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SUBCELLULAR DISTRIBUTION OF A LABELLED 2-HALOGENOALKYLAMINE IN GUINEA-PIG *VAS DEFERENS*

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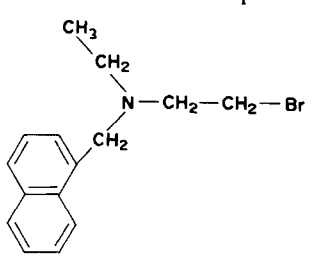
Abstract—Guinea-pig *vasa deferentia* were incubated with a 2-halogenoalkylamine (^{14}C-SY28) either alone or after exposure to the alpha receptor blocking agent, phentolamine. A quantitative evaluation was made of the distribution of the covalently bound ^{14}C-SY28 both in lipid and lipid-free phases, and the distribution throughout the subcellular fractions of lipid-free homogenate was studied. Acid hydrolysis was carried out on each fraction and hydrolysates run on chromatography paper. Autoradiographic studies of these runs showed ^{14}C-SY28–amino acid complexes, identical to those found previously, distributed unequally throughout the subcellular fractions. The effects of phentolamine protection of the alpha receptor, from binding by ^{14}C-SY28 are discussed.

THE 2-HALOGENOALKYLAMINE group of compounds are powerful alkylating agents which are capable of covalently, and therefore irreversibly, binding to several sites, including adrenergic alpha receptors.[1,2] One member of this group, *N*-(2-Bromoethyl)-*N*-ethyl-*N*-1-naphthylmethylamine HBr (SY28) has been shown to bind to both protein and proteolipid in smooth muscle of guinea-pig *vas deferens*.[3,4] Previous work on subcellular distribution of labelled 2-halogenoalkylamines has been on cat basal ganglia and brain stem,[5] where binding to a proteolipid in the fraction containing nerve ending membranes was of particular importance. In other work carried out,[6] using subcellular fractions of rabbit aortic strip, preliminary observations were made comparing the distribution of ^{14}C-dibenamine alone, or after a protecting dose of adrenaline.

The present study was concerned with the distribution of ^{14}C-SY28 within the subcellular fractions of guinea-pig *vas deferens* following exposure to the drug, and the effects of the competitive alpha receptor blocking agent, phentolamine, on that distribution. Hydrolysis of each subcellular fraction was subsequently carried out to compare the occurrence of ^{14}C-SY28–amino acid complexes in each fraction.



METHODS

Male guinea-pigs weighing 350–450 g were used, and, after stunning and bleeding, the vasa were quickly removed and stripped.[7] Two guinea-pigs were used for each experiment and vasa from each pig used for both the control and test groups. The vasa were blotted, weighed and cut into 2–3 mm sections then placed in tubes and washed twice with normal saline. Both test and control groups were subjected to the same procedure subsequently, except that the test group was exposed to 10^{-3} g/ml phentolamine mesylate for 5 min at 37° prior to exposure to ^{14}C-SY28. The concentrations of ^{14}C-SY28 (sp. act. 25 mCi/m-mole) used were 10^{-6} g/ml ($2{\cdot}68 \times 10^{-6}$ M), 2×10^{-6} g/ml ($5{\cdot}36 \times 10^{-6}$ M) and 5×10^{-6} g/ml ($1{\cdot}34 \times 10^{-5}$ M).

Following exposure to the labelled drug, both groups were washed, 12 times, with 5 ml volumes of normal saline, once every 5 min. Lipids were extracted with 8 ml volumes of chloroform–methanol (2:1) for 45 min at 37° or for 4 min at 70°. The vasa were prepared for subcellular fractionation by homogenising in 0·25 M sucrose (containing 0·001 M EDTA) in a proportion of 30 ml/g tissue weight. A Tri-R homogeniser was used, having a glass mortar and teflon pestle, with an ice bath being used to cool the homogenate. After 4 min homogenisation, the material was subjected to 2 min in an M.S.E. ultrasonic disintegrator, again using an ice bath. The procedure for subcellular fractionation was based on the method of Li *et al.*[8] The homogenate was spun in an M.S.E. Superspeed Centrifuge, using a 10 × 10 ml 50,000 rev/min max. speed rotor, a thermostat maintaining the rotor temperature at 4°. The speeds used were: 800 *g* (10 min)—Nuclei and cell debris; 7000 *g* (10 min)—Mitochondria; 15,000 *g* (10 min)—Lysosomes; 100,000 *g* (90 min)—Microsomes;—Supernatant.

The pellets from each fraction were re-suspended in 1 ml phosphate buffer (pH 6·5) and 0·1 ml samples from each used for scintillation counting, by standard techniques, on a Tracerlab 100 apparatus (scintillation solution: 2,5-diphenyloxazoline (PPO) 4 g, *p*-di[2-(5-phenyloxazole)]-benzene (POPOP) 0·1 g, toluene 700 ml, 2-ethoxyethanol 300 ml).

Protein content was measured on a Unicam S.P.600 spectrophotometer, using Biuret reagent.

Samples of the subcellular fractions were subjected to digestion with papain followed by hydrolysis with 6N HCl. Chromatography and autoradiography were carried out as described previously.[3]

RESULTS AND DISCUSSION

Distribution of labelled SY28. Table 1. shows the distribution of labelled SY28 between the lipid and non-lipid fractions of *vas deferens* after exposure to various doses of the drug. These values lie intermediate between those observed by Dikstein *et al.*[9] who used boiling chloroform:methanol and found 80 per cent labelled dibenamine in the lipid phase, and those of Yong *et al.*[10] who homogenised their tissue in the solvent at room temperature, and found 20–23 per cent labelled dibenamine in their lipid phase. Yong *et al.* tried to further extract with boiling solvent, but found no further release of radioactive drug in the extract. They concluded that incomplete extraction would not account for the difference in percentage of labelled drug extracted by the respective laboratories. In the present study it was found that differ-

TABLE 1. PERCENTAGE RADIOACTIVITY WITHIN LIPID EXTRACTS OF GUINEA-PIG *vasa deferentia* FOLLOWING 20 min EXPOSURE TO VARYING DOSES OF ^{14}C-SY28.

Doses of ^{14}C-SY28	% Lipid bound ^{14}C-SY28
10^{-6} g/ml	47·6 ± 3·9% (8)
2×10^{-6} g/ml	57·2 ± 4·0% (8)
5×10^{-6} g/ml	64·1 ± 3·3% (8)

Mean values ± S.D.
Number of experiments for each value shown in brackets.
Results were calculated using the formula: % lipid bound ^{14}C-SY28 = (L/L + L.F.H.) × 100 where: L = total radioactivity in the lipid extract; L.F.H. = total radioactivity in the lipid free homogenate.

ent extraction procedures, i.e. 70° (boiling) for 4 min or 37° for 45 min, produced the same level of extraction of labelled drug in the lipid phase. However, results show that the concentration of the drug used has a significant bearing on the amount of drug extracted in the lipid phase. Axelrod *et al.*[11] and Brodie *et al.*[12] have suggested that lipid acts as a storage site for 2-halogenoalkylamines, therefore, as the non-lipid sites become saturated with the drug, further increases in the concentration of the drug administered would simply increase the levels found in the lipid phase. Since Dikstein *et al.* used a concentration of 3×10^{-5} M dibenamine, whilst Yong *et al.* used 10^{-5} M dibenamine, then this may account for their conflicting results.

The subcellular distribution of ^{14}C-SY28 in lipid-free *vas deferens* is shown in Table 2, which also shows the distribution of protein throughout the fractions. The percentage of protein in the total tissue weight showed a mean of 20·8 ± 1·5 per cent in 8 experiments. It is clear (Table 2) that a large percentage of protein is found in the supernatant, but that very little ^{14}C-SY28 is found in this fraction. If the supernatant fraction is disregarded and the distribution of protein and labelled drug between the other fractions calculated, then a much closer correlation between drug and protein distribution is seen (Table 2, columns 3 and 4). This perhaps reflects the non-specific binding properties of the 2-halogenoalkylamines and complements the electron autoradiographic studies of Graham *et al.*[15]

TABLE 2. PERCENTAGE RADIOACTIVITY AND PROTEIN FOUND IN EACH SUBCELLULAR FRACTION OF LIPID-FREE GUINEA-PIG *vas deferens* FOLLOWING 20 min EXPOSURE TO 2×10^{-6} g/ml ^{14}C-SY28.

Subcellular fraction	1 Distribution of activity (%)	2 Distribution of protein (%)	3 Distribution of activity (%)	4 Distribution of protein (%)
Nuclear	71·4 ± 4·9	33·9 ± 5·8	75·7 ± 6·1	78·3 ± 3·8
Mitochondrial	13·9 ± 3·8	5·6 ± 0·8	15·7 ± 4·4	12·7 ± 2·4
Lysosomal	3·6 ± 0·9	2·3 ± 0·7	3·9 ± 0·9	5·0 ± 1·6
Microsomal	4·2 ± 1·6	1·7 ± 0·7	4·6 ± 1·6	4·0 ± 1·3
Supernatant	7·6 ± 0·9	56·4 ± 6·6		

Mean values ± S.D. from eight experiments.
Columns 1 and 2 distribution within all fractions. Columns 3 and 4 distribution disregarding the supernatant fraction.

Distribution of labelled SY28 in the presence of phentolamine. In experiments where protection of alpha receptors with phentolamine, prior to incubation with ^{14}C-SY28, were carried out, a consistent reduction in the levels of activity found in the total lipid-free homogenate was seen. The mean percentage reduction in count from 11 experiments was 18·7 ± 4·5 per cent, where 10^{-3} g/ml phentolamine was used to inhibit the binding of 10^{-6} g/ml ^{14}C-SY28. Table 3 shows the reduction in radioactivity in each subcellular fraction due to phentolamine pre-treatment. The nuclear and mitochondrial fractions were the sites of the greatest degree of protection by phentolamine, which is to be expected if the alpha receptor is a constituent of the cell membrane as thought by many workers,[14-16] since fragments of the cell membrane are brought down in these fractions.

TABLE 3. Counts/min per mg OF PROTEIN IN SUBCELLULAR FRACTIONS OF CONTROL AND TEST GROUPS OF LIPID-FREE GUINEA-PIG *vas deferens* AND THE PERCENTAGE REDUCTION IN COUNT DUE TO PHENTOLAMINE

Subcellular fraction	Control	Phentolamine pretreated	Mean percentage reduction in count due to phentolamine (%)
Nuclear	1380 ± 207	1042 ± 188	24·6 ± 4·7
Mitochondrial	1139 ± 440	833 ± 193	28·1 ± 6·9
Lysosomal	647 ± 208	571 ± 261	5·1 ± 4·1
Microsomal	752 ± 222	794 ± 290	+9·6 ± 2·6
Supernatant	73 ± 42	74 ± 18	1·4 ± 4·3

Mean values ± S.D., from nine experiments.

The percentage reduction in counts due to phentolamine are the mean results of the percentage reductions calculated for each experiment to indicate the consistency in the levels of reduction.

+ Indicates an increase not a reduction in the radioactivity count following phentolamine pretreatment.

It is interesting to note that the microsomal fraction consistently showed an increase rather than decrease in the level of activity following phentolamine pre-treatment. This may be explained by the fact that the microsomal fraction contains noradrenaline storage vesicles which may be a major site for the inhibition of noradrenaline uptake by 2-hologenoalkylamines.[17] Since phentolamine does not act on these sites, then, by blocking other SY28 binding sites, it thereby increases the effective concentration of SY28 available for binding to the storage vesicles, and thus increases the activity found in the microsomal fraction.

Acid hydrolysis of ^{14}C-SY28-bound subcellular fractions. In a previous paper[3] it was shown that chromatography and autoradiography of acid hydrolysates of total lipid-free *vas deferens* resulted in three consistently occurring spots, due to drug-amino acid complexes. This technique was therefore used to study each subcellular fraction. Figure 1 shows the result of such an experiment where the three spots due to the drug-amino acid complexes are seen in the nuclear and mitochondrial fractions, but only two spots are seen in the lysozomal and microsomal fractions. The R_f values of these amino-acid-^{14}C-SY28 complex spots are as shown in Table 4. These values are almost identical with those found for the total lipid-free homogenate.[3]

Figure 2 shows the results of a similar experiment, but where pre-treatment with 10^{-3} g/ml phentolamine was carried out. A reduction in the complexes is seen par-

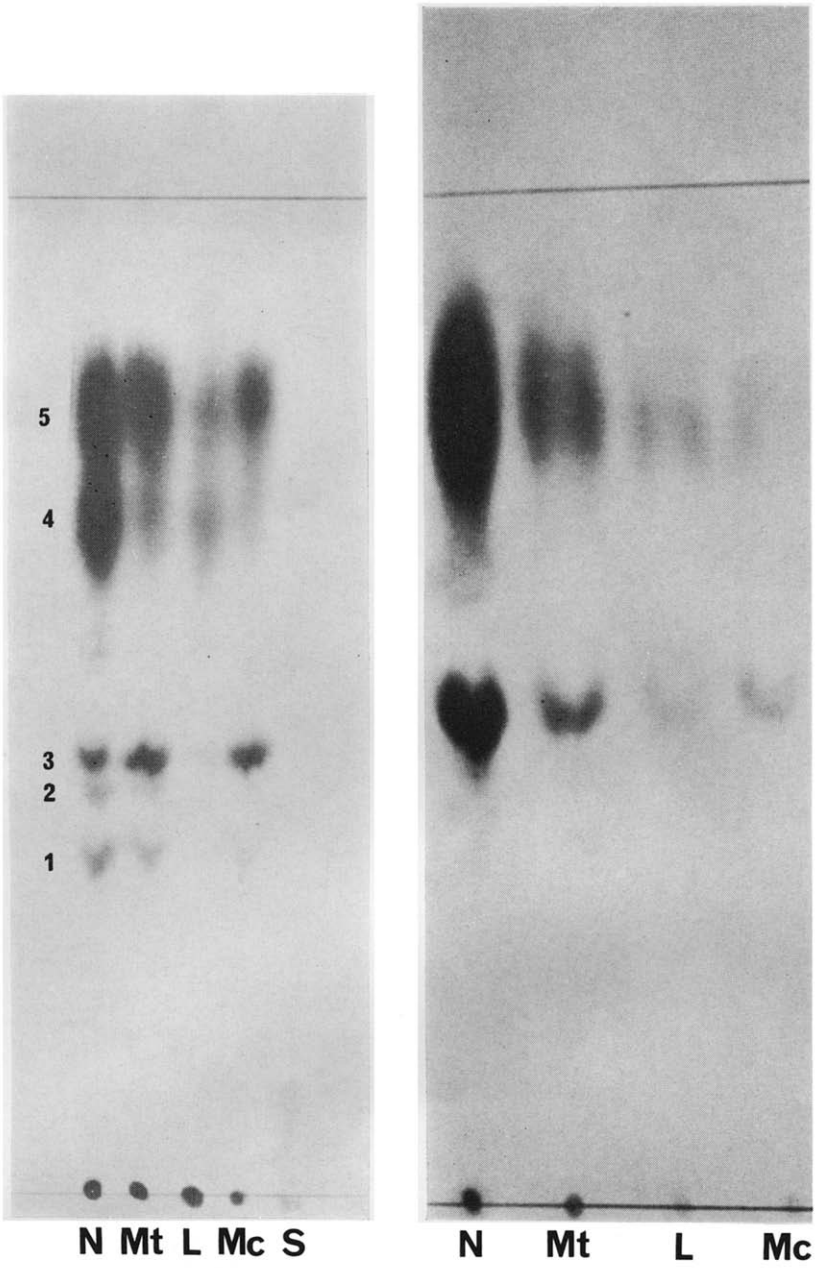


FIG. 1. Autoradiograph of paper chromatograph of acid hydrolysates of subcellular fractions of lipid-free guinea-pig *vas deferens* incubated with ^{14}C-SY28. N—nuclear fraction; Mt—mitochondrial fraction; L—lysozomal fraction; Mc—microsomal fraction; S—supernatant. The radioactive spots are designated 1–5.

FIG. 2. Autoradiograph of paper chromatograph of acid hydrolysate of subcellular fractions of lipid-free guinea-pig *vas deferens* incubated with ^{14}C-SY28 after pre-treatment with phentolamine. N—nuclear fraction; Mt—mitochondrial fraction; L—lysozomal fraction; Mc—microsomal fraction.

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TABLE 4. MEAN R_f VALUES (±S.D.) OF AMINO ACID–^{14}C-SY28 COMPLEXES FOUND IN HYDROLYSATES OF SUBCELLULAR FRACTIONS OF GUINEA-PIG *vas deferens*

Spot number (see Fig. 1)	R_f value
1	32·7 ± 1·1
2	40·0 ± 1·2
3	44·3 ± 0·7
4	73·6 ± 0·4
5	84·7 ± 0·8

Results from eight experiments.

ticularly with spots 1 and 2. The amino acids involved in the complexes which comprise spots 1 and 2 are histidine, arginine and methionine.[3] Since phentolamine is a relatively specific blocker of the alpha receptor site whereas 2-haloalkylamines are not it would seen reasonable to suppose that only a fraction of the ^{14}C-SY28 bound was inhibited by pre-treatment with phentolamine.

Though these results may throw some light on to the possible binding site of 2-halogenoalkylamines within the alpha receptor, the non-specificity of the 2-halogenoalkylamines must always be considered before any firm conclusions, as to the nature of the alpha receptor, can be reached.

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