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Binding of N-(2-bromo ethyl)-N-ethyl-N^1-naphthylmethylamine HBr (SY28) to the proteins of guinea-pig vas deferens

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Summary

1. Vasa deferentia from guinea-pigs were exposed to ^{14}C-SY28 HBr ($5{\cdot}32 \times 10^{-6}$M) washed, lipid extracted, and broken up.

2. The tissue was digested with papain, hydrolyzed with 6 N HCl, and gel filtration performed.

3. Aliquots at each stage were used for paper chromatography and autoradiography.

4. Three labelled spots (in addition to unbound 2-halogenoalkylamine) were located from acid hydrolysate and a fourth from gel filtrate.

5. Selected amino-acids were complexed with ^{14}C-SY28 and the procedure repeated. The R_F values were compared with those of the same amino-acids with and without 6 N HCl.

6. Histidine, aspartic acid, arginine and serine gave a significant degree of binding of ^{14}C-SY28.

7. The theoretical implications are discussed.

Introduction

The 2-halogenoalkylamine group of compounds are powerful alkylating agents with a wide spectrum of pharmacodynamic action. The principal feature is antagonism of certain effects of noradrenaline, adrenaline, dopamine, 5-hydroxytryptamine, histamine, and acetylcholine (Graham, 1962 ; Chapman & Graham, 1967). Despite the multiplicity of agonists involved, they have a common feature, namely the capacity to cause contraction of smooth muscle which is antagonized. Inevitably there has been speculation as to possible sites of attachment of the reactive aziridinium ions formed from 2-halogenoalkylamines (Graham, 1957) to receptive nucleophilic groupings in the cell and, in their time, sulphydryl, amino, carboxylic acid, and phosphatic radicles have been favoured (Ross, 1950 ; Harvey & Nickerson, 1954 ; Belleau, 1958, 1959) as the binding site. Takagi, Akao & Takahashi (1965) and Takagi & Takahashi (1968) recovered isotopically labelled dibenamine from subcellular fractions of intestinal smooth muscle and concluded that a large percentage of this compound was bound to protein. In the central nervous system Fiszer & De Robertis (1968) and De Robertis & Fiszer (1969) located labelled 2-halogenoalkylamine largely in a proteolipid which was extractable by a

chloroform:methanol mixture. Graham (1960) has shown that labelled SY28 added to serum binds preferentially to albumin. Graham & Katib (1966a, b) and Mottram & Graham (1970) obtained evidence that these compounds bind to bioactive peptides and that appropriate treatment with trypsin of a smooth muscle blocked by SY28 may restore agonist activity and will remove from it at the same time a ninhydrin positive extract which contains labelled compound. These indications that protein is an important binding site for 2-halogenoalkylamines in tissue and that recovery of the drug is possible led us to repeat this with labelled SY28 and guinea-pig vas deferens in the hope of achieving some degree of analysis of the binding material and the complex of the drug with it.

Methods

The vasa were removed from ten guinea-pigs of 350–450 g wt, stripped (Bentley & Sabine, 1963) and incubated for 25 min at 37° C in gassed Huković's solution (Huković, 1961) containing ^{14}C-SY28 HBr $5{\cdot}32 \times 10^{-6}$M. The compound was labelled in the methylene group of the naphth-1-ylmethyl moiety in a strength of 25 mCi/mmol and kept as a stock solution of $1{\cdot}33 \times 10^{-2}$M in acid alcohol, from which it was diluted with 0·9% NaCl solution immediately before use. The intact vasa were then washed three times with an excess of Huković's solution, rough dried and dropped into approximately 10 ml of chloroform:methanol 2:1 v/v (Analar) at 70° C for 2 minutes. The supernatant was decanted, the vasa washed three times with Huković's solution (10 ml) and the residual tissues roughly broken up in a Tri-R homogenizer (Teflon pestle and glass mortar) at 4° C for 5 minutes. The residue was spun at 2,500 *g* for 10 min, the supernatant decanted and the tissue resuspended by shaking in phosphate buffer pH 6·5 made up to a volume of 10 ml/g. Papain (0·1 ml; cryst × 2 BDH) was added and sufficient cysteine HCl to give a final concentration of 3×10^{-3}M. The mixture was then incubated at 60° C for 36 hours. Finally, concentrated HCl was added in an amount calculated to give 6 N HCl. Acid hydrolysis was then carried out in a sealed pyrex tube at 110° C for 18 hours. Samples of 0·1 ml were used to determine total radioactivity by a standard scintillation counting technique on a Tracerlab 100 apparatus.

Chromatography

(1) Ascending paper chromatograms of samples of the papain and the acid digests were prepared by running them for 8 h on Whatman 3 MM paper with butanol:acetic acid:water (50:12:25 v/v) as solvent. The dried papers were left in the dark for 90 days in contact with Ilford Industrial G X-ray film which was then processed to record autoradiography.

(2) Column chromatography was performed at room temperature by passing aliquots of the 2 acid hydrolysates through (a) 30 cm columns of Dowex 50 × 2 ion exchange resin (Hirs, Moore & Stein, 1956) or (b) 30 cm columns of Sephadex G10 gel. Two millilitre aliquots of each eluate were collected. Samples (0·1 ml) of these eluates were used for paper chromatography and autoradiography scintillation counting, as above. The eluent for Dowex resin was initially 0·2 N citrate buffer of pH 3·1 after which an exponential gradient was applied using 2 N sodium citrate-acetate buffer of pH 5·1; that for Sephadex gel was distilled water followed after 80 ml with M pyridine:0·5 M sodium chloride.

Amino-acids

Aqueous solutions of 50:50 v/v (10^{-2}M) of the L-isomers of selected amino-acids and ^{14}C-SY28 were incubated at 37° C for 30 min, made up to 6 N HCl and chromatography and autoradiography were performed as above. R_F values of all spots were recorded from the centre of the spot. The individual amino-acids were run in the same system at 10^{-2}M and R_F values recorded after ninhydrin spray.

Results

Paper chromatography and autoradiography of papain digests and acid hydrolysates

Ascending paper chromatography and autoradiography of aliquots of papain digests gave four spots. The value of one (R_F 0·858) was identical with that of un-

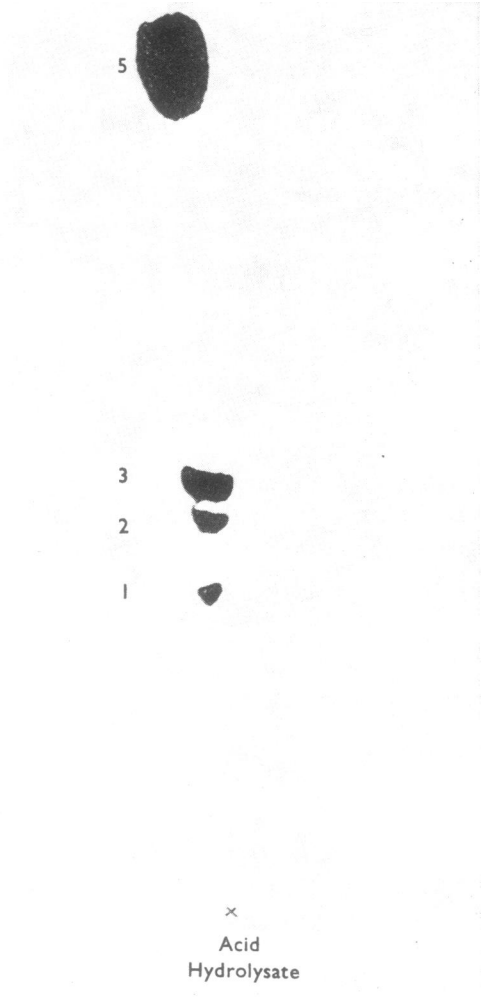

FIG. 1. Hydrolysate of lipid-free guinea-pig vas pretreated with ^{14}C-SY28. There are three spots on the autoradiograph after paper chromatography. No. 5 is unchanged 2-halogenoalkylamine, so numbered to conform with Fig. 4.

bound ^{14}C-SY28 and is attributed to free 2-halogenoalkylamine. The R_F of the other three spots varied according to the duration of the digestive process, but if acid hydrolysis had been performed three consistent spots were located in addition to that of R_F 0·858. The R_F values of these are listed in Table 1A and shown in Fig. 1.

Column chromatography of acid hydrolysates

Figure 2 is a graphic record of the counts per minute per ml (c.p.m./ml) of eluate (citrate buffer) samples after filtration of 0·3 ml of the acid hydrolysate of digested vas deferens pretreated with ^{14}C-SY28 through Dowex 50 × 2. The activity emerged at the end of the run which implies that the material was strongly cationic. The results of paper chromatography on this eluate were unsatisfactory; this was attributed to interference by the citrate buffer.

Figure 3 is a graphic record of c.p.m./ml of eluate (water) samples after filtration through Sephadex gel. Most of the ninhydrin positive material was found in the first 40 ml of eluate and was shown by ninhydrin, autoradiography and scintillation counting to be due to uncomplexed (unlabelled) amino-acids. The labelled complexes followed in the eluate volume 30–50 ml. The final elution with pyridine-

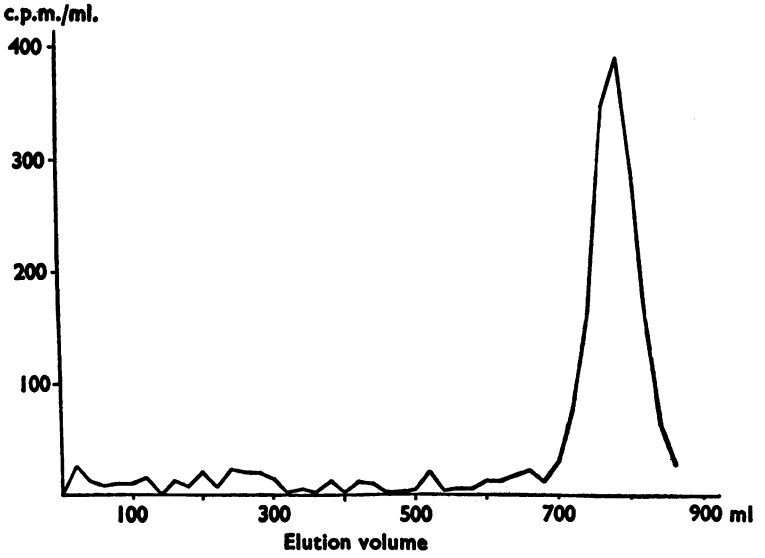

FIG. 2. Eluate from a 30 cm column of Dowex × 2 of hydrolysate of guinea-pig vas pretreated with ^{14}C-SY28.

TABLE 1. *Mean* RF *values from nine runs each* (±S.D. *of mean*) *from ascending paper chromatogram and autoradiogram from twenty vasa* (*ten guinea-pigs treated with* ^{14}*C-SY28*)

	A	B
Spot No.	R_F value	R_F value
1	0·325±0·008	0·330±0·004
2	0·400±0·009	0·404±0·009
3	0·443±0·012	0·439±0·005
4		0·496±0·009
5	0·858±0·015	0·853±0·014

Five aliquots subjected to: A, papain digest and acid hydrolysis; B, Sephadex G10 filtration of A.

sodium chloride applied after the passage of 80 ml of water displaced a further small amount of labelled cationic activity.

Paper chromatography of active eluates

Figure 4 shows the results of ascending chromatographic autoradiography on the eluate samples which contain activity and are illustrated in Fig. 3. The greater of the two radioactive peaks contains the ^{14}C-SY28-amino-acid complexes, with some unbound ^{14}C-SY28; the lesser peak containing only ^{14}C-SY28, brought off by the pyridine eluent (see **Methods**). Four labelled spots additional to that due to unbound 2-halogenoalkylamine (R_F 0·853) recurred consistently. Their R_F values are listed in Table 1B. A fifth spot of R_F 0·743 (No. 4A of Fig. 4) occurred inconsistently. The first three values agree closely with the R_F values in Table 1A which are those of the complexes before filtration but an additional spot of R_F 0·496 has appeared.

Candidates for ^{14}C-SY28-amino-acid complexes

Table 2 lists the R_F values of the selected amino-acids tested in this system and the mean $R_F \pm$ S.D. of the complexes of the same amino-acids with ^{14}C-SY28, the control values being determined after ninhydrin spraying and the complex values after autoradiography. No complexes were visible with leucine, isoleucine, phenylalanine or valine but they may have been masked by unbound ^{14}C-SY28 which formed a large spot. The possible candidates for binding of SY28 to amino-acids

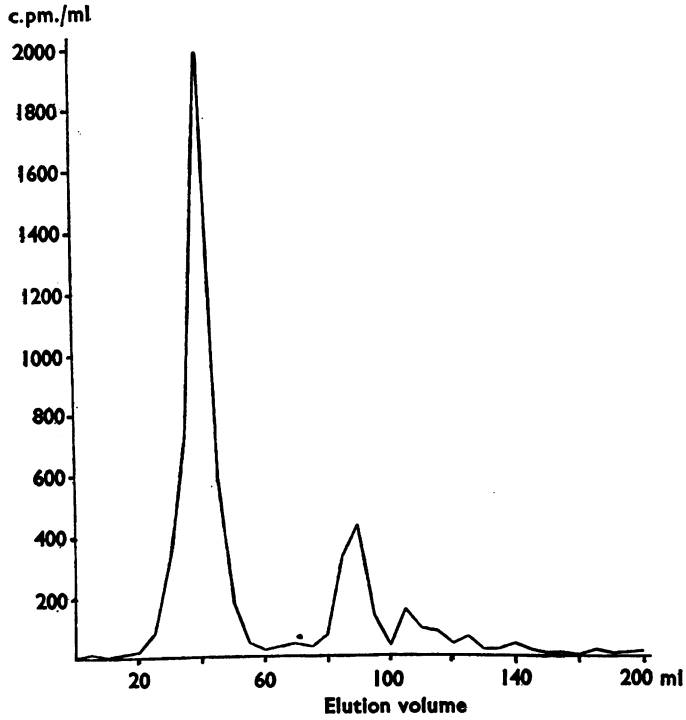

FIG. 3. Eluate from a column of Sephadex G10 gel of hydrolysate of guinea-pig vas pretreated with ^{14}C-SY28. The greater peak is a mixture of amino-acid-SY28 complex and SY28. The lesser peak is unchanged 2-halogenoalkylamine.

derived from acid hydrolysis of the digest from guinea-pig vas treated with SY28 are listed in Table 3.

Discussion

The 2-halogenoalkylamines attach covalently to their binding sites (Graham & Lewis, 1954) and it may be predicted with confidence that digestion with papain and hydrolysis with 6 N HCl will not disrupt such a bond. The three components located by paper chromatography of papain digest, which varied with the length of exposure to the enzyme, probably indicate binding of ^{14}C-SY28 to peptides. The size of these might be expected to vary with the degree of digestion. Acid hydrolysis on the other hand may be expected to complete the breakdown of these peptides into their component amino-acids and the consistency of the R_F values (Table 1A) implies that this has occurred and that ^{14}C-SY28 has bonded with individual amino-acids.

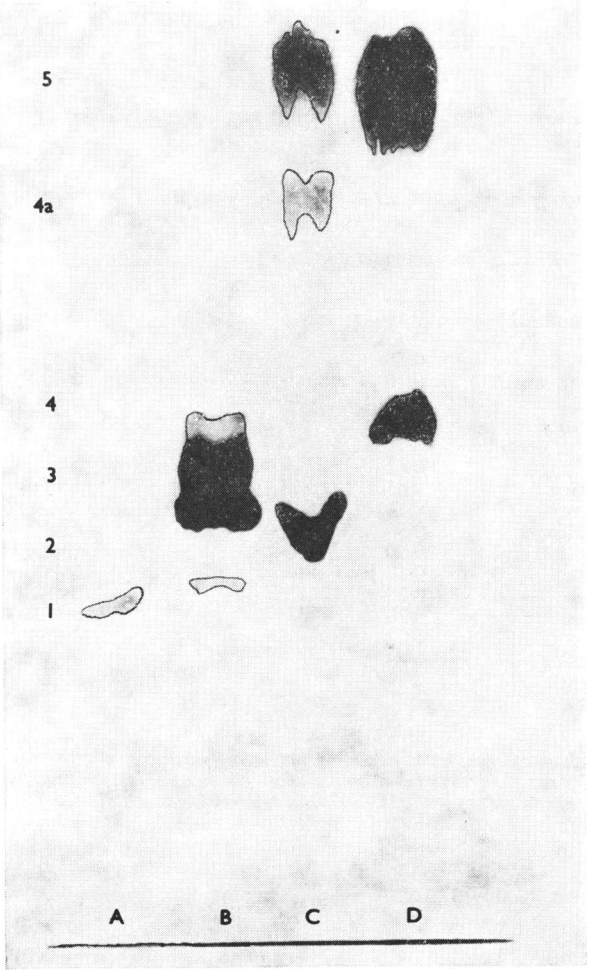

FIG. 4. Autoradiograph of paper chromatogram of the eluate of the greater peak shown in Fig. 3. There are four spots (amino-acid-SY28 complexes) in addition to unchanged 2-halogenoalkylamine (No. 5). Four eluate samples are shown: A=25–30 ml; B=31–35 ml; C=36–40 ml; D=41–45 ml incl.

If SY28 only binds to some amino-acids the HCl hydrolysates must contain many amino-acids not coupled with the 2-halogenoalkylamine. These were removed by gel filtration in which water is used as eluent, but not demonstrably by Dowex 50 × 2 in which citrate is used as eluent because this buffer interferes with subsequent paper chromatography of the complexes. Sephadex G10 is a weak cationic exchange resin (Eaker & Porath, 1967) which explains why the larger molecules of SY28-complex (cationic) came through the column after the unbound amino-acids and not before as one might have expected on the grounds of molecular weight. The resulting eluate gave four spots following paper chromatography, three of them identical in R_F value with those found in the acid hydrolysate before filtration and a fourth (R_F 0·496) which had attained its true position on the paper presumably because it was now unimpeded. The significance of the faint and inconsistent spot of R_F 0·743 is obscure but it may have been an association product of SY28 such as an ethanolamine complex.

Because it was not possible to split the bound halogenoalkylamine from its site and characterize the amino-acid, the drug was incubated with individual amino-acids and the complexes thus obtained were compared with those recovered from the tissue. The main objection to this approach is that *in vitro* the compound may bond with free $-NH_2$ and $-COOH$ groups, which are normally involved in peptide linkage,

TABLE 2. R_F *values of amino-acids and mean* $R_F \pm$ S.D. (*mean of five runs*) *of complexes with* ^{14}*C-SY28*

R_F of amino-acids (stated)		R_F of amino-acid-^{14}C-SY28 complex ± S.D. of mean ($n=5$)
Alanine	0·34	0·436 ± 0·002
Arginine	0·19	0·406 ± 0·005
Aspartic Acid	0·23	0·438 ± 0·007
Cysteine	0·15	0·464 ± 0·006
Glutamic Acid	0·30	0·442 ± 0·009
Glycine	0·18	0·472 ± 0·005
Histidine	0·16	0·333 ± ·003 0·391 ± 0·002
Hydroxyproline	0·29	0·425 ± 0·003
Iso-Leucine	0·62	
Leucine	0·65	
Lysine	0·14	0·365 ± 0·6 0·445 ± 0·003
Methionine	0·50	0·391 ± 0·002
Phenylalanine	0·60	
Proline	0·37	0·456 ± 0·002
Serine	0·23	0·438 ± 0·003
Threonine	0·28	0·450 ± 0·006
Tryptophan	0·52	0·67001
Tyrosine	0·44	0·68005
Valine	0·49	

TABLE 3. *A.* R_F *values of complexes derived from acid hydrolysis of guinea-pig vas pretreated with* ^{14}*C-SY28* (*for details see Table* 1) *and B amino-acids candidates for the binding site(s) and the* R_F *of their complexes with* ^{14}*C-SY28 in* 6 N *HCl* (*for details see Table* 2)

A	B	
0·325 ± 0·008	Histidine	(0·333 ± 0·003)
0·400 ± 0·009	Arginine	(0·406 ± 0·005)
	Histidine	(0·391 ± 0·002)
	Methionine	(0·391 ± 0·002)
0·443 ± 0·012	Alanine	(0·436 ± 0·002)
	Aspartic acid	(0·438 ± 0·007)
	Glutamic acid	(0·442 ± 0·009)
	Lysine	(0·445 ± 0·003)
	Serine	(0·438 ± 0·003)
	Threonine	(0·450 ± 0·006)

whereas this will only occur *in vivo* if these radicles are terminal on a peptide chain. This limitation applies particularly to those amino-acids (glycine, alanine, leucine, etc.) which contain alkyl side chains. Histidine (R_F 0·325) is the sole candidate for spot 1; it is also a candidate for spot 2 (R_F 0·400). Mixtures of this amino-acid with ^{14}C-SY28 always result in the formation of two spots of equal autographic intensity, probably due to the presence of two atoms in the imidazoline ring apt for electrophilic substitution. Rocha e Silva (1969) discusses the possibility of histidine being the primary binding site for histamine; Graham & Lewis (1953) have shown that SY28 is a powerful antihistamine. Arginine has a guanidyl group and inductive and mesomeric effects would produce a strong negative charge on the =NH group. Ionic bonding with halogenoalkyliminium would then become covalent. The third candidate for spot 2, methionine, has a CH_3-S grouping. The spot with methionine was an intense one, indicating strong binding. The halogenoalkylamines have an affinity for −SH groups (Ross, 1950); the CH_3-S in methionine may have been alkylated.

The third spot (R_F 0·443) was less specific. There are six possible candidates (see Table 3). Alanine seems unlikely for the reason stated; lysine gives two complexes *in vitro* and the one recovered *in vivo* (R_F 0·445) is of low intensity. It may be due to SY28 binding with −COOH groups (Graham & Katib, 1966b; Belleau, Di Tullio & Godin, 1969) normally involved in peptide linkage. Aspartic and glutamic acids are dicarboxylic amino-acids and provide a −COOH group which is not involved in peptide linkage. Serine and threonine contain suitable −OH groups but steric hindrance in the latter might be expected to reduce the likelihood of its being involved. The complexes with aspartic and glutamic acids were affected by the presence of HCl and if this was omitted, or washed out, as during gel filtration, the R_F value of the aspartic acid complex rose to 0·499 which accounts for the additional fourth spot (R_F 0·496) of the G10 eluate.

It is felt that the significant amino-acids involved in complexes derived by acid hydrolysis of lipid free vas deferens pretreated with ^{14}C-SY28 are histidine, aspartic acid, arginine and serine.

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