Biochemical Pharmacology. Vol. 28. pp. 393–395.

0006-2952/79/0201-0393 $02.00/0

INTERACTIONS OF AN HOMOLOGOUS SERIES OF CARBAMATES WITH PHOSPHATIDYLCHOLINE LIPOSOMES

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(*Received* 28 *April* 1978; *accepted* 5 *July* 1978)

Abstract—In an attempt to ascertain how membrane composition influences the passive intestinal absorption of an homologous series of aliphatic carbamates, permeability of these compounds into a model membrane system of phosphatidylcholine liposomes has been investigated. The leakage of $^{22}Na^+$ previously incorporated into these microvesicles has been measured to establish effects on permeability. All the carbamates were found to destabilise the liposomes at a concentration of 10^{-3} M, as indicated by increased permeability of the membranes to cation. This destabilisation was not linearly related to the partition coefficients of the carbamates. It was found that the increasing destabilisation produced by the carbamates as the homologous series was ascended was due to increased incorporation of the carbamates into the liposome membrane.

The importance of membranes in living cells is paramount. The basic structure of all biological membranes appears to be similar, consisting of a biomolecular lipid leaflet in which are embedded globular protein units [1]. Despite this similarity between membranes, studies on the absorption of a series of aliphatic carbamates from rat gut have demonstrated marked differences between the profile of absorption of the series in the small intestine and that in the stomach and colon [2, 3], despite the fact that in each instance only passive absorption appears to be involved. In the small intestine a parabolic relationship between absorption rate and log P of the carbamate is observed, butyl carbamate displaying the optimal absorption rate, whereas in the stomach and the colon a linear relationship between rate of absorption and log P (log octanol/buffer apparent partition coefficient) is noted. In view of the anaesthetic properties of the carbamates a possible explanation lies in their ability, which may be selective, to modify membrane properties.

In order to investigate this a simple membrane model was sought. Such a model for studying the effects of substances on the permeability of lipid membranes has been developed by Bangham *et al.* [4, 5]. This model system consists of enclosed vesicles formed by sonicating or mechanically agitating a suspension of phospholipid. These lipid vesicles or liposomes are of special interest since their limiting structure is also of bimolecular thickness. Such liposomes are thought to consist of completely closed single or concentric shells of bilipid membranes separated by aqueous compartments into which radioactive salts or other materials can be incorporated.

The phosphatidyl choline membrane was selected as a model to establish the link between log P of the aliphatic carbamates and their membrane modifying and penetrating ability into lipid membranes.

MATERIALS AND METHODS

Carbamates of general structure R-O-CO-NH_2. Methyl (Koch-Light Laboratories Ltd., Bucks., England), ethyl (BDH Ltd., Poole, Dorset, England), n-propyl and n-butyl (Kodak Ltd., Liverpool, England) were obtained commercially and were reagent grade. The purity of these chemicals was at least 99 per cent as determined by GC. t-Butyl (1,1-dimethylethyl; m.p. 104–6°), t-pentyl (2,2-dimethylpropyl; m.p. 80°), n-pentyl (m.p. 53–54°), t-hexyl (3,3-dimethylbutyl; m.p. 55–56°), n-hexyl (m.p. 59°), n-heptyl (m.p. 62°) and n-octyl (m.p. 67°) carbamate were synthesised by the Chemistry Division of the Chemical Defence Establishment, Porton Down, England.

Radiolabelled compounds. [^{14}C]-carbonyl labelled ethyl carbamate was supplied by Fluorochem Ltd., Glossop, Derby, England, specific activity 31.4 mCi/mol.; 354 μCi/mg. n-Butyl, n-hexyl and n-octyl carbamate (specific activity 1.5 mCi/mol) all ^{14}C-labelled in the carbonyl group of the ester linkage were synthesised by the Chemistry Division, Chemical Defence Establishment. The radiochemical purity of each carbamate was found to be not less than 99 per cent as checked by TLC and subsequent liquid scintillation counting of scraped sections of the whole plate. Isotonic ^{22}NaCl was obtained from the Radiochemical Centre, Amersham, England.

Determination of ^{22}Na *leakage from liposomes.* Liposomes were prepared using grade I egg lecithin (BDH) which was stored as a 30 mM solution in chloroform at −20°. Purity of the lecithin was checked by TLC

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before each experiment. Silica gel G plates were run in chloroform:methanol:water:ammonia (75:30:4:0.5) sprayed with 50 per cent sulphuric acid and developed by heating at 110° for 15 min [6]. The egg lecithin always ran as one spot (R_f value 0.61).

For the preparation of liposomes 50 μmol of lecithin were taken to dryness in a 25 ml round bottomed flask. The dried films were then shaken with 0.9 ml 160 mM ^{22}NaCl (25 μCi/ml) and 0.1 ml 160 mM Tris–HCl (pH 7.4) for 5 min. The flask was exhaustively flushed with nitrogen before shaking to prevent oxidation of the lipid. The resulting suspension was then sonicated for 60 min in a Pulsatron 50 bath (Kerry Ultrasonics Ltd., Herts., England). The temperature of the bath water was kept below 28° and the flask flushed with nitrogen every 10 min. TLC showed no presence of lysolecithin. After sonication the lipid was allowed to equilibrate at room temperature and under nitrogen for 24 hr before use. Untrapped ^{22}Na was removed by column chromatography; the 1 ml sample of liposomes was passed over a column of 3 g of hydrated Sephadex G 50 prepared in non-radioactive 144 mM NaCl–16 mM Tris–HCl (buffered saline, pH 7.4). Liposomes were eluted in the 12th–13th 1 ml fraction from the column and these fractions were pooled and diluted to 25 ml with buffered saline (7), giving a liposome preparation containing 2 μmol lipid/ml.

Series of dialysis bags (8/32 Visking tubing), previously washed, tied at one end and left soaking overnight in buffered saline were then prepared containing 1 ml of the liposome suspension. Further aliquots were taken for estimation of the total liposome-^{22}Na content by direct gamma counting using an LKB Wallac 80,000 counter. Liposome-containing dialysis bags were then placed in 10 ml screw-top test tubes containing 8 ml of buffered saline or various concentrations (10^{-3}–10^{-7} M) of carbamates in buffered saline. Tubes were then rotated at 37° at 10 rev./min. Tube contents (diffusate) were removed at intervals for ^{22}Na gamma counting to permit calculation of sodium leakage from the liposomes.

Determination of ^{14}C radiolabelled carbamate uptake by liposomes. The preparation of liposomes (containing 2 μmol lipid/ml) and experimental procedure was exactly as previously described except that ^{22}NaCl was replaced by unlabelled 160 mM NaCl. Carbamate solutions (10^{-3} M) were made containing 0.05 μCi ^{14}C-labelled carbamate per ml of buffered saline. After rotation at 37° for 21 hr samples were taken from inside and outside the dialysis bag for scintillation counting. Dialysis bags containing 1 ml buffered saline were used as controls, so that the uptake by lipid could be calculated.

Liquid scintillation counting. A Packard 3375 Liquid Scintillation Counter was used. Efficiency of sample counting was determined by automatic external standardisation and was between 70 and 85 per cent. A dioxan-based scintillation cocktail (10 ml per sample) was used comprising:- naphthalene (BDH Ltd., scintillation grade) 216 g; 2,5-diphenyloxazole, PPO (Koch-Light Labs. Ltd.) 7.2 g; 1,4-di [2-(5-phenyloxazolyl)]-benezene, POPOP (Koch-Light Labs. Ltd.) 0.2 g; ethyleneglycol monoethyl ether (Koch-Light Labs. Ltd.) 300 ml and 1,4-dioxan (Koch-Light Labs. Ltd.) 1500 ml.

RESULTS AND DISCUSSION

The effect of the series of aliphatic carbamates on the permeability of phosphatidylcholine liposomes to $^{22}Na^+$ was determined by measuring the leakage of $^{22}Na^+$ previously entrapped inside the liposomes. Using a 21 hr equilibration time, the leakage of sodium from the liposomes in the presence of a range of concentrations of each carbamate (10^{-3} M-10^{-7} M) was measured. At a concentration of 10^{-3} M all the carbamates significantly increased the percentage of sodium leakage above that of the control. These values are shown in Table 1. Methyl, ethyl and n-propyl carbamate increased the leakage by a similar and comparatively small amount (<20 per cent increase). However, n-butyl to n-octyl carbamate caused a progressive increase in the leakage rate as the homologous series was ascended. The overall non-linear dependency of the increase in leakage rates upon the octanol/buffer apparent partition coefficients of the carbamates is shown in Fig. 1 (log P values were from reference [2]).

Except for n-heptyl and n-octyl carbamates, which, at a concentration of 10^{-4} M brought about a small increase in sodium leakage, no carbamate was found to produce a significant increase in sodium leakage over the concentration range 10^{-4} to 10^{-7} M (Table 1). Neither was any significant stabilisation of the liposomal membrane (reflected by a decrease in entrapped sodium leakage) produced by any carbamate at any concentration studied.

Table 1. The effect of carbamate homologues on sodium leakage from lecithin liposomes after 21 hr

Carbamate concentration (M)	Control sodium leakage*	Carbamate used* methyl	ethyl	n-propyl	n-butyl	n-pentyl	n-hexyl	n-heptyl	n-octyl
10^{-3}	100	109 ± 3	111 ± 6	115 ± 4	135 ± 5	188 ± 7	229 ± 21	332 ± 41	404 ± 55
	(19.4)	(21.2)†	(21.5)†	(22.3)†	(26.2)†	(36.5)†	(44.4)‡	(64.4)‡	(78.4)†
10^{-4}	100	98 ± 5	100 ± 4	101 ± 6	108 ± 5	104 ± 2	109 ± 7	112 ± 5	129 ± 9
	(19.4)	(19.0)	(19.4)	(19.6)	(20.9)	(20.2)	(21.1)	(21.7)‡	(25.0)‡

Mean ± s.d. of 3 experiments.

*Results are expressed as a percentage of control leakage (control = 100). Figures in parentheses represent leakage as a percentage of total liposomal entrapped sodium. Levels of significance are shown (using 't' test) comparing these "percentage of total" values with controls. †$P < 0.01$, ‡$P < 0.001$, otherwise no significant difference.

Concentrations of 10^{-5}, 10^{-6} and 10^{-7} M of all carbamates showed no significant difference from controls.

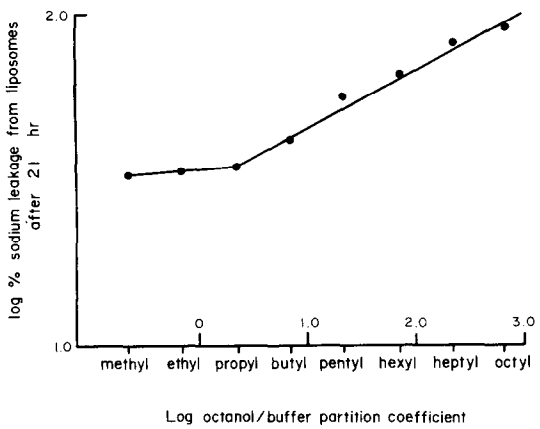

Fig. 1. The relationship between the octanol/buffer partition coefficient of the carbamate series (methyl to octyl) and their effect (at 10^{-3} M) on the sodium permeability of phosphatidylcholine liposomes.

Table 2. Distribution of carbamates into dialysis bags containing unlabelled liposomes after 21 hr equilibration

Carbamate (concn.)	Distribution ratio*	Lecithin/buffer† partition coefficient
ethyl (10^{-3} M)	0.90 ± 0.02	—
n-butyl (10^{-3} M)	1.02 ± 0.01	0.007
n-hexyl (10^{-3} M)	1.30 ± 0.01	0.146
n-octyl (10^{-3} M)	4.57 ± 0.04	1.278

mean ± S.D. of three determinations.

$$\text{distribution ratio} = \frac{[\text{carbamate in dialysis bag (1 ml)}]}{[\text{carbamate in dialysate (8 ml)}]}.$$

†lecithin/buffer partition coefficient calculated as

$$\frac{\mu\text{mol carbamate associated with 1 } \mu\text{mol lecithin}}{\mu\text{mol carbamate in 1 ml buffer}}.$$

Incorporation of the carbamates into the lipid membranes was investigated using four radiolabelled carbamates and following their distribution into dialysis bags containing unlabelled liposomes or buffer controls. At the end of the 21 hr incubation period in the control experiments, the radiolabelled carbamates were found to be evenly distributed throughout the total buffer volume available. Less than 1 per cent of the total activity was found bound to the dialysis bag. The distribution of Me from carbamates between Me dialysate and the dialysis bag containing liposomes, and the apparent lecithin/buffer partition coefficients are shown in Table 2.

As was found in the control experiments, n-butyl carbamate was evenly distributed throughout the total volume, but this was not the case for the other carbamates. The concentration of ethyl carbamate was higher in the dialysate and thus the presence of liposomes in the sac appeared to influence the distribution of this compound between the inside and outside of the dialysis sac. The two other carbamates tested (n-hexyl and n-octyl) were both found in greater concentrations inside the dialysis sac than in the dialysate.

It would thus appear that as the chain-length of the carbamate is extended, these compounds are "concentrated" inside the liposome-containing dialysis sac, probably by dissolving into the liposome membranes. The values for the lecithin/buffer partition coefficients shown in Table 2 show that this parameter increases an order at a time from butyl to hexyl to octyl carbamate. This "concentration" of the longer chain-length carbamates in turn causes destabilisation of the liposome membranes. The results for the distribution of ethyl carbamate are somewhat anomalous and require further investigation, although it should be remembered that this compound only marginally increased liposome membrane destabilisation at 10^{-3} M and the low distribution ratio would account for this. The explanations for the proportionately much greater uptake and liposome damage experienced with longer alkyl carbamates is presumably a result of their better ability to intercolate between the stacked phospholipid molecules.

The *in vitro* everted small intestine absorption studies of Houston, *et al.* [2] showed that with increasing chain-lengths, the amounts of carbamate tissue-bound (expressed as percentages of that lost from the mucosal fluid), could be represented as a curve similar in shape to that shown in Fig. 1. They found that at chain-lengths up to n-butyl, the percentages tissue-bound were relatively constant. However, at longer chain-lengths, carbamates tended to remain in the tissue rather than pass along the concentration gradient into the serosal fluid. It would thus appear that phosphatidylcholine liposomes are a good model for this aspect of intestinal absorption.

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