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UPTAKE OF INORGANIC LEAD *IN VITRO* BY ISOLATED MITOCHONDRIA AND TISSUE SLICES OF RAT RENAL CORTEX

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Abstract—Slices of rat renal cortex were shown to take up Pb^{2+} during incubation *in vitro*; Pb^{2+} was also shown to enter mitochondria within the slices. The uptake of Pb^{2+} by isolated mitochondria was inhibited by N_3^-, La^{3+} and ruthenium red. A steady state of uptake was attained within 60 sec. The concentration dependence of uptake was complex; maximum uptake was attained at 25 μM and inhibition ensued at higher concentrations. A substantial inhibitor-resistant component of Pb^{2+} uptake was noted, especially at medium Pb^{2+} concentrations greater than 25 μM, and these concentrations also inhibited respiration state 3. The effects on respiration were reduced if the mitochondria had been preincubated with ruthenium red. Slices of renal cortex incubated at 1° in medium with various concentrations of Pb^{2+} showed two fractions of uptake, one saturating at 50–100 μM external Pb^{2+} and the other at 150–200 μM. Subsequent incubation for 60 min at 25° led to further uptake at all concentrations. Upon isolation of mitochondria from incubated slices, significant amounts of Pb^{2+} were detected in the mitochondria within 5 min of addition of Pb^{2+} (200 μM), with maximum attained at 30 min. Electron microscopy of slices showed electron-dense particles, apparently of Pb^{2+}, in the cortical cells but the greatest concentration was deposited in the basement membranes. The results indicate the importance of the basement membrane in limiting access of Pb^{2+} to cortical cells, and of mitochondria in accumulating Pb^{2+} once it is in the cells. They also illustrate the importance of interactions between Pb^{2+} and Ca^{2+}.

Renal cortex exhibits important manifestations of acute plumbotoxicity and accumulates high concentrations of Pb^{2+} *in vivo* (reviewed in Ref. 1). Lead entering renal cortical cells *in vivo* is deposited particularly in the nuclei [2–4], but electron-dense deposits of Pb^{2+} are also found in the cytoplasm and mitochondria, especially when the cells show signs of morphological damage [4].§ The importance of mitochondria in the nephrotoxic effects of Pb^{2+} is indicated by abnormalities of the structure and function of these organelles [5].

There have been few direct studies *in vitro* of the transport and distribution of Pb^{2+} in intact cells and isolated mitochondria. Pounds and co-workers [6, 7] have studied the fluxes and steady-state distribution of Pb^{2+} in cultured hepatocytes, concluding that much of the Pb^{2+} is in a mitochondrial compartment. On indirect grounds (displacement of Ca^{2+} by Pb^{2+}), a mechanism was proposed for the entry of Pb^{2+} into synaptosomes [8]. Mitochondria isolated from heart actively accumulate Pb^{2+} [9], and electron-dense deposits indicative of Pb^{2+} accumulation have been observed in mitochondria isolated from liver [10]. Lead ions are known to inhibit the accumulation of Ca^{2+} by various types of isolated mitochondria [9, 11–13], while inhibitors of mitochondrial Ca^{2+} accumulation protect O_2 consumption against inhibition by Pb^{2+} [14, 15]. These results suggest that Pb^{2+} might itself be taken into mitochondria by the system normally transporting Ca^{2+}.

As far as we are aware, no observations have been made of the transport of Pb^{2+} by isolated mitochondria of renal cortex, while the only work on Pb^{2+} uptake by intact kidney cells *in vitro* is that of Vander *et al.* [16], who showed that kidney slices (both cortical and medullary) accumulate Pb^{2+} from medium concentrations of 1 μM and less. This external concentration of Pb^{2+} is not one which produces biochemically detectable toxic effects in the slices, for which 50–100 μM is required [17]. The experiments described below show that isolated cortical mitocondria accumulate Pb^{2+} by an energy-dependent mechanism and that Pb^{2+} entering tissue slices of the cortex can also be taken up rapidly by the mitochondria *in situ*.

METHODS

All experiments were done with male, Sprague–Dawley rats with body weights of 250–400 g (Zivic-Miller, Allison Park, PA). They were fed *ad lib.* on a stock diet of tap water. The animals were killed by decapitation, their kidneys were removed and placed on a Petri dish cooled with ice, and the cortices were dissected free of medullary material.

Experiments with isolated mitochondria

Mitochondria were isolated from the pooled cortices of two to three rats [18]. The medium for

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the preparation contained 250 mM mannitol, 75 mM sucrose, 2.5 mM Tris (hydroxymethyl) aminomethane (Tris) and 0.1 mM EDTA, pH 7.4; this is referred to as MS medium. After isolation, EDTA was removed from the mitochondria by washing three times with MS medium from which EDTA was omitted. The organelles were finally resuspended in the incubation medium, which contained 300 mM mannitol, 5 mM KCl, 2.5 mM Tris (pH 7.4) and 0.2% (w/v) bovine serum albumin ("essentially fatty acid-free"; Sigma Chemical Co., St. Louis, MO). The final volume of each preparation was 2.5 to 3.0 ml (13–15 mg protein/ml). The respiratory control ratio (RCR) [19] of each preparation was determined with a Clarke-type O_2 electrode, using pyruvate *plus* L-malate as substrate, and the mitochondria were only used for further work if the RCR exceeded 5.0.

Mitochondrial uptake of Pb^{2+} was studied at 25°, usually with $^{210}Pb^{2+}$ as radioactive tracer. The mitochondria were preincubated for 1 min in the incubation medium at a concentration of 1–2 mg protein/ml. Uptake was initiated by addition of different concentrations of $Pb(NO_3)_2$ (1–50 μM) labeled with $^{210}Pb^{2+}$ (0.3 μCi/μmole) and was terminated at intervals by rapid filtration of 0.1 ml of the incubation suspension through a cellulose acetate filter (0.45 μm pore diameter). The mitochondria retained on the filters were immediately washed with 15 ml of ice-cold incubation medium to which had been added KCN (100 mM) and unlabeled Pb $(NO_3)_2$ (100 μM), a procedure which took 2–3 sec. The filters were then transferred to vials for counting radioactivity.

In a few experiments, the Pb^{2+} content of the mitochondria was estimated by anodic stripping voltammetry. In this case, Pb^{2+} was omitted from the medium used to wash the filters.

Experiments with tissue slices

Slices (0.2 to 0.3 mm thick) cut from the renal cortices of one to three rats [20] were pooled in 30 ml of Ringer medium at 1°. Within 2 min, the slices were transferred, in lots of 100–150 mg wet weight, to a series of Erlenmeyer flasks maintained at 1°, each containing 3 ml Ringer with the required concentrations of Pb^{2+}. The Ringer medium contained (mM): Na^+, 140; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1.2; Cl^-, 157; SO_4^{2-}, 1; Tris, 10; and glucose, 10; the pH was 7.0. To this were added concentrations of $Pb(NO_3)_2$ up to 0.2 mM (the maximal Pb^{2+} concentration attainable in this medium); $NaNO_3$ was added to give a constant nitrate concentration of 0.4 mM. The slices were incubated in these media at 1° for up to 90 min, slices being taken for analysis at intervals. Slices to be studied at 25° were preincubated for a standard time of 90 min at 1°, during the last 10 min of which the incubation vessels were gassed with O_2 and stoppered. Incubation at 25° was then carried out in a shaking water bath.

Slices were collected for analysis by tipping the entire contents of an incubation flask onto hardened filter paper (Whatman No. 54) which was supported on a sintered glass funnel under suction. The contents of two to three flasks were collected at each sampling time. The slices were blotted gently and transferred to tared weighing bottles for analysis of Pb^{2+} by anodic stripping voltammetry.

In experiments to determine the uptake of Pb^{2+} into mitochondria *in situ*, slices were preincubated for 60 min at 1° in Ringer medium without Pb^{2+} before being transferred to other flasks at 25°; each flask contained 3 ml of pre-oxygenated medium with 200 μM $Pb(NO_3)_2$ labeled with $^{210}Pb^{2+}$ (0.3 to 0.5 μCi/μmole). The incubation was terminated by filtration, as above, except that the contents of five flasks (approximately 0.7 g wet wt slices) were pooled on a single, large filter. The slices were rinsed once on the filter with MS medium (see above) and were transferred to a Potter–Elvehjem homogenizer containing 15 ml MS medium with the addition of 3 μM ruthenium red and 0.1 mM ethyleneglycolbis-(amino-ethylether)tetra-acetate (EGTA). The latter agents were present to prevent uptake of Pb^{2+} into mitochondria upon homogenization of the tissue [21, 22]. Mitochondria were then isolated as described above. They were resuspended in 1.0 to 1.5 ml MS medium, and 0.1 to 0.2-ml aliquots were taken for analysis of protein and radioactivity, each in triplicate.

Analytical methods

Radioactivity. Filters bearing isolated mitochondria labeled with $^{210}Pb^{2+}$ were transferred to counting vials and dissolved in 1 ml ethylene glycol monomethyl ether. A toluene-based scintillation mixture (10 ml) was added, and radioactivity was determined by liquid scintillation spectrometry (Packard Tri-Carb Liquid Scintillation Spectrometer, model B-2450) using a series of external standards to correct for quenching.

In the case of mitochondrial suspensions isolated from incubated tissue slices, samples (0.1 to 0.2 ml) were dried in an oven at 105° overnight and then extracted with 2.5 ml of 0.1 N HNO_3 for 16 hr. Radioactivity in the acid extract was determined as above.

Mitochondrial protein. Samples of mitochondrial suspensions were assayed for protein by a biuret method [23].

Slice dry weight. Slices in tared weighing bottles were dried for not less than 4 hr at 105° and then weighed.

Lead assay by anodic stripping voltammetry. Whole filters bearing isolated mitochondria, or small pieces (2–3 mg) taken in triplicate from each of the dried tissue slices, were digested at 200° in equal parts by volume of $HNO_3/HClO_4/H_2SO_4$. When the digest was clear and colorless (1–8 hr digestion), it was cooled and diluted. The samples, together with blanks and a series of standards, were assayed in a four-cell anodic stripping voltammeter (ESA Corp., model 2014) connected to a recorder (Honeywell, model 1057). Plating was carried out for 25 min at a potential of −780 mV. Lead standards were made up from stock solutions ("Atomic absorption standards", Fisher Scientific Inc.), covering two ranges, viz. 10, 60 and 160 ppm, and 2, 4 and 6 ppm.

Electron microscopy

Mitochondria were studied by fixing and sectioning centrifugation pellets, while tissue slices were col-

lected directly from incubation vessels and transferred to fixative immediately. In either case, the fixative was 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) containing $CaCl_2$, at 4°. The samples were post-fixed in OsO_4 and embedded in EPON 812. The sustained sections were examined with a Philips EM 300 electron microscope. Three representative samples were examined from each piece of tissue or pellet, using up to ten sections per sample.

Expression of results

Results are given as mean ± S.E.M. (number of observations). Differences between treatments were examined for statistical significance by Student's *t*-test, a P value of 0.05 or less being accepted as significant.

Table 1. Effects of inhibitors on the uptake of Pb^{2+} by isolated mitochondria*

Inhibitor	Pb^{2+} uptake (nmoles/mg protein)
None	3.64 ± 0.37
La^{3+} (100 μM)	0.67 ± 0.26
La^{3+} (200 μM)	0.43 ± 0.16
N_3^- (1 mM)	0.86 ± 0.12
Ruthenium red (1 μM)	0.91 ± 0.14
La^{3+} (100 μM) plus ruthenium red (1 μM)	0.65 ± 0.12

* Conditions of incubation were as for Fig. 1, except that the concentration of Pb^{2+} in the medium was 10 μM (specific activity of $^{210}Pb^{2+}$, 0.3 μCi/μmole). Uptake of $^{210}Pb^{2+}$ was measured over 10 sec. Each value is the mean ± S.E.M. of ten to twelve observations.

RESULTS

Mitochondria

The kinetics of uptake of $^{210}Pb^{2+}$ were studied at several concentrations of Pb^{2+}, each in the presence and absence of 1 mM N_3^-, an inhibitor of electron transfer, or 2 μM ruthenium red. When the medium contained 10 μM Pb^{2+}, there was a rapid uptake during the first 10 sec and a maximal mitochondrial content of 13.5 ± 1.2 nmoles Pb^{2+}/mg protein was attained after approximately 30 sec (Fig. 1). Analogous results were obtained at all other external concentrations of Pb^{2+}, and examples at two lower concentrations (2 and 3 μM) are illustrated in Fig. 2. Ruthenium red and N_3^-, singly or in combination, reduced the uptake of Pb^{2+} to a similar extent (Figs. 1 and 2), as did 0.1 mM La^{3+} (Table 1). Since ruthenium red [24] and La^{2+} [25] are known to inhibit mitochondrial accumulation of Ca^{2+} rather specifically, we conclude that a large fraction of the Pb^{2+} uptake was both energy-dependent and similar to the mechanism accumulating Ca^{2+}.

At each external concentration of Pb^{2+}, the active transport was calculated as the difference between the uptake in the absence and presence of ruthenium red (see Figs. 1 and 2). In Fig. 3, both the active and the passive accumulation of Pb^{2+} during the first 60 sec (i.e. that taken up in attaining the steady-state level) are shown for 5–150 μM external Pb^{2+}. The kinetic picture of active uptake is complex, for at concentrations less than 25 μM the curve tends to be sigmoidal, while at 50 μM and above there was inhibition of the active uptake. The sigmoidal region of the kinetic curve is more clearly seen in Fig. 4, where the initial rate of uptake (in the first 10 sec) has been plotted against Pb^{2+} concentrations of 1–25 μM. The concentration of Pb^{2+} giving half-maximal rate of uptake was 12.5 μM, but the occurrence

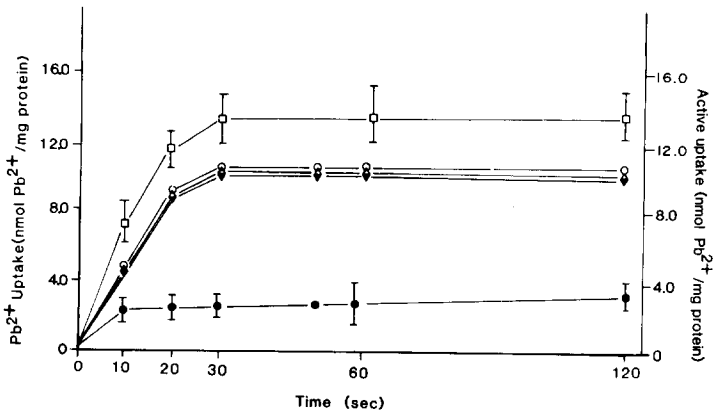


Fig. 1. Time-course of the uptake of Pb^{2+} by isolated mitochondria of kidney cortex. Mitochondria were preincubated for 1 min in the mitochondrial incubation medium (see Methods) at 25° and uptake was initiated by addition of 10 μM Pb^{2+} as nitrate, labeled with $^{210}Pb^{2+}$ at a specific activity of 0.3 μCi/μmole. At the intervals specified, 0.1-ml samples of the reaction mixture were taken and the mitochondria collected by rapid filtration followed by washing with a reaction-stopping solution (see Methods). The uptake in parallel incubations was inhibited with 1 mM N_3^-, 2 μM ruthenium red, or both agents together. These three treatments each gave virtually identical inhibition of uptake, as shown by the difference curves representing active uptake: (□) total uptake; (●) uptake in presence of ruthenium red; other lines represent the active uptake determined as the differences between total uptake and uptake in the presence of (○) ruthenium red, (△) N_3^-, and (▼) ruthenium red *plus* N_3^- (right-hand ordinates).

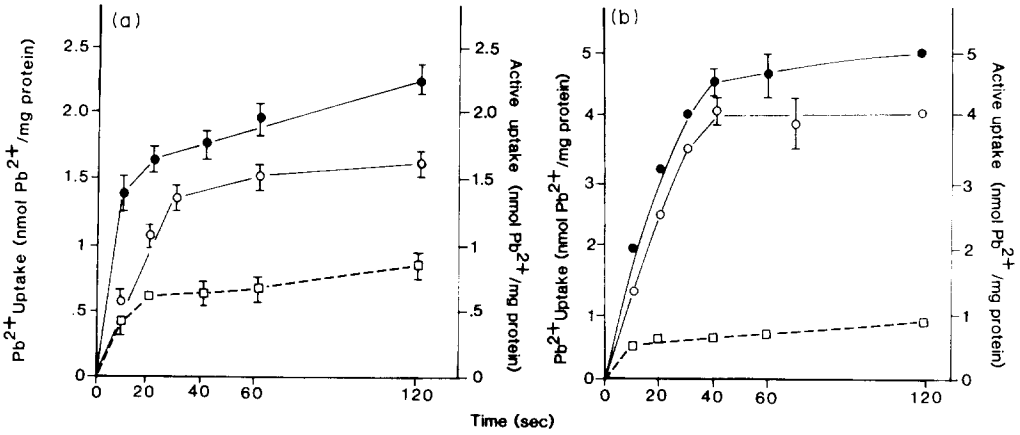


Fig. 2. Time-course of the uptake of Pb^{2+} by isolated mitochondria of kidney cortex incubated with (a) 2 μM and (b) 3 μM Pb^{2+}. Parallel incubations were conducted with (--□--) and without (—●—) 2 μM ruthenium red in the preincubation mixture. The line with open circles (—○—) is the difference between uptake with and without ruthenium red and is taken as an estimate of the active uptake of Pb^{2+} (right-hand ordinates). Procedures were as in Fig. 1.

of inhibition of the transport at concentrations above 25 μM makes this a minimal estimate of the K of the transport process *per se*. Figure 3 also shows that there was a substantial passive uptake of Pb^{2+} which reached a maximum at 50 μM external Pb^{2+}. At this point, the passive uptake exceeded the active component.

In experiments with 50 μM external Pb^{2+}, the accumulation was also followed by determination of lead by anodic stripping voltammetry. The time-course of uptake and the inhibition by ruthenium red were similar to those measured by uptake of $^{210}Pb^{2+}$ (not shown).

It seemed likely that the inhibition of active transport of Pb^{2+} at an external Pb^{2+} concentration of 50 μM, or higher, was due to inhibition of energy metabolism by the metal itself. Concentrations of 20 μM Pb^{2+} and higher significantly reduced respiration in the presence of ADP as phosphate acceptor, i.e. state 3 [19], and drastically reduced the respiratory control ratio (RCR; Table 2). These effects appeared to require entry of Pb^{2+} into the mitochondrial matrix for the respiration and RCR were partially protected if the mitochondria had been preincubated with ruthenium red (Table 2). That the protection was only partial may be explained by the large passive component of Pb^{2+} uptake (Fig. 3).

Electron microscopy of the renal cortical mitochondria, fixed after 3 min of incubation at 25°, shows them to be well preserved in the orthodox or inter-

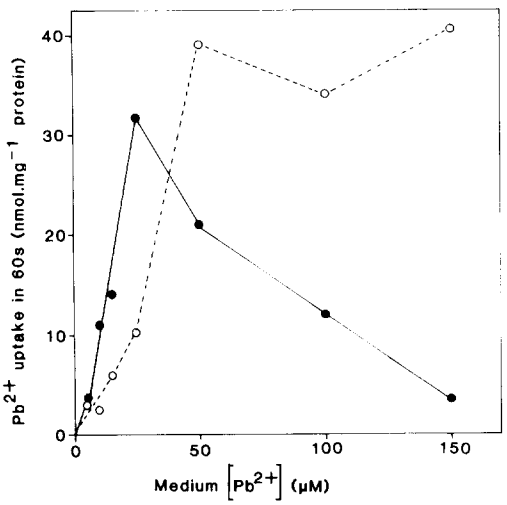


Fig. 3. Concentration dependence of the active and passive uptake of $^{210}Pb^{2+}$ by isolated mitochondria in media containing 5–150 μM Pb^{2+}. The uptake illustrated took place during the first 60 sec after addition of Pb^{2+}, in experiments similar to those of Figs. 1 and 2. Passive (○) and active (●) uptakes were taken to be the amounts resistant to and inhibited by 3 μM ruthenium red respectively.

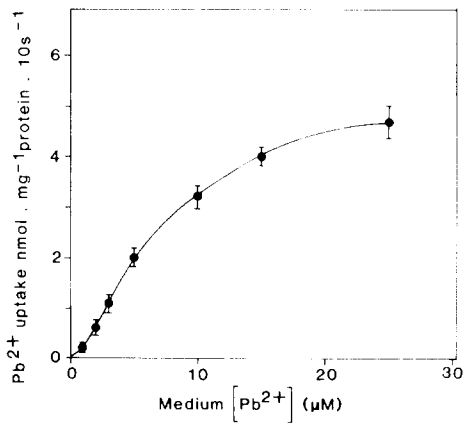


Fig. 4. Concentration dependence of the initial rate of active uptake of $^{210}Pb^{2+}$ by isolated mitochondria from media containing 1–25 μM Pb^{2+}. Results are from the same series of experiments as Fig. 3, but the uptake illustrated was that which occurred during the first 10 sec after addition of Pb^{2+}.

Table 2. Effect of Pb^{2+} on respiratory activity of renal cortical mitochondria in the presence and absence of ruthenium red*

Pb^{2+} in medium (μM)	State 3 respiration		Respiratory control ratio	
	Control	+ Ruthenium red (2.5 μM)	Control	+ Ruthenium red (2.5 μM)
0	272 ± 19	263 ± 15	5.2 ± 0.9	5.2 ± 0.6
10	244 ± 27	249 ± 16	3.5 ± 0.4	4.2 ± 0.4
20	205 ± 18	218 ± 13	2.8 ± 0.3	3.3 ± 0.3
40	57 ± 9	129 ± 8	1.0 ± 0.1	1.4 ± 0.1

* To the mitochondrial incubation medium (see Methods) were added 10 mM pyruvate, 1 mM L-malate, 10 mM inorganic phosphate and mitochondria (1–2 mg protein/ml). Lead was added at the concentrations indicated before addition of the phosphate. Where used, ruthenium red was preincubated with the mitochondria for 1 min prior to addition of Pb^{2+}. The respiratory control ratio and state 3 respiration were determined by making additions of 0.36 mM ADP [26]. Rates of respiration are expressed as μatom O/mg protein · min^{-1}; mean ± S.E.M.

mediate conformations [27], with only about 15% showing morphological damage; they contained no intrinsically electron-dense structures in the unstained sections (Fig. 5a). After 2 min at 25° in the presence of 5 μM Pb^{2+}, however, 30–50% of the organelles showed damage which varied from moderate swelling to complete disorganization. Some of these mitochondria (5–8%) showed large, electron-dense granules which were preferentially associated with the cristae or vesicular membranes and presumably consisted of Pb^{2+} deposits (Fig. 5b).

Cortical slices

The passive entry of Pb^{2+} into renal cortical slices was studied by incubating slices at 1° in Ringer solution (pH 7.0) containing 200 μM Pb^{2+}. A significant entry was already evident after 1 min and the maximal level was attained after approximately 45 min (Fig. 6). The extracellular water content of such slices, as determined by inulin distribution, is 1.3 kg water/kg dry weight [17], and the quantity of Pb^{2+} contained in this water, assuming a concentration equal to that of the medium, is 260 μmoles/kg dry weight. During incubation, this value was attained by 2–3 min (Fig. 6), so that further entry of Pb^{2+} either represents binding to extracellular structures or entry into the cells. The concentration dependence of the uptake during 90 min at 1° (Fig. 7) suggests that two systems may be involved, one saturating at 50–100 μM external Pb^{2+} and the other at 150–200 μM. Subsequent incubation of the pre-loaded slices for 60 min at 25°, in the presence of the same concentrations of Pb^{2+}, led to a further uptake. The final content again showed two steps, but the component seen at lower concentrations was already saturated at 20 μM Pb^{2+} while the other failed to show a plateau over the range of concentrations studied. The findings appear to indicate a substantial metabolism-dependent component of the two Pb^{2+} uptake systems of the intact slices.

To study the ability of mitochondria within tissue slices to take up Pb^{2+}, slices were preincubated for 60 min at 1° and then transferred to medium at 25° containing 200 μM Pb^{2+}, labeled with $^{210}Pb^{2+}$. Slices were removed at intervals and mitochondria isolated in the presence of ruthenium red and EGTA (see Methods). Significant amounts of Pb^{2+} were detected in the mitochondria after 5 min of incubation of the slices and the maximum was attained after 30 min (Fig. 8).

The uptake of $^{210}Pb^{2+}$ by mitochondria in the slices appeared to involve a calcium-sensitive step, possibly a Ca^{2+} transporting mechanism at the plasma membrane or mitochondrial membrane, for uptake was approximately doubled when the preincubation at 1° and incubation at 25° were carried out in a calcium-free Ringer solution (Table 3). Addition of EGTA (0.2 mM) to the medium lacking Ca^{2+} reduced the uptake of Pb^{2+} by 70% (Table 3), presumably due to the avid chelation of Pb^{2+} by EGTA [28].

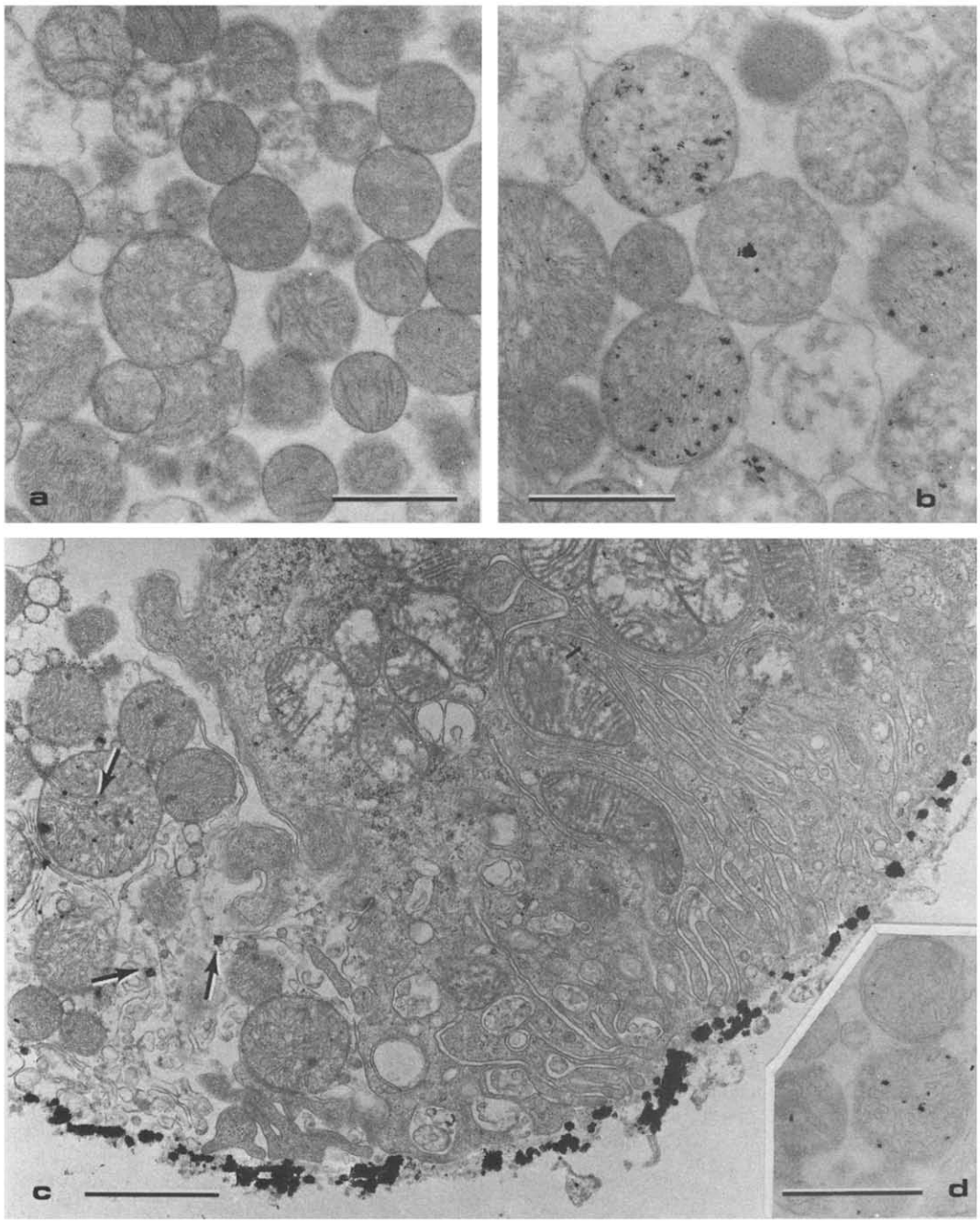
To obtain a further indication of the distribution and effects of Pb^{2+} in renal cortical slices, we examined the ultrastructure after incubation for 90 min at 1° and 60 min at 25° in medium containing 200 μM Pb^{2+}. An unstained preparation (Fig. 5c) shows two adjacent cells and basement membrane of a proximal tubule. One of the two cells is generally well preserved, as judged by the microtubules, basal infoldings and uniform electron density of the cytoplasmic ground substance, although some of its mitochondria show a modest degree of swelling. This cell shows small, highly electron-dense deposits scattered through the cytoplasm and some of the mitochondria. The other cell is in an advanced stage of disorganization and contains several, large electron-dense particles in the cytoplasm and mitochondria (Fig. 5d). However, it is clear that the largest amount of the dense material is associated with the basement membrane (Fig. 5c), a finding similar to that in rats treated with Pb^{2+} *in vivo*.* Figure 5d shows an enlargement of electron-dense particles in the matrix of mitochondria in the orthodox conformation. Electron-dense particles of the types described above were not found in tissue slices incubated without Pb^{2+}.

* M. A. Russo, S. C. Kapoor and G. D. V. van Rossum, manuscript submitted for publication.

DISCUSSION

Our results show that Pb^{2+} was taken up by mitochondria isolated from renal cortex, that it entered the intact cortical cells and, once in the cells, was taken up by mitochondria *in situ*. The accumulation of Pb^{2+} by the kidney mitochondria was dependent on energy since a large part of it was prevented by respiratory inhibitors, although there was also a substantial energy-independent component at higher concentrations of Pb^{2+}. These findings are similar to those with heart mitochondria [9]. The respiration-dependent portion of the Pb^{2+} uptake by kidney mitochondria was inhibited by La^{3+}, as with liver, heart and brain mitochondria [9, 11, 12], and by ruthenium red. The latter agent is a rather specific inhibitor of mitochondrial Ca^{2+} accumulation [24, 29] and this, together with the Pb^{2+}-induced inhibition of Ca^{2+} accumulation by the kidney mitochondria [13], suggests that these two cations are actively accumulated by the same transport system.

The active component of Pb^{2+} uptake showed a complex kinetic picture. The sigmoidal part of the curve at low external Pb^{2+} may indicate a degree of cooperativity in the transport system, but another possibility is that Pb^{2+} had to compete for occupation of the transport sites with residual Ca^{2+} in the preparation. At high concentrations there was inhibition



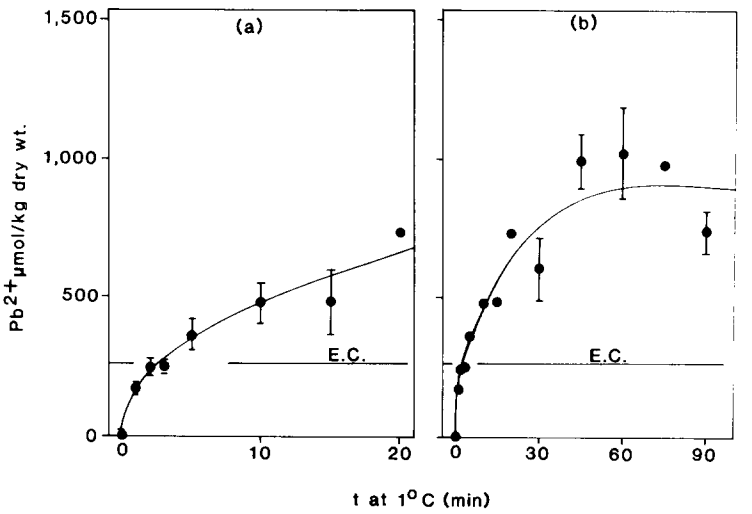


Fig. 6. Time-course of entry of Pb^{2+} into slices of renal cortex during incubation at 1°. (a) Uptake during the first 20 min. (b) Uptake during the full 90-min incubation. Incubation was in Tris-buffered Ringer solution (see Methods) containing 200 μM $Pb(NO_3)_2$. Each point is the mean ± S.E.M. of eight observations. Lead in the tissue was analyzed by anodic stripping voltammetry. E.C., quantity of Pb^{2+} calculated to be present in free solution in the extracellular compartment (see text and legend to Fig. 7).

of uptake; we attribute this to inhibition of energy-conserving reactions by the Pb^{2+} itself, after it has entered the matrix. This is not a contradictory situation, for the energy metabolism of mitochondria is less sensitive to Pb^{2+} than is Ca^{2+} accumulation [11] and we have shown here that 25 μM Pb^{2+}, which permitted maximal energy-dependent uptake of Pb^{2+} (Fig. 3), only partially inhibited energy metabolism, e.g. respiration in state 3 was only reduced by 26% [30]. Thus, as the concentration of Pb^{2+} is increased, a balance is struck between the increasing transport activity due to an increasing degree of saturation of the carrier by Pb^{2+}, and the decreasing availability of energy. The partial protection of respiratory activity by ruthenium red (Table 2; see also Ref 14) indicates that Pb^{2+} must enter the mitochondria for energy metabolism to be inhibited. Since ruthenium red totally inhibits active accumulation of Pb^{2+}, its failure to afford complete protection to the respiratory activity must have been due to the large amount of Pb^{2+} that entered the mitochondria passively at the concentration used (40 μM). The even larger passive uptake at higher external concentrations could similarly account for the complete inhibition of active transport of Pb^{2+} by inhibiting the energy metabolism more completely.

Fig. 5. Unstained electron micrographs of isolated mitochondrial and tissue slices of renal cortex. (a) Control, isolated mitochondria after incubation for 2 min at 25° without Pb^{2+}. After incubation, the mitochondria were sedimented by centrifugation for 2 min at 10,000 *g* at room temperature (18°). The pellet was fixed and embedded (see Methods). The micrograph shows a transverse section through the central region of a pellet and represents the appearance most frequently seen in the preparations. The following points should be noted. There is little contamination with non-mitochondrial components. The mitochondria are mainly in orthodox or intermediate configurations and have well-preserved, or slightly dilated cristae. However, 15–18% of the mitochondria show varying degrees of morphological damage. Magnification: ×22,000. (b) Isolated mitochondria after incubation with 5 μM Pb^{2+}. These mitochondria are from the same preparations as those in (a), and differ from the latter mainly in that most are in the intermediate configuration, some 30–50% show signs of damage, and about 10% of these contain extremely electron-dense granules. The granules, which are not seen in the control pellet (a), are variously localized inside, or on the surface of the mitochondria, with preferential association with membranes. Magnification: ×24,000. (c) renal cortical slice after incubation for 90 min at 1° and 60 min at 25° in the presence of 200 μM Pb^{2+}. Parts of two cells of a proximal tubule are illustrated. That on the right is well-preserved, with microtubules, well-maintained in-foldings of the baso-lateral membrane, uniform density of the cytoplasmic ground substance, and modest swelling of some mitochondria. This cell shows a certain number of isolated, very electron-dense particles both in the mitochondria and cytoplasm. The left-hand cell, by contrast, is in an advanced stage of disorganization and contains the very electron-dense particles (arrows) both in mitochondria and in membranous vesicles. Most marked, however, is the large number of very big electron-dense particles attached to, or within, the basement membrane of the tubule; such particles are not seen in slices incubated without Pb^{2+}. Magnification: ×18,000. (d) Detail of mitochondria in a slice incubated with 200 μM Pb^{2+}, as in (c). The mitochondria show groups of very electron-dense particles, especially associated with the internal mitochondrial membranes. The particles are in all respects similar to those seen in the isolated mitochondria of (b).

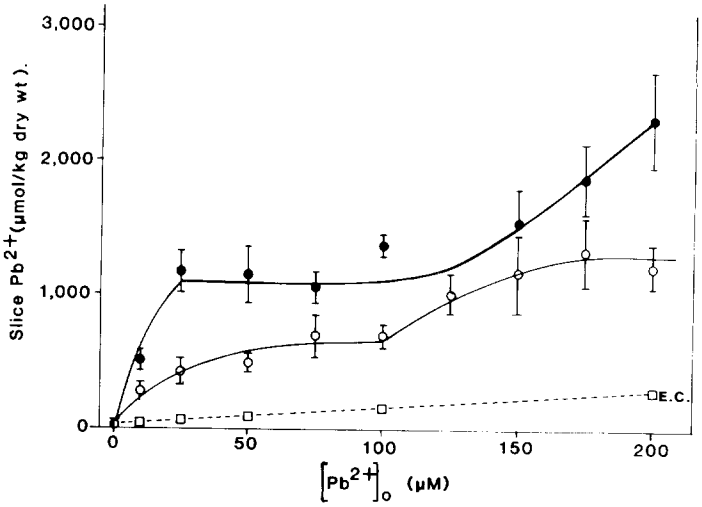


Fig. 7. Concentration dependence of Pb^{2+} uptake into slices of renal cortex. The slices were incubated in the appropriate concentrations of Pb^{2+} for 90 min at 1°, at which time samples were collected for analysis (○). The vials were gassed with O_2, and the incubation of the remaining slices was continued for a further 60 min at 25° in the same concentrations of Pb^{2+} (●). Also shown is the Pb^{2+} content of the slices calculated to be present in the extracellular water (□); this was calculated from the quantity of extracellular water, assuming that the latter contains diffusible Pb^{2+} at the same concentration as the bulk medium (see text). For further details of incubation conditions, see Methods. Each point is the mean of six observations; at the concentrations 0, 50, 100 and 200 μM Pb^{2+} a larger series of observations (N = 18) was conducted in extra experiments, with mean values that did not differ significantly from those shown.

The results with tissue slices suggest a rapid entry of Pb^{2+} into the intracellular compartment, with rapid uptake of some lead by the mitochondria; an apparent steady state was attained after about 45 min. The additional uptake taking place during incubation at 25° suggests a dependence on metabolism. An indication of the regions of the tissue reached by Pb^{2+} is provided by the electron-dense deposits seen by electron microscopy. These provide only a minimum indication of the distribution of Pb^{2+} for they must be confined to those regions in which Pb^{2+} is present at concentrations high enough to exceed the solubility products of the precipitated materials; diffusible forms of Pb^{2+} will clearly also be present and are presumably more widely distributed. Since most tubules in renal cortical slices have closed lumina,* access of Pb^{2+} from the incubation medium to the cells must be predominantly from the baso-lateral borders. But the basement membranes contained much deposited Pb^{2+} and clearly limited the passage of Pb^{2+} to the baso-lateral cellular membranes. Nevertheless, Pb^{2+} appeared in the mitochondria (as shown both by electron-dense deposits and by the tracer experiments) and, to a smaller extent, the cytoplasm of both damaged and relatively intact cells, so that it was clearly able to penetrate the plasma membranes. The experiments with $^{210}Pb^{2+}$ show that this occurred within 5 min.

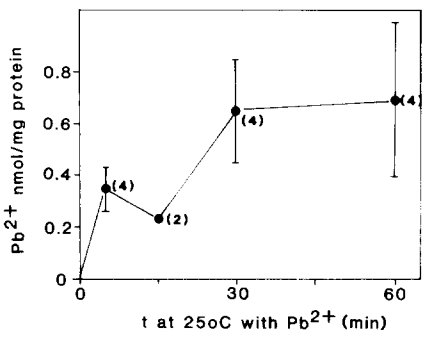


Fig. 8. Time-course of the appearance of $^{210}Pb^{2+}$ in mitochondria *in situ* during the incubation of kidney cortex slices at 25°. Slices were preincubated for 60 min at 1° without Pb^{2+}. At time zero, slices were transferred to flasks containing pre-oxygenated, Tris-buffered Ringer solution and 200 μM Pb^{2+} labeled with $^{210}Pb^{2+}$ (0.3 μCi/μmole). At the times indicated, slices were collected by filtration, rapidly washed, homogenized in the presence of ruthenium red and EGTA, and their mitochondria isolated (see Methods for details).

The entry of Pb^{2+} into mitochondria *in situ* was increased markedly when the medium did not contain Ca^{2+}. The most likely explanation is that Ca^{2+} and Pb^{2+} competed with each other at a transport site, either at the plasma membrane or the mitochondrial membrane. The latter site has been shown above to be one at which the two ions share a common carrier, and interactions at the plasma membrane have been noted in brain and intestine [31, 32].

The existence of two classes of uptake sites for Pb^{2+} in tissue slices, as indicated by the concentration dependence of its entry (Fig. 7), may be explained in several ways: (a) the distribution between viable and severely damaged cells, the latter containing

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Table 3. Uptake of $^{210}Pb^{2+}$ into mitochondria in intact slices of renal cortex; effects of medium Ca^{2+}*

	Pb^{2+} content of mitochondria (nmoles/mg protein)	
	Preincubation at 1° 1.2 mM Ca^{2+}	Ca^{2+}-free
Medium Ca^{2+} for incubation at 25°:		
1.2 mM Ca^{2+}	0.29	
Ca^{2+}-free		0.63
Ca^{2+}-free + EGTA (0.2 mM)		0.19

* Slices were preincubated in Ringer with or without Ca^{2+} for 60 min. They were then transferred to medium at 25° containing 0.2 mM Pb^{2+}, with or without Ca^{2+} and EGTA. Mitochondria were isolated from the slices after 30 min at 25°. Other details were as for Fig. 8.

more Pb^{2+}, (b) occurrence in both cells and extracellular regions (the most obvious example of the latter being the basement membranes), and (c) distribution between subcellular sites in viable cells (e.g. mitochondria and cytosol). There may also be differences between the cells of the various segments of the nephrons. It is clear that there exist many more than two physiological or structural compartments of Pb^{2+} in slices so that the two sites detected by the curves of Fig. 7 probably each have several components. Indeed, the difference between the higher affinity uptake at 1° (saturating at 50–100 μM external Pb^{2+}) and at 25° (saturating at 20 μM) may indicate that an additional component, with high affinity, contributes to this uptake at 25° but is not seen at 1°. The failure of the lower affinity step to saturate at 25° could be due to the activation of a very low affinity transport system, but the electron microsopic findings exemplified by Fig. 5c make it more likely that the high external concentrations of Pb^{2+} (i.e. 100–200 μM) result in damage to the plasma membranes at 25° and thus allow Pb^{2+} more ready access to the cytoplasm and mitochondria.

Our results with renal cortical slices are generally similar to those of Vander *et al.* [16], who further showed that Pb^{2+} uptake by cortical slices is reduced by a number of inhibitors of energy metabolism. The highest external concentration of Pb^{2+} used by these authors was 1 μM and this gave a maximal slice content of 40 μmoles Pb^{2+}/kg wet wt. Based on the water content of our slices [17], this may be converted to 160 μmoles/kg slice dry wt, and comparison with our results (Figs. 6 and 7) shows that 1 μM external Pb^{2+} is far from sufficient to saturate the slices with Pb^{2+}. In other work we have shown that at least 50 μM external Pb^{2+} is required for a significant inhibition of energy metabolism or K^+ transport to be produced in the slices [17].

Further comparison of our results may be made with those of Pounds *et al.* [7] on isolated hepatocytes in culture. These cells showed an initial, rapid uptake of Pb^{2+} which continued for about 4 hr at a rate of approximately 0.4 mmole/kg dry weight · hr^{-1} (recalculated by assuming a liver protein content of 0.6 g/g dry weight; [33]) from a medium containing 3 μM Pb^{2+}. The nearest comparable conditions in our work are for a medium concentration of 10 μM, at which we found an uptake of 0.28 mmole/kg dry weight during 90 min at 1° and of 0.23 mmole/kg during a subsequent 60 min at 25°. Considering that our work was with slices, with longer average diffusion paths for Pb^{2+} to the plasma membranes than with isolated cells, the overall rates of uptake in the two studies seem to be remarkably comparable.

From subsequent study of the inhibitor sensitivity of $^{210}Pb^{2+}$ efflux from the hepatocytes, Pounds *et al.* [7] concluded that one kinetic compartment, with an exchange half-time of 800 min, included the mitochondrial Pb^{2+}. This appears to be a much longer half-time than that shown by the uptake of $^{210}Pb^{2+}$ into mitochondria of the slices, where an apparent steady-state was approached by 60 min (Fig. 8). The much shorter uptake time we used than the 18 hr used by Pounds *et al.*, together with the absence of phosphate from our medium, renders comparison difficult. However, it is important to note that the much smaller amount of Pb^{2+} taken up during our experiments was sufficient for it to appear in the intact cells in sufficient quantities to be deposited as particulates and to damage the mitochondrial structure and function *in situ* [17].

In conclusion, Pb^{2+} enters renal cortical cells through a number of barriers. The basement membrane appears to be a major barrier restricting the entry of Pb^{2+} into tubular cells, but the plasma membrane also limits influx, as indicated by the greater deposition of Pb^{2+} particles in cells that were morphologically damaged. Once in the cells, Pb^{2+} was rapidly taken up by mitochondria, apparently by the calcium-transporting system, in quantities sufficient to show deposition of particles and damage to mitochondrial structure and energy-conserving function.

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REFERENCES

1. R. A. Goyer and B. C. Rhyne, *Int. Rev. expl. Path.* **12**, 1 (1973).
2. S. S. Blackman, *Bull. Johns Hopkins Hosp.* **58**, 384 (1936).
3. P. Galle and L. Morel Maroger, *Nephron* **2**, 273 (1965).
4. R. A. Goyer, D. L. Leonard, J. F. Moore, B. C. Rhyne and M. R. Krigman, *Archs environ. Hlth* **20**, 705 (1970).
5. R. A. Goyer, A. Krall and J. P. Kimball, *Lab. Invest.* **19**, 78 (1968).
6. J. G. Pounds and R. A. Mittelstaedt, *Science* **220**, 308 (1982).
7. J. G. Pounds, R. Wright and R. L. Kodell, *Toxic. appl. Pharmac.* **66**, 88 (1983).
8. E. K. Silbergeld and H. S. Adler, *Brain Res.* **148**, 451 (1978).
9. M. Scott, K. M. Hwang, M. Jurkowitz and G. P. Brierley, *Archs Biochem. Biophys.* **147**, 557 (1971).
10. J. R. Walton, *Nature, Lond.* **243**, 100 (1973).
11. D. R. Parr and E. J. Harris, *Biochem. J.* **158**, 289 (1976).
12. G. W. Goldstein, *Brain Res.* **136**, 185 (1977).
13. S. C. Kapoor and G. D. V. van Rossum, *Biochem. Pharmac.* **33**, 1771 (1984).
14. D. Holtzman, K. Obana and J. Olson, *J. Neurochem.* **34**, 1776 (1980).
15. S. C. Kapoor, G. D. V. van Rossum and K. J. O'Neill, *Pharmacologist* **22**, 173 (1980).
16. A. J. Vander, D. R. Moun, J. Cox and B. Johnson, *Am. J. Physiol.* **236**, F373 (1979).
17. G. D. V. van Rossum, S. C. Kapoor and M. S. Rabinowitz, *Archs Toxic.*, in press.
18. D. Johnson and H. A. Lardy, *Meth. Enzym.* **10**, 94 (1967).
19. B. Chance and G. R. Williams, *J. biol. Chem.* **217**, 383 (1955).
20. G. D. V. van Rossum and S. A. Ernst, *J. membr. Biol.* **43**, 251 (1978).
21. G. D. V. van Rossum, K. P. Smith and P. Beeton, *Nature, Lond.* **260**, 335 (1976).
22. S. Foden and P. J. Randle, *Biochem. J.* **170**, 615 (1978).
23. K. W. Cleland and E. C. Slater, *Biochem. J.* **53**, 547 (1953).
24. C. L. Moore, *Biochem. biophys. Res. Commun.* **42**, 298 (1971).
25. L. Mela, *Archs Biochem. Biophys.* **123**, 286 (1968).
26. R. W. Estabrook, *Meth. Enzym.* **10**, 41 (1967).
27. C. R. Hackenbrock, *J. Cell Biol.* **30**, 269 (1966).
28. L. Meites, *Handbook of Analytical Chemistry*, 1st Ed., p. 1–45. McGraw-Hill, New York (1963).
29. P. W. Reed and F. L. Bygrave, *Biochem. J.* **140**, 143 (1974).
30. S. C. Kapoor, *Ph.D Thesis*, Temple University (1981).
31. C. S. Kim, L. A. O'Tuama, S. L. Cookson and J. D. Mann, *Toxic. appl. Pharmac.* **52**, 491 (1980).
32. K. M. Six and R. A. Goyer, *J. Lab. clin. Invest.* **76**, 933 (1970).
33. G. D. V. van Rossum, *Biochem. J.* **129**, 427 (1972).