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The dual role of cadmium on leucine transport in human erythrocytes

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THE DUAL ROLE OF CADMIUM ON LEUCINE TRANSPORT IN HUMAN ERYTHROCYTES

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Summary

Cross-membrane transport of ^{14}C-leucine was measured by means of liquid scintillation counting. Both influx and efflux rates of Leu were determined by disappearance and exodus of ^{14}C-Leu into the external medium. A saturable and non-saturable components of Leu transport were observed in human erythrocytes.

Maximum uptake rate (Jmax) of Leu by the saturable component increased at low Cd^{++} concentrations between 5-20 ppm. The uptake by the non-saturable component was inhibited by Cd^{++}. Diffusion constant (Kd) and transport constant (Kt) of Leu influx decreased logarithmically as the Cd^{++} concentration was increased. The presence of intracellular Cd^{++} caused a decrease in Leu efflux rate; whereas, extracellular Cd^{++} had no effect.

In general, Cd^{++} appeared to affect the transport protein asymmetrically, depending on its presence at the inner or outer side of the membrane.

INTRODUCTION

World wide annual emissions of cadmium from natural sources (such as airborne soil particles, volcanic aerosols, forest fires, vegetation, etc...) were estimated at 8.4×10^5 kg (Nriagu, 1980). In the United states alone, the total amount of cadmium dispersed from anthropogenic sources is about 3.5×10^7 kg/year (U.S. EPA, 1975b).

The first major use of cadmium, as CdS in paint pigments, occured well over 60 years after its discovery in 1817. Current commercial applications of cadmium include electroplating, enamel pigments, plastic stabilizers, nickel-cadmium batteries, alloys, solar cells, luminescent materials, solders, and control rods of nuclear reactors. The combined annual discharge of cadmium in southern California's municipal effluents and San Francisco Bay has been estimated at 50 tons (Schafer, 1976) and 2 tons (CBE Report, 1983) respectively.

Cadmium is highly toxic to human beings (Gleason et al., 1969). During World War II, cadmium oxide fumes, which cause chemical pneumonitis, were considered as a potential weapon (Gafafer, 1964). Long-term human exposure to cadmium is associated with gastrointestinal disturbances (Hise and Fulkerson, 1973; Friberg et al., 1974; Friberg, 1978), lung insufficiency (Friberg, 1950; Smith et al., 1976), kidney impairment and proteinurea (Kazantzis et al., 1963; Lauwerys et al., 1974, 1976), osteomalacia (Adams et al., 1969; Pujol et al., 1970; Kazantzis, 1978), anosmia (Friberg, 1950) and yellowing of teeth (Princi, 1947). In addition, cadmium was shown to be a mutagen and

carcinogen in animals (Furst and Schlauder, 1977; Rivedal and Sanner, 1981; Rubin and Kroll, 1981; Takenaka et al., 1983), and a potential carcinogen causing lung and renal cancer in humans (Potts, 1965; Kolonel, 1976). In the diet, the highest concentration of cadmium is found in grain, cereals, oyster, crab and whale meat (Ishizaki et al., 1970; Reynolds and Reynolds, 1971; Fox, 1976). In the United States, an average diet contains about 50 ug Cd/meal (Duggan and Corneliussen, 1972). Cadmium is also present in cigarettes, which contain approximately 1 ug/cigarette when inhaled (Menden et al., 1972). The average daily intake of cadmium by Americans was estimated at 200-500 ug (Schroeder et al., 1967). Little evidence exists indicating that cadmium plays a physiological role in any organism. Recent data on rat growth rates suggest the possibility that cadmium may be an essential element for this species only (Schwarz and Spallholz, 1976). Intake of cadmium at a level of 10-20 ug/day has been shown to induce maximum cardiovascular changes. However, cadmium may be apparently inert at dosages of 50-100 ug intake/day due to the production of the detoxifying protein, metallothionein (Kopp et al., 1982).

Cadmium ion is presumably transported across the cell membrane by active transport mechanisms rather than by simple diffusion (May et al., 1977). It has been observed that the rapid clearance of Cd++ from plasma after venous injection resulted partly from its subsequent transfer to the rat red cells (Lucis et al., 1969). In one study with mice (Nordberg, 1972), maximum cadmium levels in red cells were reached within 4 hours. It has been suggested that cadmium may compete for binding sites with calcium or zinc-binding protein (Washko and Cousins,

1977; Frazier and Kingsley, 1977). Apparently no study has been done on the transport kinetics of cadmium in any mammalian cell system, nor have the effects of cadmium on the cell surface enzymes been examined.

Several membrane bound ATPases were found to be involved in the transport of inorganic ions (Barany and Barany, 1959; Stracher and Chan, 1961; Skou, 1963; Hille, 1968), which, in turn, also regulate cellular transport of small water-soluble metabolites. For example, the co-transport of Na+ and amino acids in human red blood cells (Spencer, 1969; Alvarado et al., 1970), and the transport of glucose in rabbit ileum (Goldner et al., 1969; Frizzell et al., 1973) had been observed. Previous studies on cells of brain, liver, and kidney in mammals indicated that Cd++ inhibited the Na+ ATPase by interacting with essential sulphydryl groups (Simon et al., 1947; Bader et al., 1970; Mustafa and Cross, 1971). It is likely that inhibition of membrane ATPase may interfere with the transport process.

Recent studies on the amino acid transport system in several marine organisms have indicated a reduction in the uptake rate of amino acids when divalent cations such as Cd++, Cu++, Mg++, Hg++ and Fe++ were present (Rice and Chien, 1977; Ellory et al., 1981; Viarengo et al., 1981; Lee et al., 1983; Chien et al., 1984). A similar decreasing trend in the uptake rate of glycine in marine mussels, Mytilus edulis, with increasing cadmium concentration was observed by Marelich et al. (1984). Cadmium exposure caused a decrease in the transport constant (Kt), indicating an increase in affinity between the transport site and glycine. At present, the nature of interaction between Cd++ and the amino acid transport components remains obscure.

The purpose of this study is to determine the kinetics of cadmium uptake, and to investigate the effect of cadmium on the transport of leucine into human erythrocytes (RBC). Five experiments were conducted: (1) The uptake kinetics of Cd++ by RBC. (2) Leu uptake rate was monitored in the presence of the extracellular Cd++ as well as (3) the Leu uptake affected by intracellular Cd++ in pre-loaded RBC. (4) Outward transport of Leu was also studied by observing the efflux of cellular Leu affected by extracellular Cd++, as compared to (5) the efflux of Leu in the presence of intracellular Cd++. ^{109}Cd and ^{14}C-Leu were used as radioactive tracers to monitor the subtrate concentration.

MATERIALS and METHODS

Freshly packed human red blood cells (RBC) were purchased from the Irwin Memorial Blood Bank (San Francisco, California). The RBC's were washed twice in isotonic solution (150mM NaCl, 15 mM Tris-HCl, and 5 mM glucose), centrifuged, and the buffy coat discarded. The red cells were stored in synthetic plasma (140 mM NaCl, 15 mM KCl, 20 mM Tris-HCl, and 5 mM glucose) at 4°C, and were used within one week. Before each experiment, erythrocytes were separated from the synthetic plasma by centrifugation at 2,000 g for 3 minutes.

Concentrations of substrates in the media were adjusted proportionally with added radio-isotope dosages. Therefore, both cold leucine (Sigma Chemical Company) and ^{14}C-leucine (50 uCi/ml) (Amersham UK) were used to prepare leucine solutions. Cadmium solutions contained both cold $CdCl_2$ and ^{109}Cd (123 uCi/ml) (Amersham UK). Synthetic plasma, leucine and Cd++ solutions were adjusted to pH 7.4 (at 37°C) with diluted HCl or NaOH. Iso-osmolality at various leucine and Cd++ concentrations was maintained by adjusting the concentration of NaCl in the media.

Uptake experiments (1, 2 & 3):

The uptake rate of leucine and Cd++ into the red cells was determined by measuring the decrease in radioactivity of the external medium, which contained either ^{14}C-leucine (.005 uCi/ml) or ^{109}Cd (.006 uCi/ml). A 500 ul suspension of washed erythrocytes (hematocrit = 77%) was incubated in media containing the appropriate concentration of

leucine (in leucine uptake experiments) or cadmium (in the cadmium uptake experiments) at 37°C. When necessary, RBC's were pre-loaded with Cd++ before incubation in leucine media. Incubation was stopped after 20-30 minutes by adding 1 ml of ice-cold n-dibutylphthalate (Ferreira and Lew, 1976) to the cell suspension, and immediately centrifuged at 7,000 g for 5 minutes. The decrease in radioactivity (cpm) from the media (above the n-dibutylphthalate layer) was determined as follows. A 200 ul sample from the incubating medium was added to 3.5 ml Scintisol (Isolab Inc) and analyzed with a Searle Analytic 6892 liquid scintillation counter (LSC). Uptake rates were reported in mole/liter cell water/hour.

Efflux experiment (4 & 5):

In the leucine exodus experiment, the cells were pre-loaded with either leucine (.012 mol/1) or both leucine and Cd++ (0-110 ppm). The extracellular leucine and Cd++ were removed by washing with isotonic buffer and efflux of leucine into the external medium measured. A 1 ml suspension of washed erythrocytes (hematocrit = 70% - 90%) was incubated in leucine and Cd++ solution from 10 to 60 minutes at 37 °C. The cells were then washed and centrifuged at 5,000 g for 2 minutes. The supernatant was removed by siphoning, and the inside wall of centrifuge tube was carefully wiped with absorbent tissue. The red cell pellet was re-incubated in normal synthetic plasma at 37°C. After 5 to 15 minutes, radioactivity of the incubating medium was measured by LSC.

Extraction of ^{14}C-leucine and ^{109}Cd:

Intracellular leucine and Cd++ were determined by measuring the radioactivity level within the RBC by lysing them in 1 ml of 5% Triton X-100 in water, deproteinizing in 2-3 ml trichloroacetic acid (50 g/1), and centrifuged at 7,000 g for 10 minutes. Hemolysate radioactivity was monitored by LSC. Blank values, the measure of radioactivity of interstitial fluid in packed RBC, were obtained by mixing cell samples with ^{14}C-leucine or ^{109}Cd at 0°C. The cells were quickly separated by centrifugation and the supernatant siphoned off. Net entry of leucine and Cd++ were calculated by subtracting the hemolysate radioactivity from the blanks. In uptake experiments, the mean recovery rate was 97% ($r^2 = 0.99$). Cellular Cd++ and leucine were determined by same methods for both influx and efflux experiments.

Determination of cell water:

Cell water contents were estimated by drying the pellet of red cells to constant weight in an aerated oven at 110°C. Mean values of cellular water were found to be 52% of cell weight.

RESULTS

Saturable and non-saturable constituents were observed in Cd++ and leucine transport by human erythrocytes (Fig. 1a,1b,2). The mean, maximum uptake rate (Jmax) of Cd++ was 3.4×10^{-4} mol/l/h. At 0 ppm Cd++ exposure, the mean Jmax of leucine influx was 3.8×10^{-4} mol/l/h. When extracellular Cd++ was 5 and 20 ppm, Jmax of Leu uptake increased 116% and 122% respectively (Fig. 3). However, at 50 ppm Cd++ Jmax differed little from the control value (Fig. 3).

The uptake rate (J) of leucine at physiological concentration in serum (2×10^{-4} M) was 3.1×10^{-4} mol/l/h. At 5 ppm of intracellular Cd++, leucine uptake rate was increased to 151% as compared to an increase of J to 244% when Cd++ was present extracellularly (Fig. 4). Above 5 ppm Cd++, the uptake rate decreased with increasing extracellular Cd++. The reverse relationship was observed when intracellular Cd++ was increased (Fig. 4).

In the non-saturable system, the entry rate of Cd++ and leucine increased linearly with increasing substrate concentration (Fig. 1a,1b,2). The mean values of the diffusion constant (Kd) and transport constant (Kt) of leucine influx are listed in Table 1. Kd and Kt of leucine transport decreased logarithmically with increasing extracellular concentration of Cd++ (Fig. 5a,6).

The mean efflux rate of leucine in pre-loaded red cells (0.012 mol/l Leu) was 6.4×10^{-7} mol/h. Efflux rate remained constant despite the increases in external Cd++ (Fig. 7). However, efflux rate decreased logarithmically as cellular Cd++ concentration increased (Fig. 7). The greatest drop in efflux rate (67%) occured near the 5

ppm Cd++ level. Above this level, efflux rate decreased slowly (Fig. 7).

DISCUSSION

Two different modes of transport, saturable and non-saturable, of amino acids have been reported for human, sheep, and rabbit erythrocytes (Winter and Christensen, 1964; Young et al., 1980). The present study on leucine uptake supports the presence of these two distinctive uptake pathways in human red blood cells (Fig. 2). The influx of cadmium also demonstrated similar characteristics (Fig. 1). Since the saturable components of both the leucine and divalent Cd++ transport system showed a limited transport rate (Jmax), they could operate according to the carrier-mediated model in which the substrate molecule forms complex with a carrier-protein and, then, is translocated through the bilipid-layer membrane. However, its non-saturable component behaved more like a channel-mediated structure through which the substrate was translocated. Jmax value can not be determined for this mode of transport. The relationship between the uptake rate and substrate concentration of the non-saturable component was linear. Its diffusion constant (Kd) was estimated as the slope of the uptake curve (Table 1).

In the presence of extracellular Cd++, Jmax of leucine uptake initially increased at low Cd++ concentration, but gradually decreased to the level of the control when Cd++ concentration approached near 50 ppm (Fig. 3). This finding contradicts earlier reports on marine invertebrates (i.e. Rice and Chien, 1977; Viarengo et al., 1981; Lee et al., 1983) that Cd++ reduces the amino acid uptake rate. It is possible that Jmax of leucine uptake in human erythrocytes may decrease

even further at Cd++ levels above 50 ppm. However, the uptake rate of leucine at physiological concentration in serum (2 x 10^{-4} mol/l Leu) did not fall below the level of controls when the cells were exposed to 100 ppm Cd++ (Fig. 4). As has been suggested by several reviewers (Vallee and Ulmer, 1972; McAuliffe and Murray, 1972; Friedberg, 1974; Blundel and Jenkins, 1977), Cd++ reacts strongly with proteins, enzymes, and a wide range of other biological molecules. The enhancement of leucine uptake may be due to (1) Cd++ directly interacts with the transport protein and makes it more efficient. (2) The presence of Cd++ indirectly results in a secondary effect on the transport component, such as complexing with glutathione (Li and Manning, 1955; Rabenstein et al., 1983). Such a mechanism has been postulated to function in the transport of amino acids (Young et al., 1976). And (3) the availability of new transport sites is considered.

It has been postulated that Cd++ is attached to thiol groups in glutathione and cysteinyl residues; to imidazole groups in histidinyl residues, and also to keto and phosphate groups. Cadmium interaction probably induces changes in structural conformation and affects the reactive site of the transport protein (Cherian and Nordberg, 1983). As was pointed out, the Na+ ATPase is inhibited by Cd++ at the sulphydryl group(s) (Simon et al., 1947; Mustafa and Cross, 1971), and the co-transport of Na+ and amino acids is affected. However, the uptake rate of leucine was not reduced because leucine transport in human erythrocytes is not Na+-dependent (Young et al., 1980). Therefore, different effect in the uptake rate of Leu was observed, contradicting other results of previous studies with systems which may

be Na+ sensitive. The opposite effect occured as low Cd++ concentration caused an increase in the Leu uptake rate (Fig. 3). According to Wang and Gilpin (1983), several amino acids form complexes with Cd++ (e.g. octahedral complex between three molecules of glycine and a cadmium ion). Thus, uptake of leucine would be more efficient when the Leu-Cd++ complex was transported by a second, saturable system.

Both intracellular and extracellular Cd++ increased the uptake of leucine; except the uptake rate further increased as intracellular Cd++ increased (Fig. 4). Hoare (1972), in his study of leucine transport in human erythrocytes, found: (1) a single carrier system mediates both the exchange and net fluxes of leucine, (2) that its binding to the carrier increases the rate of carrier re-orientation, and (3) the carrier is, at equilibrium, about equally distributed between the two sides of membrane. Hence, the uptake rate of leucine increased, it is assumed that the re-orientation of the carrier on the outer membrane is much faster than that of the carrier-subtrate complex at the inner side, and intracellular Cd++ is supected to induce such an effect by interfering with this orientation process. As a consequence, Cd++ may have shifted the equilibrium and availability of the carrier toward the extrinsic side of the membrane.

The affinity between the carrier protein and its substrate in the saturable system increased significantly when Cd++ was present (Table 1). This effect may be caused by conformational change of the transport protein as it interacts with Cd++ as suggested by Rice and Chien (1977). However, it is possible that the new Kt value(s)

describes the affinity of a different transport system, as suggested earlier where the Leu-Cd++ complex was being translocated. In this case, this new Kt value(s) was not affected by Cd++ and remained constant despite the increase in Cd++ level (Table 1). A similar effect was observed for the diffusion constant (Kd) of leucine influx in the non-saturable pathway. Possible chelation between amino acids and Cd++ (Wang and Gilpin, 1983) may explain the decrease of Kd (Table 1); and the Leu-Cd++ complex could not migrate through the same channel as did leucine. Instead, this complex species may have entered the red cells via a different route that possessed a lower Kd characteristic. However, data from the efflux experiments discounted this possibility.

It can be argued that Leu-Cd++ complex could exit by the same channel that it uses to diffuse into the cells. Yet, efflux rates of pre-loaded leucine decreased as intracellular Cd++ was increased. Again, similar to leucine influx, the reduction in efflux rate may have been due to the chelating process, which was absent when Cd++ was only present outside the cells. Under such conditions, the efflux rate of cellular leucine remained constant regardless of the increasing extracellular Cd++ level.

Cadmium acts both as a stimulant and repressor in leu uptake; at low Cd++ concentration the uptake of Leu was enhanced (Fig. 3,4). As the Cd++ concentration was increased, the Leu uptake rate levelled off and gradually decreased to the level of control (Fig. 3). Hence, we observed an inversion effect known as hormesis (Furst, 1985). Furthermore, earlier data from a time exposure experiment (Malerich et al., 1984) reported that the inversion effect of Cd++ on the amino acid

uptake appeared to be dependent on Cd++ dosage and not on time exposure to Cd++.

The experimental results indicated that Cd++ reduced the Leu transport by the non-saturable component. However, at physiological leucine concentration in serum this system contributes very little to the total Leu uptake (Fig. 2), as compared to the saturable system alone. Since low Cd++ level stimulated the enhancement of the saturable pathway, the efficiency of Leu uptake was not seriously affected. Our data suggested that short-term exposure to 20 ppm Cd++ or less is unlikely to interfere with the Leu uptake in human erythrocytes. Further studies should be done to elucidate the mechanism and nature of interactions between cadmium and the leucine transport components.

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TABLE 1

Kinetic parameters describing the uptake of leucine by human erythrocytes at various Cd++ levels.

[Cd] (ppm)	Jmax (mol/l/h)	Kt (mol/l)	Kd (1/h)
0	$(3.8 \pm .9) \times 10^{-4}$	$(1.0 \pm .2) \times 10^{-4}$	5.8 ± 1.3
5	$(8.1 \pm .7) \times 10^{-4}$	$(2.8 \pm .2) \times 10^{-5}$	$3.8 \pm .9$
20	$(8.3 \pm 1.5) \times 10^{-4}$	$(2.4 \pm .4) \times 10^{-5}$	$2.0 \pm .7$
50	$(4.3 \pm .7) \times 10^{-4}$	$(2.9 \pm .5) \times 10^{-5}$	$1.9 \pm .5$

Jmax and Kt values of the saturable component were calculated according to the Lineweaver-Burk linear transformation. Kd values of the non-saturable component were estimated as the slopes of the diffusion curves. Each value represents the mean + its standard deviation (n=5).

LEGENDS

Fig. 1. A: The uptake rate of Cd++ by human erythrocytes at different extracellular Cd++ concentrations . The uptake curve describes the influx of Cd++ by the (Y) saturable component with the mean Jmax = $(3.4 \pm 1.2) \times 10^{-4}$ mol/l/h and the mean Kt = $(1.1 \pm .4) \times 10^{-4}$ mol/l, and (D) non-saturable component with the mean Kd = 2.8 ± .7 1/h. The zig-zag axes represents the condensed scales. B: The uptake curve has been resolved into the (Y) saturable and (D) non-saturable component. E represents the transport of Cd++ by both components. At Cd++ concentration below 1×10^{-4} M, difussion of Cd++ across the membrane may not occur.

Fig. 2. The uptake rate of leucine in human erythrocytes at various Cd++ levels by the (Y) saturable component and (D) non-saturable component. Below physiological leucine concentration in serum (2×10^{-4} M), Leu uptake in the control occurred mainly by the saturable component. The non-saturable component may not function below 2×10^{-4} M Leu. In the saturable pathway Cd++ generally enhanced the Leu uptake rate. Increasing the Cd++ concentration inhibited the non-saturable component. This could be demonstrated as change in slope (Kd) of the uptake curves (Fig. 5a). The zig-zag axis represents a condensed scale.

Fig. 3. Maximum uptake rate (Jmax) of Leu at various Cd++ concentrations.

Fig. 4. Leu uptake at the physiological Leu concentration (2×10^{-4} M) in synthetic plasma at various Cd++ concentrations. Mean uptake rate of the control (at 0 ppm Cd++) was $(3.1 \pm .8) \times 10^{-4}$ mol/l/h.

Fig. 5. A: Diffusion constant (Kd) of the non-saturable component for Leu transport at various Cd++ concentrations. B: Analysis of Leu uptake by the non-saturable component, according to the procedure of Akedo and Christensen (1962). Distribution ratios (c.p.m./l cellular water : c.p.m/l external medium) are plotted against 1/[Leu], showing different intercepts for the Leu uptake curves at various extracellular Cd++ concentrations. If Cd++ exerts no effects on the Leu uptake, all uptake curves should intercept y-axis at a same point. The experimental results showed decreasing intercepts, which indicated Cd++ inhibits the non-saturable component and vary its diffusion constant, Kd.

Fig. 6. Transport constant (Kt) of the saturable component for Leu transport at various Cd++ concentrations. Kt decreased with increasing Cd++ concentration from 0-20 ppm, indicating an increase in affinity between the transport protein and Leu.

Fig. 7. Efflux of Leu at different Cd++ concentrations. Mean efflux rate of the control (at 0 ppm Cd++) was $(6.4 \pm .3) \times 10^{-7}$ mol/h. Efflux rate was measured within 5 minute period. Note: preliminary data suggested that prolonged incubation of RBC in media up to 15 min did not result in the alteration of the efflux rate.

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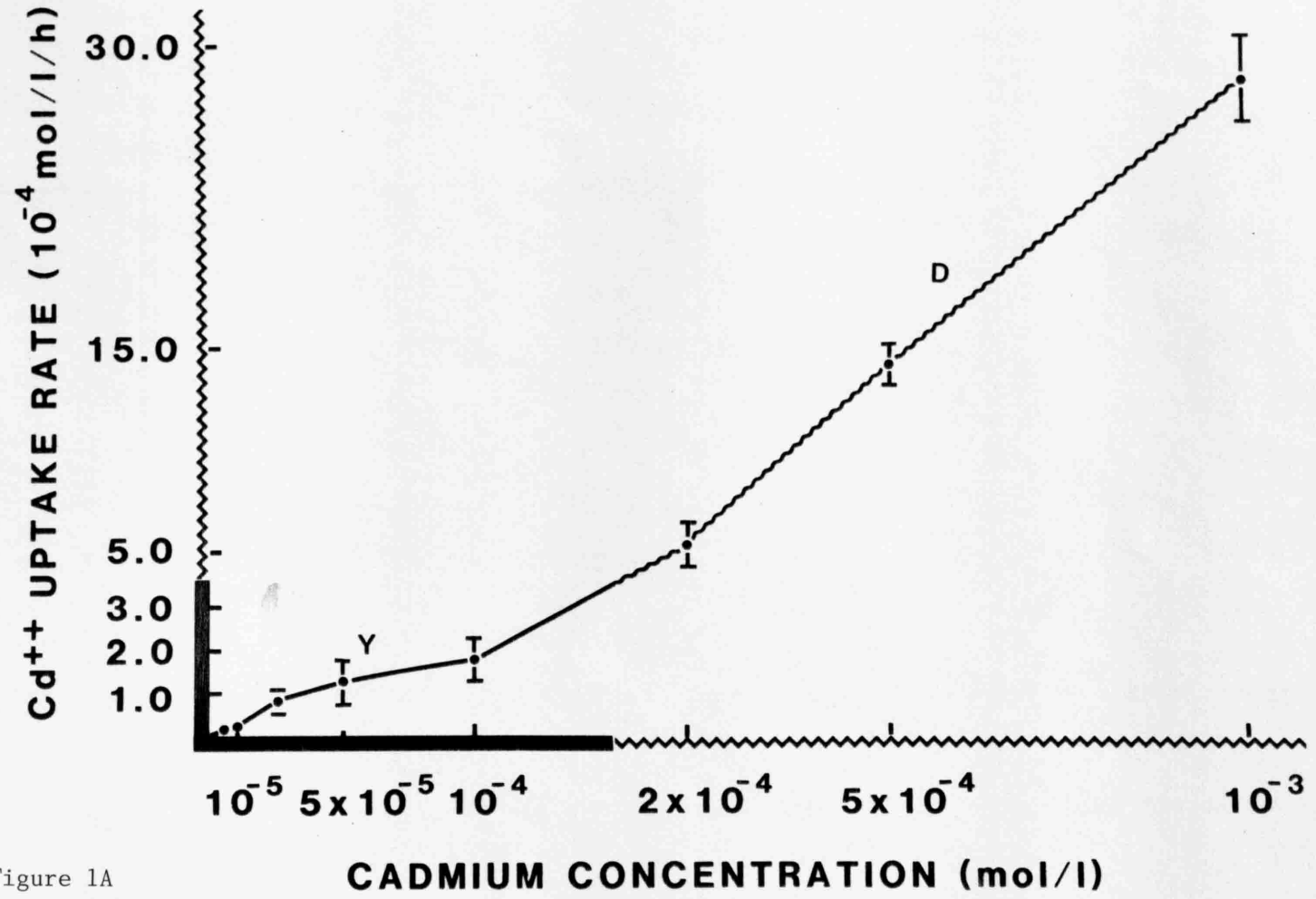
Cd++ UPTAKE RATE (10-4 mol/l/h)
30.0
15.0
5.0
3.0
2.0
1.0
Y
D
10-5
5x10-5
10-4
2x10-4
5x10-4
10-3
CADMIUM CONCENTRATION (mol/l)

Figure 1A

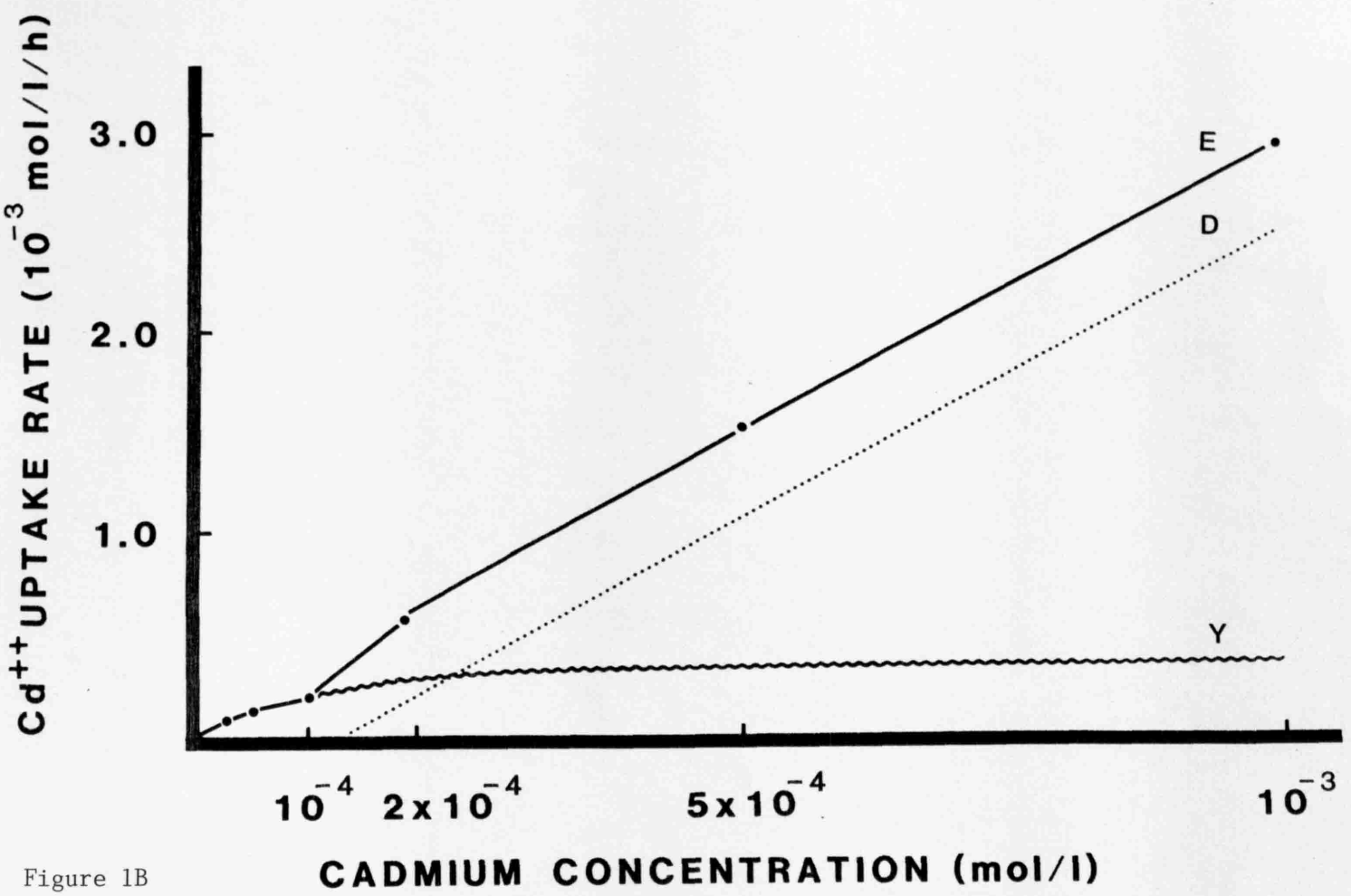
Cd++ UPTAKE RATE (10-3 mol/l/h)
3.0
2.0
1.0
E
D
Y
10-4
2x10-4
5x10-4
10-3
CADMIUM CONCENTRATION (mol/l)

Figure 1B

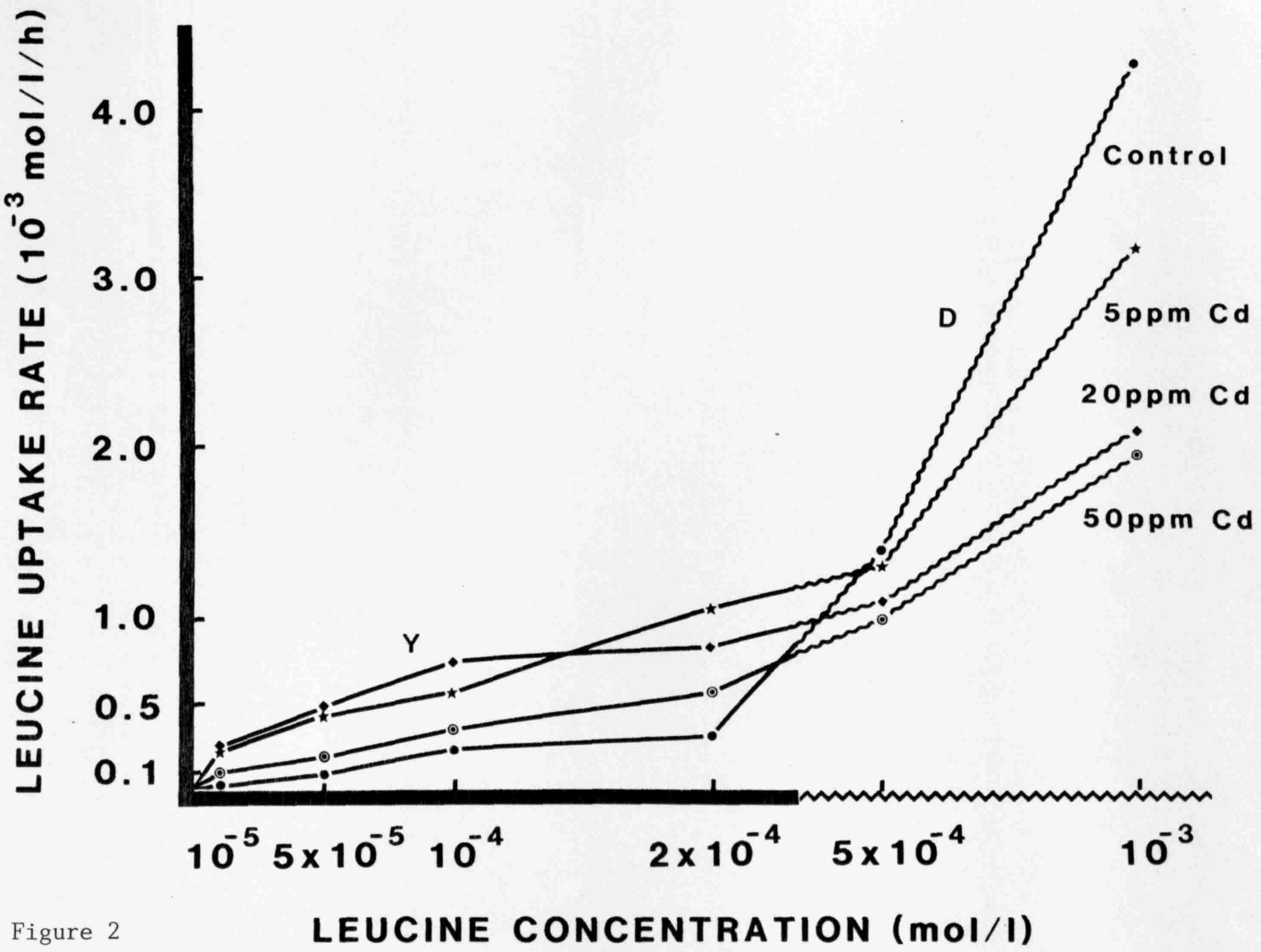
LEUCINE UPTAKE RATE (10^{-3} mol/l/h)
4.0
3.0
2.0
1.0
0.5
0.1
Control
5ppm Cd
20ppm Cd
50ppm Cd
D
Y
10^{-5} $5x10^{-5}$ 10^{-4} $2x10^{-4}$ $5x10^{-4}$ 10^{-3}
LEUCINE CONCENTRATION (mol/l)

Figure 2

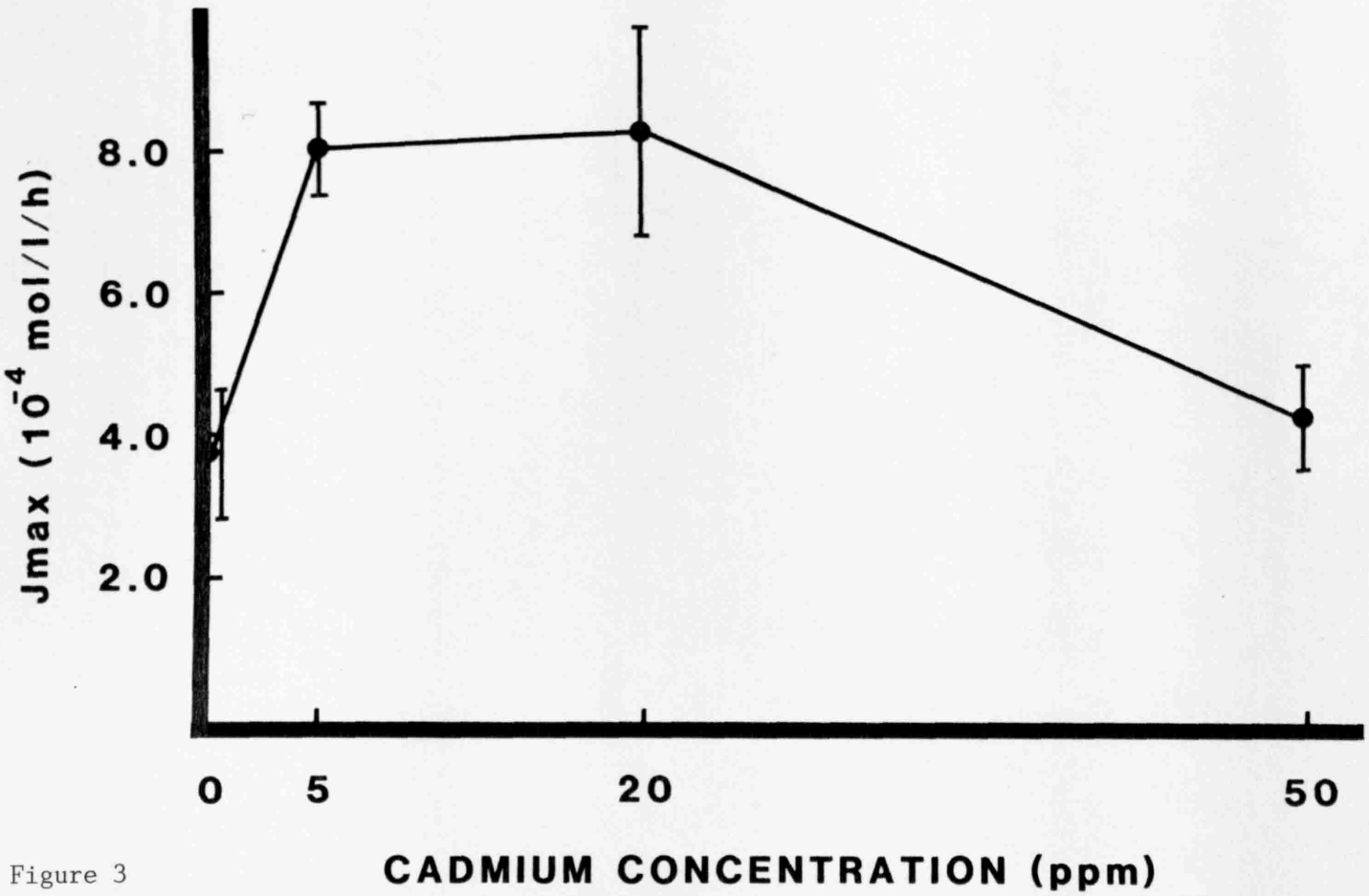

Figure 3

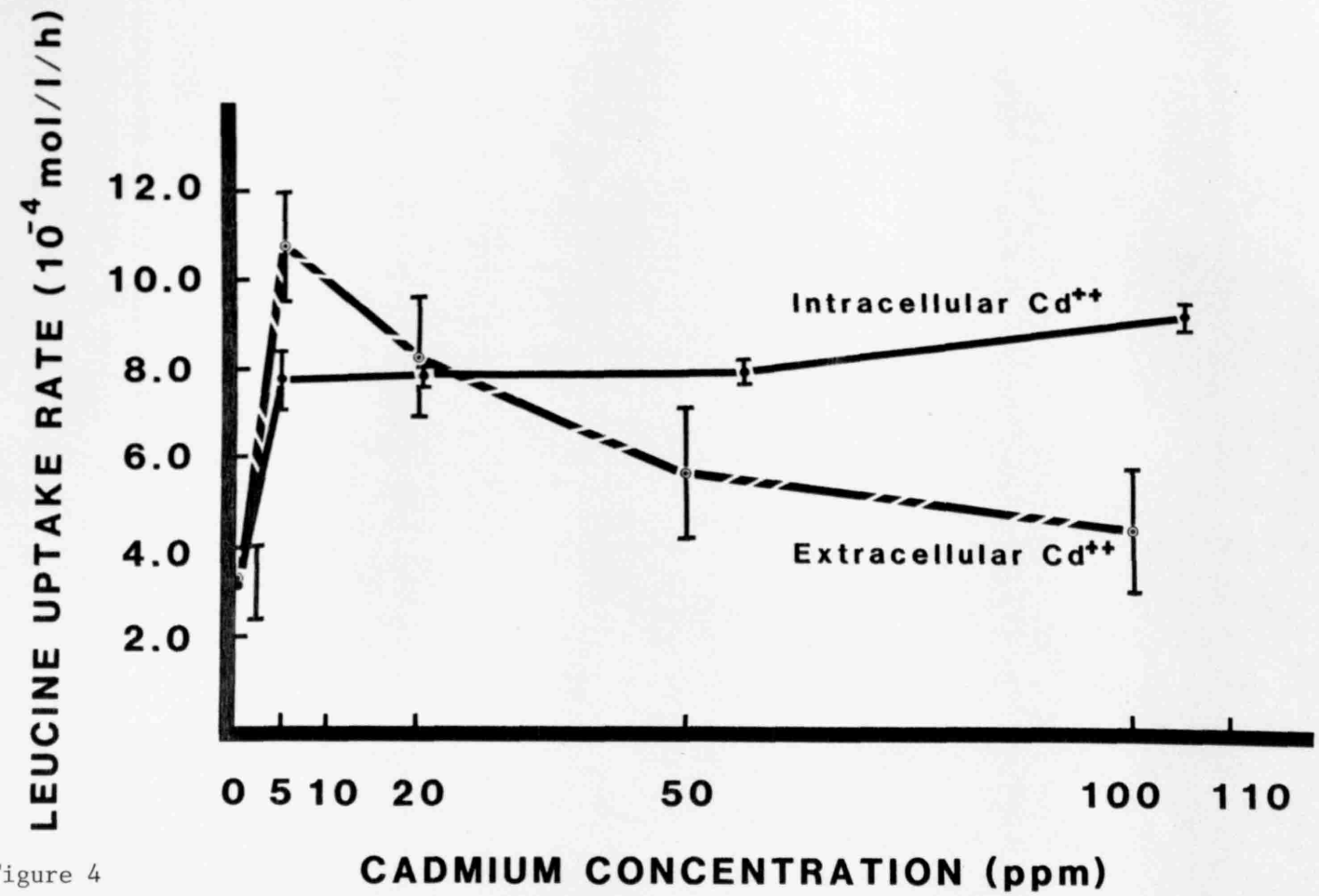
LEUCINE UPTAKE RATE (10^{-4} mol/l/h)
12.0
10.0
8.0
6.0
4.0
2.0
Intracellular Cd^{++}
Extracellular Cd^{++}
0 5 10 20 50 100 110
CADMIUM CONCENTRATION (ppm)

Figure 4

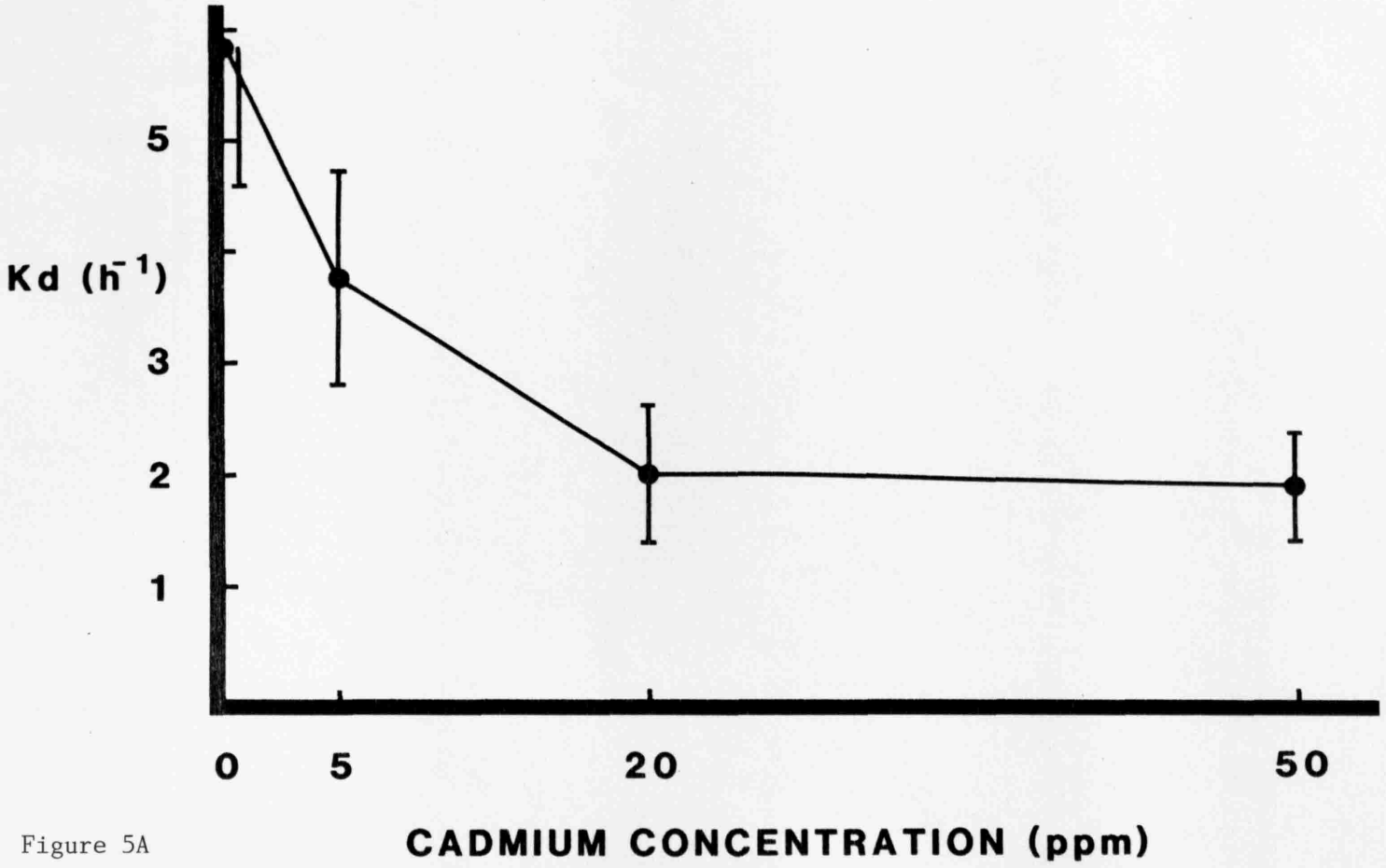

Figure 5A

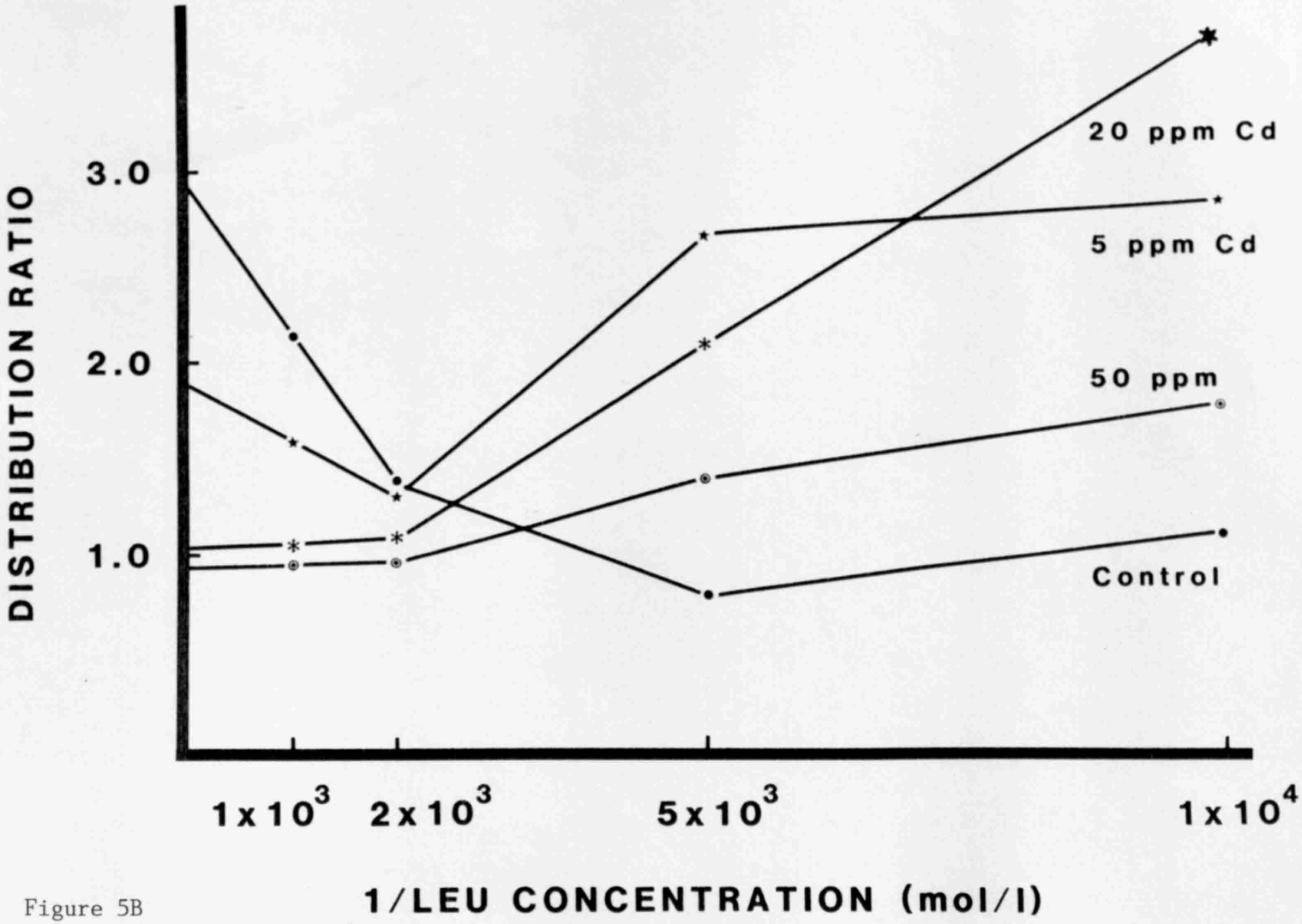
DISTRIBUTION RATIO
3.0
2.0
1.0
20 ppm Cd
5 ppm Cd
50 ppm
Control
1x10^3
2x10^3
5x10^3
1x10^4
1/LEU CONCENTRATION (mol/l)

Figure 5B

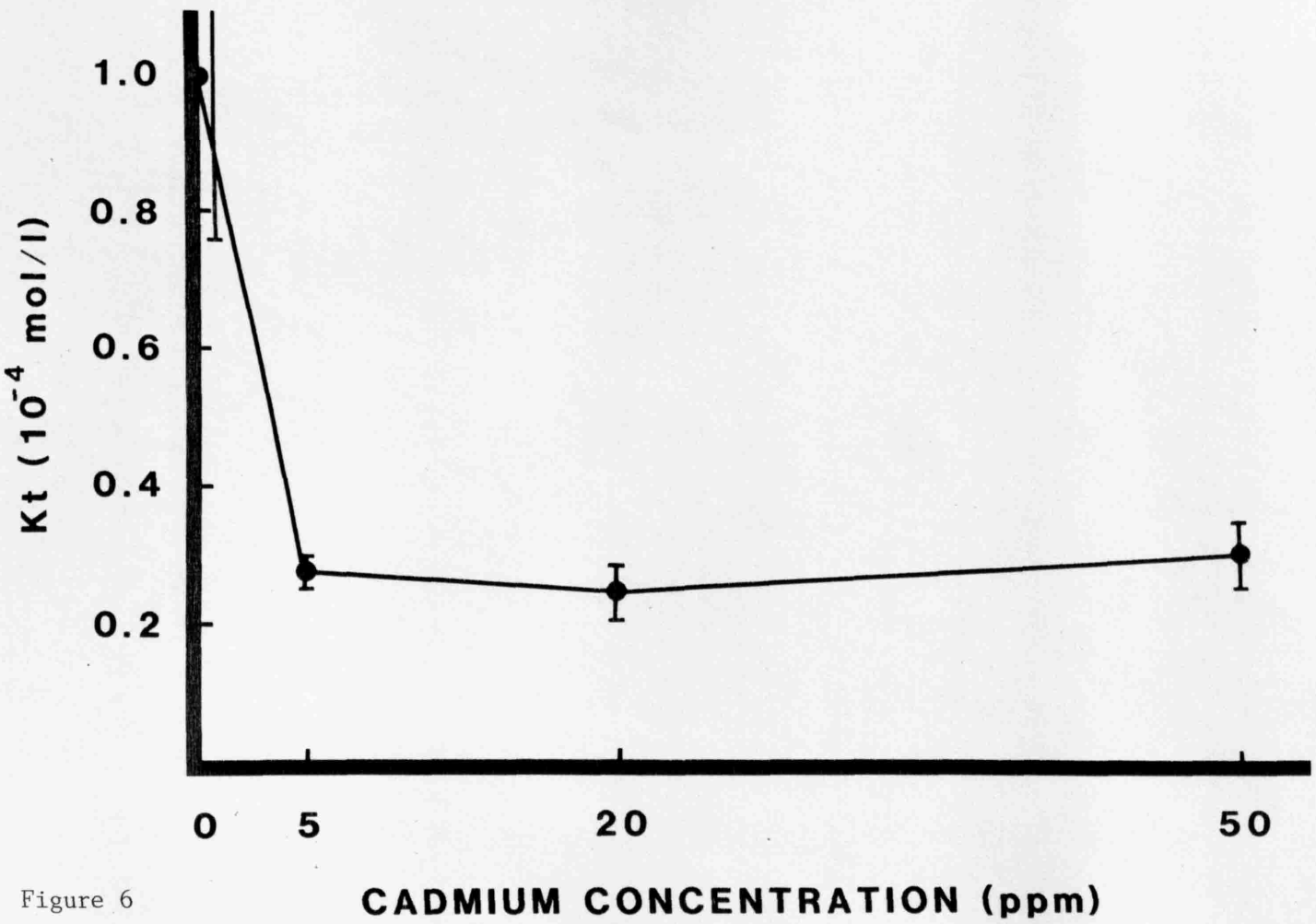

Figure 6

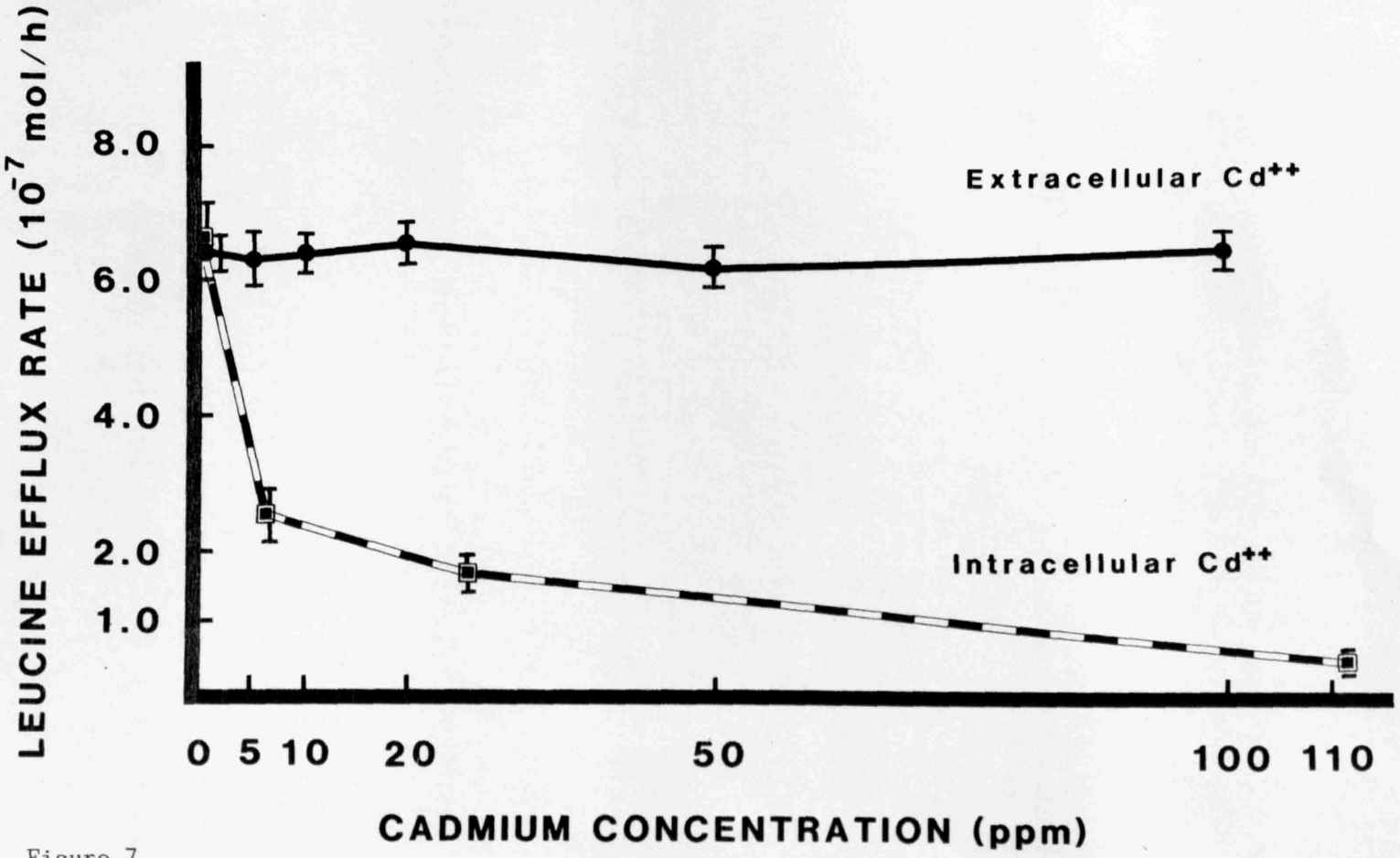
LEUCINE EFFLUX RATE (10^{-7} mol/h)
8.0
6.0
4.0
2.0
1.0
Extracellular Cd^{++}
Intracellular Cd^{++}
0 5 10 20 50 100 110
CADMIUM CONCENTRATION (ppm)

Figure 7