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THE ACCUMULATION OF PUTRESCINE INTO SLICES OF RAT LUNG AND BRAIN AND ITS RELATIONSHIP TO THE ACCUMULATION OF PARAQUAT

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Abstract—The diamine putrescine inhibits accumulation of the herbicide paraquat into slices of rat lung. Putrescine was also shown to be accumulated into rat lung slices by an uptake process which obeyed saturation kinetics. The apparent K_m for the process was 7 μM with a V_{max} of 330 nmoles/g wet wt of lung/hr. The uptake of putrescine was enhanced when the slices were incubated in sodium deficient medium and was inhibited by iodoacetate (1 mM) together with KCN (1 mM), rotenone (100 μM), and by paraquat. Putrescine was not accumulated by slices of liver, kidney, heart or spleen to concentrations much above that in the medium. It was accumulated however by a KCN sensitive process into brain slices although the accumulation was less than that which occurred in lung slices. Lung slices taken from rats given an amount of paraquat known to damage both type I and type II lung alveolar epithelial cells were less able to accumulate putrescine or paraquat than lung slices taken from control rats. This reduction in uptake was similar for both compounds. These data have led us to conclude that (1) the process in the lung and brain which accumulates paraquat is that which is normally responsible for the uptake of putrescine in particular and diamines in general, and (2) it is the alveolar type I and type II cells of the lung which possess a receptor for the active uptake of the diamine putrescine.

The herbicide paraquat (1,1′-dimethyl-4,4′-bipyridilium) has been shown to be accumulated into the lung in comparison with other tissues [1, 2]. This accumulation has been shown to obey saturation kinetics and to be energy dependent [1]. The mechanism or system which is responsible for this accumulation of paraquat is not known although it has been shown [3] that it is not the system reported for the uptake of 5-hydroxytryptamine into the lung [4, 5]. Indirect evidence has been provided to implicate the alveolar type I and type II epithelial cells as the compartment into which paraquat is accumulated [3, 6]. Several endogenous and exogenous compounds have been shown to reduce the accumulation of paraquat into lung slices [7] suggesting that the uptake process for paraquat may be rather non-specific. As a consequence of our search for compounds which would more effectively reduce the uptake of paraquat into the lung we found that the diamine putrescine was a very effective inhibitor of paraquat uptake. We have undertaken studies, therefore, to determine (1) whether putrescine was itself accumulated selectively into the lung, and (2) if the uptake process for putrescine was the same as that for paraquat.

MATERIALS AND METHODS

Materials

[^{14}C]Methyl paraquat dichloride (30 mCi/mmole) and [1,4-^{14}C]putrescine dihydrochloride (116 mCi/mmole) were purchased from the Radiochemical Centre, Amersham. Analytical grade paraquat dichloride (99% pure) was supplied by Plant Protection Division, ICI Ltd., Surrey, U.K. Putrescine dihydrochloride and rotenone were purchased from Sigma Chemical Co., Dorset, U.K., ouabain (Strophanthin G), potassium cyanide (KCN) and iodoacetate from B.D.H. Ltd., Dorset, U.K., and halothane from Pharmaceuticals Division, ICI Ltd., Cheshire, U.K. Soluene 350 (a tissue solubiliser), Dimilume and Instagel (scintillation cocktails) were purchased from Packard Ltd.

Methods

Preparation of tissue slices. Male Alderley Park Wistar derived specific pathogen free rats (body weight 180–220 g) were killed with halothane, the organs removed and slices prepared by hand using a "valet strip" blade (Gillette Surgical Ltd.). Only slices with two cut surfaces were used. As soon as each slice was cut it was placed in a Krebs–Ringer phosphate medium or a sodium deficient medium (when appropriate) at room temperature.

Uptake of paraquat and putrescine into tissue slices. Tissue slices (25–60 mg) were weighed and then incubated in 3.0 ml of modified Krebs–Ringer phosphate containing NaCl (130 mM), KCl (5.2 mM), $CaCl_2$ (1.9 mM), $MgSO_4$ (1.29 mM), Na_2HPO_4 (10 mM) and glucose (11 mM). The pH of the buffer was adjusted to 7.4 with HCl. When sodium deficient buffer was required, sucrose (260 mM) was used to replace the NaCl (130 mM) and thereby maintain osmolality. To measure the uptake of paraquat, 0.1 μCi of [^{14}C]paraquat was added to each 3 ml of incubation medium already containing 10 μM of unlabelled paraquat. To measure the uptake of putrescine, 0.1 μCi [^{14}C]putrescine was added to each 3 ml of medium already containing the required concentration of unlabelled putrescine with the exceptions that when 1 μM and 0.55 μM putrescine concentrations were required, [^{14}C]putrescine alone was

added to give the correct final concentration. Its accumulation into the tissues was measured after 15, 30 and 60 min of incubation in the presence or absence of KCN (1 mM). Incubations were carried out under air in a shaking water bath at 37° and 140 shakes per minute.

To determine the effect of 100, 10 and 1 μM putrescine on the uptake of 10 μM paraquat the lung slices were incubated for 2 hr. When the effect of metabolic inhibitors, ouabain or sodium free medium was studied the uptake of putrescine was measured at 15, 30 and 60 min. With sodium free medium a "paired study" was undertaken in which the uptake of putrescine in the presence or absence of sodium was measured in slices taken from the same rats. The ability of 1 mM, 100 μM and 10 μM paraquat to reduce the uptake of 1 μM or 10 μM [^{14}C]putrescine was determined after 60 min, as was the effect of pre-incubating the slices for 30 min in 10 μM, 100 μM and 1 mM aminoguanidine before measuring the uptake of 10 μM [^{14}C]putrescine. To determine whether it was necessary for putrescine to be present in the incubation medium to inhibit the uptake of paraquat, lung slices were pre-incubated in 100 μM putrescine for 2 hr and then transferred to fresh medium containing 10 μM [^{14}C]paraquat and the uptake of paraquat measured after 30 min, 1 hr and 2 hr.

Measurement of paraquat and putrescine in tissue slices and medium. Tissue slices were removed from the incubation medium and washed by brief immersion in KRP. They were carefully blotted, dissolved in 1 ml Soluene 350 and 10 ml Dimilume added. The radioactivity was determined by liquid scintillation spectrometry. A sample of the medium (0.1 ml) was made up to 1 ml with water and 10 ml Instagel scintillator added. The radioactivity was measured as described above. The counting efficiency was determined by the addition of an internal ^{14}C standard and all counts were then expressed as disintegrations per minute. The slice to medium ratio was calculated as the ratio of ^{14}C present per unit weight of slice to the ^{14}C present in the equivalent volume of medium. From this the amount of paraquat or putrescine present in the slice was calculated.

The validity of using radioactivity as measures of paraquat and putrescine. (i) [^{14}C]*Paraquat*: Paraquat has been shown not to be metabolised in the rat [8, 9]. When ^{14}C label was extracted from lung slices which had been incubated in medium containing [^{14}C]paraquat, the extracted radioactivity was eluted from a G25 Sephadex column in the same fraction as pure [^{14}C]paraquat [10]. Thus, ^{14}C radioactivity has been used as a measure of paraquat.

(ii) [^{14}C]*Putrescine*: The metabolism of putrescine was investigated by measuring the production of $^{14}CO_2$. When lung slices were incubated for up to 4 hr in ^{14}C-labelled putrescine, less than 0.1% of the label initially present in the medium was released as $^{14}CO_2$. After 1 hr incubation, 95 per cent of the label extracted from the lung slice was dialysable and none was precipitated with 50% w/v TCA.

The extraction of putrescine. With lung and brain tissue (the tissues into which [^{14}C]putrescine accumulates most effectively) the ion exchange method of Rosenblum and Russell [11] was used to recover the label from the slice as follows: one hour after incubation in 1 μM [^{14}C]putrescine (0.35 μCi), two washed slices were homogenised in 1.5 ml 5% w/v ice cold TCA, kept on ice, and agitated gently. The homogenate was centrifuged at 2000 *g* for 15 min at 4° and the precipitate discarded. The TCA supernatant was neutralised with 1 M NaOH, the volume of neutralised sample noted and 0.1 ml was taken to measure radioactivity as described above for the medium. 1.0 ml of neutralised supernatant was applied to an ion exchange column (Dowex 50W-X8 200–400 mesh) and 1.8 ml fractions collected. A sample (0.2 ml) was taken from every alternate fraction and dispensed into a scintillation vial containing 0.8 ml water. Instagel (10 ml) was added and the radioactivity determined as above. The percentage radioactivity found in each fraction was calculated and this was compared with the results obtained when [^{14}C]putrescine was added directly to an ion exchange column. The ^{14}C label extracted from the tissue slices was eluted in similar fractions to those using the [^{14}C]putrescine standard, with 95% of the label present in the peak fractions (Fig. 1). We have used, therefore, the presence of ^{14}C label in lung and brain slices as a measure of putrescine.

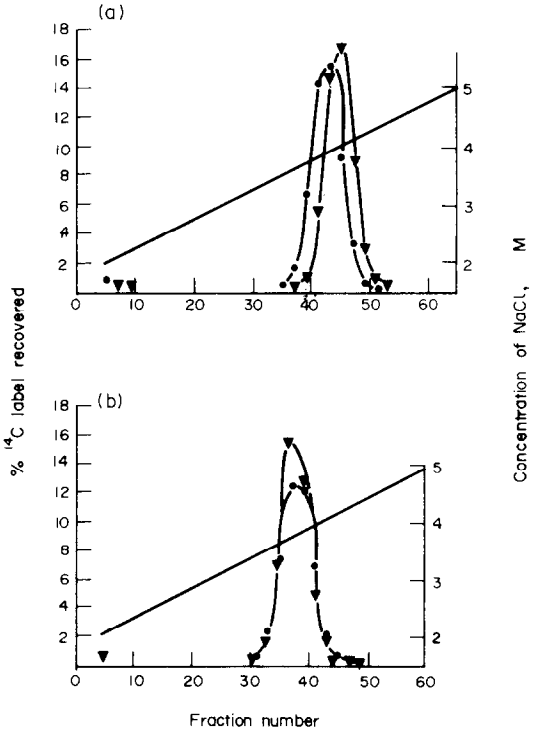

Fig. 1. Lung slices were incubated at 37° for 1 hr in KRP containing 1 μM [^{14}C]putrescine, and the slices were then prepared for the separation of the ^{14}C metabolites by ion exchange chromatography (see Materials and Methods). The columns were eluted with a 2 to 5M linear NaCl gradient in 0.05 M sodium potassium phosphate buffer adjusted to pH 7.2. The elution profile for standard [^{14}C]putrescine is shown by (▼) whilst the elution profile for the ^{14}C label extracted from either the lung or brain slice is shown by (●) and is an average of 2 experiments which gave identical profiles. (a) Is profile for the lung and (b) is for brain.

RESULTS AND DISCUSSION

The discovery that paraquat is actively accumulated into rat lung tissue both *in vivo* [12] and *in vitro* [1] initiated our search for compounds which might inhibit this process. It has been proposed [2, 13] that it is the concentration of paraquat in the lung as compared with other tissues which is responsible for the selective damage which occurs in the lung when rats are dosed with paraquat. We previously reported from this laboratory that a number of endogenous and exogenous compounds reduce the accumulation of paraquat into lung slices [7] and we have subsequently evaluated the effect of many others. Amongst the most effective inhibitors of paraquat accumulation we found, were a series of diamines. We have investigated the relationship between one such endogenous diamine, putrescine, and the lung in more detail.

The effect of putrescine on the accumulation of paraquat into rat lung slices

The accumulation of paraquat (10 μM) into lung slices was reduced by the presence of putrescine in the incubation medium and the extent of this reduction was dependent on the concentration of putrescine (Table 1). Several possible explanations for this inhibition of paraquat accumulation are possible and three have been considered: (1) that putrescine damaged or destroyed the cells into which paraquat accumulates; (2) that putrescine accumulated into the lung cells to very high concentrations and thereby prevented the accumulation of paraquat; or (3) that putrescine competed with paraquat at a site of uptake.

Table 1. The effect of putrescine on the accumulation of paraquat into rat lung slices

Medium concentration		Accumulation of paraquat
Paraquat	Putrescine	(% of control)
10 μM	—	100
10 μM	100 μM	21.0 ± 1.5 (4)
10 μM	10 μM	46.8 ± 5.5 (4)
10 μM	1 μM	97.4 ± 16.9 (4)

Slices of rat lung were incubated at 37° in KRP glucose medium containing [^{14}C]paraquat together with one of the above concentrations of putrescine. The amount of paraquat accumulated into the slice was calculated from the S/M ratio after 2 hr incubation. The results are expressed as mean ± S.E.M. Individual values from slices incubated in the presence of putrescine were expressed against a mean value for slices incubated in 10 μM paraquat of 74.2 nmoles/g wet wt lung/2 hr. Number of determinations are in parentheses.

The first possibility was considered unlikely since it has been shown that putrescine does not affect the ability of lung slices to (a) consume oxygen [14], (b) oxidise glucose [14], (c) release proteins from the slice into the medium [14], (d) synthesise fatty acids [15], or (e) maintain lung NADPH levels [16]. We have taken these parameters to be indices of cellular viability. That the second possibility is not responsible was shown by pre-incubating lung slices in putrescine for 2 hr and then transferring the slices to fresh medium containing [^{14}C]paraquat. In this experiment there was no inhibition of the uptake of paraquat compared with lung slices preincubated in the absence of putrescine (Table 2). (We have shown below that the incubation of lung slices in putrescine results in the accumulation of high concentrations of the diamine in the slice.) It is, therefore, likely that putrescine inhibits the accumulation of paraquat into the slice by competing at the site of uptake.

Accumulation of putrescine into lung slices

We have described (Methods) the measures taken to establish that the presence of ^{14}C label in the slice corresponds to the level of putrescine. Also, amino guanidine (10 μM, 100 μM or 1 mM) an inhibitor of the enzyme diamine oxidase (which is responsible for the metabolism of putrescine [17]) did not affect the amount of radioactivity present in the slice. This indicates that the radioactivity present has not been metabolised and further supports the use of ^{14}C label as a measure of putrescine.

Putrescine (10 μM) accumulated into lung slices in a linear manner over a period of 1 hr (Fig. 2). This accumulation was reduced when the metabolic inhibitors rotenone (100 μM) or iodoacetate (1 mM) in combination with KCN (1 mM) were present in the incubation medium (Fig. 2). Ouabain (100 μM) did not significantly depress the uptake of putrescine and the use of a sodium free medium slightly enhanced putrescine accumulation (Fig. 2). The inhibition of putrescine into lung slices by metabolic inhibitors is consistent with their effect on the accumulation of paraquat [1]. Also, ouabain which did not depress putrescine accumulation (Fig. 2) does not reduce paraquat [1]. The enhancement of accumulation by sodium deficient medium is qualitatively

Table 2. The effect of preincubating rat lung slices in putrescine on the accumulation of paraquat by the slices

Treatment	No. of observations per time point	Slice to medium ratio 0.5 hr	1 hr	2 hr
Control	4	2.2 ± 0.3	3.9 ± 0.4	6.2 ± 0.7
Putrescine	4	1.9 ± 0.2	3.3 ± 0.2	6.0 ± 0.3

Slices of rat lung were preincubated at 37° for 2 hr in KRP glucose medium with or without 100 μM putrescine, and then transferred to fresh KRP glucose medium containing 10 μM [^{14}C]paraquat. The level of paraquat in the slice was determined at 0.5, 1 and 2 hr by radiochemical techniques and a slice to medium ratio determined. The results are expressed as the mean ± S.E.M.

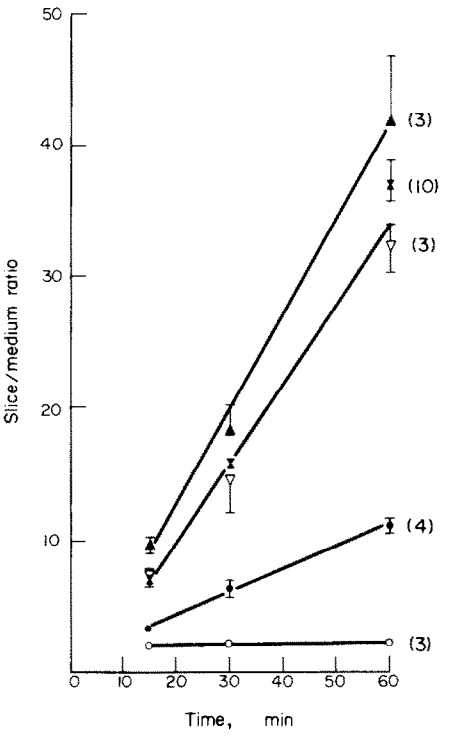

Fig. 2. Slices of rat lung were incubated at 37° in KRP glucose medium containing 10 μM [^{14}C]putrescine. The accumulation of putrescine into the slice was measured after 15, 30 and 60 min incubation(×—×). The experiment was carried out using sodium free medium (▲—▲), in the presence of ouabain (100 μM) (▽—▽), rotenone (100 μM) (●—●) or KCN (1 mM) plus iodoacetate (1 mM) (○—○). The results are expressed as the mean ± S.E.M. with the number of measurements in parentheses.

Table 3. The effect of paraquat on the accumulation of putrescine

Medium concentration Putrescine	Paraquat	Accumulation of putrescine (% of control)
1 μM	—	100
1 μM	1 mM	15.5 ± 2.2
1 μM	100 μM	63.6 ± 5.0
1 μM	10 μM	83.5 ± 14.3
10 μM	—	100
10 μM	1 mM	35.5 ± 0.7
10 μM	100 μM	92.4 ± 9.6
10 μM	10 μM	122-4 ± 14.9

Slices of rat lung were incubated at 37° in KRP glucose medium containing either 1 μM or 10 μM [^{14}C]putrescine. The S/M ratio for putrescine was determined for each treatment after 60 min incubation. The amount of putrescine present in the slices incubated in the presence of paraquat is expressed as a percentage of the mean amount present in the slices incubated in the absence of paraquat. The control putrescine S/M ratios for 1 μM and 10 μM were 81.9 and 22.7 respectively. The results are expressed as mean ± S.E.M.

Table 4. The accumulation of putrescine into rat lung slices

Concentration of putrescine in medium (μM)	Rate of accumulation (nmoles/g wet wt lung/hr)
0.55	24 (6)
3.9	106 (13)
10	244 (47)
33	302 (79)
100	318 (91)

Slices of rat lung were incubated at 37° in KRP glucose medium containing various concentrations of [^{14}C]putrescine (see above). The amount of putrescine in the slice was measured after 15, 30 and 60 min of incubation and from this the rate of accumulation was determined using a weighted linear regression model against time. The results are expressed as mean with 95% confidence limits in parentheses. Three determinations were made for each time point with each concentration.

the same both for putrescine (Fig. 2) and paraquat [3]. However, the accumulation of paraquat was enhanced approximately 3-fold whereas the enhancement of putrescine accumulation, while statistically significant, by paired t test analysis ($P < 0.05$), was only marginal. At present we are unable to offer a satisfactory explanation for this quantitative difference.

The accumulation of putrescine into lung slices was increasingly reduced when increasing concentrations of paraquat were present in the incubation medium (Table 3). Also when the concentration of putrescine decreased (Table 3). These observations (from 1 μM to 10 μM) the ability of a given concentration of paraquat to inhibit the accumulation of putrescine decreased (Table 3). These observations support our suggestion that there is competition between paraquat and putrescine for a site of uptake into the lung. The concentration of paraquat required to inhibit the accumulation of putrescine was much greater than that of putrescine required to inhibit the uptake of paraquat. An explanation for this can probably be found in the apparent kinetic constants which can be derived for the accumulation of paraquat and putrescine. Using a range of concentrations of putrescine in the incubation medium the rate of accumulation was measured and found to be linear. Using these data (Table 4) an apparent K_m of 7 μM and V_{max} of 330 nmoles/g wet wt/hr was calculated. The apparent K_m for the accumulation of paraquat is 70 μM with a V_{max} of 300 nmoles/g wet wt/hr [1]. Thus there is an order of magnitude difference in the apparent K_m for the uptake process for these compounds.

The compartment into which putrescine is accumulated

It has previously been shown that the uptake of paraquat into lung slices prepared from lungs with damaged type I and type II alveolar epithelial cells is reduced [3]. The damage to these alveolar epithelial cells was produced by the intravenous administration of 65 μmoles of paraquat/kg body wt and the extent of damage to the epithelium assessed at

Table 5. The accumulation of paraquat and putrescine into lung slices taken from rats previously dosed with paraquat

Time after dosing with paraquat (hr)	Accumulation (as % of control)	
	Paraquat	Putrescine
2	97.6 ± 9.6 (12)	85.7 ± 6.7 (7)
24	69.4 ± 8.9 (12)*	66.2 ± 0.8 (12)†

Rats were dosed s.c. with 108 μmoles/kg of paraquat, or saline and killed at 2 or 24 hr after dosing. Slices of lung from each rat were incubated at 37° in KRP glucose medium containing 10 μM [^{14}C]paraquat or 10 μM [^{14}C]putrescine and the accumulation of each compound studied at 15, 30 and 60 min. Using a linear regression model for the time course studied in each rat we calculated the nmoles of compound accumulated/g wet wt/hr and from this data obtained % of control, which is expressed as mean ± S.E.M. with the figures in parentheses being the number of animals studied. Control rate of accumulation for paraquat was 44.0 nmoles/g wet wt/hr and for putrescine was 488 nmoles/g wet wt/hr.

* Statistically different to controls at the $P < 0.02$ level.

† Statistically different to controls at the $P < 0.01$ level.

various times after dosing [6]. Using a similar protocol we dosed rats with paraquat and removed the lungs at 2 hr and 24 hr after dosing. The accumulation of putrescine (10 μM) and paraquat (10 μM) was measured in lung slices prepared from the paraquat dosed and saline injected rats. Two hours after dosing when there was no evidence of damage to the alveolar epithelial cells as determined by electron microscopy [18], there was no reduction in the accumulation of putrescine or paraquat into the slices. By 24 hr after dosing, when there was good morphological evidence that the alveolar type I and type II cells were damaged [18], the accumulation of both putrescine and paraquat was reduced by approximately 35 per cent (Table 5). These results confirm those already obtained with paraquat [3] and support the conclusion that the compartment into which paraquat and also putrescine is accumulated is at least in part the type I and type II alveolar epithelial cells.

The uptake of putrescine into various rat tissues slices

It has been shown that tissue slices prepared from various organs of the rat do not accumulate paraquat to the same extent as lung slices [2]. Slices of lung incubated in 1 μM paraquat accumulated approximately 5 nmoles paraquat/g wet wt/hr whereas brain cortex, which is the next most effective accumulator of paraquat, accumulated only 1 nmoles/g wet wt/hr [2]. The adrenal, kidney, muscle, liver, heart and spleen accumulated approximately 0.5 nmole/g wet wt/hr. However, whether this was an energy dependent uptake was not established [2]. Even if paraquat was accumulated by these tissues it occurred at a much reduced rate in comparison with the lung. We found that the relative accumulation of putrescine into various rat tissues was essentially the same as that described for paraquat. When lung slices were incubated in 1 μM putrescine the rate of accumulation was 82 nmoles/g wet wt/hr (Table 3) whereas the next most effective tissue was the brain with 8.7 nmoles/g wet wt/hr (Table 6). The liver, kidney, heart and spleen accumulated approximately 1 nmole/g wet wt/hr (Table 5). With all these tissue slices we have shown the accumulation to be reduced by KCN but with the exception of the lung and brain the rate of accumulation was extremely small. Thus, there appears to be selective accumulation of putrescine into lung and brain slices in comparison with other tissues studied.

The relationship of the accumulation of putrescine to that of paraquat

When paraquat was shown to accumulate selectively into the lung, the uptake system was considered novel [1, 3]. We started with the assumption that the uptake process responsible for the accumulation of paraquat was required to accumulate some endogenous compound(s). The fact that putrescine is (1) selectively accumulated into lung

Table 6. The accumulation of putrescine into rat tissue slices and the effect of KCN

Tissue	Concentration of putrescine in medium	KCN present in medium (1 mM)	Accumulation into tissue S/M ratio at times of incubation		
			15 min	30 min	60 min
Liver	1 μM	−	0.48 ± 0.02 (9)	0.61 ± 0.05 (9)	0.75 ± 0.14 (9)
	1 μM	+	0.37 ± 0.03 (5)	0.46 ± 0.02 (5)	0.52 ± 0.03 (5)
Kidney cortex	1 μM	−	0.78 ± 0.04 (10)	0.94 ± 0.03 (10)	1.15 ± 0.05 (10)
	1 μM	+	0.59 ± 0.03 (5)	0.80 ± 0.06 (5)	0.97 ± 0.05 (5)
Spleen	1 μM	−	0.45 ± 0.03 (10)	0.62 ± 0.04 (10)	1.10 ± 0.11 (10)
	1 μM	+	0.27 ± 0.01 (5)	0.33 ± 0.01 (5)	0.37 ± 0.02 (5)
Heart	1 μM	−	0.61 ± 0.002 (10)	0.74 ± 0.02 (10)	0.85 ± 0.02 (10)
	1 μM	+	0.51 ± 0.03 (5)	0.57 ± 0.03 (5)	0.62 ± 0.04 (5)
Brain cortex	1 μM	−	1.61 ± 0.18 (3)	3.53 ± 0.45 (8)	8.71 ± 0.98 (8)
	1 μM	+	—	1.04 ± 0.06 (5)	1.32 ± 0.18 (5)
	10 μM	−	1.35 ± 0.15 (3)	2.22 ± 0.10 (3)	3.43 ± 0.34 (8)
	10 μM	+	—	1.02 ± 0.09 (4)	1.15 ± 0.1 (4)

Slices of the above tissues were incubated in KRP glucose medium containing 1 μM or 10 μM [^{14}C]putrescine, and the amount of ^{14}C label accumulated was measured at 15, 30 and 60 min, using radiochemical techniques. The results are expressed as the mean ± S.E.M. with the number of observations in parentheses.

and brain slices by an energy dependent, sodium independent system, (2) able to compete with paraquat for the uptake site, and (3) accumulated into the same cellular compartment as paraquat (i.e. the type I and type II alveolar epithelial cells) leads us to propose that there exists in the lung an uptake process for the diamine putrescine which is responsible for the uptake of paraquat. This may well be a process responsible for the accumulation of a number of diamines since a range of these compounds will inhibit the uptake of paraquat into lung slices (R. Krieger, personal communication). It is of interest that an uptake process for the polyamines spermidine and spermine has already been described in brain cortex slices [19] and this process was found to be sodium independent. It seems possible that the uptake process in the brain for polyamines may well be similar for that we have described for the diamine putrescine in the lung.

At present we cannot explain why lung and brain slices selectively accumulate putrescine (and probably other diamines). That the process occurs *in vivo*, however, can be argued from the analogy with paraquat which is known to be accumulated both *in vitro* and *in vivo*. If it is a diamine transport system which paraquat uses then the accumulation of diamines from the plasma should be demonstrable *in vivo*. Current studies are testing this hypothesis.

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