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STRUCTURAL REQUIREMENTS OF COMPOUNDS TO INHIBIT PULMONARY DIAMINE ACCUMULATION

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Abstract—The diamine, putrescine, is accumulated into slices of rat lung by a temperature and energy dependent process similar to that responsible for the uptake of cadaverine, the polyamines spermidine and spermine, and the herbicide paraquat. Structure–activity studies using monoamines and diaminoalkanes, amino acids and guanidino compounds, have shown that in order to inhibit the pulmonary accumulation of putrescine, chemicals should possess at least one but preferably two nitrogen-containing cationic groups. In the series of α, ω-diaminoalkanes studied, the inhibitory potential increased with increasing chain length, reaching a plateau at 1,7-diaminoheptane. These observations together with the fact that putrescine is a good substrate for the uptake system (K_m 15 μM, V_{max} 704 nmoles/g wet wt/hr) suggest that effective inhibitors require at least four methylene groups between their cationic centres and that diamines with more methylene groups may fold to give this separation. With both the monoamines and the α,ω-diaminoalkanes, changes in the free energies of interaction suggest that the observed increases in inhibitory potential with increasing chain length are due to increased hydrophobic bonding, which is a consequence of the addition of methylene groups to the alkyl chain. Furthermore, the ability of compounds to inhibit putrescine uptake appears to be related to their propensity to bind with the appropriate site for putrescine. Steric hindrance of this ionic interaction by the quaternisation of the cationic centres of the inhibitors with methyl groups, results in a total loss of measurable inhibitory activity. Also, the introduction of anionic carboxyl groups into inhibitors results in a loss of inhibitory potential, probably due to ionic repulsion.

The antileukaemic drug, methylglyoxal-bis-guanylhydrazone (MeGAG), and its congeners, were some of the most potent inhibitors of putrescine uptake studied. Our findings suggest similarities between the uptake system for putrescine into the lung with other uptake systems described for MeGAG and certain polyamines.

The endogenous polyamines, spermidine (*N*-[3-aminopropyl]-1,4-diaminobutane) and spermine (*N*,*N*′-bis-[3-aminopropyl]-1,4-diaminobutane), and their diamine precusor, putrescine (1,4-diaminobutane), have been the subject of increasing interest, due to a growing appreciation of their physiological importance. The polyamines have regulatory roles in tissue growth and interact with nucleic acids, so exhibiting a variety of effects upon macromolecular synthesis, expression and metabolism [1–3].

Recently, we have described in both rat and human lung the presence of a saturable, energy dependent uptake system for these polyamines [4–6]. This is apparently identical with that previously described for the accumulation into lung of the herbicide paraquat [4, 7]. Thus the accumulation of paraquat into the lung results from its transport by a process normally responsible for the uptake of the endogenous polyamines [6, 8].

Similar polyamine accumulation systems have previously been described in rat, rabbit and mouse brain [4, 9, 10]. This accumulation of spermidine and spermine [10] has been shown to be sodium independent, so resembling the system described for putrescine accumulation by both rat brain and lung [4]. Also, in addition to acting as a growth factor in human fibroblasts [11], putrescine is accumulated by a temperature sensitive system and this can be inhibited by cadaverine, spermidine and spermine [11], suggesting an uptake mechanism similar to that described for the lung [6, 12].

The antileukaemic agent, methylglyoxal-bis-guanylhydrazone (MeGAG), markedly inhibits putrescine-activated *S*-adenosylmethionine decarboxylase [13]. In addition, the antileukaemic action of MeGAG *in vivo* [14] and its accumulation by human leukaemic leukocytes and rabbit reticulocytes *in vitro* [15, 16] is inhibited by polyamines. These results suggest that polyamines and MeGAG may interact by competing for a common uptake mechanism. This hypothesis is further tested in the present study using the diamine and polyamine uptake system in lung slices. In particular, we have investigated some of the structural requirements for inhibition of the accumulation system by determining the ability of a number of compounds to inhibit the pulmonary uptake of putrescine.

We have concluded from these studies that two nitrogen containing cationic centres separated by a minimum of four methylene groups are required for a compound to be an effective inhibitor of the pulmonary diamine and polyamine accumulation system. MeGAG was found to be a potent inhibitor of pulmonary putrescine accumulation suggesting sim-

ilarities between the pulmonary uptake system for diamines and uptake systems described for MeGAG in other cell types such as leukocytes [15].

MATERIALS AND METHODS

Materials. (1,4-^{14}C) Putrescine dihydrochloride (113 mCi/mmol) was obtained from Amersham International PLC, Bucks, U.K. Aquasol Scintillation cocktail was obtained from New England Nuclear, Edinburgh, U.K. Ethylenediamine dihydrochloride, 1,3-diaminopropane dihydrochloride, putrescine dihydrochloride, 1,5-diaminopentane (cadaverine) dihydrochloride, 1,6-diaminohexane, 1,7-diaminoheptane dihydrochloride, 1,12-diaminododecane, spermidine trihydrochloride, spermine tetrahydrochloride, ornithine hydrochloride, lysine hydrochloride and *n*-propylamine were all purchased from Sigma Chemical Co, Dorset, U.K. 1,8-Diaminooctane, 1,9-diaminononane, 1,10-diaminodecane, *n*-pentylamine, agmatine sulphate, and methylglyoxal-bis-guanylhydrazone monohydrate were obtained from the Aldrich Chemical Co., Gillingham, U.K. Hydrazinium sulphate and *n*-butylamine were purchased from BDH Chemicals Ltd, Poole, Dorset, U.K. Arginine was purchased from Koch Light Labs Ltd, Colnbrook, U.K. Dimethylglyoxal-bis-guanylhydrazone (NSC 68120), ethylglyoxal-bis-guanylhydrazone (NSC 76509), pentane dialdehyde-bis-guanylhydrazone (NSC 67322) and *p*-phenylene-bis-guanylhydrazone (NSC 66832) were kindly supplied by Dr. V. L. Narayanan, National Cancer Institute, USA. Halothane (Fluothane) was kindly supplied by the Pharmaceuticals Division, ICI PLC. All other reagents were of analytical grade or otherwise of the highest grade available.

Animals. Male, Alderley Park Wistar derived specific pathogen free rats (body wt 170–300 g) were used throughout.

Methods. The accumulation of putrescine into rat lung slices was determined by the method of Smith and Wyatt [4] with minor modifications as described below.

Preparation of lung slices. The rats were anaesthetised with halothane. On the cessation of respiratory movements, the heart and lungs were removed and perfused, via the pulmonary artery, with 10 ml of either a modified Krebs–Ringer phosphate solution (KRP) [4] or a HEPES buffered salt solution (HBS), containing potassium chloride (5 mM), magnesium chloride (1 mM), sodium chloride (111 mM), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid) (2.5 mM) and sodium dihydrogen orthophosphate. $2H_2O$ (9.6 mM). Both buffers contained glucose (11 mM) and their pH adjusted to pH 7.4. The lobes were then removed and washed by immersion in the appropriate buffer. Slices (0.5 mm thick) were then prepared using a McIlwain tissue chopper (Mickle Labs, U.K.).

Accumulation of putrescine into tissue slices. Freshly prepared lung slices, excluding those with only one cut surface, were weighed into lots (10–30 mg) and placed into pre-incubated Erlenmeyer flasks. Each flask contained either 3 ml of modified KRP or HBS buffer, with 0.1 μCi (1,4-^{14}C) putrescine dihydrochloride diluted by the addition of the unlabelled salt to give the desired putrescine concentration and also a known concentration of inhibitor. Incubations were carried out at 37°C under air, in a shaking water bath at 60–70 cycles per minute. The accumulation of putrescine was studied at predetermined time intervals. For incubations which contained a putrescine concentration of 1 μM, time intervals of either 5, 15, and 30 or 10, 20 and 30 min were employed, whereas for concentrations of 10 μM putrescine, time intervals of 15, 30 and 60 min were used. The accumulation of putrescine by the slices was linear with respect to time over the period studied. Lung slices from a single rat were used in any one experiment and at least three rats were studied per inhibitor. When sodium deficient medium was required, sucrose was substituted for sodium chloride to maintain osmolality. Choline was not used for this purpose as it was to be investigated as a possible inhibitor of putrescine accumulation.

Measurement of putrescine uptake. In order to determine the accumulation of labelled putrescine by rat lung slices, the incubated slices were removed at the appropriate time and washed by brief immersion in fresh KRP or HBS buffer. The slices were then carefully blotted, solubilised in 0.5 M potassium hydroxide (200 μl) and neutralised by the addition of 0.5 M hydrochloric acid (200 μl) and distilled water (100 μl) before the addition of scintillation cocktail (10 ml). The radioactivity was determined by liquid scintillation spectrometry. Pre- and post-incubation media samples (100 μl) were taken and made up to 500 μl with distilled water, and the radioactivity determined as above. Counting efficiency was determined by the use of an external ^{14}C standard and all counts were expressed as disintegrations per minute. The slice to medium ratio was calculated as the ratio of radioactivity present per unit wet weight of slice to the radioactivity present in the equivalent volume of medium. From the specific activity of the preincubation medium, the amount of putrescine accumulated by the lung slices was calculated. The radioactivity in the lung associated with putrescine has been shown by chromatography to be due to unmetabolised putrescine [4].

From studies employing the incubation of lung slices with ^3H_2O and by the extrapolation from graphs of the uptake of putrescine it has been determined that lung contains approximately 0.8 ml water/g wet weight which is accessible to putrescine by diffusion. This value was, therefore, employed to establish a zero time, diffusion factor which was subsequently included in the derivation of rates of accumulation by linear regression analysis.

Calculation of inhibitory potencies. In order to discriminate between the inhibitory potencies of structurally related compounds, I_{50} values (the concentration of inhibitor required to reduce the control putrescine accumulation rates by 50%) were derived using the method of Ross and Krieger [17]. Lung slices from each animal were used to determine the accumulation of putrescine in the absence of inhibitor in order to eliminate inter-animal variation and at least four different concentrations of inhibitor were employed per study.

RESULTS

In preliminary studies, various compounds were tested in order to determine their ability to block the pulmonary accumulation of putrescine. The first potential inhibitor of putrescine accumulation considered was choline, a precursor of phosphatidyl choline, the major phospholipid component of pulmonary surfactant [18]. Choline is not only accumulated into brain tissue by a high affinity, sodium dependent uptake system [19] similar to that described for spermine [20], but also influences the transport of compounds into kidney slices [21]. These observations, together with the knowledge that surfactant synthesis and putrescine accumulation both occur in type II cells of the lung [4, 22] suggested that choline may be a potential inhibitor of pulmonary putrescine uptake. However, choline even at a concentration of 1 mM did not significantly inhibit the accumulation of putrescine (10 μM) (Table 1), suggesting that the uptake of choline was not linked directly to that of putrescine. Neither ornithine (1 mM), the precursor of putrescine in mammalian polyamine biosynthesis, nor lysine (1 mM), displayed any significant inhibitory effect on putrescine (10 μM) accumulation (Table 1). In contrast to the results with the amino acids, the monoamines, *n*-propylamine and *n*-butylamine, inhibited putrescine accumulation (Table 1). Substitution of the amino group by a hydroxyl group, as illustrated by *n*-butanol (Table 1) resulted in the loss of the inhibitory ability. This observation and the poor inhibitory potency of the weak bases aniline (1 mM) and *m*-phenylene-diamine (1 mM) (Table 1) compared to the monoamines, suggest the importance of a charged amino group on the molecule in order for it to inhibit the pulmonary accumulation of putrescine. The endogenous polyamines, spermidine and spermine, were more effective inhibitors than the monoamines and were shown to be good inhibitors of the pulmonary uptake of putrescine (Table 1), confirming previous observations (6).

Thomas *et al.* (23, 24) demonstrated the ability of Na^+ and certain amino acids to react co-operatively to inhibit the transport of cationic amino acids into various cell types. Smith *et al.* have also shown that putrescine [4] and paraquat [12] accumulation into rat lung slices was not only sodium independent, but was enhanced by sodium deficient media. Therefore the influence of Na^+ on the inhibitory activity of the monoamines was also investigated by employing normal and sodium deficient media. We found that the extent to which the pulmonary accumulation of putrescine was inhibited by both *n*-propylamine and *n*-butylamine was similar in either normal or sodium deficient medium (Table 2).

The inability of the amino acids, L-ornithine and L-lysine to inhibit pulmonary putrescine accumulation (Tables 1 and 3) suggested that the presence of a carboxyl group may interfere with inhibitory potency. Support for this was provided by the findings that cadaverine and agmatine were more potent inhibitors of putrescine accumulation than the corresponding amino acids, lysine and arginine (Table 3). The very marked loss of the inhibitory potential of the basic amino acids (compared to either their decarboxylated products or also the respective monoamines) may be attributed to the presence of

Table 1. Preliminary studies of the inhibition of putrescine (10 μM) accumulation into rat lung slices by various compounds

Compound	Concentration (mM)	(%) Inhibition of putrescine uptake
Choline	0.01	10 ± 5
	0.1	−22 ± 3
	1.0	8 ± 1
L-Ornithine	0.01	1 ± 13
	1.0	6 ± 19
L-Lysine	0.01	17 ± 17
	1.0	1 ± 14
n-Propylamine	0.1	18 ± 14
	10.0	85 ± 2*
n-Butylamine	0.1	18 ± 12*
	1.0	69 ± 3*
	10.0	92 ± 1*
Butan-1-ol	1.0	3 ± 8
Aniline	1.0	−11 ± 12
m-Phenylene-diamine	1.0	−2 ± 1
Spermidine	0.01	7 ± 16
	1.0	91 ± 1*
Spermine	0.01	−26 ± 10
	1.0	92 ± 2*

Slices of rat lung were incubated in HBS (pH 7.4) containing 10 μM putrescine (0.1 μCi/3 ml vol). Inhibitors were present at time zero and incubated for 15, 30 and 60 min at 37°. Results were calculated by linear regression analysis and were expressed as a percentage of the appropriate control uptake rate (mean ± SEM = 166.2 nmoles/g wet wt/hr ± 16.3, N = 15).

* Significantly < control rate of putrescine accumulation ($P > 0.05$) by paired Student's *t*-test.

Table 2. The influence of sodium deficient medium upon the inhibition by n-butylamine and n-propylamine of putrescine (10 μM) accumulation by rat lung slices

Incubation medium	Accumulation of putrescine (nmol/g wet wt/hr)	(%) Control
Control + Na^+	136 ± 22	100 ± 16
1 mM *n*-Butylamine + Na^+	53 ± 3	39 ± 2
1 mM *n*-Propylamine + Na^+	92 ± 24	67 ± 18
Control − Na^+	211 ± 30	100 ± 14
1 mM *n*-Butylamine − Na^+	81 ± 8	38 ± 3
1 mM *n*-Propylamine − Na^+	179 ± 22	85 ± 10

Slices of rat lung were incubated in HBS medium (+Na^+) or modified HBS medium (−Na^+), where NaCl had been replaced by sucrose to maintain osmolality for sodium deficient incubations. Inhibitors were present at zero time and slices incubated at 37° for 15, 30 and 60 min. Accumulation of putrescine was calculated as in Table 1. Results expressed as the mean ± S.E.M. for 3 animals.

an anionic group in the molecule rather than to the overall reduction in charge.

Having established some of the general structural properties required for inhibitors of the accumulation system, a more detailed study using structurally similar compounds and determining I_{50} values as a measure of their inhibitory potential was implemented (Table 4).

We confirmed that the pulmonary accumulation of putrescine obeys saturation kinetics (K_m 15 μM, V_{max} 704 nmoles/g wet wt/hr) in agreement with previous results [6]. In order to realise the maximal inhibitory effect of the compounds, a non-saturating substrate concentration of putrescine (1 μM) was employed. The accumulation of putrescine at this concentration was shown to be both temperature sensitive (Fig. 1), being almost completely inhibited at 4°, and also in agreement with our previous observations [4] was inhibited by rotenone and potassium cyanide. These results further support the suggestion that pulmonary polyamine uptake is an energy dependent process.

Amongst the compounds selected for further study were the α,ω-diaminoalkanes, because of their ability to inhibit the accumulation of putrescine and cadaverine [6], and also their inhibitory effect on the uptake of paraquat [17]. These diamino compounds were more potent inhibitors of putrescine accumulation than the corresponding monoamines. These findings suggest that for more effective inhibition,

Table 3. Inhibition of pulmonary putrescine (1 μM) accumulation by selected amino acids and their decarboxylated derivatives.

Compound	Structure at pH 7.4	I_{50} (μM)
Ornithine	$H_3\overset{+}{N}CH_2)_3CH\overset{+}{N}H_3$ $\vert$ COO^-	> 1000
Lysine	$H_3\overset{+}{N}(CH_2)_4CH\overset{+}{N}H_3$ $\vert$ COO^-	> 1000
Cadaverine (1,5-diaminopentane)	$H_3\overset{+}{N}(CH_2)_5\overset{+}{N}H_3$	25.6 ± 7.8
Arginine	$H_2N-C(=\overset{+}{N}H_2)-NH(CH_2)_3CH(\overset{+}{N}H_3)(COO^-)$	> 1000
Agmatine	$H_2N-C(=\overset{+}{N}H_2)-NH(CH_2)_4\overset{+}{N}H_3$	4.9 ± 0.7

Slices of rat lung were incubated in HBS or in KRP glucose medium (pH 7.4) containing 1 μM putrescine (0.1 μCi/3 ml vol). The inhibitors were present at zero time and incubated either for 10, 20 and 30 min or for 5, 15 and 30 min at 37°C in a shaking water bath. I_{50} values were determined using the method of Ross and Krieger [17], each animal acting as its own control. Results are expressed as the mean ± S.E.M., at least 3 animals being employed per compound studied.

Table 4. The inhibitory potencies of various amino compounds to reduce putrescine (1 μM) accumulation into rat lung slices and their estimated differences in free energy of interaction with the postulated receptor site for uptake

Compound	I_{50} (μM)*	Differences in $\Delta F_1 - \Delta F_2$† $-CH_2-$(KCal/mol)
Monoamines		
n-Propylamine	725 ± 72	
		0.88
n-Butylamine	175 ± 32	
		0.58
n-Pentylamine	69 ± 10	
Cysteamine	35.3 ± 7.6	
Diamines		
Hydrazine	> 1000	
1,2-Diaminoethane	373.7 ± 115.9	
		0.68
1,3-Diaminopropane	124.0 ± 37.0	
		0.49
1,5-Diaminopentane	25.6 ± 7.8	
		0.53
1,6-Diaminohexane	10.7 ± 0.8	
		0.39
1,7-Diaminoheptane	5.7 ± 1.6	
		−0.21
1,8-Diaminooctane	8.0 ± 1.7	
		0.29
1,9-Diaminononane	5.0 ± 1.3	
		−0.20
1,10-Diaminodecane	6.9 ± 1.1	
		0.23
1,12-Diaminododecane	3.3 ± 1.0	
Cystamine	22.0 ± 3.6	
Methoniums		
Trimethonium	> 1000	
Tetramethonium	> 1000	
Pentamethonium	> 1000	

Slices of rat lung were incubated in HBS or KRP glucose solution (pH 7.4) containing putrescine (1 μM) (0.1 μCi/3 ml volume).

* I_{50} values were calculated as described in legend to Table 3. Results expressed as mean ± S.E.M.

† The mean difference in the free energy of interaction of a, α-diaminoalkanes for the addition of one $-CH_2-$moiety.

compounds should contain at least two postively charged nitrogen-containing groups. The inhibitory potency of the α,ω-diaminoalkanes increased markedly with increasing chain length, particularly up to 1,5-diaminopentane or 1,6-diaminohexane (Table 4). Increasing the chain length of the monoamines also increased their inhibitory potency (Table 4). Thus the distance between the charged groups and possibly the concomitant increase in lipophilicity are of importance in determining the inhibitory potential of the compound.

Further indication of the importance of the nature of the inter-cationic region of a diamine with respect to its inhibitory potential was obtained using cystamine (Table 4). This diamine, which contains six atoms between its cationic nitrogen groups, two of which are sulphur atoms, was found to be a less effective inhibitor than α,ω-diaminoalkanes of similar maximal intercationic distance. It was also of interest to note that cysteamine, the mono-amino precusor of cystamine was a markedly better inhibitor than the corresponding aliphatic monoamine (Table 4). However, it has been shown (Smith and Wyatt unpublished data) that under these incubation conditions cysteamine is oxidised to the disulphide cystamine. Thus the inhibitory effect attributed to cysteamine is largely a consequence of cystamine formation.

Quaternization of the amino groups of the α,ω-diaminoalkanes with methyl groups to form the methonium derivatives caused a complete loss of measurable inhibitory potential (Table 4). This loss of activity is most likely due to masking of the cationic groups by the surrounding methyl groups, thus preventing their ionic binding to the putative receptor site.

The anti-tumour agent, MeGAG, and related

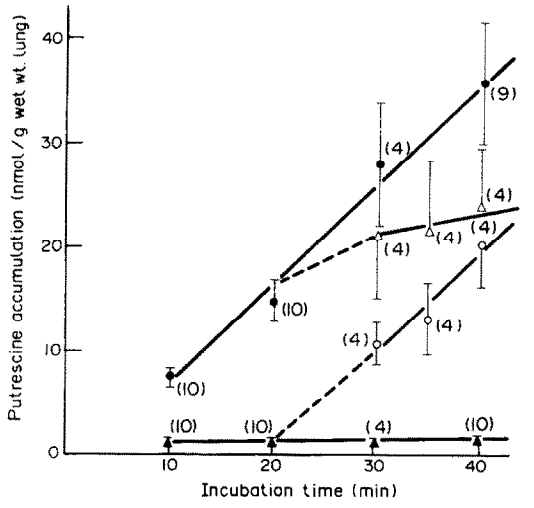


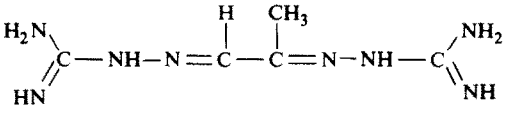
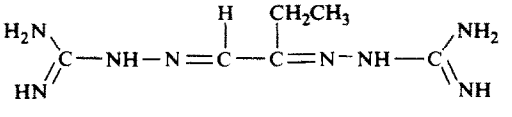
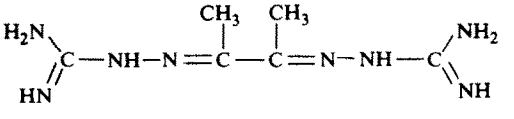
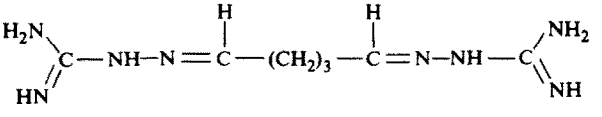
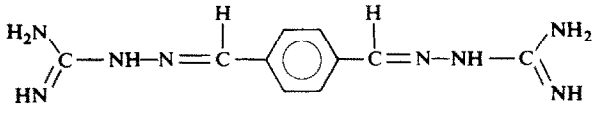
Fig. 1. Slices were incubated in KRP glucose medium containing 1 μM ^{14}C labelled putrescine at (a) 37° for up to 40 min (●—●), (b) 4° for up to 40 min (▲—▲), (c) at 37° for 20 min then 4° for 20 min (△—△) and (d) at 4° for 20 min then 37° for 20 min (○—○). The accumulation of putrescine into the slices was measured at the incubation times shown. The results are expressed as mean ± S.E.M. with the number of measurements at each time point shown in parenthesis.

derivatives were found to be very potent inhibitors of pulmonary putrescine accumulation (Table 5). Both methyl- and ethyl-glyoxal-bis-guanylhydrazones were the most effective inhibitors of putrescine accumulation. Structural alterations to the central glyoxal region (Table 5) caused only a slight decrease in inhibitor potency although these compounds were as potent inhibitors of putrescine accumulation as the majority of the α,ω-diaminoalkanes examined (Table 4).

DISCUSSION

Although mammalian transport systems display a high degree of structural specificity, compounds resembling endogenous substrates, are often transported by the same carrier systems. This has been well documented for several systems including the monoamine uptake system in the lung [25] and the base transport system in the kidney [26]. More recently, similarities in the accumulation of putrescine into rat lung slices with the uptake of cadaverine, the polyamines, spermidine and spermine, and paraquat [4, 6, 7] have led to the suggestion that these compounds are transported by a common mechanism. In this study, we have investigated the

Table 5. The inhibitory potencies of methylglyoxal-bis-guanylhydrazone and its cogeners against the accumulation of putrescine (1 μM) by rat lung slices

Compound	Structure	I_{50} (μM)
Methylglyoxal-bis-guanylhydrazone (MeGAG)		1.0 ± 0.2
Ethylglyoxal-bis-guanylhydrazone		2.3 ± 1.1
Dimethylglyoxal-bis-guanylhydrazone		9.5 ± 3.6
Pentane dialdehyde-bis-guanylhydrazone		7.5 ± 1.8
p-Phenylene-bis-guanylhydrazone		6.7 ± 2.3

Slices of rat lung were incubated in KRP glucose solution (pH 7.4) containing putrescine (1 μM) (0.1 μCi/3 ml vol). I_{50} values were calculated as described in the legend to Table 3. Results are expressed as mean ± S.E.M. Control rate of uptake was 60 ± 5.1 nmoles/g wet wt/hr ± S.E.M.

structural requirements for inhibition of the putrescine accumulation system into rat lung slices, and by inference for the uptake of structurally related compounds.

The studies of the inhibition of putrescine accumulation into lung slices, indicated that compounds required at least one basic group for inhibitory action and that dicationic bases were better inhibitors than monocations. The lack of inhibitory action of the amino acids ornithine, lysine or arginine not only indicated that a carboxyl group adjacent to one of the cationic groups prevented inhibition but also that the uptake system was different from that reported for the uptake of diamino acids by Ehrlich ascites tumour cells [27]. The lack of effect of sodium deficient medium, upon the percentage inhibitory potential of *n*-butylamine or propylamine (Table 2) further separated this system from that described for the diamino acid uptake system described by Thomas *et al.* [23, 24]. Evidence that the ability of a compound to inhibit putrescine accumulation was dependent on the presence of a charged cationic group was illustrated by the lack of inhibition of *n*-butanol compared to *n*-butylamine (Table 1). The basicity of the molecule was also apparently of importance as aniline (pKa 4.69 at 20°) [28] and *m*-phenylenediamine (pKa_1 5.11, pKa_2 2.50 at 20°) [28] are weak bases compared to *n*-butylamine (pKa 10.78 at 20°C) (28) and were also much weaker inhibitors of pulmonary putrescine uptake (Table 1).

The presence of more than one cationic group markedly increased the ability of compounds to block putrescine accumulation as illustrated by the α,ω-diaminoalkanes (Table 4) and the polyamines spermidine and spermine (Tables 1 and 4). The inhibitory potential of both the α,ω-diaminoalkanes and the monoamines increased with increasing chain length (Table 4). This was particularly marked in the diamino series up to 1,7-diaminoheptane. It appears that a distance of four methylene units or approximately 6.6Å [29] between cationic nitrogen containing groups is required for a compound to be a good inhibitor of, and possibly a suitable substrate for, the pulmonary diamine and polyamine uptake system. This is supported by the observations that (1) putrescine is a more effective inhibitor of pulmonary paraquat accumulation than cadaverine [17], (2) putrescine is a better substrate than cadaverine for the uptake system [6] and (3) lower members of the series were much poorer inhibitors of putrescine accumulation (Table 4). The relative inhibitory potencies of the diamines, found in these experiments, compare well with those reported by Ross and Krieger [17] for the inhibition of paraquat uptake into rat lung slices. This provides further evidence for a common system of accumulation for paraquat and putrescine in agreement with previous suggestions [4].

Inhibition of putrescine accumulation was not restricted to simple amines. Other dicationic compounds containing guanidino groups [agmatine, MeGAG and derivatives (Tables 3 and 5)] were also effective. MeGAG was studied because it inhibits both putrescine-activated *S*-adenosyl-methionine decarboxylase [13] and the uptake of MeGAG by both human leukaemic leukocytes and the mouse leukaemic L1210 cell line is competitively inhibited by polyamines [15, 30]. MeGAG and related derivatives were found to be potent inhibitors of the pulmonary accumulation of putrescine (Table 5). These results suggested marked similarities between the uptake system for polyamines in the lung with other uptake systems in a number of different cell types, such as the energy dependent uptake system for the accumulation of MeGAG in human leukocytes and rabbit reticulocytes [15, 16]. Mandel and Flintoff [31] has also suggested that putrescine, spermidine and MeGAG share a common uptake system in Chinese hamster ovary cells. Competitive inhibition of putrescine and spermidine accumulation by MeGAG has been demonstrated using fibroblasts from Swiss 3T3 mice [32]. These studies suggest that, in a number of diverse systems, the polyamines and MeGAG may compete for a common uptake system. In one study, the structural requirements described to inhibit the uptake of MeGAG into human leukaemic leukocytes [15] appear to be very similar to those described in the present study to block putrescine accumulation in the lung, although monoamines were reported not to inhibit this particular system. It would therefore appear that the uptake system described for polyamines by the lung is similar if not identical to uptake systems by which polyamines are known to be accumulated by other tissues and cells. It may be possible to exploit the similarities in these uptake systems for therapeutic purposes.

It was of interest that MeGAG and structurally-related analogues were all good inhibitors of pulmonary putrescine accumulation. Small changes in the structure of MeGAG's central glyoxal region have profound effects on its antileukaemic activity [33]. Thus of the compounds studied in the present investigation, only MeGAG retains its antileukaemic activity [33]. This is in marked contrast to the structural criteria of the guanylhydrazones required for inhibition of polyamine uptake, which was far less rigorous than those involved in anti-leukaemic activity.

From the apparent kinetics for the accumulation of putrescine, and from the structure–activity studies for its inhibition, it is possible to infer certain properties about the nature of the receptor for uptake [34]. Since the K_m for the uptake of putrescine is known, it is possible to determine the overall change in free energy of interaction of putrescine with its receptor from the equation

$$\Delta F = RT \ln K_m \quad (1)$$

[Where ΔF = change in free energy, T = absolute temperature (K), R = Universal gas constant (1.986 cal/deg mole), K_m = Michaelis constant]

Solution of this equation gives a value of ΔF 6.8 Kcal/mole for the interaction of putrescine with its receptor for uptake at 37°, suggesting that the major binding force involved was ionic.

As described by Webb [35], the relationship between free energy of interaction and inhibitory potential for a competitive inhibitor may be expressed as

$$\Delta F = RT \ln K_i \quad (2)$$

[Where K_i is the inhibition constant]

The difference in binding energy between two structurally related inhibitors may be shown to be

$$\Delta F_1 - \Delta F_2 = RT \ln \frac{(I_1)_{50}}{(I_2)_{50}} \quad (3)$$

where $(I_n)_{50}$ (M) are equipotent inhibitor concentrations required to give 50% inhibition. If the two inhibitors are closely related, this calculated difference in binding energy will be due predominantly to differences in their structure and independent of external forces. However, if, for example, a polar group if substituted for a non-polar group the calculated difference will only give a poor estimate of the difference in interaction energies. Therefore, the effect of adding methylene ($-CH_2-$) groups to the diamines may be considered accurately but the effect of removal of $-NH_2$ groups may only be estimated.

From a consideration of differences in binding energy (Table 4) certain conclusions may be drawn. The increased inhibitory potency of the higher α,ω-diaminoalkanes compared to lower members of the series, e.g. 1,3-diaminopropane would appear to be due to hydrophobic bonding by the addition of CH_2- groups of the higher members of the series. The observations that the inhibitory potency increases markedly after 1,3-diaminopropane (Table 4) and that putrescine is a particularly good substrate for the uptake system suggests that there should be a minimum of 4 carbon atoms between the cationic groups on the inhibitor for effective competition for the receptor site. The binding energies of the long chain diamines ($n = 8$–10) are only marginally greater than that of 1,6-diaminohexane suggesting that the larger molecules may fold before they are able to bind to a receptor site and block uptake.

Further information about the nature of the putrescine-receptor interaction may be obtained from the Hill equation [34].

$$\log \frac{v}{V\max - v} = n \log [S] - \log K \quad (4)$$

[Where n is an indication of the number of substrate-receptor binding sites, S is the concentration of putrescine substrate and K is the dissociation constant]

A graph of $\log \frac{v}{(V_{max} - v)}$ against $\log S$ (Fig. 2) for putrescine accumulation into rat lung slices from both the present data and from our previous studies [4] yields a value of $n = 1$, indicating a 1:1 putrescine-receptor interaction for uptake.

In summary, these studies have extended our understanding of the pulmonary accumulation of putrescine and indicated that, in order to inhibit this accumulation, compounds should have a specific structure or shape, including:

(1) One but preferably two charged nitrogen containing cationic groups in order to form ionic bonds with a putative receptor site governing putrescine accumulation.

(2) A distance equivalent of 4–7 methylene units between the cationic centres—longer alkyl chains may fold to give this separation and may also be good inhibitors.

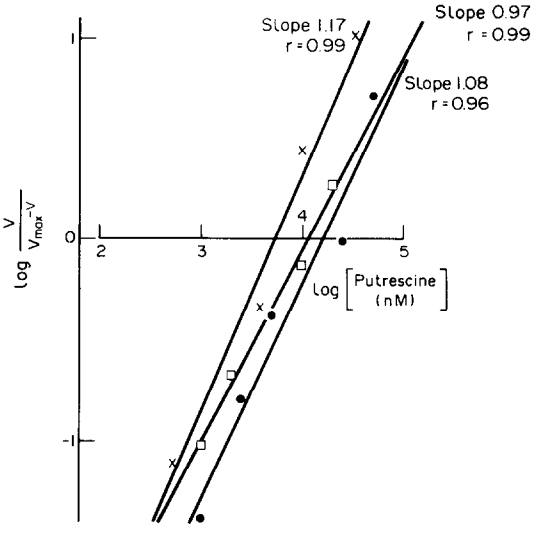


Fig. 2. Hill plot of the accumulation of (^{14}C)-labelled putrescine by rat lung slices. Results plotted are those obtained in these studies using male Alderley Park Wistar derived specific pathogen free rats (●—●) and University of Surrey Wistar rats (□—□), data not shown, for the accumulation of putrescine by rat lung slices. Previous results obtained by Smith and Wyatt [4] are also indicated (X—X). Concentration range putrescine employed 1–100 μM. (V = rate of accumulation of putrescine/g wet wt lung/hr.)

(3) No carboxyl groups adjacent to a cationic centre.

(4) Steric factors or ionic interaction resulting in the hindered access to the cationic binding site, results in the loss of inhibitory potency, as illustrated by the alkylmethonium and amino acids respectively.

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