REFERENCES

1. J. Rouffy, *Thérapie* **30**, 815 (1975).
2. C. Baggio, G. Briani, R. Fellin, S. Martini, M. R. Baiocchi and C. Crepaldi, *Artery* **5**, 486 (1979).
3. P. Saba, E. Pagliai, A. Scalabrino, F. Galeone, F. Giuntoli, G. Guidi, S. Morini and G. Lavoratti, *Curr. ther. Res.* **27**, 677 (1980).
4. C. Roze, P. Cuchet, M. Souchard, C. Vaille and C. Debray, *Eur. J. Pharmac.* **43**, 57 (1977).
5. J. K. Reddy, in *Drugs Affecting Lipid Metabolism* (Eds. R. Fumagalli, D. Kritchevsky and R. Paoletti), p. 301. Elsevier/North-Holland Biomedical Press, Amsterdam (1980).
6. R. K. Berge and O. M. Bakke, *Biochem. Pharmac.* **30**, 2251 (1981).
7. R. K. Berge. S. Skrede and M. Farstad, *FEBS Lett.* **124**, 43 (1981).
8. J. K. Reddy, D. L. Azarnoff and C. E. Hignite, *Nature, Lond.* **238**, 397 (1980).
9. T. A. Miettinen, in *Lipid Pharmacology* (Eds. R. Paoletti and C. J. Glueck), Vol. 2, p. 83. Academic Press, New York (1976).
10. B. J. Kudchodkar, S. S. Sodhi, L. Horlick and D. T. Mason, *Clin. Pharmac. Ther.* **24**, 354 (1978).
11. S. M. Grundy, H. Y. I. Mok, L. Zech and M. Berman, *J. Lipid Res.* **22**, 24 (1981).
12. R. K. Berge, A. Aarsland, O. M. Bakke and M. Farstad, *Int. J. Biochem.* (in press).
13. C. DeDuve, B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, *Biochem. J.* **60**, 604 (1955).
14. O. C. Ingebretsen and M. Farstad, *J. Chromat.* **202**, 439 (1980).
15. J. K. Reddy, D. L. Azarnoff and C. R. Sirtori, *Archs int. Pharmacodyn. Thér.* **234**, 4 (1978).
16. P. B. Lazarow, *J. biol. Chem.* **253**, 1522 (1977).
17. I. Batosek, A. Guaitani and A. Guindani, *Pharmacology* **13**, 212 (1975).
18. J. K. Batra, H. G. Raj and T. A. Venkitasubramanian, *Experientia* **36**, 1311 (1980).
19. M. N. Cayen, W. T. Robinson, J. Dubuc and D. Dvornik, *Biochem. Pharmac.* **28**, 1163 (1979).
21. L. A. Carlson, M. Danielson, I. Ekborg, B. Klintemar and G. Rosenhamer, *Atherosclerosis* **28**, 81 (1977).
21. T. Flatmark, E. N. Christiansen and H. Kryvi, *Eur. J. Cell. Biol.* **24**, 62 (1981).
22. A. J. Cohen and P. Grasso, *Fd Cosmet. Toxic.* **19**, 585 (1981).

Biochemical Pharmacology, Vol. 31, No. 23, pp. 3933–3936, 1982.
Printed in Great Britain.

0006-2952/82/233933-04 $03.00/0

Analysis of the displaceable binding of the hypotensive drug R 28935 in rat brain

(*Received* 25 *May* 1982; *accepted* 23 *July* 1982)

A number of reports describe the pronounced hypotensive activity of R 28935, the racemic mixture of erythro 1-{1-[2-(1,4-benzodioxan-2-yl)-2-hydroxyethyl]-4-piperidyl}-2 benzimidazolinone (Fig. 1) in various animal models [1–7]. The hypotensive effect of R 28935 is probably of central nervous origin and stereospecific, since the threo form R 28914 is much less potent [2, 4, 5, 7, 8]. The central mechanism via which R 28935 induces hypotension is still unknown. The involvement of central α-adrenoceptors has been considered unlikely based on the observations that α-adrenoceptor-blocking agents, like yohimbine and piperoxan [2–4, 6] or phentolamine and tolazoline [1], were ineffective in inhibiting the hypotensive action of R 28935. However, it was found subsequently that the selective antagonist of α_1-adrenoceptors prazosin [9] strongly diminished the hypotension induced by R 28935 [7, 10, 11]. As a consequence thereof, central α_1-adrenoceptors have been suggested to play a role in the mechanism of action of R 28935. Although R 28935 possesses affinity for central [8] and peripheral α_1-adrenoceptors [7, 12], it is still very doubtful whether these sites represent the primary targets for R 28935 to cause hypotension, since, within a series of benzodioxane derivatives structurally related to R 28935, α_1-adrenoceptor affinity bears no relationship to hypotensive activity [7, 8].

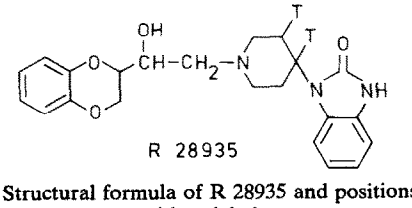

Fig. 1. Structural formula of R 28935 and positions of the tritium labels.

In the present study we have analyzed the specific binding of tritium-labeled R 28935 [13] to rat brain membranes in our efforts to learn more details of its mechanism of action. It was the main goal of the present investigation to establish whether the specific binding sites of [^{3}H]R 28935 identified in rat brain were associated with distinct receptor sites, possibly responsible for the initiation of the hypotensive action of this drug.

Materials and methods. Erythro [^{3}H]R 28935 (sp. act. 9 Ci/mmole) as well as unlabeled erythro R 28935 and threo R 29814 were gifts of Janssen Pharmaceutica (Beerse, Belgium). A number of benzodioxane derivatives structurally related to R 28935 indicated by the notation "R" (for structures see Ref. 8) were also provided by this company. Other drugs were obtained from normal commercial sources or the pharmaceutical companies of origin. All other chemicals were reagent grade.

Adult male Wistar rats (200–250 g) were decapitated, their brains (minus cerebella) removed and homogenized in 20 vol. (w/v) of 50 mM ice-cold Tris–HCl buffer (pH = 7.7 at 25°) using a Brinkman Polytron. The homogenate was centrifuged at 50,000 *g* for 10 min at 4°, resuspended in fresh buffer and centrifuged again. The final pellet containing the crude brain membranes was suspended in Tris–HCl buffer to give a concentration of 4 mg protein/ml and kept on ice until used. Protein concentration was assayed according to Lowry *et al.* [14].

Standard binding assays were run by incubating 500 μl of rat membrane suspension (see earlier) at 25° for about

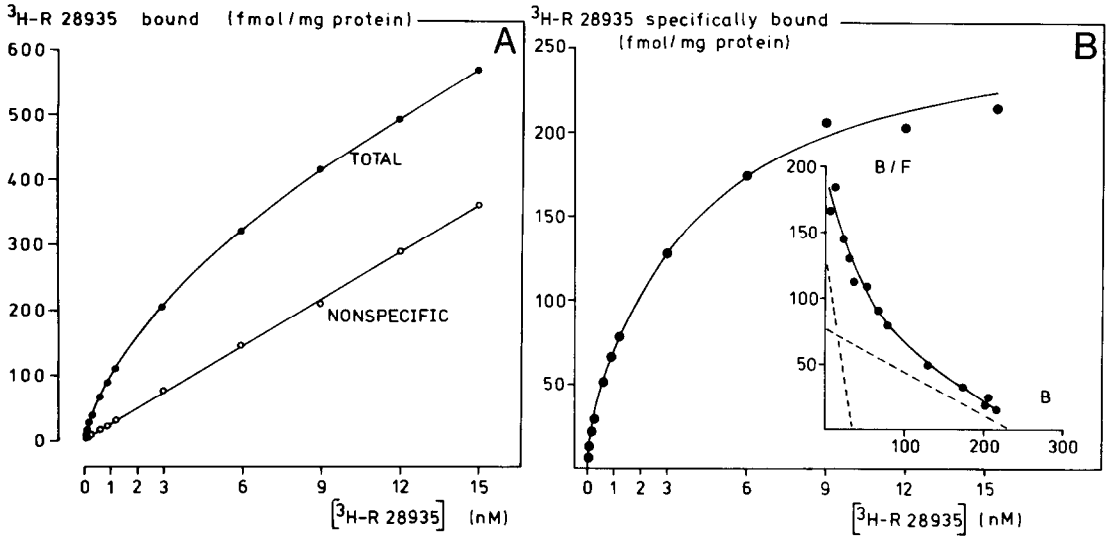

Fig. 2. Binding of [^{3}H]R 28935 to rat brain membranes as a function of the radioligand concentration. For details of performance see Materials and Methods. Non-displaceable binding was determined by addition of 10 μM of threo R 29814. Displaceable binding was calculated as the difference between total and non-displaceable binding. Data represent mean values of six separate saturation experiments performed in duplicate. A: Total and non-displaceable (non-specific) binding. B: Displaceable (specific) binding. Inset: Scatchard plot of the displaceable binding. Ordinate: Bound/free (fmoles/mg protein/nM). Abscissa: Bound (fmoles/mg protein).

45 min with [^{3}H]R 28935 (sp. act. 9 Ci/mmole; 0.05–15 nM) with occasional shaking in a total volume of 1 ml of incubation buffer. In displacement experiments, the inhibition of the specific binding of [^{3}H]R 28935 (0.4 nm) was determined in the presence of various concentrations of non-radioactive competitors. Incubations were terminated by addition of 4 ml of ice-cold buffer followed by rapid filtration onto Whatman GF/B glass fibre filters positioned over a vacuum and washed with 3 × 4 ml ice-cold buffer. Radioactivity collected on the filters was counted in 10 ml of Instagel (Packard-Becker) at an efficiency of 35–40% after 24 hr storage at room temperature. Displaceable (specific) binding of erythro [^{3}H]R 28935 was defined as binding remaining after subtraction of non-displaceable (non-specific) binding determined in the presence of 10 μM of threo R 29814. Blank values measured in the presence of 10 μM unlabeled R 28935 were similar to those assayed after addition of excess R 29814. The affinity of drugs for the displaceable binding sites of [^{3}H]R 28935 was expressed by the concentration inhibiting this binding by 50% (IC_{50}) calculated from the displacement curves by log probit analysis.

Results. The displaceable as well as the non-displaceable binding of [^{3}H]R 28935 was linear at protein concentrations between 0.5 and 4 mg/ml (data not shown). Total binding depended on the concentration of [^{3}H]R 28935 and non-displaceable binding increased linearly up to 15 nM, the highest concentration studied (Fig. 2A). The displaceable binding increased monophasically at concentrations up to about 3 nM, but deviated from a simple hyperbolic function at higher concentrations (Fig. 2B). The binding isotherm was resolved into two components of binding using non-linear regression analysis (Fig. 2B). The high and low binding components had maximal numbers of binding sites (B_{max}) of 30 and 230 fmoles/mg protein and approximate equilibrium dissociation constants (K_D) of 0.25 and 3 nM, respectively.

The association of [^{3}H]R 28935 (1 nM) to its binding sites at 25° was at equilibrium after 10 min with half-maximal binding attained at about 1 min and was stable for at least 60 min. The observed initial rate constant (k_{ob}) was 0.43 min^{-1} for 1 nM. Upon adding unlabeled threo R 29814 (10 μM) to an equilibrated mixture of brain membranes and [^{3}H]R 28935 (1 nM), the radioligand was biphasically displaced. There was a rapid initial displacement with a $t_{\frac{1}{2}}$ of about 2 min followed by a slower phase with a half-life of about 16 min.

The displaceable as well as the non-displaceable binding of [^{3}H]R 28935 (0.4 nM) was unaffected by NaCl (150 mM), guanosine triphosphate (GTP) (10 μM) or $MgCl_2$ (10 mM) in excess above 0.5 mM of EDTA. The sulfhydryl reagent *N*-ethylmaleimide (1 mM) did not significantly influence the non-displaceable binding of [^{3}H]R 28935, but reduced the displaceable binding by 54 ± 6% (mean ± S.E.M., N = 5).

Effects of various drugs on the displaceable binding of [^{3}H]R 28935 (0.4 nM) were evaluated in order to characterize the binding sites. Compounds closely related to R 28935 (for structures see Ref. 8) all ultimately caused 100% inhibition. Table 1 lists the IC_{50} values of the various derivatives. Unlabeled R 28935 was most potent with an IC_{50} value of 9 nM. No marked stereoselectivity was observed, since the threo form R 29814 was only 3.5 times less active. Derivatives of R 28935 were generally potent displacers of the displaceable [^{3}H]R 28935 binding. Their IC_{50} values varied approximately 150-fold (Table 1).

Hypotensive activity of erythro R 28935, threo R 29814 and their congeners has been determined following i.v. administration to anaesthetized normotensive rats [8] and expressed by the dose (nmoles/kg) eliciting a 30% decrease in mean arterial pressure (EC_{30}). The relationship between hypotensive activity (log EC_{30}) and affinity for [^{3}H]R 28935 binding sites (log IC_{50}), shown in Fig. 3, is very modest ($r^2 = 0.60$).

Certain compounds such as pimozide, methysergide, haloperidol and (−)-propranolol exhibited some affinity for the sites identified by [^{3}H]R 28935, but none of the pharmacological classes of drugs tested systematically inhibited the [^{3}H]R 28935 binding (Table 1). The α-sympatholytic drugs prazosin and phentolamine clearly biphasically inhibited the binding of 0.4 nM of [^{3}H]R 28935. At low concentrations (10^{-10}–10^{-7}M) of these antagonists,

Table 1. Inhibition of displaceable [^{3}H]R 28935 binding to rat brain membranes

Compound	IC_{50} (nM)	Compound	IC_{50} (nM)
R 28935	9	(−)-Propranolol	3700
R 22499	20	Apomorphine	6000
R 29814	32	Phentolamine	6500
R 22497	35	Prazosin	10,500
R 24315	55	Mepyramine	$>10^5$
R 23707	70	Atropine	$>10^5$
R 36240	100	(+)-Bicuculline	$>10^5$
R 31480	170	Metiamide	$>10^5$
R 29677	320	Naloxone	$>10^5$
R 24388	1400	Tubocurarine	$>10^5$
Pimozide	670	Strychnine	$>10^5$
Methysergide	1350	Glutamate	$>10^5$
Haloperidol	1900	Glycine	$>10^5$
Yohimbine	3200	(−)-Norepinephrine	$>10^5$

Rat brain membranes were incubated with 0.4 nM of [^{3}H]R 28935 and nine increasing concentrations of unlabeled competitors in duplicate, as described in Materials and Methods. IC_{50} values (concentrations inhibiting 50% of displaceable binding) were calculated by log probit analysis. The mean value of four separate determinations is reported.

about 20% of the displaceable binding was displaced.

Discussion. The present study has allowed the identification of displaceable binding sites of [^{3}H]R 28935 in membranes from rat brain which have all the characteristics of a structure-recognition site. [^{3}H]R 28935 bound with high affinity with at least two saturable components. It was rapid and reversible. The binding of [^{3}H]R 28935 was not affected by Na^+, Mg^{2+} and GTP. The modulatory role of mono- and divalent cations as well as of guanine nucleotides has been established in the binding affinity of agonists in β-adrenergic [15], α-adrenergic [16, 17], dopamine [18, 19], serotonin [20] and opiate [21] systems. Thus it may be conceived that the displaceable binding of [^{3}H]R 28935 is in a way associated with an antagonist interaction.

One of most important findings of this study is, however, that the inhibition of [^{3}H]R 28935 binding was hardly stereoselective. Furthermore, the *in vitro* affinity for the sites identified by [^{3}H]R 28935 was only weakly correlated with the hypotensive activity. Although a relationship between *in vitro* affinity and *in vivo* effectiveness may be hampered by many factors, the lack of correlation and the absence of stereoselectivity of the inhibition argue against the conclusion that the displaceable binding sites of [^{3}H]R 28935 in rat brain bear a relationship with distinct receptor sites responsible for the hypotensive effect. In addition, the accessibility to the displaceable high-affinity binding sites of [^{3}H]R 28935 was limited to structural analogues of R 28935. No other class of drugs investigated showed high-affinity inhibition.

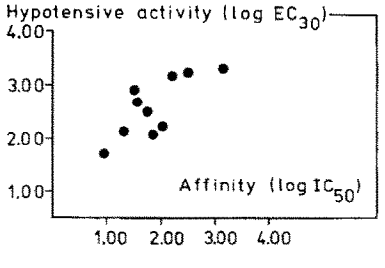

Fig. 3. Relationship between hypotensive activity quantified after intravenous administration to anaesthetized normotensive rats (log EC_{30}, Ref. 8) and inhibition of [^{3}H]R 28935 binding to rat brain membranes (log IC_{50}) for erythro R 28935, threo 29814 and eight structurally related derivatives listed in Table 1.

The selective α_1-adrenoceptor antagonist prazosin [9] as well as the non-selective α_1/α_2-sympatholytic drug phentolamine inhibited a small portion of the [^{3}H]R 28935 binding at low concentrations, suggesting that part of the displaceable binding of [^{3}H]R 28935 occurred to α_1-adrenoceptors. The *in vivo* and *in vitro* affinity of R 28935 for α_1-adrenoceptors is appreciable, whereas its affinity for α_2-adrenoceptors is low [7, 8, 12]. However, it is not likely that this small portion of central α_1-adrenoceptors represents the site of action of R 28935 and thus explains the interaction between prazosin and the hypotensive effect of this drug [7, 10, 11], because phentolamine, which is not effective *in vivo* [1], also displaced this binding.

In summary, the displaceable binding of the potent centrally acting hypotensive drug [^{3}H]R 28935 (sp. act. 9 Ci/mmole) to membranes from rat brain was found to be of high affinity, rapid and reversible with at least two saturable components. Binding was not affected by Na^+, Mg^{2+} and GTP. The displaceable binding of [^{3}H]R 28935 was effectively inhibited by its structurally related derivatives, but was hardly stereoselective and not accessible for other known drug classes. Part of the binding occurred to α_1-adrenoceptors. A relationship with only limited statistical value ($r^2 = 0.60$) was derived between affinity for the [^{3}H]R 28935 sites and the hypotensive activity. It is concluded that the sites identified by [^{3}H]R 28935 display a character which does not fit with any known receptor site or with a pharmacological effect. The present study shows the presence of pharmacologically irrelevant structure-recognition sites for this [^{3}H]-labelled ligand in membranous brain tissue preparations.

Acknowledgements—The authors are most indebted to Janssen Pharmaceutica (Beerse, Belgium) for generous gifts of [^{3}H]R 28935, R 28935, R 29814 and derivatives. The non-linear regression analysis of the binding data by Dr J. R. Rodriguez de Miranda is gratefully acknowledged.

Department of Pharmacy
Division of Pharmacotherapy
University of Amsterdam
Plantage Muidergracht 24
1018 TV Amsterdam
The Netherlands

PIETER B. M. W. M. TIMMERMANS*
YVETTE M. HARMS
HARRY D. BATINK
PIETER A. VAN ZWIETEN

REFERENCES

1. L. Finch, *Eur. J. Pharmac.* **33**, 409 (1975).
2. P. A. van Zwieten, *Archs Int. Pharmacodyn. Thér.* **215**, 104 (1975).
3. D. Wellens, J. M. van Nueten and P. A. J. Janssen, *Archs Int. Pharmacodyn. Thér.* **213**, 334 (1975).
4. D. Wellens, A. de Wilde, A. van Bogaert, P. P. van Bogaert, L. Wouters, R. S. Reneman and P. A. J. Janssen, *Archs Int. Pharmacodyn. Thér.* **215**, 91 (1975).
5. D. Wellens, L. Snoeckx, R. de Reese, R. Kruger, A. van de Water, L. Wouters and R. S. Reneman, *Archs Int. Pharmacodyn. Thér.* **215**, 119 (1975).
6. D. G. Taylor and M. J. Antonaccio, *Clin. exp. Hypertension* **1**, 103 (1978).
7. P. B. M. W. M. Timmermans, F. P. Slothorst-Grisdijk, H. Y. Kwa and P. A. van Zwieten, *Archs Int. Pharmacodyn. Thér.* **255**, 309 (1982).
8. P. B. M. W. M. Timmermans, F. P. Slothorst-Grisdijk, J. E. van Kemenade, A. M. C. Schoop, H. D. Batink and P. A. van Zwieten, *Archs Int. Pharmacodyn. Thér.* **255**, 321 (1982).
9. D. Cambridge, M. J. Davey and R. Massingham, *Br. J. Pharmac.* **59**, 514P (1977).
10. P. B. M. W. M. Timmermans and P. A. van Zwieten, *Eur. J. Pharmac.* **61**, 385 (1980).
11. P. J. Beckett and L. Finch, *Br. J. Pharmac.* **74**, 190P (1981).
12. P. E. Hicks and C. Waldron, *Br. J. Pharmac.* **74**, 844P (1981).
13. I. van Wijngaarden, A. G. Knaeps, A. Pluym and W. Soudijn, *J. Lab. comp. Radiopharmac.* **14**, 307 (1978).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. *J. biol. Chem.* **193**, 265 (1951).
15. L. T. Williams and R. J. Lefkowitz, *J. biol. Chem.* **252**, 5295 (1977).
16. D. C. U'Prichard and S. H. Snyder, *J. biol. Chem.* **253**, 3444 (1978).
17. H. Glossmann and R. Hornung, *Naunyn-Schmiedeberg's Archs Pharmac.* **314**, 101 (1980).
18. N. R. Zahniser and P. B. Molinoff, *Nature, Lond.* **275**, 453 (1978).
19. I. Creese, T. B. Usdin and S. H. Snyder, *Molec. Pharmac.* **16**, 69 (1979).
20. S. J. Peroutka, R. M. Lebovitz and S. H. Snyder, *Molec. Pharmac.* **16**, 700 (1979).
21. S. R. Childers and S. H. Snyder, *Life Sci.* **23**, 759 (1978).

* To whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 31, No. 23, pp. 3936–3938, 1982.
Printed in Great Britain.

0006-2952/82/233936-03 $03.00/0

Alterations in phospholipid methylation in rat brain synaptosomal membranes produced by ethanol *in vitro* and *in vivo*

(*Received* 17 *May* 1982; *accepted* 4 *August* 1982)

The effects of alcohols on the CNS are considered by many to depend on entry into the synaptic membrane lipid bilayer with a consequent change in the physical properties of the membrane [1, 2]. This physical change, often referred to as a 'fluidisation', may then disrupt the activity of the various membrane-associated proteins and lipids responsible for synaptic transmission. Among the proteins which are likely to be most affected by such an action are enzymes which are located within the membrane bilayer and which have as their substrates the lipids of the bilayer. The enzymes responsible for the progressive methylation of phosphatidylethanolamine to phosphatidylcholine are examples of this kind of enzyme [3, 4] and the fact that they appear to be coupled to receptor protein activation [5] and calcium transport [5] make them of considerable interest in relation to synaptic function.

Current concepts of the mechanism of development of tolerance to alcohols indicate that the fluidisation which is produced in synaptic membranes is reduced when these are taken from ethanol-tolerant animals [6] and that this is due to some alteration in the lipid composition of these membranes [7–9]. If this is so one would expect the activity of membrane-bound enzymes also to be changed in synaptic membranes from ethanol-tolerant animals. We have therefore undertaken investigations of the effects of ethanol *in vitro* on the activity of phospholipid-methylating enzymes in synaptosomal preparations of control rats, as well as those from rats made tolerant and physically dependent on ethanol.

Methods and results

Male Sprague–Dawley rats (200–250 g) obtained from Charles River U.K. were used in these experiments. Rats were killed by decapitation, and whole brain dissected in the cold and synaptosomes prepared by the method of Cotman [10]. Synaptosomes were then incubated either in 10 mM HEPES buffer (pH 7.4) containing 4 mM dithiothreitol (DTT), 6% sucrose, 5 mM $MgCl_2$, 40 μM $CaCl_2$, or in 'artificial CSF' [11] for 1 hr at 37° in the presence of 200 μM *S*-adenosyl-L-methionine (SAM) containing 1 μCi of *S*-adenosyl-L-([^{3}H]methyl)-methionine [15 Ci/mmole (Radiochemical Centre, Amersham, U.K.)] as the methyl donor. At the end of this period synaptosomal lipids were extracted with chloroform : methanol : HCl (2 : 1 : 0.02 v/v/v) followed by repeated washings with 0.1 M KCl in 50% methanol. Aliquots of the chloroform phase were then used for scintillation counting. Alternatively, lysed synaptosomes (vigorous shaking in distilled water for 1 hr at 4°) were subjected to filtration (0.45-μm Millipore filters) before being taken for scintillation counting. These meth-