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THE ACCUMULATION AND LOCALISATION OF PUTRESCINE, SPERMIDINE, SPERMINE AND PARAQUAT IN THE RAT LUNG

IN VITRO AND *IN VIVO* STUDIES

I. WYATT, A. R. SOAMES, M. F. CLAY* and L. L. SMITH†

Central Toxicology Laboratory, Imperial Chemical Industries PLC, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, and * Medical Research Council Pneumoconiosis Unit, Llandough Hospital, Penarth, South Glamorgan, CF6 1XW, U.K.

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Abstract—Putrescine was accumulated into the isolated perfused rat lung by a temperature dependent process. The uptake obeyed saturation kinetics for which an apparent K_m of 14 μM and V_{max} of 48 nmol/g wet wt/hr was derived. After rats were dosed subcutaneously with [^{14}C]putrescine, it was accumulated in the lung to concentrations greater than that in the plasma with the highest amount found between 3 and 12 hr. From 3 hr after dosing until 24 hr, there was a progressive increase in ^{14}C label incorporated into spermidine, indicating that putrescine was converted to spermidine. Using autoradiographic techniques in lung slices the [^{3}H]oligoamines were found in the alveolar epithelial type II, Clara and very probably the alveolar type I cells. With [^{3}H]paraquat, the presence was detected only in the alveolar type II cells. Likewise, in the isolated perfused rat lung or following s.c. dosing of rats with [^{3}H]putrescine the radiolabel was located only in the alveolar type II cell. We have suggested that the most likely explanation for the differences in localisation of label between *in vitro* and *in vivo* studies resulted from the use of [^{3}H] label of different specific activity. Consequently we have concluded that the cell types with the ability of accumulate paraquat and oligoamines were the alveolar epithelial type I and type II cells and Clara cells.

When the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium) is ingested, it is capable of damaging the lung of both man [1–3] and various species of experimental animal [4–6]. Although the toxicity of paraquat can involve damage to several vital organs, the lung is often the most severely affected, resulting in the death of the patient or experimental animal as a consequence of anoxia. At least part of the explanation of this organ selective toxicity is the ability of the lung to accumulate [7, 8] and retain paraquat [9–11]. In this way the lung achieves higher concentrations of the bipyridyl compared with other organs [8]. Recently, we found that the process responsible for paraquat accumulation in lung is one which is able to accumulate the endogenous polyamines, putrescine, spermidine and spermine [12, 13]. The accumulation system has been further characterised using rat lung slices [14] and the human lung has been shown to have a similar uptake system [15].

The ability of the isolated perfused lung or the lung *in vivo* to accumulate putrescine in a similar manner to lung slices has not previously been described. Although it is well established in other systems that ornithine is converted by ornithine decarboxylase to putrescine and that this in turn is converted to spermidine and then spermine [16, 17], the fate of accumulated putrescine in the lung has not been reported. However, since paraquat is taken up both in lung slices [7, 8] and by the lung *in vivo* [18], we have speculated that by analogy the polyamines would be accumulated *in vivo*. We have therefore attempted to (1) established whether the diamine putrescine is accumulated into the isolated perfused lung and the lung *in vivo*, (2) determine the fate of the putrescine accumulated into the lung, (3) determine the cell type(s) into which putrescine, spermidine, spermine and paraquat are accumulated *in vitro*, and (4) determine the cell type(s) into which putrescine is accumulated *in vivo*.

MATERIALS AND METHODS

Materials

[1,4-^{14}C]Putrescine dihydrochloride (116 mCi/mmol), [^{14}C]spermidine trihydrochloride (120 mCi/mmol), [^{14}C]spermine tetrahydrochloride (112 mCi/mmol), [1,4n-^{3}H]putrescine dihydrochloride (19 Ci/mmol) and [methyl ^{3}H]paraquat dichloride (3 Ci/mmol) were purchased from Amersham International PLC (Amersham, Bucks, U.K.). [^{3}H]-Spermidine trihydrochloride (31 Ci/mmol) and [^{3}H]spermine tetrahydrochloride (29 Ci/mmol) were purchased from New England Nuclear Ltd (Southampton, U.K.). Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, heptane diamine, *o*-phthaldialdehyde and Tween 80 were all obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Paraquat dichloride (99.9% pure) was a sample from Imperial Chemical Industries PLC (Bracknell, Berkshire, U.K.). Halothane BP was supplied by Imperial Chemical Industries PLC (Pharmaceuticals Division,

† To whom correspondence should be addressed.

Macclesfield, Cheshire, U.K.). Span 60 was purchased from Honeywell-Atlas Ltd (Carshalton, Surrey, U.K.). 1-Octanesulfonic acid (sodium salt) was obtained from Kodak Ltd (Kirby, Liverpool, U.K.). Dowex 50W-X8 (200–400 mesh) H^+ form was supplied by Bio-Rad Laboratories Ltd (Bromley, Kent, U.K.). An ultrasonic disintegrator (150 W) was supplied by MSE Ltd (Crawley, Sussex, U.K.). The tissue solubiliser Soluene 350 and scintillator Dimilume were purchased from Packard Instruments Ltd (Amersham, Bucks, U.K.), whilst the scintillator Fisofluor "MPC" was obtained from Fisons PLC (Loughborough, Leicestershire, U.K.). A μ-Bondapak C_{18} column (3.9 mm × 300 mm; 10 μm particle) was supplied by Waters Associates (Inst) Ltd (Northwich, Cheshire, U.K.). Millipore filters Millex AA (0.8 μm) and Millex GS (0.22 μm) were purchased from Millipore UK Ltd (Harrow, Middlesex, U.K.). Sodium pentobarbitone "Sagatal" was supplied by May & Baker Ltd (Dagenham, U.K.).

Animals

Male Alderley Park, Wistar-derived, specific pathogen-free rats (body weight approximately 200 g) were used for the lung slice and *in vivo* studies. Male Albino Oxford strain rats (body weight 250–450 g) were used in the isolated perfused lung studies.

Isolated purfused lung

Preparation. The apparatus and surgical procedure had many of the features in the methods described by Rosenbloom and Bass [19] and by Neimeier and Bingham [20]. Guyton *et al.* [21] and Russell *et al.* [22] showed that the period for which the IPL remained oedema free could be prolonged by limiting the input pressure of the perfusate and raising the intra-alveolar air pressure respectively. Consequently, modifications were made to the methods so that:

(1) The perfusate pressure in the pulmonary artery was 16 cm H_2O, pulsing to 20 cm H_2O.
(2) A collecting cannula was tied into the left atrium and the perfusate pressure at this point in the circulation was maintained at 4 cm H_2O.
(3) During its dissection and transfer to the artificial thorax the lung was kept partially inflated by a small positive intratracheal pressure. Thereafter this pressure was raised to +10 cm H_2O and the lungs were ventilated by a cyclical pressure of −1 to −4 cm H_2O applied to the pleura.

The perfusate was prepared from Dulbecco's modification of Eagles Medium (DMEM)* with glutamine and also contained 4.5% w/v bovine serum albumin [23], penicillin (100 units/ml), streptomycin (100 μg/ml) and gentamycin (20 μg/ml). When the perfusate was equilibrated (at 37°) against the cylinder gas (40% v/v O_2, 9.5% CO_2 and 50.5% N_2) the pH was 7.45.

Accumulation of putrescine. The ability of the isolated perfused lung to accumulate putrescine was studied with 1 μM [^{14}C]putrescine (113 mCi/mmol) in the perfusate for 30, 60 and 120 min with three lungs per time point. The effect of temperature (7°) on the accumulation of 1 μM [^{14}C]putresceine was investigated during 120 min perfusion with three lungs per treatment. To determine if the accumulation of putrescine obeyed saturation kinetics the concentration of putrescine (0.5, 1, 5, 10 and 50 μM) in the perfusate was varied.

The identification of the cell types involved in accumulation was determined using autoradiographic techniques on two lungs perfused with 1 μM [^{3}H]putrescine for 2 hr. The flow rate of the perfusate was monitored for all perfusions at 0, 30, 60 and 120 min and was between 10–20 ml/min. Samples of medium (1 ml) were taken at these time points and frozen at −40°. At the end of the perfusion, the lungs were perfused with 10 ml of putrescine free DMEM at 37°, and frozen at −40°. Prior to freezing the lungs perfused with [^{3}H]putrescine, the left lobe was removed, sliced on the McIlwain tissue chopper (0.5 mm), divided into five equal portions of known weight and each placed in 5 ml of modified Karnovsky's fixative [24] in readiness for autoradiography. In order to determine the fate of radiolabel in the lung, two lungs were perfused with 1 μM [^{14}C] or [^{3}H]putrescine, for 2 hr.

Measurement of putrescine in the lung and perfusate. The level of radiolabel in the lung and perfusate was determined using a liquid scintillation spectrometer and the amount of putrescine accumulated by the lung was determined as described by Smith and Wyatt [12].

The in vivo *distribution of putrescine in the plasma and the lung*

Preparation of a slow release putrescine formulation. This was prepared based on information obtained from Becker [25]. The emulsion consisted of 10% w/w aqueous phase containing [^{14}C] or [^{3}H]putrescine, 11% w/w Span 60, one drop of Tween 80 and was made up to 100% w/w with liquid paraffin (s.g. 0.83–0.89). The mixture was emulsified by sonication for 30 sec on a MSE 150 W ultrasonic disintegrator with a 3/8 in. probe, at maximum output. This dosing solution was given s.c. at 0.2 ml/100 g body weight equivalent to 518 μmol putrescine/kg for all studies. The dosing solution (0.1 ml) was made up to 10 ml with toluene and 20 μl of this dilution was added to 10 ml of Fisofluor and the radioactivity measured in a liquid scintillation spectrometer. The level of putrescine in the dosing solution was checked using the HPLC method of Seiler and Knodgen [26] and the specific activity of the dosing solution was then determined.

The measurement of [^{14}C] *in the lung and plasma.* Rats were dosed s.c. with 518 μmol [^{14}C]putrescine/kg (0.0565 μCi/μmol) and killed with an overdose of halothane at 1, 3, 6, 12.5 and 24 hr after dosing, with five rats per time point. Immediately upon cessation of breathing, blood was taken by cardiac puncture into a lithium heparin tube and stored on ice. The lung was then gently perfused free of blood with 10 ml saline via the pulmonary artery, the right anterior lobe weighed, dissolved in Soluene 350, and

* Abbreviations used: TCA, trichloroacetic acid; PCA, perchloric acid; KRP, Krebs–Ringer phosphate with glucose; DMEM, Dulbecco's modification of Eagles Medium (with glutamine); oligoamines, putrescine, spermidine and spermine.

the level of radiolabel determined. The remainder of the lung was weighed and stored at −70°. Plasma was separated from the blood, the level of radioactivity determined and the remainder of the plasma frozen at −70°.

Control rats were dosed s.c. with an emulsion formulation containing no putrescine and five were killed 1, 12.5 and 24 hr after dosing. The lungs and plasma were treated in an identical manner to the putrescine dosed rats.

The distribution and localisation of [^{3}H]*putrescine in the lung.* Male rats were dosed s.c. with 518 μmol [^{3}H]putrescine/kg (1.62 μCi/μmol) in the slow release emulsion and the lungs removed 6 and 24 hr after dosing (three and two rats studied respectively). The lungs were perfused with 10 ml of saline, the left lung removed for autoradiographic studies—sliced on a McIlwain tissue chopper (0.5 mm) and known weights placed in 5 ml of modified Karnovsky's fixative [24]. The remainder of the lung was used to determine the fate of the [^{3}H] label.

The fate of [^{14}C] *and* [^{3}H] *label in the plasma and lung.* Equal volumes of plasma and ice-cold 0.4 M $HClO_4$ were mixed, placed on ice for 30 min, centrifuged at 10,000 *g* for 2 min in a microfuge, and the level of radiolabel in the supernatant determined using a liquid scintillation spectrometer. Lung tissue homogenate (20% w/v) was prepared in ice-cold 5% w/v TCA or 0.2 M $HClO_4$ with a vortex homogeniser. The homogenates were stored on ice for 30 min with occasional mixing, centrifuged at 1000 *g* for 20 min at 4°, and the supernatant removed. The level of radioactivity in the supernatant was measured and the radiolabel distribution in the supernatant determined using the ion exchange method of Rosenblum and Russell [27]. Approximately 85% of the radiolabel from the isolated perfused lung was eluted as putrescine. Thus the presence of [^{14}C] or [^{3}H] label in the perfused lung has been used as a measure of putrescine.

Analysis of putrescine, spermidine and spermine in plasma and lung

The lung $HClO_4$ extracts were passed though a Millex AA filter (0.8 μm) followed by a Millex GS filter (0.22 μm) whilst the plasma extracts were only passed through a 0.22 μm filter. The levels of putrescine, spermidine and spermine in the lung and plasma filtrate were measured using the HPLC post column derivatisation technique of Seiler and Knodgen [26]. Dilution of all samples was carried out in the HPLC buffer A and they all contained an internal standard of heptane diamine.

The accumulation of [^{3}H]*oligoamines and paraquat by lung slices*

Lung slices and Krebs–Ringer Phosphate (KRP) were prepared as described by Smith and Wyatt [12]. The slices were incubated in 3 ml KRP containing either 1 μCi of [^{3}H]putrescine, spermidine or spermine and 1 μM of the corresponding unlabelled compound, or 3 μCi [^{3}H]paraquat and 10 μM of unlabelled paraquat. The oligoamines were incubated for 1 hr, whilst paraquat was incubated for 2 hr, in a shaking water bath at 37° and 70 rpm. The level of radiolabel was determined in one group of slices as previously described [12], whilst the remainder was immersed in modified Karnovsky's fixative [24] in preparation for autoradiography.

Autoradiographic studies

Slices of lung which had been immersed in modified Karnovsky's fixative [24] overnight were washed in three changes of sucrose phosphate buffer, post fixed in 1% Millonig's osmium tetroxide for 30 min and dehydrated through a graded series of acetones. One series of slices were not post fixed in 1% Millonig's osmium tetroxide, but were otherwise treated similarly. Fifty microlitres of water was added to the desiccated slices, followed by 1 ml Soluene 350 and 10 ml of Dimilume. The level of radiolabel was determined so that the loss of label following fixation and dehydration could be determined (osmium tetroxide appears to compromise the scintillator). The remaining series of slices for each compound were infiltrated with araldite and embedded at 60° overnight. Autoradiographs were prepared from 1 μm sections using Ilford K2 nuclear emulsion. The sections were developed, after two weeks exposure, with half strength Kodak D19 for 7 min at 20° and fixed in 30% w/v sodium thiosulphate. The sections were stained with 1% w/v toluidine blue.

RESULTS

Accumulation of putrescine in isolated perfused lung

When lungs were perfused with 1 μM [^{14}C]putrescine at 37°, putrescine was accumulated in a linear, time-dependent manner over a period of 2 hr (Fig. 1). When the lungs were cooled to 7°, the uptake was abolished (Fig. 1). By varying the concentration of putrescine in the perfusate (0.5 μM to 50 μM), it was possible to demonstrate that the accumulation obeyed saturation kinetics, and an apparent K_m of 14 μM with a V_{max} of 48 nmol/g wet weight lung/hr was calculated (Table 1). The apparent K_m for accumulation is similar in both lung slices and the perfused lung although V_{max} in the perfused lung is much lower (Table 1).

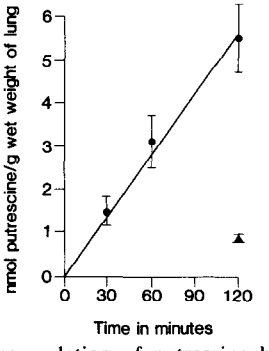

Fig. 1. The accumulation of putrescine by the isolated perfused lung. The lungs were perfused as described in the Materials and Methods section. The perfusate was 1 μM [^{14}C]putrescine at 37° (●–●–●), or 7° (▲). At the end of the perfusion, the lungs were perfused with putrescine free medium, weighed, dissolved in Soluene and the level of radioactivity determined. Using the specific activity of the perfusing medium the amount of putrescine in the lung was determined. The results are expressed as the mean ±SEM with three observations per point.

Table 1. Kinetic constants for the accumulation of putrescine into the perfused rat lung

Concentration of putrescine in perfusate (μM)	Rate of accumulation (nmol/g wet wt lung/hr)	Apparent K_m (μM)	V_{max} (nmol/g wet wt/hr)
0.5	1.7		
1	3		
		14	48
5	15		
10	19		
50	82		
	*Lung slice (hand cut)	7	330
	*Lung slices (tissue chopped)	13	723

The perfused lungs were prepared as described in the Materials and Methods section. The lungs were perfused with the above concentrations of [^{14}C]putrescine for 1 hr at 37°. At the end of the perfusion, the lungs were perfused with 10 ml of putrescine free medium, weighed, dissolved and the level of radioactivity determined. Using the specific activity of the perfusate the concentration of putrescine in the lung was determined. The apparent K_m and V_{max} were determined using the method of Lineweaver–Burke,† plotting 1/v against 1/c. A regression line on the plot gave a correlation coefficient of 0.99.

* Smith *et al.* [13]; † Lineweaver and Burke [39].

Accumulation of putrescine into the lung in vivo

When rats were dosed s.c. with [^{14}C]putrescine (518 μmol/kg) in a slow release formulation the level of radioactivity in the plasma and lung rose rapidly, peaking approximately 5 hr after dosing and thereafter the concentration continued to fall until completion of the experiment at 24 hr (Fig. 2). However, when the lungs and plasma were treated with PCA the amount of ^{14}C in the supernatant of the treated plasma was reduced whereas the amount of label in the lung remained unchanged (Fig. 2). The concentration in the lung increased from approximately 40 nmol/g wet weight to approximately 120 nmol/g wet weight (Fig. 3). The level in the plasma increased from zero to approximately 45 nmol/ml by 3 hr and by 24 hr after dosing it had returned to control levels (Fig. 3). The increase in the lung concentration was entirely accounted for by the presence of [^{14}C]putrescine which reached levels of approxi-

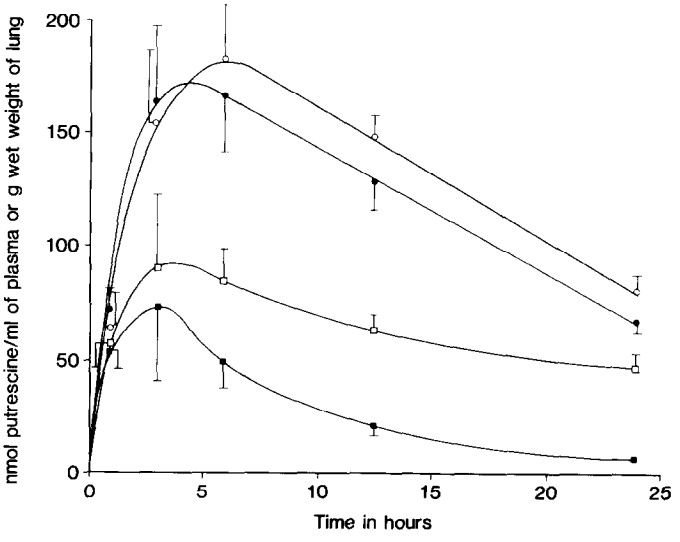

Fig. 2. The level of radiolabel in the lung and plasma following a s.c. dose of 518 μmol [^{14}C]putrescine/kg. Male rats were dosed s.c. with 518 μmol of [^{14}C]putrescine (0.0565 μCi/μmol)/kg in a slow release formulation, and the animals were killed with an overdose of fluothane at 1, 3, 6, 12.5 and 24 hr after dosing (five animals per time point). The levels of ^{14}C in the lung and plasma before and after addition of perchloric acid (PCA) were determined using radiochemical techniques. The level of ^{14}C detected was converted to nmol of putrescine assuming the label was putrescine. The profiles are as follows: ○-○-○, lung dissolved in soluene; ●-●-●, PCA supernatant of lung; □-□-□, plasma; and ■-■-■, PCA supernatant of plasma. The results are expressed as the mean ± SEM.

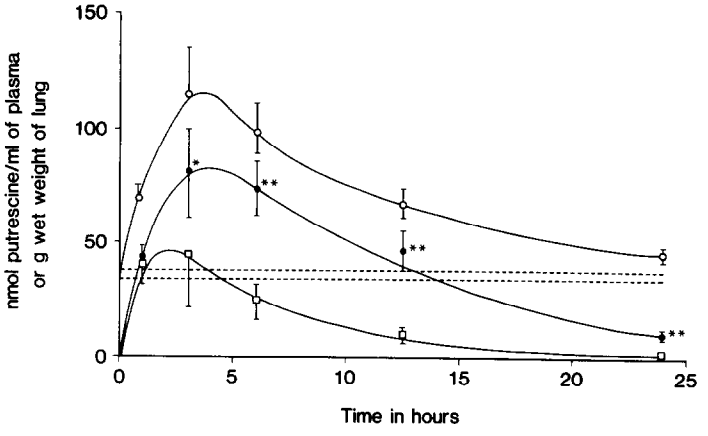

Fig. 3. The level of putrescine in the lung and plasma following a s.c. dose of 518 μmol [^{14}C]putrescine/kg. Male rats were dosed s.c. with 518 μmol of [^{14}C]putrescine (0.0565 μCi/μmol)/kg in a slow release formulation, and the animals were killed with an overdose of fluothane 1, 3, 6, 12.5 and 24 hr after dosing, with five rats per time point. Control rats were dosed with the ingredients that made up the slow release formulation and were killed at 1, 12.5 and 24 hr with five rats per time point. The level of putrescine in the plasma and lung was determined using the HPLC post column derivitisation method of Seiler and Knodgen [26]. The level of [^{14}C]putrescine in the lung was determined using the ion exchange technique of Rosenblum and Russell [27]. The profiles are as follows: ○–○–○, concentration of putrescine in the treated lung; ●–●–●, concentration of [^{14}C]putrescine in the treated lung; □–□–□, concentration of putrescine in the treated plasma, and the dotted bar across the graph is the level of putrescine in the control lung (35.6 ± 1.5 nmol/g wet weight of lung for 15 observations). The results are expressed as the mean ± SEM. Significantly different to plasma level, *$P > 0.05$, **$P > 0.01$ by paired t test.

mately 75 nmol/g wet weight, indicating that [^{14}C] putrescine was present in the lung at twice the concentration found in the plasma (Fig. 3). By 24 hr after dosing, the lung concentration of [^{14}C]putrescine fell to very low levels and the total concentration of putrescine returned to control values (Fig. 3).

The levels of polyamines in the plasma and the lung

No putrescine, spermidine or spermine was detected in the plasma of control rats, which is in agreement with Saeki *et al.* [28]. After dosing with [^{14}C]putrescine (518 μmol/kg) no spermidine or spermine was detected in the plasma, which is consistent with other *in vivo* studies [29, 27]. The lung from control rats contained 35.6 ± 1.5 nmol/g wet weight of putrescine (Fig. 3), whilst spermidine ranged from 760 to 850 nmol/g wet weight and spermine from 400 to 500 nmol/g wet weight (Table 2). These levels are similar to those detected by Morton *et al.* [30]. The levels of spermidine or spermine found in the lung were similar to controls (Table 2).

Table 2. The concentration of spermidine and spermine in control lungs and lungs from rats dosed with [^{14}C]putrescine

Time after dosine (hr)	nmol/g wet weight of lung				
	Control		Treated		
	Spermidine	Spermine	Spermidine	[^{14}C]Spermidine	Spermine
1	764 ± 30	395 ± 19	770 ± 25	ND	380 ± 20
3			819 ± 28	15.3 ± 6.3	474 ± 19
6			805 ± 26	14.3 ± 8.8	432 ± 20
12.5	766 ± 26	477 ± 17	770 ± 19	21.5 ± 3.1	432 ± 11
24	847 ± 22	500 ± 42	816 ± 28	30.4 ± 2.4	534 ± 43

ND Not detected.

Male rats were dosed s.c. with 518 μmol [^{14}C]putrescine (0.0565 μCi/μmol)/kg in a slow release formulation. At 1, 3, 6, 12.5 and 24 hr after dosing, the level of spermidine and spermine in the lung was determined using the HPLC post column derivatisation method of Seiler and Knodgen [26]. The level of [^{14}C]spermidine in the lung was determined using the ion exchange separation of Rosenblum and Russell [27]. Control animals were dosed with the slow release formulation lacking in putrescine and the levels of spermidine and spermine in the lung determined, 1, 12.5 and 24 hr after dosing.

The results are expressed as the mean ± SEM with five observations per time point.

Table 3. The percentage distribution of ^{14}C in putrescine, spermidine or spermine in the lung

Time (hr)	Not retained on column (unidentified)	Putrescine	Spermidine	Spermine
1 (5)	34.1 ± 4.0	57.2 ± 2.1	ND	ND
3 (5)	36.4 ± 2.5	47.0 ± 3.7	10.3 ± 5.3	ND
6 (5)	45.7 ± 4.9	43.7 ± 2.9	8.6 ± 5.6	ND
12.5 (5)	34.7 ± 4.5	35.3 ± 4.2	16.8 ± 1.4	ND
24 (4)	27.0 ± 6.2	14.6 ± 2.1	46.6 ± 3.9	ND

ND Not detected.

Male rats were dosed s.c. with 518 μmol [^{14}C]putrescine (0.0565 μCi/μmol)/kg in a slow release formulation. At 1, 3, 6, 12.5 and 24 hr after dosing the radiolabel in the lung was measured. The presence of ^{14}C label in putrescine, spermidine and spermine was determined using the ion exchange method of Rosenblum and Russell [27].

The results are expressed as the mean ± SEM with the number of observations in parenthesis.

The fate of ^{14}C labelled putrescine in the lung

The distribution of ^{14}C label in putrescine, spermidine and spermine was determined at various times after dosing (Table 3). One hour after dosing, approximately 60% of the ^{14}C label was found in putrescine whereas none was detected in spermidine or spermine (Table 3). As the time after dosing increased, the amount of ^{14}C label present as putrescine decreased so that by 24 hr after dosing only 15% of the label in the lung was putrescine (Table 3). Concomitantly the percentage ^{14}C label present in the lung as spermidine increased with time, so that by 24 hr approximately 45% of the ^{14}C label was in spermidine (Table 3). During the course of the study no ^{14}C label was found in spermine and between 27% (24 hr) and 46% (6 hr) of the label was present as unknown compounds.

Even at 24 hr when the percentage ^{14}C label in the form of spermidine is maximal (Table 3) only 30 nmol of [^{14}C]spermidine was present although the total lung spermidine content is over 800 nmol/g wet weight (Table 2).

Autoradiographic studies

Retention of the [^{3}H] label. It has previously been demonstrated that lung slices do not metabolise putrescine, spermidine or spermine [12, 13], and paraquat has been shown not to be metabolised by the rat [31, 32]. Thus, the measurement of radioactivity was taken as a measurement of the compound. We found that greater than 80% of the putrescine, spermidine and spermine accumulated by the lung slice, the isolated perfused lung, or the lung *in vivo* was retained during fixation and dehydration, whilst only 5% of the paraquat accumulated by the lung slice was retained.

Disposition of ^{3}H label in the lung. The disposition of [^{3}H]putrescine was found to be similar to that of [^{14}C]putrescine both 6 and 24 hr after dosing (cf. Tables 3 and 4).

The autoradiographs of lung slices incubated with [^{3}H]putrescine, [^{3}H]spermidine or [^{3}H]spermine showed that the polyamines were selectively taken up by the dark staining Clara cells of the respiratory bronchiole in comparison to the paler staining ciliated cells (Figs 4b, 4d, 5b). There was also selective labelling of the alveolar type II cells with some less pronounced labelling of the remainder of the alveolar tissue (Figs 4a, 4c, 5a). In the case of spermidine and spermine, the less pronounced labelling appeared to be associated with the air side of the alveolar tissue (probably type I cells) although the cell type involved

Table 4. The percentage distribution of ^{3}H in putrescine, spermidine or spermine in the lung

Time after dosing (hr)	Not retained on column (unidentified)	Putrescine	Spermidine	Spermine
6 (3)	36.6 ± 2.7	41.3 ± 3.5	4.2 ± 0.2	ND
24 (2)	40.7 (37.7–43.6)	5.9 (4.6–7.1)	39.2 (38.8–39.5)	ND

ND Not detected.

Male rats were dosed s.c. with 518 μmol [^{3}H]putrescine (1.62 μCi/μmol)/kg using a slow release formulation. At 6 and 24 hr after dosing the amount of radiolabel in the trichloroacetic acid extract of the lung was measured, and the percentage distribution of the ^{3}H label in putrescine, spermidine and spermine determined using the ion exchange separation of Rosenblum and Russell [27]. The results are expressed as the mean ± SEM or the range of results with the number of observations in parenthesis.

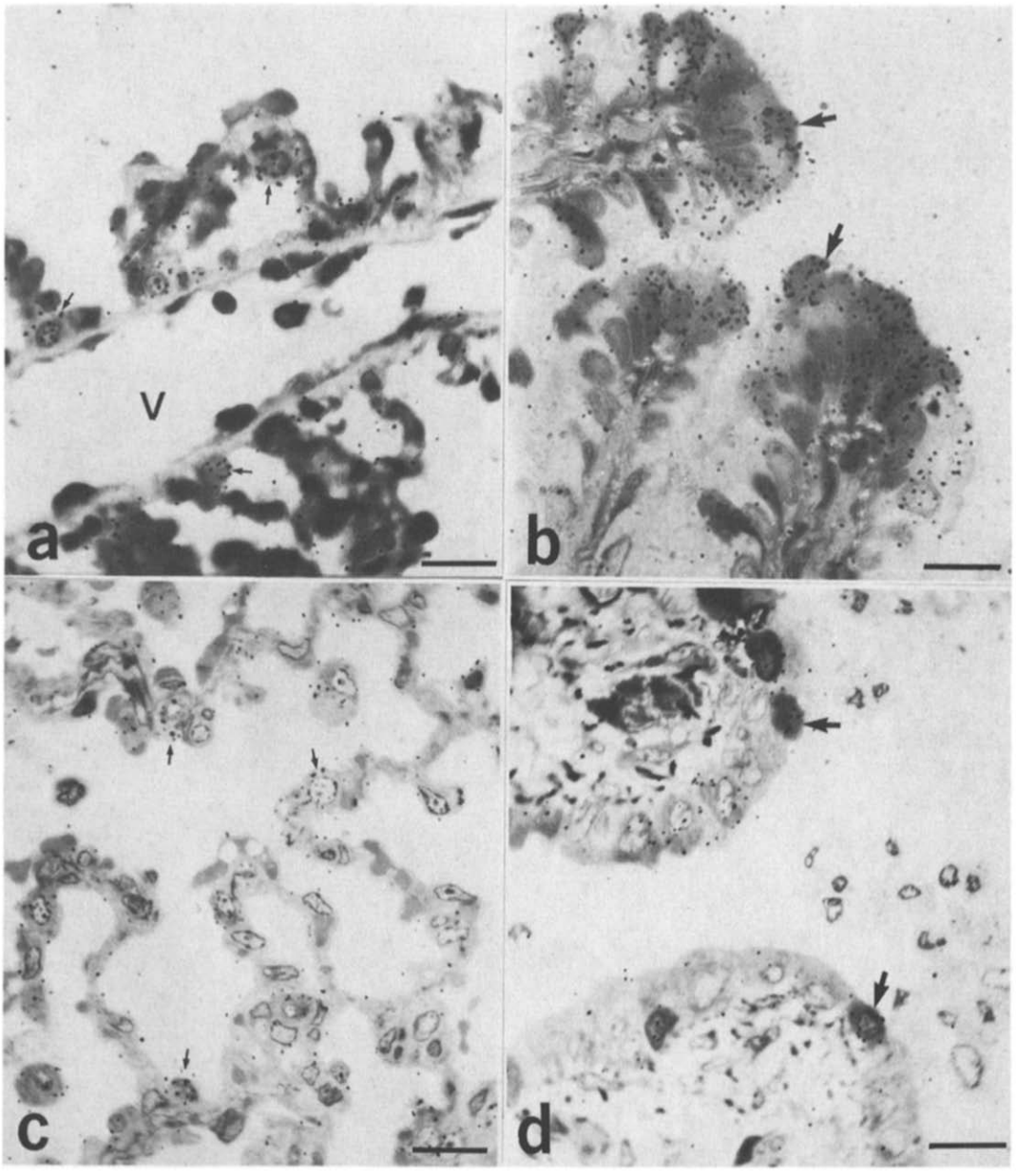

Fig. 4. (a) Lung incubated *in vitro* with putrescine, 1 μM. Type II cells (↗) labelled about a blood vessel (V) in which the endothelial cells are unlabelled. Light labelling is seen on the remainder of the alveolar surface. (b) Lung incubated *in vitro* with putrescine, 1 μM. Clara cells (➚) heavily labelled in bronchiolar tissue. Pale ciliated cells were only lightly labelled. (c) Lung incubated *in vitro* with spermidine, 1 μM. Type II cells (↗) labelled. Light labelling seen on remainder of the alveolar surface. (d) Lung incubated *in vitro* with spermidine, 1 μM. Clara cells (➚) labelled and ciliated cells unlabelled. Magnification, ×1300; bar = 10 μm.

could not be identified at the resolution of the light microscope. There was no labelling of connective tissue or vascular tissue.

In the autoradiographs prepared from the lung perfused with [^{3}H]putrescine the labelling was almost entirely confined to the alveolar type II cells (Fig. 5c). Similarly when rats were dosed s.c. with [^{3}H]putrescine the labelling was detected over the alveolar type II cells (Fig. 5d). In general there were few cells labelled following *in vivo* dosing and in the case of the perfused lung or the lung *in vivo* there was, in contrast to the results obtained with lung slices, no labelling of the Clara cells.

With [^{3}H]paraquat the autoradiographs of lung slices showed labelling in the alveolar type II cells. No other cell types were labelled (Fig. 6).

DISCUSSION

The identification of an oligoamine uptake system in the lung resulted from a search for endogenous compounds which could be accumulated by an active

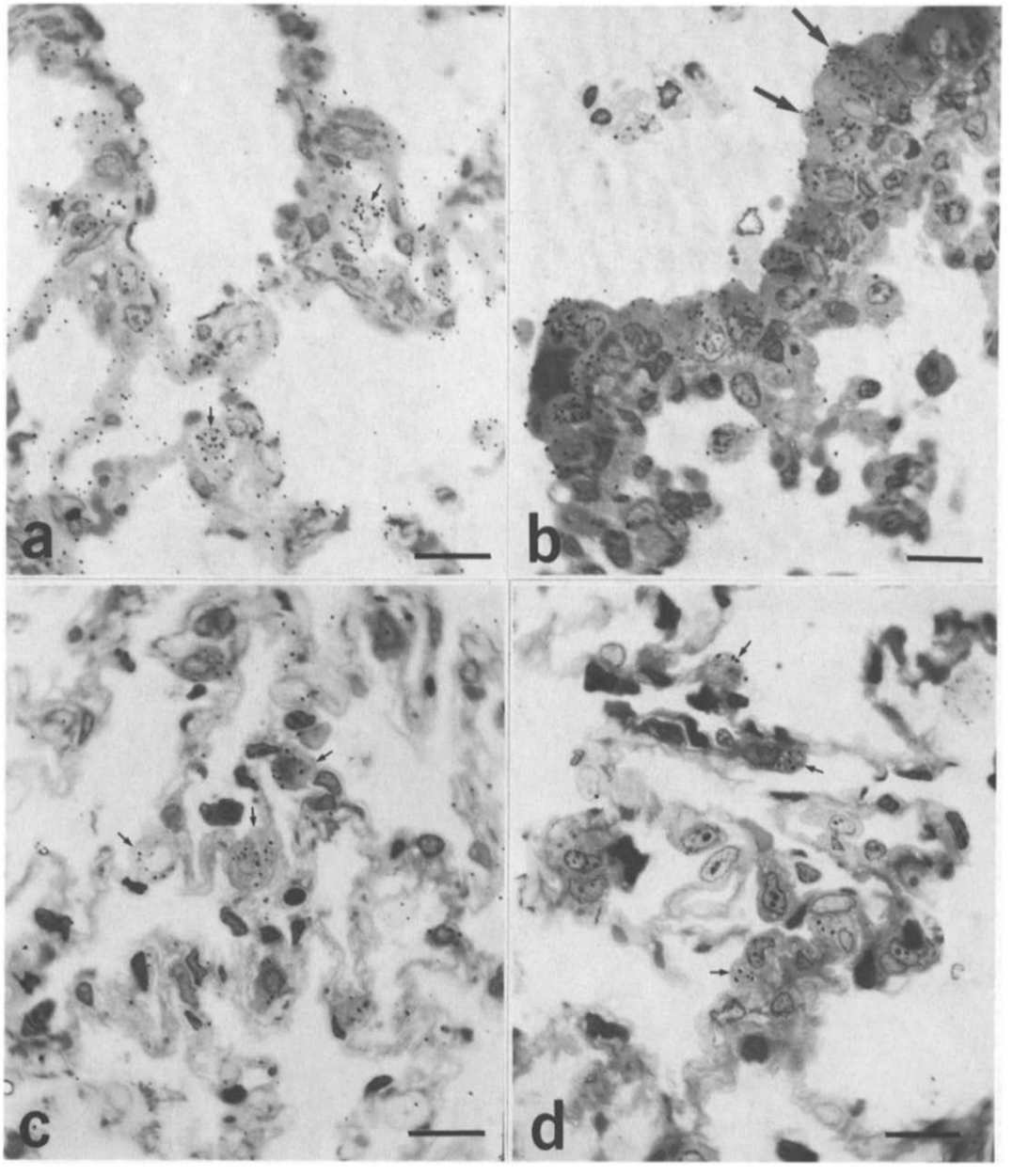

Fig. 5. (a) Lung incubated *in vitro* with spermine, 1 μM. Type II cells (↗)labelled. Light labelling seen in the remainder of the alveolar surface. (b) Lung incubated *in vitro* with spermine, 1 μM. Clara cells (↗) labelled and ciliated cells only lightly labelled. (c) Lung perused with putrescine, 1 μM. Type II cells (↗) labelled. (d) Lung treated *in vivo* with putrescine, 518 μmol/kg, 6 hr after dosing. Type II cells (↗) labelled. Magnification, ×1300; bar = 10 μm.

transport process that was originally described for the uptake of paraquat [7, 8]. The identification and characterisation of this pulmonary oligoamine uptake system has been investigated using lung slices [12, 13]. By analogy with paraquat, which is also accumulated by the lung *in vivo* [8], oligoamines accumulated by the lung slices might also be expected to accumulate *in vivo*.

Using an isolated perfused lung technique, we have shown that putrescine can be accumulated by the lung in a time-dependent, temperature-dependent manner which obeys saturation kinetics (Fig. 1, Table 1). Compared with lung slices, the perfused lung probably provides a more physiological model for the study of the uptake of putrescine. Also, it has the advantage that it avoids the complication of metabolic activity associated with other organs. The kinetic constants derived using the perfused lung compared with those from lung slices indicate that the apparent K_m for the uptake of putrescine is very similar although different V_{max} values were obtained (Table 1).

The difference in the V_{max} obtained with the perfused lung may reflect the greater contact of putrescine with alveolar tissue in slices as compared with the contact via the vasculature of perfused lung.

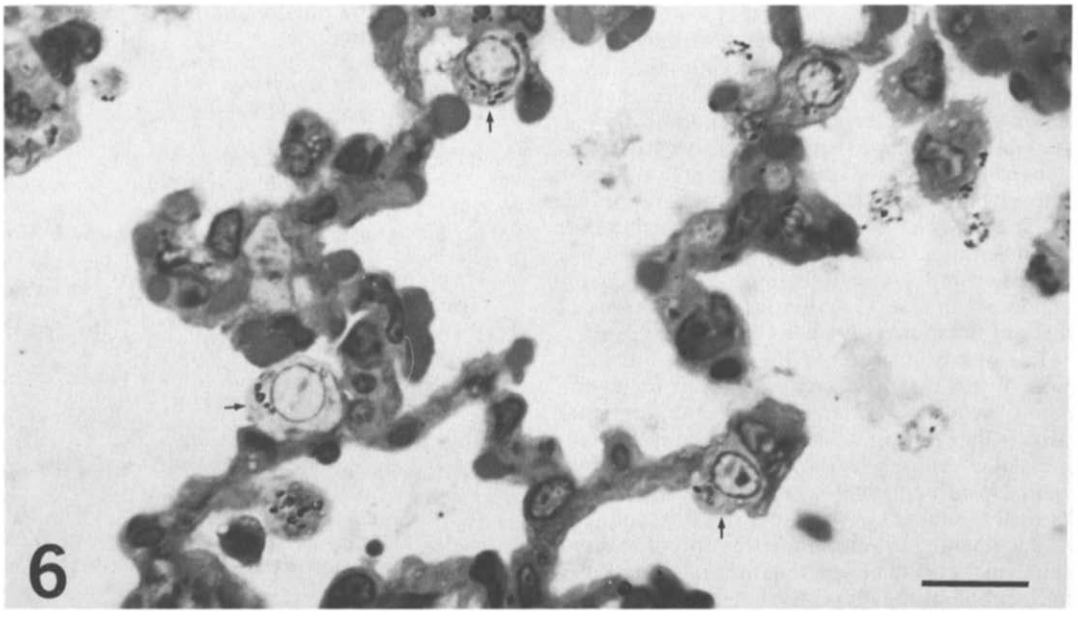

Fig. 6. Lung incubated *in vitro* with paraquat, 10 μM. Type II cells (↗) labelled. Magnification, ×1700; bar = 10 μm.

When rats were dosed s.c. with [^{14}C]putrescine the amount of [^{14}C] label in the plasma was different to the total concentration of free putrescine (Fig. 2). This effect is probably the result of the catabolism of putrescine by either the monamine oxidase [33] or diamine oxidase systems [34]. Conversely in the lung there was little or no evidence of catabolism of putrescine (Fig. 2). However, the concentration of [^{14}C]putrescine in the lung was much less than the total amount of ^{14}C label present (cf. Figs 2 and 3). This indicates that a significant proportion of the ^{14}C label was present as a metabolite of putrescine.

We found that the lung *in vivo* is capable of accumulating putrescine to concentrations in excess of that in plasma (Fig. 3). This accumulation of putrescine into the lung may in fact underestimate the ability of the lung to take up the diamine since approximately 90 nmol of putrescine appears to have been metabolised to other compounds (cf. Figs 2 and 3). Assuming that this metabolism took place in the lung, then the most meaningful comparison would be the level of putrescine in the plasma versus the total amount of ^{14}C label found in the lung.

The importance of putrescine metabolism is demonstrated by examining the fate of the ^{14}C label present in the lung. Six hours after dosing, 44% of the ^{14}C label is putrescine (75 nmol/g wet weight) but by 24 hr it has fallen to only 15% (10 nmol/g wet weight). Over this time course there has been a concomitant increase in the percentage and level of ^{14}C present in the lung as spermidine from 9% to 47% and from 14 to 30 nmol/g wet weight. Thus, it appears that the loss of ^{14}C label from putrescine can account for the increase of ^{14}C in spermidine. This conversion of putrescine to spermidine has not been previously described in the lung although its metabolism has been studied in other systems [16, 17, 35]. During the 24 hr period after dosing no ^{14}C label was detected in spermine which is predicted from the known biosynthetic pathway. This absence of ^{14}C label in spermine may be explained by the ability of putrescine to inhibit spermine synthase [17].

We found that in lung slices the label from [^{3}H]putrescine, spermidine or spermine were taken up into the Clara cells whereas the adjacent ciliated cells remain almost entirely unlabelled (Figs 4b, 4d, 5b). Also the alveolar type II cells were heavily labelled (Figs 4a, 4c, 5a). With [^{3}H]spermidine and [^{3}H]spermine there was considerable labelling over other areas of the alveolar tissue. This appeared to be on the periphery of the tissue and may well indicate that these labelled compounds are localised within the type I epithelial cell. However, with the resolution of the light microscope it is not possible to identify precisely the cell type in which the label is present.

With the perfused lung system the only significant labelling was found selectively in the type II cell (Fig. 5c) as was the case when rats were dosed s.c. with putrescine (Fig. 5d). These results with the perfused lung or lung *in vivo* may reflect the delivery of putrescine through the capillary endothelial network unlike the delivery with lung slices where the compound has direct access to the alveolar epithelium. However, it is also possible that the difference in the results may be attributable to the difference in the specific activity of the labelled compound which can be presented to the lung in these different systems. With the lung slice technique it was possible to present labelled compound of high specific activity whereas with the perfused lung and the lung *in vivo* this was not the case.

When lung slices were incubated *in vitro* with [^{3}H]paraquat only the alveolar type II cells were labelled (Fig. 6). However, since only 5% of the label present in the lung survived processing for autoradiographic evaluation, the results from this experiment have to be treated with extreme caution. At best it can be said that it seems probable that the alveolar type II cell is capable of accumulating

paraquat, but whether other cell types in the lung also have this ability cannot be determined from this study. Nevertheless, when the autoradiographic results from the paraquat study are taken in conjunction with those from the oligoamines, it seems reasonable to conclude that the transport process for the oligoamines and, by analogy, paraquat is present in the alveolar type II cells and probably in the alveolar type I cell and Clara cell. Since these are the primary target cells for paraquat toxicity [36, 37], the relationship between the presence of an oligoamine accumulation system and cellular specific toxicity of paraquat is further enhanced.

The selective nature of the uptake and localisation of this system has consequences for understanding the toxicity of paraquat. Most authors when reporting the concentration of paraquat in the lung express their results in terms of the gramme wet weight. However, if only a small proportion of the cells in the lung (i.e. type I and type II epithelial cells and Clara cells) are in fact the site of accumulation then there is a danger that the real intracellular concentration of the bipyridyl in the target cells will be underestimated.

The reason why the alveolar epithelial cells possess this transport process is at present unknown but it has been suggested that the oligoamines are necessary for the transformation of alveolar type II cells into alveolar type I cells [38]. However, it must be emphasised that it would be premature to conclude that the transport process is present primarily for the accumulation of oligoamines. A characterisation of this accumulation system in the lung [14] has revealed some of the structural requirements necessary for a molecule to be transported. From these data it is possible to predict other endogenous substrates which may use this transport process and preliminary results from our laboratory have indeed confirmed that this uptake process is not selective for polyamines.

At the outset of these studies, we asked four questions. It is now possible to conclude:

(1) That the diamine putrescine is accumulated into the perfused lung and into the lung *in vivo*.

(2) That following accumulation, putrescine uses the normal biosynthetic pathway to form spermidine.

(3) The accumulation of putrescine, spermidine, and spermine in lung slices occurs in the alveolar type II cell, Clara cell and very probably the alveolar type I cell. By analogy, it seems likely that paraquat also is accumulated into these cell types although it was located only in the type II cell. Similarly, in the isolated perfused lung, putrescine was located only in the alveolar type II cell.

(4) The cell types into which putrescine is accumulated *in vivo* is certainly the alveolar type II cell although other cells may be involved.

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