased to 67 and 51 % for A_3 and A_4. The Einstein-Stokes radius of the monomer, calculated from the position of the last peak, was 3.56 nm for native albumin and A_1, 3.63 nm for A_2, 4.20 nm for A_3 and 4.70 nm for A_4.

Plasma disappearance rate of the radioactivity

Thirty-two female Wistar rats weighting 200 to 300 g were anesthesized with Inactin (100 mg/kg, intraperitoneally). The jugular vein and carotid artery were catheterized using PE 50 tubing. Five i.u. of heparin sulfate (Novo) were given through the venous catheter. The bladder was catheterized with a PE 10 tubing.

The content of 1 vial of ^{125}I albumin was dissolved in 2 ml of distilled water containing 10 mg/ml of unlabelled protein of the same kind, and was dialyzed overnight against 0.15 M NaCl / 0.005 M phosphate buffer (pH 7.8).

Six 10 µl aliquots were pipetted to serve as standards for the plasma, urine and organ counting. Free ^{125}I of the injectate was measured on two of the reference samples as the activity of the supernatant after precipitation with 10 % trichloracetic acid (TCA). It was always found to be less than 5 %.

0.25 ml of the solution, i.e. 2.5 mg of albumin labelled with 15 to 30 µCi (0.55 to 1.10 MBq) of ^{125}I, were injected into the jugular vein. The syringe was weighted before and after injection. 3 to 400 µl of blood were drawn from

the arterial catheter into heparinized tubes 10, 30, 60, 120 and 180 minutes after injection. Two 20 µl aliquots of plasma were made to 1 ml with distilled water and counted in a Philips automatic gamma counter. Two 20 µl aliquots were precipitated with 2 ml of 10 % TCA; the precipitate was sedimented at 6000g and 1 ml of the supernatant was counted. The plasma protein bound ^{125}I concentration (PB ^{125}I) was calculated, in percentage of the activity (% IA) per ml, as :

$$PB\ ^{125}I\ (\%\ IA/ml) = \frac{50 \times 0.010 \times 1.00638 \times (cpm\ plasma - 2.02\ cpm\ supernatant)}{injected\ weight \times cpm\ standard\ (10\ \mu l)} \times 100$$

1.00638 is the density of physiologic saline (to convert the injected weight into an injected volume). The plasma radioactivity concentration was normalized for a body weight of 250 g.

The urine was collected throughout the experimental period to measure the total excreted radioactivity. The free ^{125}I was determined by TCA precipitation.

Tissue distribution of the albumins

Five animals injected with native albumin were killed at 1 h and five at 3 h. Four animals injected with A_1, three injected with A_2 and five injected with A_3 were killed at 3 h. Five animals injected with A_4 were killed at 1 h and five at 3 h.

The lungs, liver, spleen and kidneys were dissected free, rapidly washed with saline and blotted dry. Fragments of the organs were weighted and counted in a 1 - 2 ml geometry, while other pieces were immediately homogenized in 5 ml of cold 10 % TCA with an Ultra Turrax; 1 ml of this homogenate was centrifuged at 6000g in triplate, and the radioactivity of the supernatant and sediment was determined to calculate the percentage of free ^{125}I. For native albumin and A_4, the percentage of free ^{125}I was not determined for the animals used in the distribution study; it was measured, at 1 and 3 h after injection, in a separate experiment on eight rats (two animals per albumin and time).

The total and protein bound ^{125}I per gram of tissue was calculated in percentage of the injected activity. As the plasma radioactivity concentration remaining in the circulation at the time of death was very different for the native and the different cationic albumins, the results were expressed in two ways : First, as the apparent distribution volume of protein bound ^{125}I, that is the organ protein bound ^{125}I (% IA/g) divided by the plasma protein bound radioactivity (% IA/ml). Second, for cationized albumins, as the organ protein bound activity outside the apparent distribution volume of native albumins, that is, substracting from the tissue cationic albumin radioactivity, the product of its plasma protein bound activity by the apparent distribution volume of native albumin.

In two experiments with A4, one kidney and a fragment of the liver were homogenized in distilled water; the homogenate was centrifuged at 8200g to estimate the % of insoluble activity.

Binding of highly cationized albumin to the red cells

As it was shown in vitro that cationized albumins with a pI higher than 9.5 bind to the erythrocytes, we wanted to estimate the importance of this binding in vivo. The activity bound to the red cells was measured in six animals injected with A3. In two of them, 1 ml of blood taken 3 h after injection was centrifuged at 4000g; the cells were washed once with 1 ml saline, hemolysed with 1 ml distilled water and counted. In four rats, 400 to 600 µl of blood were drawn in tubes containing 0.5 ml dibutylphtalate and one drop of heparin sulfate at 10, 30, 60, 120 and 180 minutes. The blood was spun at 4000g for 15 minutes; the supernatant was removed and counted separately from the cells, allowing to calculate the ratio α of cell activity to plasma activity. This ratio α and the plasma activity concentration were then used to estimate, at each time, the percentage of the injected activity bound to the erythrocytes, that is α times the activity in the plasma of 1 ml of blood. The activity in the plasma of 1 ml of blood is that in 1 ml of plasma times the plasmacrit ($\sim$ 50 %).

Long term retention of radioiodine after the intravenous injection of ^{131}I albumins

Three groups of five rats were injected into a tail vein under light ether anesthesia with native ^{131}I albumin (group 1), ^{131}I-A1 (group 2) or ^{131}I-A3 (group 3). The amount of albumin and activity injected were the same as for the plasma clearance and organ distribution study. One rat had to be discarded in group 2 and in group 3 because of a partial paravenous injection. The rats received 0.05 % NaI in their drinking water to block the thyroid. The animals were counted between 2 NaI(Tl) crystals of a whole body counter (Nuclear Entreprise) at 15 minutes, 16, 24, 48, 72, 96, 120, 168, 264, 336, 432 and 480 h. The results were corrected for the physical half life of ^{131}I and expressed as the percentage of the whole body counts at 15 minutes (R_{15}) or at 24 h (R_{24}).

RESULTS

Evolution of the plasma radioactivity

From 10 minutes to 3 h after injection, the protein bound plasma radioactivity was significantly lower for all cationized albumins than for the native protein (Student's test). The plasma concentration was the lowest for the albumins with a pI higher than 9.5 . Between 30 minutes and 3 h, the plasma concentration could be adjusted with single exponentials for the five proteins (Fig. 1). The slopes of these exponentials, and their standard error, were calculated by a linear regression of the logarithm of the plasma activity concentration on the time (10). They were : -0.0036 $\pm$ 0.0007, -0.0065 $\pm$ 0.0005, -0.0124 $\pm$ 0.0014, -0.0139 $\pm$ 0.0018 and -0.0208 $\pm$ 0.032 min^{-1} for native albumin and for A_1 to A_4 respectively. Thus, after 30 minutes, the plasma disappearance rate of cationized albumins was still higher than that of native albumin. Free iodine concentration in plasma was less than 0.1 % IA at 10 minutes and reached a plateau around 0.45 % IA per ml at 1 h, for all injected albumins.

Urinary excretion of the radioactivity

The excreted radioactivity at 3 h varied from 1.4 % IA (SD : 0.8; n = 4) for A_3 to 7.4 % IA (SD : 4; n = 4) for A_1; the difference between the groups was not statistically significant. Free iodine accounted for more than 98 % of the urinary activity for cationized albumins and for 90 % of the urinary activity after native albumin injection. This high amount of non-proteic activity precluded any precise calculation of the proteins urinary clearances.

Organ distribution of the albumins

The apparent distribution volumes of native albumin were 0.10 to 0.12 ml/g for the liver, spleen and kidney, and 0.16 ml/g for the lung. As shown in Fig. 2, the apparent distribution of the protein bound ^{125}I in the organs increased with

increasing pI. No significant differences were found by Student's test between A_3 and A_4, both with a pI higher than 9.5.

The percentage of free iodine in the tissues 3 h after injection was the highest for native albumin (from 32.5 % in the spleen to 42 % in the kidney) and the lowest for the cationic albumins with a pI above 9.5 (10 % in the spleen and liver, 20 % in the kidney and 38 % in the lung).

The calculated protein bound ^{125}I outside the distribution space of native albumin also increased with pI (Table 1), with a step increase between A_2 and A_3. The highest tissular concentrations were found in the liver and spleen. Tissue protein bound radioactivity was lower at 3 h than at 1 h (Table 2).

When the liver or kidney of animals injected 1 h previously with A_4 were homogenized in distilled water and spun at 8200g, 60 % of the total radioactivity and 90 % of the protein bound activity were found in the sediment.

Activity bound to the red cells

After the injection of A_3, the ratio of red cell activity to plasma activity α increased from 0.23 $\pm$ 0.09 (n = 3) at 10 minutes to 0.53 $\pm$ 0.05 (n = 4) at 3 h. Assuming a plasmacrit of 50 %, one can thus calculate that roughly 0.10 % of the injected activity was bound to the erythrocytes of 1 ml of blood throughout the experiment. At 3 h, we also found 0.10 % of the injected activity bound to the washed sediment of 1 ml of blood.

Whole body retention of radioiodine after intravenous injection of radiolabelled cationized albumin

As compared with native albumin, a larger amount of both cationized albumins A_1 and A_3 was degraded during the first 24 h : R_{15} at this time was 0.50 $\pm$ 0.019 for native albumin, 0.21 $\pm$ 0.008 for A_1 and 0.26 $\pm$ 0.036 for A_3 (Fig. 3a). Thereafter, the degradation of cationized albumins was slower than that of native albumin, since R_{24} was at all times significantly higher for A_1 and A_3 (Fig. 3b). The long term behaviour of the two cationized samples was very similar, in spite of their different pI.

DISCUSSION

The faster removal of cationized albumins from the plasma can be attributed to several mechanisms : excessive urinary loss, increased escape to the extracellular space, binding to cells and basement membrane and intracellular uptake and degradation.

A higher urinary clearance of cationic albumins has been shown by Purtell et al. (1). In our experiments, most of the urinary radioactivity was free ^{125}I, and a precise estimation of the protein clearance was impossible. Nevertheless, our urinary data show that a higher urinary loss of cationized albumin was not responsible for its faster plasma removal, since the total ^{125}I urinary output was not different between the group, and the percentage of non-protein bound iodine was higher for cationic albumins.

There are few available data to support the idea of a higher capillary permeability for cationized than for native albumin. The only direct evidence of an increased transport of positively charged molecules across the capillary walls was obtained for the kidney glomerulus : DEAE dextrans (11) and cationized proteins (1, 12, 13) were shown to be filtered to a greater extent than neutral dextrans or native proteins with the same molecular size. These findings were attributed to the fact that basic molecules would cross more easily the negative charge barrier of the GBM (4, 11). These observations on the glomerulus cannot be extrapolated to non-fenestrated or diaphragm-fenestrated endothelia. Indeed, Pietra et al. have presented recently data showing that, in the lung, negatively charged dextran sulfate molecules were transported to the lymph more readily than neutral dextran molecules with the same radius (14). However, Michel and Turner have shown recently (15) that the osmotic force developed in frog mesenteric capillaries by succinylated myoglobin, which bears negative charges at the pH of the plasma, is higher than that induced by native myoglobin, which has an isoelectric point of approximately 8 (16); this is an indirect argument to think that the negatively charged proteins are retained more efficiently in the capil-

lary beds.

Even if an increased transport to the extracellular fluid is in part responsible for the higher plasma clearance of cationic albumins, it cannot be the only mechanism : indeed, the distribution volume of the cationized albumins with the higher pI was larger than that expected for extracellular fluid, and most of the protein bound radioactivity was found in a water insoluble fraction of the tissue homogenate. We must thus admit that cationized albumin is bound to cells or basement membranes, or is internalized into the cells.

Binding of cationic ferritin to basement membrane was found in the kidney glomerulus by Farquhar et al. (4). Lambert et al. found that cationized albumin was bound to the GBM in a similar fashion. Binding of the polycation polyethylene imine (MW 30000 - 40000) was observed in the basement membranes of the lung and choroid plexus capillaries, and in the lamina elastica of blood vessels by Schurer et al. (17).

Cell membrane binding of cationic probes has been shown to be a general phenomenon (18, 19, 20, 21). We have found that binding of cationized albumin to the erythrocytes was directly related to the pI (Vizet A., Bergmann P. : unpublished work), so that binding to cell membranes could account for the direct relation between pI and plasma disappearance rate.

Cationic probes such as cationized ferritin are rapidly internalized by endothelial cells (18) and by macrophages (19). The uptake of cationic albumin by cells of the reticuloendothelial system is suggested by the high concentration observed in the liver and the spleen. In this respect, cationized albumin would have a behaviour similar to that of heat denaturated albumin (22).

Eventually, cationic albumin binds to plasma proteins, especially fibrinogen, to form large electron dense amorphous complexes which were demonstrated in the subendothelial space of the kidney glomerulus (Lambert P.P. et al. : unpublished work). Such large complexes would also be more susceptible to macrophage uptake and degradation.

Once internalized into the cells, the cationized albumin is rapidly degraded accounting for the diminution of the tissue protein bound radioactivity between 1 and 3 h, and for the low 24 h whole body retention. However, a fraction of the cationized albumin not degraded at 24 h seems to be catabolized more slowly than native albumin. This could reflect either a decreased sensitivity to the action of proteolytic enzymes, which has been described for cytoplasmic proteins with a high pI (23), or trapping in sites not accessible to catabolism, such as basement membranes.

The very short intravascular half life of highly cationized proteins is liable to preclude the detection in the plasma of any cationic peptide which could be released by the cells and possibly play a role in some human diseases characterized by diseminated coagulation, unless it is continuously secreted.

TABLE 1

PROTEIN BOUND ^{125}I IN TISSUES (% IA/g) 3 H AFTER THE INJECTION OF CATIONIZED ALBUMINS (MEAN ± 1 SD) (THE ACTIVITY SUPPOSED TO BELONG TO THE DISTRIBUTION SPACE OF NATIVE ALBUMIN WAS SUBSTRACTED)

	A_1 (pI = 7.2-8.7) n = 4	A_2 (pI = 7.9-9.5) n = 3	A_3 (pI > 9.5) n = 5	A_4 (pI > 9.5) n = 5
LUNG	0.12 ± 0.15	0.15 ± 0.04^{A}	0.36 ± 0.24^{A}	0.75 ± 0.30^{B}
SPLEEN	0.24 ± 0.02^{A}	$0.56 \pm 0.17^{A,C}$	$3.05 \pm 0.52^{B,D}$	3.23 ± 0.40^{B}
LIVER	0.63 ± 0.04^{B}	0.61 ± 0.32	$3.39 \pm 0.32^{B,D}$	$2.87 \pm 0.61^{B::}$
KIDNEY	0.23 ± 0.04^{A}	0.28 ± 0.07^{A}	$0.93 \pm 0.22^{B,D}$	0.92 ± 0.13^{B}

SIGNIFICANTLY DIFFERENT FROM 0 (STUDENT'S TEST) : A : $P < 0.05$
B : $P < 0.01$

SIGNIFICANTLY DIFFERENT FROM THE ALBUMIN WITH THE IMMEDIATELY LOWER pI (STUDENT'S TEST) : C : $P < 0.05$ D : $P < 0.01$

:: n = 4

TABLE 2

EVOLUTION OF THE PROTEIN BOUND ^{125}I IN THE TISSUES (% IA/g) BETWEEN 1 H AND 3 H AFTER THE INJECTION OF A_4 (MEAN ± 1 SD)

	1 H	n = 5 Student's test	3 H
LUNG	1.01 ± 0.35	NS	0.75 ± 0.30
SPLEEN	4.51 ± 0.64	$P < 0.01$	3.23 ± 0.40
LIVER	4.20 ± 0.62	$P < 0.01$	2.87 ± 0.61 ⁂
KIDNEY	1.13 ± 0.20	NS	0.92 ± 0.13

⁂ n = 4

LEGEND OF FIGURES

Fig. 1. Plasma concentration of protein bound ^{125}I (of the injected activity per ml) after the injection of either native albumin (●) or cationized albumins (x : A_1 , pI = 7.2-8.7; o : A_2 , pI = 7.9-9.5; Δ : A_3 , pI > 9.5; □ : A_4 , pI > 9.5).

Fig. 2. Protein bound ^{125}I apparent distribution volume (ml/g) 3 h after the injection of either native albumin (A^-) or cationized albumins (A_1 , pI = 7.2-8.7; A_2 , pI = 7.9-9.5; A_3 , pI > 9.5; A_4 , pI > 9.5) a) in the lung and kidney; b) in the liver and spleen.

Fig. 3. Whole body retention of ^{131}I after the injection of either native albumin (●) or cationized albumins (x : A_1 , pI = 7.2-8.7; o : A_3 , pI > 9.5) a) expressed as a percentage of the activity immediately after intravenous injection, b) expressed as a percentage of the activity retained at 24 h (A_1 and A_3 then superpose).

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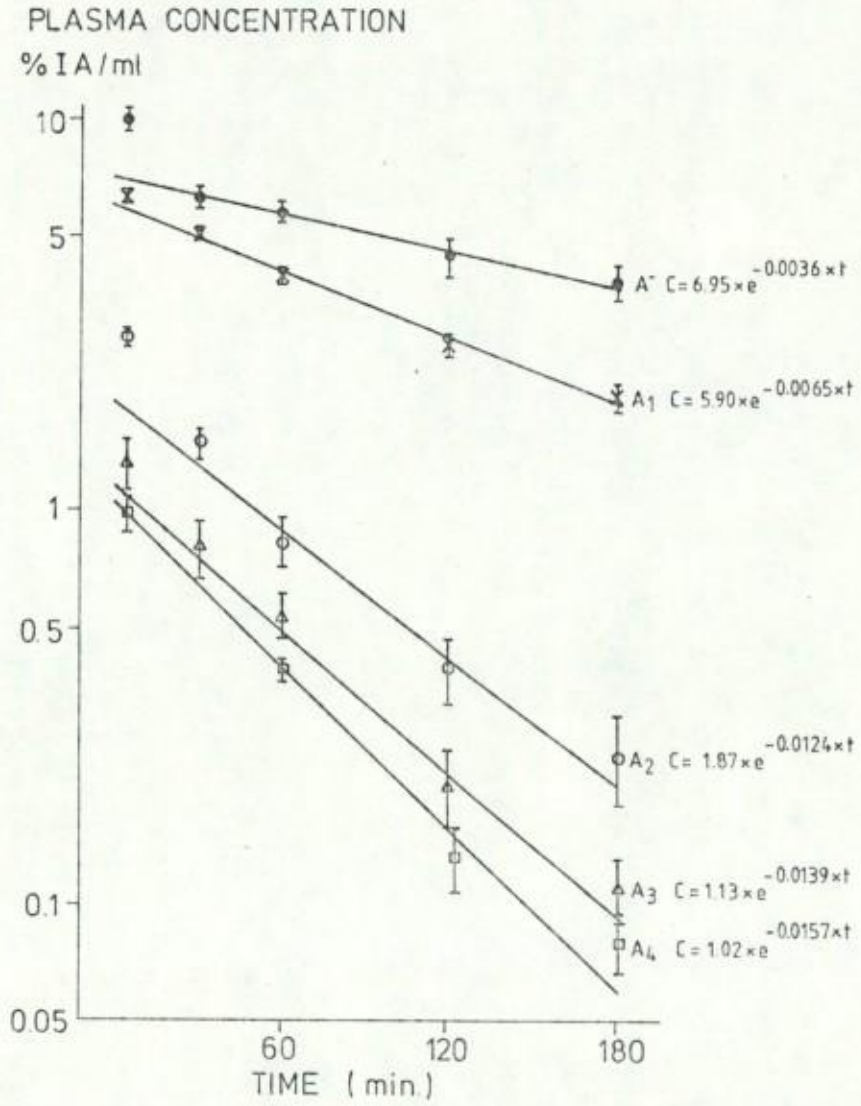
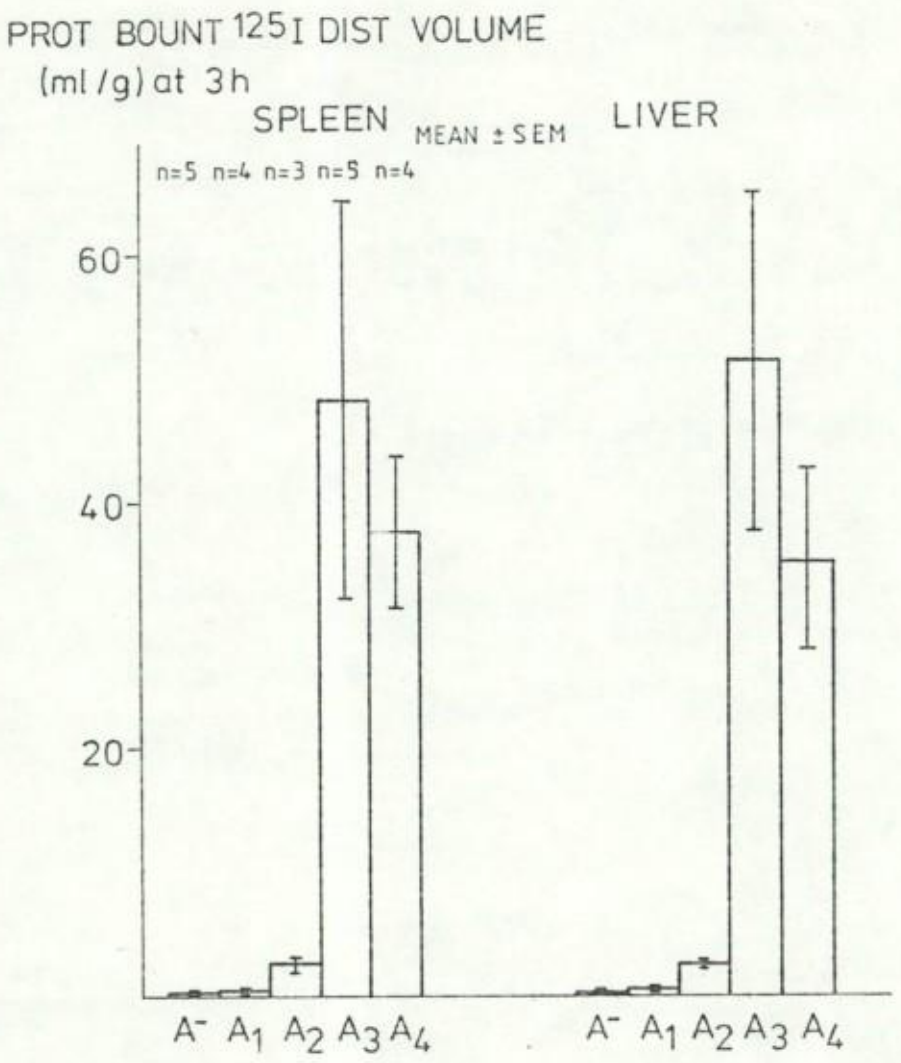
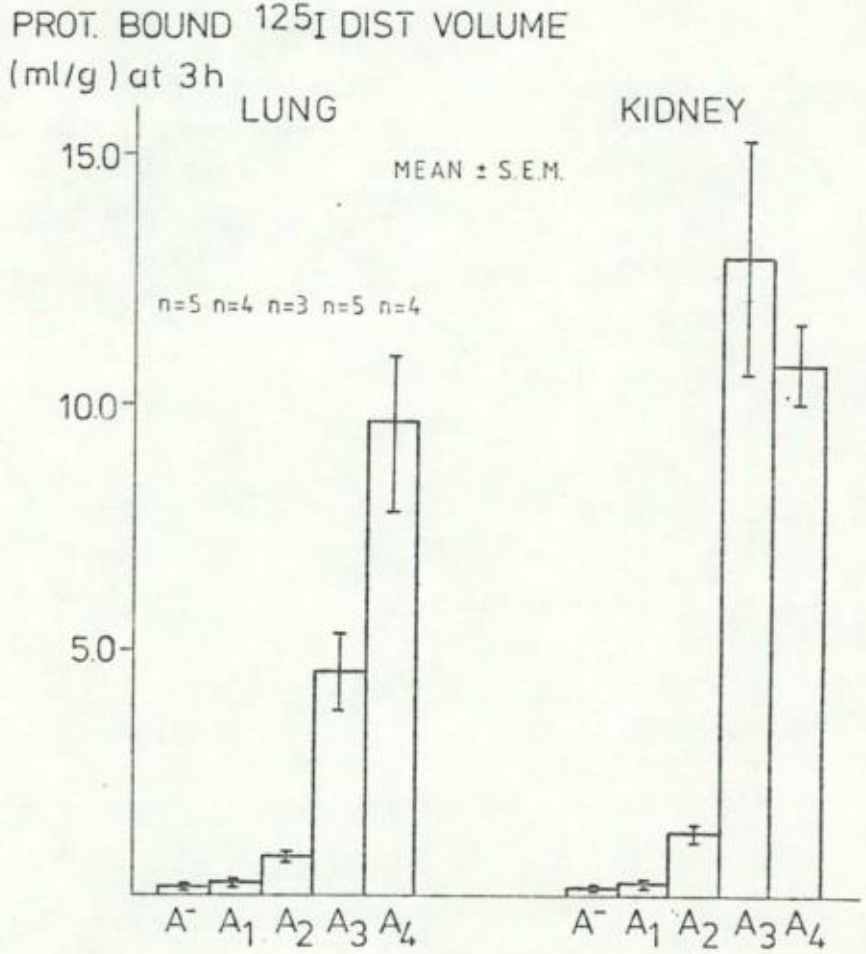

PLASMA CONCENTRATION
%IA/ml
10
5
1
0.5
0.1
0.05
60
120
180
TIME (min.)
A⁻ C=6.95×e^-0.0036×t
A1 C=5.90×e^-0.0065×t
A2 C=1.87×e^-0.0124×t
A3 C=1.13×e^-0.0139×t
A4 C=1.02×e^-0.0157×t

Fig 1

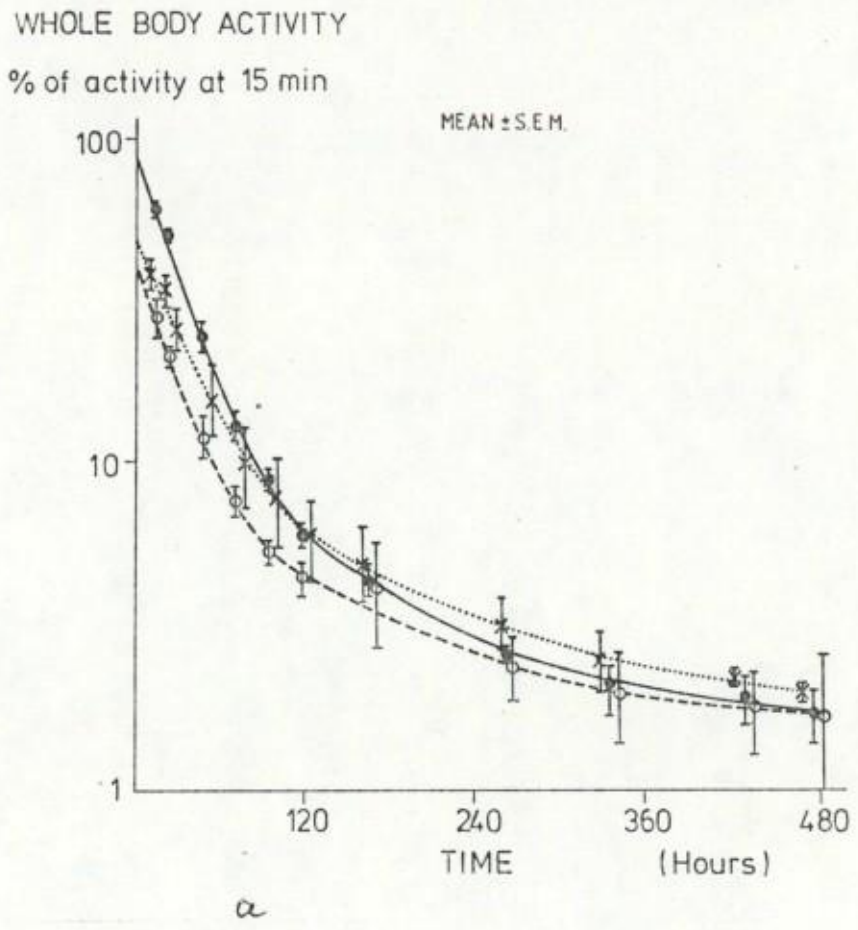

PROT BOUNT 125I DIST VOLUME
(ml/g) at 3h
SPLEEN
MEAN ± SEM
LIVER
n=5 n=4 n=3 n=5 n=4
60
40
20
A⁻ A1 A2 A3 A4
A⁻ A1 A2 A3 A4

b.

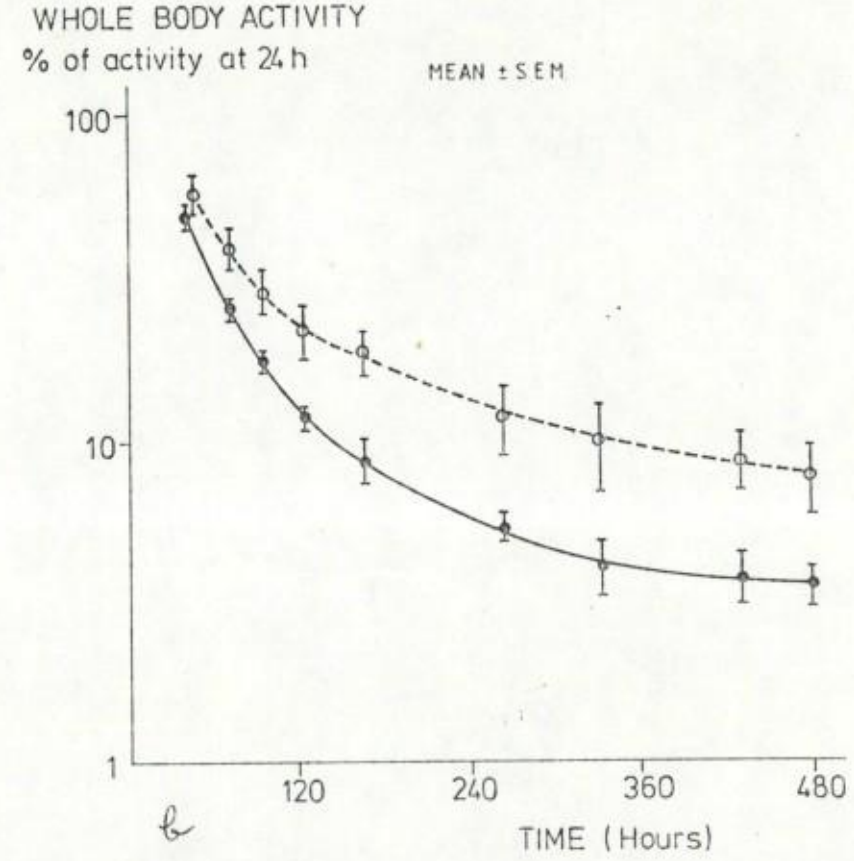

PROT. BOUND 125I DIST VOLUME
(ml/g) at 3h
LUNG
KIDNEY
MEAN ± S.E.M.
15.0
10.0
5.0
n=5 n=4 n=3 n=5 n=4
A⁻ A1 A2 A3 A4
A⁻ A1 A2 A3 A4

a.

Fig 2


WHOLE BODY ACTIVITY
% of activity at 15 min
MEAN ± S.E.M.
100
10
1
120
240
360
480
TIME
(Hours)

a


WHOLE BODY ACTIVITY
% of activity at 24 h
MEAN ± S.E.M.
100
10
1
120
240
360
480
TIME (Hours)

b

Fig 3