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ATOMIC WEAPONS RESEARCH ESTABLISHMENT

AWRE REPORT No. O - 37/66

The *in vitro* Distribution of Plutonium and Americium in Blood Serum

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The *in vitro* Distribution of Plutonium and Americium in Human Blood Serum

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SUMMARY

The binding of plutonium by transferrin has been demonstrated, whether the reaction is *in vitro* or *in vivo*. The binding of americium by serum proteins shows marked differences to plutonium binding. Distribution studies of the two actinides have been made between the protein and the non-protein components of sera. The effects of citrate ions on this distribution have been observed.

1. INTRODUCTION

The principal plutonium(IV)-binding protein of rat blood serum is transferrin [1, 2]. The serum used in the experiments described in those reports was prepared from rats which had been intravenouly injected with plutonium(IV) nitrate solution. Results reported below show the similarity of the distribution of plutonium in blood serum proteins whether the serum is prepared by *in vitro* or by *in vivo* methods. Consequently further experiments on plutonium distribution in sera have been carried out by *in vitro* techniques.

Bruenger and co-workers [3] have studied the *in vitro* distribution of plutonium(IV) in the proteins of dog blood serum. A partial resolution of the proteins was achieved, using standard methods of gel filtration [4]. It was shown that plutonium(IV) "was associated primarily with low molecular weight proteins consisting of albumin and a globulin". An earlier report [5] wherein albumin was said to combine with plutonium is judged to be erroneous, since no resolution of the albumin-globulin fraction was attempted.

Normally Am-241 is present in plutonium samples, being produced by the β decay of Pu-241. Accordingly it is of interest to compare the *in vitro* distribution of americium in blood serum proteins with that of plutonium. It has been shown that there is a difference in behaviour in the interaction of plutonium and americium with blood serum proteins.

Besides the plutonium which reacts with the proteins of sera, a smaller proportion reacts with other, low molecular weight substances present in sera. Experiments are recorded below showing the distribution of plutonium, and also of americium, between protein and non-protein components of sera, and how this distribution can be affected by the presence of competing substances such as citrate ions.

2. EXPERIMENTAL PROCEDURES

Human blood serum was used in all the studies recorded below. The serum was prepared by [redacted] of the Medical Division, AWRE. Blood was allowed to clot at room temperature, stood overnight at about 4°C, and then centrifuged. The serum was stored at -20°C until required.

A stock solution of plutonium nitrate in 2N nitric acid was heated to 65°C for 30 min with a few drops of "100-volume" hydrogen peroxide. This treatment ensured that the plutonium was in the tetravalent state. Using a capillary dropping pipette, 10 - 20 μl of the plutonium stock were added to about 5 ml of serum. The resulting mixture was kept at 0°C for 1½ h and then resolved by gel filtration through Sephadex G-200 and ion-exchange chromatography using the anionic diethylaminoethyl-Sephadex A-50. The elution pattern of the various proteins was similar to those previously reported [1] and to Figure 3 (G-200 separation) and Figure 6 (DEAE-Sephadex A-50 separation). In the absence of immunological techniques, the authenticity of the transferrin and albumin proteins was confirmed in two ways. One experiment utilised a synthetic mixture of human transferrin and human albumin (Kali pharmaceuticals). The mixture was fractionated by DEAE-Sephadex A-50 ion-exchange and the elution volumes noted. This experiment, an exact parallel of the routine separation of Fraction III, gave similar elution volumes to those of Experiments 6, 7 and 8. A second experiment utilised Fe-59 indicate the presence of the transferrin throughout the successive G-200 and DEAE resolutions of human blood serum proteins. The elution volume of the labelled transferrin compared closely with the elution volumes of transferrin in actinide-labelled experiments.

A small amount of α-globulin was noted in several DEAE-Sephadex separations; this protein was unabsorbed and manifested itself as a small diffuse peak before the transferrin was eluted. As shown in Figure 6, no radioactivity was associated with this peak. Gelotte and co-workers [4] showed that the two successive separation procedures used produced immunologically pure transferrin, but the albumin was admixed with α-globulin.

The plutonium content of eluate samples was determined by the method of delayed neutron counting following thermal neutron irradiation. The plutonium, supplied by the Radiochemical Centre, was free from other fissile elements, thus the delayed neutron counting method was highly specific for plutonium. The activity distributions are represented in Tables 1 and 2.

Using Sephadex G-50 as a medium for gel filtration, the distribution of plutonium in blood serum was determined. Sephadex G-50 excludes solute molecules having molecular weights greater than about 10000 [6]. Thus, the blood serum filtered through Sephadex G-50 produced by two fractions: a protein fraction and a diffuse fraction mainly consisting of low molecular weight sugars, steroids,

fatty acids and peptides. This type of experiment enabled one to determine the distribution of metal ions between high molecular weight proteins and low molecular weight non-proteins. This principle has been used to measure serum iron-binding capacities in clinical diagnoses [7]. The effect of citrate ions on the plutonium distribution in sera was also examined. Any plutonium/citrate complexes were eluted along with the low molecular weight components.

Some experiments were carried out without pretreating the plutonium stock solution with hydrogen peroxide. There was no apparent difference in distributional behaviour between treated and untreated plutonium. Subsequent experiments were done without treating the plutonium with peroxide.

The above series of experiments was repeated using americium instead of plutonium. The americium was a particularly pure batch obtained from the Radiochemical Centre. The americium was greater than 99.5% pure, measured by α spectrometry of Am-241. Further analysis [redacted], Chemistry Division) showed Pu-239/Am-241 = 0.00372 by atoms. Accordingly the americium radioactivity was characteristic of the Am-241 isotope. The thermal neutron fission cross section and the delayed neutron emission of Am-241 are very low compared with the values for Pu-239. Consequently delayed neutron emission counting following thermal neutron irradiation has too low an efficiency to be convenient for americium measurement. Hence a simple scintillation method was used. A measured aliquot of the eluate fractions was added to a weighed amount of copper-activated zinc sulphide scintillator in the form of a uniform layer in a squat-form 10 ml beaker. The beaker was stood in a pool of clear silicone oil on the upturned face of a photomultiplier tube. The system was then housed in a light-tight container. Following the decay of the light-induced luminescence of the scintillator, the scintillations due to the radioactivity of the aliquot were counted using conventional electronic equipment. Tests of this counting method shows it to be reproducible within ± 2 - 3%. Accuracy of counting was not of primary importance; simplicity was the main consideration.

In some experiments the activity of the Am-241 was measured by γ ray spectrometry. Samples were weighed into 3 ml test-tubes, evaporated to dryness and assayed in a well-crystal γ counter. Attempts were made to measure the Am-241 γ activity by passing the column effluents through a transparent plastic tube packed with finely powdered zinc sulphide or anthracene scintillator. The tube was taped on to the face of a photomultiplier. Unfortunately radioactivity became absorbed on the scintillator with the result that after the passage through the system of a radioactive solution a prohibitively high background count remained. The transparent scintillating plastic tube phosphor (Nuclear Enterprises NE102) proved to be insensitive to the amounts of Am-241 being used.

3. RESULTS

The distribution of plutonium in human blood serum proteins is depicted in Tables 1 and 2. The elution pattern of the proteins was similar to the patterns illustrated in Figures 3 and 6. At the G-200 gel filtration stage the recovery of plutonium was about 60%, suggesting that there may be a loss of plutonium by irreversible absorption on the dextran gel.

TABLE 1

Experiment 1. Delayed Neutron Counting of the Aliquots from the G-200 Separation of Human Blood Serum Proteins following the *in vitro* Addition of Plutonium(IV)

Aliquot Number	Net Count in 1 min
20	110
22	94
26	Nil
29	Nil
32	Nil
37	2186
39	2754
42	459
46	57
48	90

Fraction I: Aliquots 18 - 25 inclusive (macroglobulins, γ-globulins).
Fraction II: Aliquots 28 - 33 inclusive (γ-globulins, α-globulins).
Fraction III: Aliquots 37 - 45 inclusive (transferrin, albumin).

TABLE 2

Experiment 1. Delayed Neutron Counting from the DEAE-Sephadex Resolution of Fraction III, Table 1.

Aliquot Number	Net Count in 1 min
5	43
6	3006
7	2348
8	201
9	79
10	26
14	4

Transferrin: Aliquots 5 - 7 inclusive.
Albumin: Aliquots 8 - 9 inclusive.

The results recorded in Table 1 show that plutonium, added *in vitro* to serum, diffuses at the same rate as the proteins of Fraction III. In addition a small quantity of plutonium migrates along with the very highest molecular weight proteins of Fraction I. The further resolution of Fraction III by ion-exchange chromatography on diethylaminoethyl-Sephadex A-50 (Table 2) showed that the bulk of the plutonium was found in the transferrin fraction. Clearly the results of this *in vitro* experiment, using human blood serum, closely resemble the results of the *in vivo* experiments on rat blood serum previously reported [1, 2].

Mention has been made of the presence of a small amount of plutonium in a fourth, low molecular weight, fraction [1]. The results of experiments designed to measure the amount of plutonium combined with serum constituents other than proteins are listed in Table 3. Normally (Experiments 2 and 3) about 90% of the recovered plutonium was found in the protein fraction, and the remainder in the lipid fraction, but Experiments 4 and 5 with added citrate ions produced the inverse effect, practically no plutonium being found in the protein aliquots. Figures 1 and 2 furnish an example of each type of plutonium distribution.

TABLE 3

Experiments 2, 3, 4 and 5. Delayed Neutron Counting of Aliquots following the G-50 Resolution of Protein-Bound and Non-Protein-Bound Plutonium

Aliquot Number	Net Count in 1 min			
	Experiment 2 Added Citrate Conc. Nil	Experiment 3 Added Citrate Conc. Nil	Experiment 4 Added Citrate Conc. 0.001M	Experiment 5 Added Citrate Conc. 0.001M
2	7	0		
3	15000	0		
4	3050	112	106	1070
5	105	13700	278	2640
6	60	14600	45	793
7	104	1560	59	292
8	593	280	78	361
9	670	39	731	639
10	211	60	15400	3400
11	82	168	19200	12700
12	92	1790	6990	
13		952	1560	2500
14		61	918	
15	26	20	515	
16		8	445	57
17	23		303	
18			233	
19		22		36
20		6		
21		17		26
23		14	43	
25	18			
Protein aliquots	2 - 4	4 - 7	3 - 6	3 - 7
Lipid aliquots	8 - 14	10 - 16	9 - 16	10 - 20

The major components of the low molecular weight fraction are fatty acids and their cholesteryl esters, sugars, organic hydroxy-acids, amino acids and smaller amounts of inorganic ions. Of the substances present, citrate, amino acids and phosphate are potential complexing agents with actinides. Thus, the citrate concentration of blood is about 10^{-4} M and the amino acid content about ten times this figure. Experiments 2 and 3 demonstrate the partition of plutonium between the competing complexing agents transferrin and, probably, citrate. It is worthy of note that the plutonium complex of the low molecular weight fraction occurred in a fairly well defined band. A substantial portion of the light-absorbing components of the lipid fraction was free from plutonium activity (Figures 1 and 2). Experiments 4 and 5 demonstrate the effectiveness of citrate ions in removing plutonium from its transferrin complex. Even citrate at a concentration of only 10^{-3} M had a most pronounced effect on the *in vitro* distribution of plutonium.

Table 4 lists the results of measuring the distribution of americium in serum proteins; the close similarity to the distribution of plutonium (Table 1) is readily apparent. The results of Experiment 6 are illustrated in Figure 3. The small amount of activity associated with the macroglobulins of Fraction I (Figure 3) was too low to be represented on the figure, but can be discerned in the results in Table 4. The results of these two experiments are comparable, as the elution volume for the various fractions show.

TABLE 4

Experiments 6 and 7. ZnS Scintillation Counting of Aliquots from the G-200 Separation of Human Blood Serum Proteins following the *in vitro* Addition of Am-241

Aliquot Number	Experiment 6		Experiment 7	
	Cumulative Volume, ml	Relative Count Rate	Cumulative Volume, ml	Relative Count Rate
4	34.3	Nil	38	262
9			70.6	211
10			77.4	2110
11			83.4	769
12	70.2	595	89.8	545
14			103.7	913
15	78.0	182	111.2	352
16			118.6	1000
17			125.9	11500
19			140.5	51100
20	102.6	302		
22	113.3	583		
23	118.3	909		
24			171.9	7440
26	130.5	15200	181.7	4500
27			189.8	2320
28	137.4	47500	197.4	3890
29			205.1	8270
30			212.9	8720
31	147.9	53900	220.7	6280
33			235.3	1300
35	160.0	16100		
37	165.8	4310		
40			277.7	488
42	183.2	1270		
46	202.3	1620		
47	207.3	6360		
50	226.9	2360		
55	258.9	485		

The further resolution of the albumin and transferrin-containing fraction was achieved using DEAE-Sephadex A-50 ion-exchanger. The results are listed in Table 5. Results of Experiment 7 are presented graphically in Figure 4. It is seen that the distribution of americium in the proteins is quite different from that of plutonium in the proteins.

TABLE 5

Experiments 6 and 7. Scintillation Counting of Aliquots following DEAE-Sephadex Resolution of Fractions III.

Aliquot Number	Experiment 6		Experiment 7	
	Cumulative Volume, ml	Relative Count Rate	Cumulative Volume, ml	Relative Count Rate
4			23.1	151
5		495	26.4	405
6		466	29.6	271; 313
7		240	32.9	228
8		297	36.3	430
9		319	39.5	1060
10		387	42.9	1065
11		5370	46.1	1681
12		1600	49.5	1664
13			52.8	4418
14			56.1	5972; 6174
15			59.6	3236; 3421
16			63.1	3772; 3747
17			66.2	676
18			69.2	466; 503
22			82.7	274
24		200		
36		193		

To check the validity of the protein separations, an experiment was done with added plutonium and americium. The results are given in Table 6 and illustrated in Figures 5 and 6. The serum used in this experiment had been dialysed against the eluting buffer of the G-200 separation stage, before the two actinides were added. Thus, the low molecular substances were removed from the serum, and there was no Fraction IV "afterpeak". The difference in distribution of plutonium and americium is clearly shown in Figure 6.

TABLE 6

Experiment 8. Distribution of Plutonium and Americium in Serum Proteins following *in vitro* Addition; Resolution by G-200

Aliquot Number	Plutonium Delayed Neutron Count in 1 min	Am-241 γ Count, cpm/g
23	40	(17)
25	268	(41)
26	346	(82)
28	76	(67)
30	30	(59)
32	51	-
36	11	(73)
39	22	253
41	403	1600
43	2001	5900
44	3056	9900
46	6635	20540
48	5143	19400
50	831	12800
53	50	-

Table 6 clearly shows the similar diffusion rates of americium and plutonium through Sephadex G-200 in presence of human blood serum. As in Experiments 6 and 7, the bulk of the americium occurs in the albumin and transferrin-containing Fraction III. A minor point of difference between the plutonium and americium distributions is that a rather greater proportion of plutonium than americium is associated with the highest molecular weight proteins of Fraction I.

The preparation of the serum of this experiment had been accompanied by some haemolysis. The first portion of Fraction II (Figure 5) was distinctly pink in colour. This elution of the haptoglobin-haemoglobin complex corresponded to the observations of [redacted] [8]. No significant amounts of plutonium or americium were noted in the pink aliquots (38 - 40, Table 6). Whilst it is attractive to conclude that there is negligible binding of the actinides with haemoglobin it must be realised that the haemoglobin liberated by haemolysis was only a small proportion of the total in whole blood. [redacted] [9] showed that the binding of plutonium to the cellular matter of blood is very low. Accordingly the work recorded here has been confined to sera rather than whole blood.

TABLE 7

Experiment 8. Distribution of Plutonium and Americium between Albumin and Transferrin: Fraction III Resolved by DEAE-Sephadex A-50

Aliquot Number	Plutonium Delayed Neutron Count in 1 min	Am-241 γ Count, cpm/g
3	(3)	(6)
4	10	(3)
5	92	(6)
6	635	(10)
7	2429	48
8	3984	880
9	2834	3620
10	544	4990
11	81	2250
12	17	2030
13	16	4770
14	33	5330
15	63	4200
16	55	2500
17	26	413
18	-	119
19	21	-

Aliquots 2 - 4 inclusive: "7S" γ-globulins from Fraction II (?).
Aliquots 4 - 10 inclusive: Transferrin.
Aliquots 12 - 17 inclusive: Albumin.

While the plutonium separated largely in the transferrin fractions, the americium had a quite different distribution. It was remarkable that the fractions having a high specific activity of americium were low in protein content. Moreover three experiments (two of which are illustrated in Figures 4 and 6) showed the same qualitative behaviour but different quantitative behaviour.

The distribution of americium between the proteins and lipids of human blood serum was studied using the Sephadex G-50 gel filtration method similar to that described for plutonium. The results given in Table 8 are in a form analagous to that used for the plutonium results.

TABLE 8

Experiment 9, 10 and 11. Scintillation Counts of Aliquots following the G-50 Resolution of Protein-Bound and Non-Protein-Bound Americium

Aliquot Number	Relative Count Rate, cpm		
	Experiment 9 Added Citrate Conc. Nil	Experiment 10 Added Citrate Conc. 0.001M	Experiment 11 DTPA Conc. 0.001M
3			119
5			26
6	1990		304
7	1440	18	9660
8	4060		12000
9			2580
10	760	120	260
11		44	823
12	396	34	137
13	100	21	
15		16	453
17	43	22	
20	69	19	
22		274	
23		1890	
25		7800	
27		4480	
28		1800	
29	57		
30		656	
35	64		
45		35	
Protein Fraction Aliquots	4 - 13	7 - 17	2 - 5
Lipid Aliquots	29 - 34	23 - 39	7 - 12

The results given in Table 8 are similar to the results obtained with plutonium distribution studies, recorded in Table 3. Graphical representations of the results of Experiments 9 and 10 are closely similar to the plutonium distributions illustrated in Figures 1 and 2 respectively. It appears that in Experiment 9 (no added complexing agent), the proportion of americium bound to

protein rather than lipids is greater than the proportion of plutonium bound to protein.

4. CONCLUSIONS AND DISCUSSION

The results of Experiment 1 and previously published work [1, 2] show the similarity of plutonium distribution in serum, whether the admixing is done *in vivo* or *in vitro*. This finding enables experiments to be confined to *in vitro* techniques, thus dispensing with the need for animal experiments in work of the nature described in this report.

The excellence of the citrate ion as a competing ligand for removing plutonium and americium from their protein complexes has been established. In this respect citrate compares favourably with diethylenetriamine-penta acetic acid (DTPA) in the removal of americium from proteins. DTPA is of proven value in the removal of the actinides deposited in the body of rats [10, 11]. [redacted] [12] found that citrate produced a slight enhancement of the urinary excretion of plutonium soon after injection of the radioelement into rats. Unfortunately the short biological half-life of citrate ions in the body, and their metabolism on the surface of the bone limit the usefullness of citric acid as an effective treatment for decorporation of actinides from the human body.

The marked difference between americium and plutonium when albumin/transferrin fractions are resolved by ion-exchange is of interest. It may be that americium forms protein complexes weaker than those of plutonium, and they do not survive the ion-exchange procedure. When americium is subjected to elution through the DEAE-Sephadex ion-exchanger under conditions identical with those used in the second stage of Experiment 8, the americium elutes at a single broad peak at an elution volume correspondingly greater than the protein elution volume. This fact indicates that the americium of Experiment 8 must have been bound chemically to something at least initially.

Another possible explanation of the peculiar americium distribution is that there may be present, along with albumin and transferrin, other proteins in small amounts so that their optical absorption is obscured by the larger albumin and transferrin absorption. Such proteins could be responsible for binding americium.

Caeruloplasmin is a copper-binding α_2-globulin present in blood serum. To check whether or not this protein was influencing the americium distribution in serum, the elution volumes of caeruloplasmin were measured on G-200 and DEAE-Sephadex A-50 columns. In the former process the peak of the caeruloplasmin elution occurs near the trough between Fractions II and III, as noted by [redacted] [9]. Figure 5 clearly shows that the americium specific activity rises to a maximum in the region of the albumin/transferrin peak. This suggests that caeruloplasmin does not influence americium binding. When caeruloplasmin

is added to a transferrin/albumin mixture and subjected to DEAE-Sephadex A-50 ion-exchange chromatography under the conditions of Experiment 8, Figure 6, the caeruloplasmin elutes along with the albumin. This fact is a possible explanation of the americium in the second peak but does not explain the reason for the disposition of the americium in the region of the transferrin. In view of the limited evidence available it would be unwise to make too definite conclusions regarding the nature of the americium species present.

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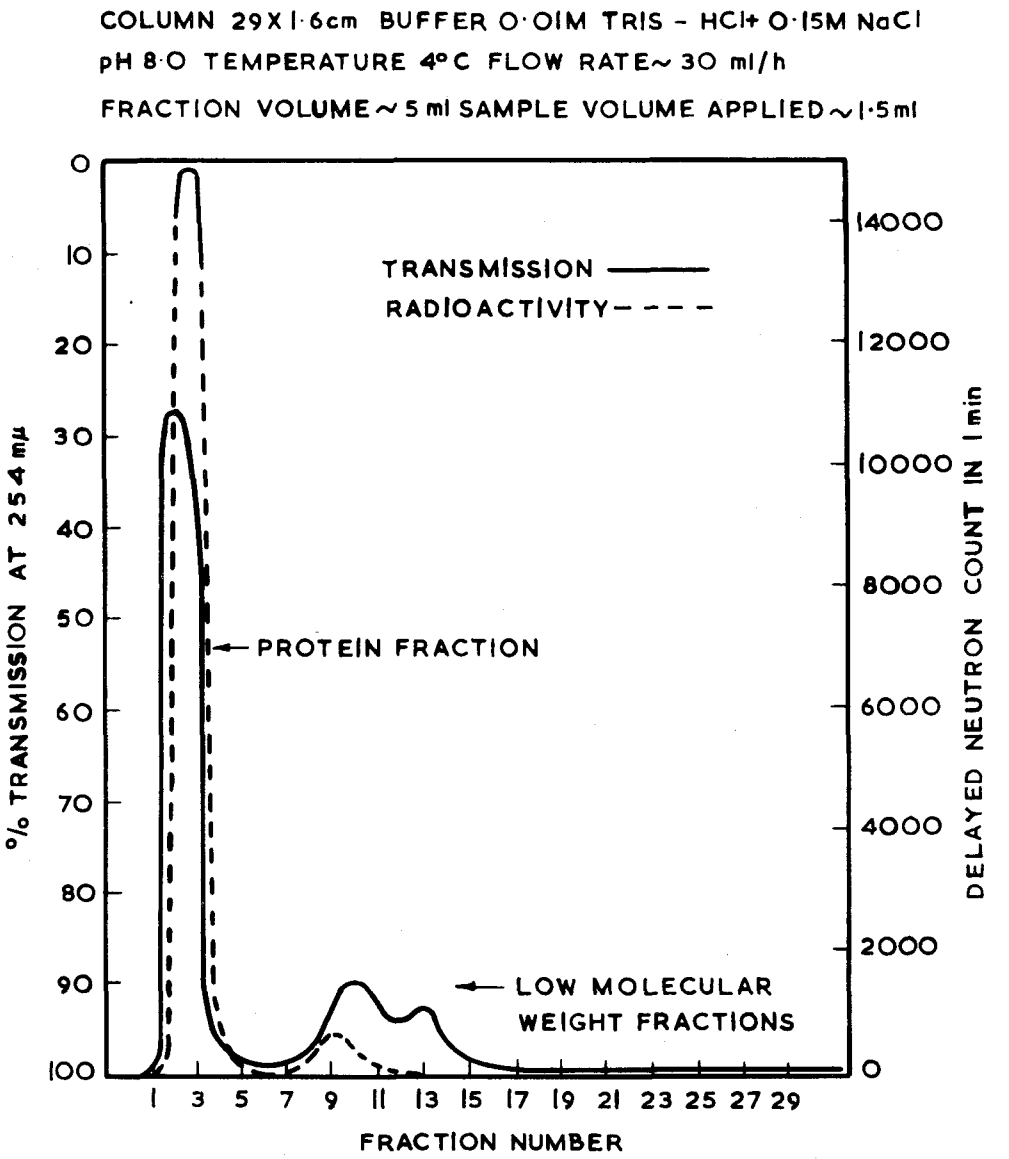

FIGURE 1. EXPERIMENT 2. SEPARATION OF PROTEIN-BOUND AND NON-PROTEIN-BOUND PLUTONIUM ON SEPHADEX G-50, CITRATE ABSENT

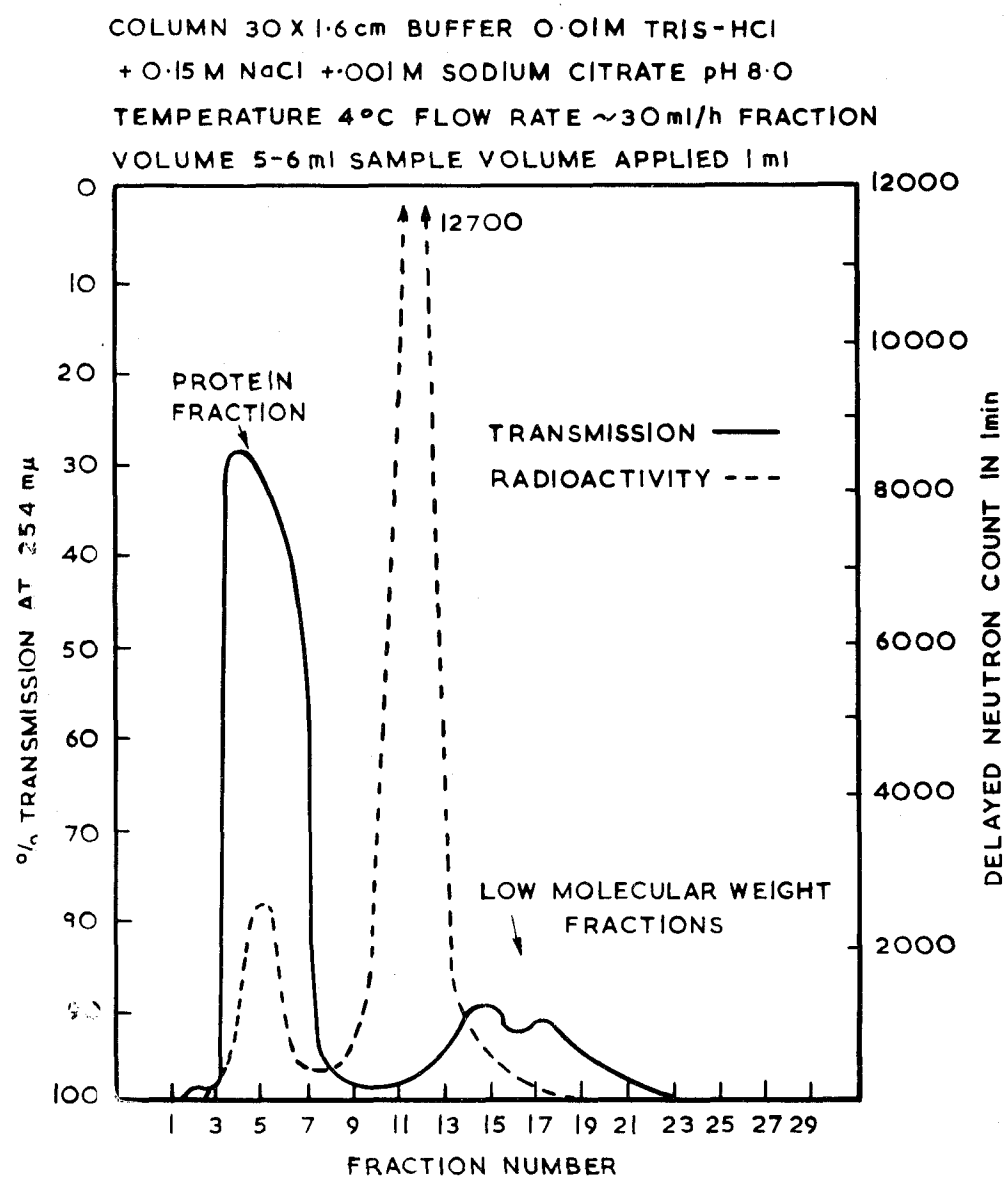

FIGURE 2. EXPERIMENT 5. SEPARATION OF PROTEIN-BOUND AND NON-PROTEIN-BOUND PLUTONIUM ON SEPHADEX G-50, CITRATE PRESENT

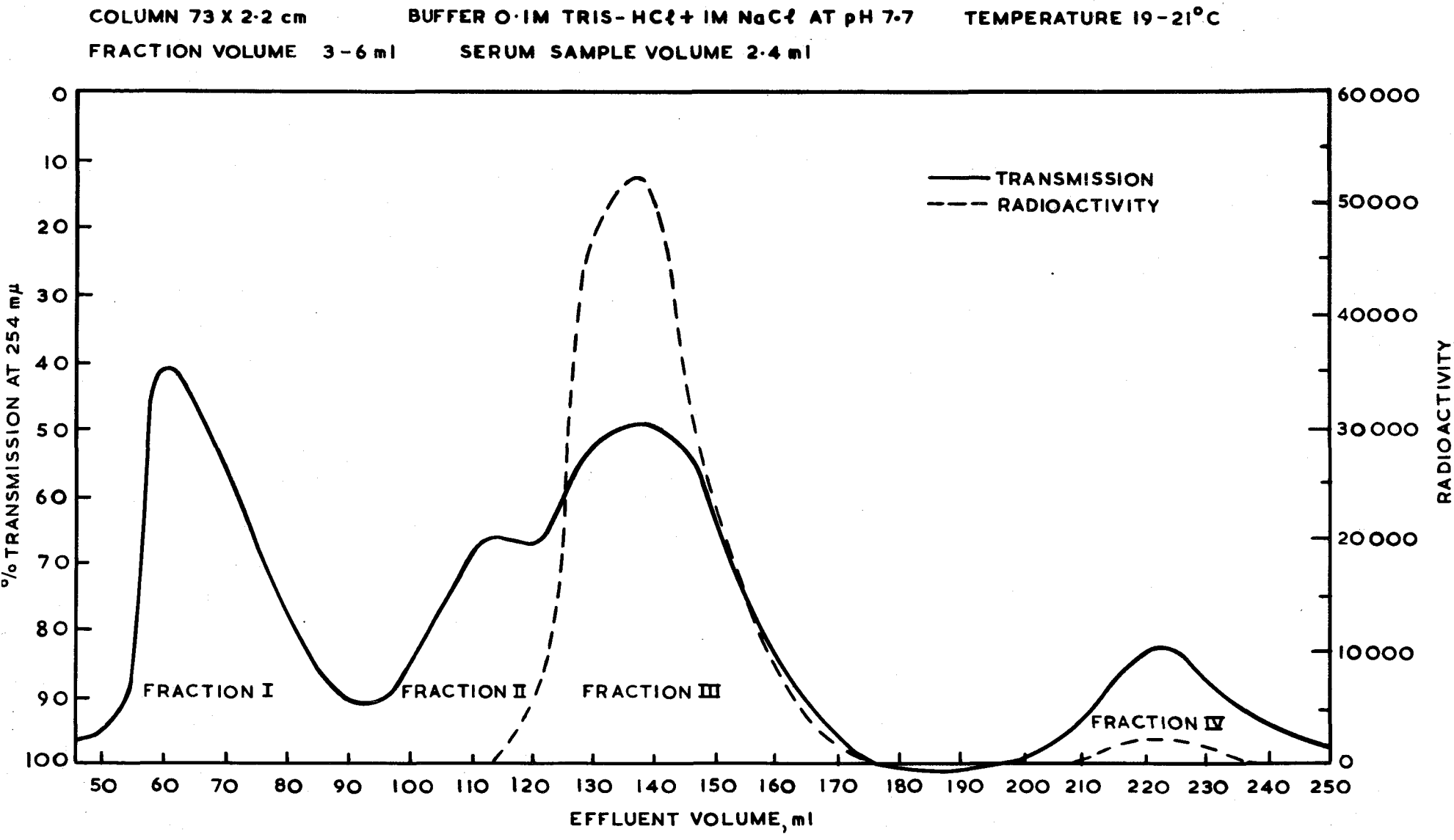

FIGURE 3. EXPERIMENT 6. DISTRIBUTION OF AMERICIUM FOLLOWING SEPARATION OF HUMAN BLOOD SERUM PROTEINS ON SEPHADEX G-200

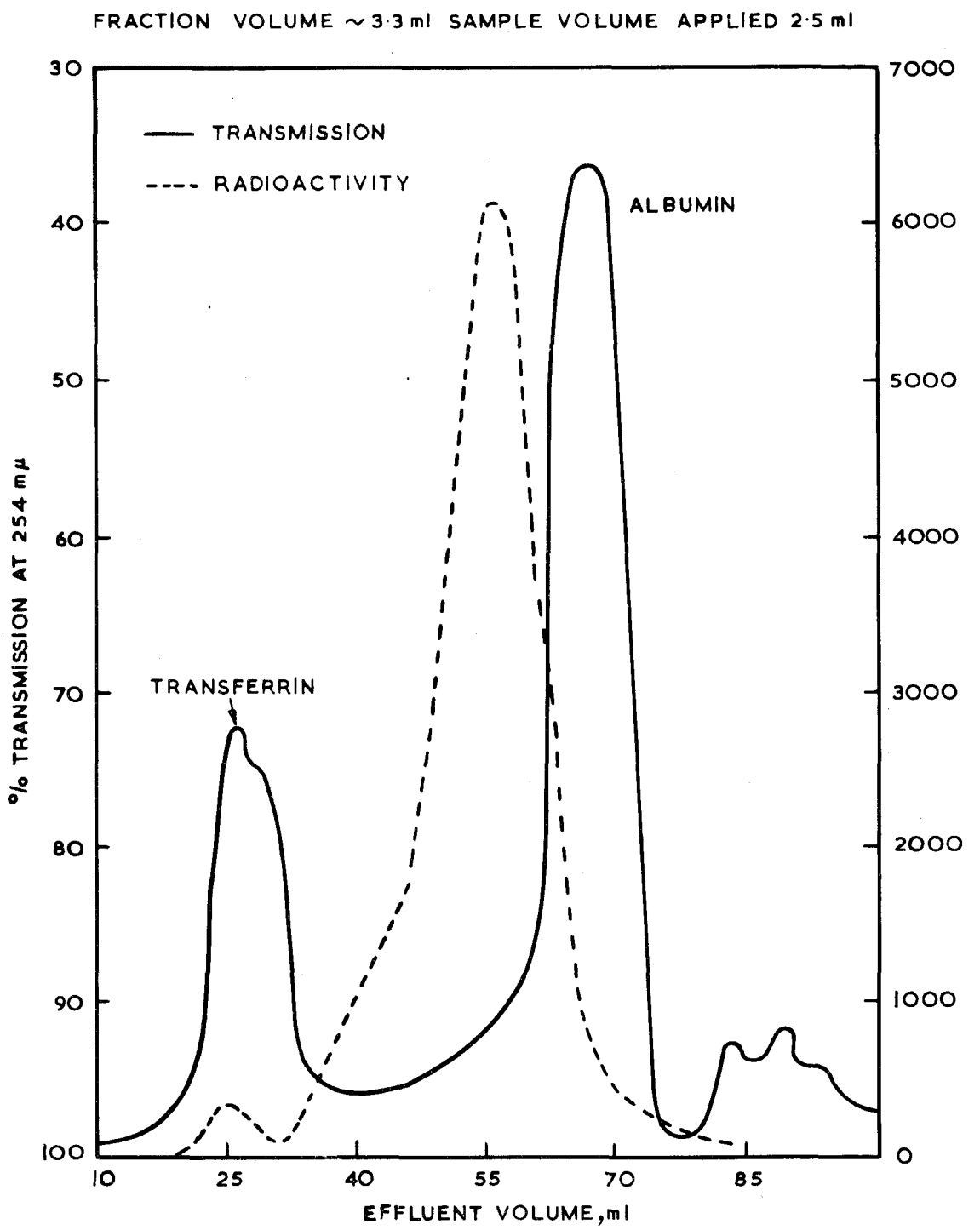

FIGURE 4. EXPERIMENT 7. DISTRIBUTION OF AMERICIUM FOLLOWING SEPARATION OF TRANSFERRIN AND ALBUMIN ON DEAE-SEPHADEX A-50

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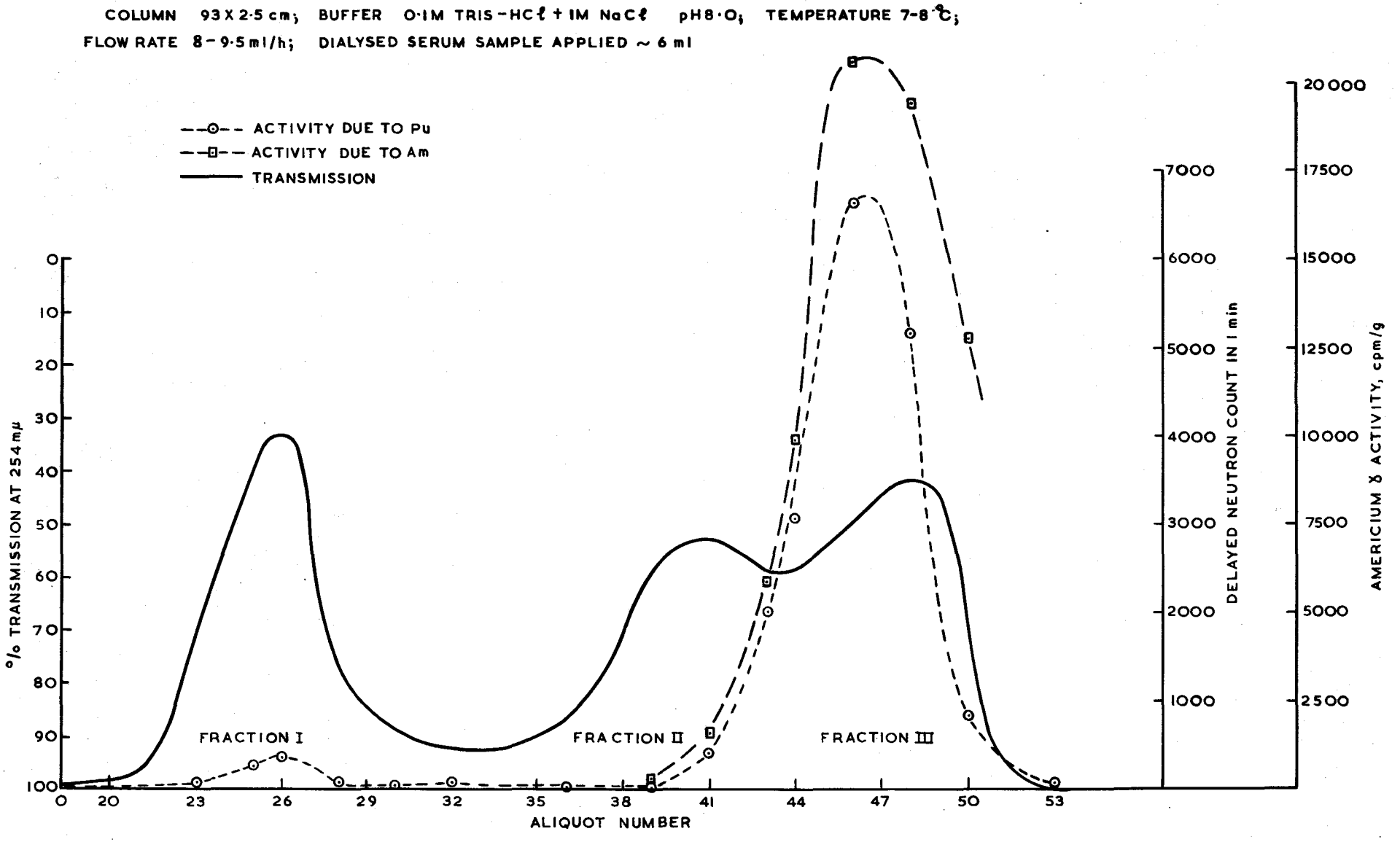

FIGURE 5. EXPERIMENT 8. SEPARATION OF HUMAN BLOOD SERUM PROTEINS ON SEPHADEX G-200

COLUMN 28 X 1·6 cm; BUFFER 0·1M TRIS-HCℓ, GRADIENT 0·1-2M NaCℓ, pH 8·0;
TEMPERATURE 7-8°C; FLOW RATE ~11·7 ml/h; FRACTION VOLUME ~4 ml;
SAMPLE VOLUME APPLIED 3·6 ml; T TRANSFERRIN; A ALBUMIN

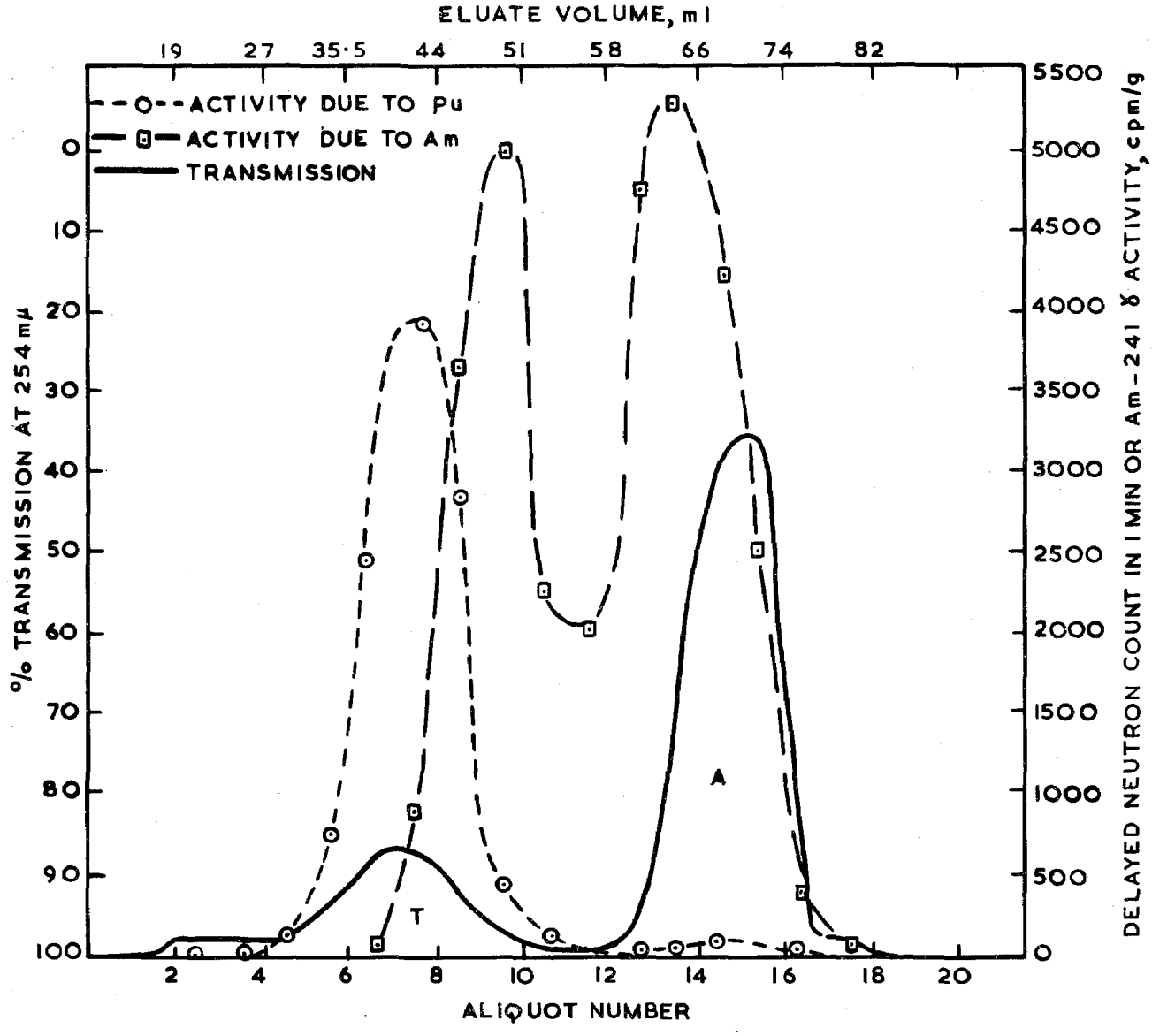

FIGURE 6. EXPERIMENT 8. RESOLUTION OF FRACTION III ON DEAE-SEPHADEX A-50

[redacted]
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