Table 1. The influence of ketamine on inhibition of acetylcholinesterase by sarin

	Ketamine concentration (mM)				
	0	0.5	1	2	5
k_i (sarin) (10^6 M^{-1} min^{-1})	2.86	1.88	1.61	0.79	0.46
(SD)	0.17	0.49	0.32	0.08	0.08
(N)	(7)	(4)	(5)	(4)	(5)

inhibition [5]. The inhibition constants were determined to $K_i = 0.52$ mM (SD 0.098) and $K_i' = 3.3$ mM (SD 0.67).

An interaction with the active site may result in a protective effect against the irreversible inhibitor sarin, in analogy with the action of several other reversible, competitive inhibitors. This was also found to be true for ketamine, as shown in Table 1. It protected against the irreversible inhibitor in a concentration-dependent manner, but the data could not be described by the most simple kinetics, $k_{obsd} = k_i/(1 + [\text{ketamine}]/K_i)$. The observed k_is were, for all concentrations of ketamine, higher than calculated from such a simple interaction only at the active site.

The influence of ketamine on aging and reactivation was studied at only one and a rather high (5 mM) concentration. From Table 2 it can be concluded that ketamine retarded both the rate of reactivation and, to a lesser extent, the rate of aging. A possible explanation for these effects is that a conformational change of the phosphonylated enzyme is induced by ketamine-binding.

Table 2. The influence of 5 mM ketamine on the aging rate constant (k_a) and the reactivation efficiency (k_2/K_d) by HI-6 of sarin-inhibited acetylcholinesterase

	k_a (min^{-1})	k_2/K_d (10^3 M^{-1} min^{-1})
Control	0.260	1.28
SD	0.008	0.21
(N)	(3)	(3)
Ketamine	0.197	0.61
SD	0.028	0.12
(N)	(3)	(3)

Mazzanti *et al.* have recently shown that the local concentration of ketamine in cholinergic synaptical membranes during anesthesia is high [2]. Their results on membrane fluidity changes and effects on acetylcholinesterase activity in combination with the results presented here give some support for the hypothesis of ketamine protection against organophosphate poisoning. Current research on acetylcholinesterase levels in ketamine-anesthetized organophosphate-intoxicated animals will throw more light on this issue. The question whether ketamine facilitates (by reducing the rate of aging), aggravates (by reducing the efficiency of reactivation by oximes) or has no effect at all on the therapeutic countermeasures in the *in vivo* situation also deserves further investigations.

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Activation of the carcinogen, 5-hydroxymethylchrysene, to the mutagenic sulphate ester by mouse skin sulphotransferase

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5-Methylchrysene (5-MCR), a well known environmental carcinogen found in tobacco smoke, shows carcinogenicity towards adult mouse skin [1] as well as towards newborn mouse skin and lung [2]. Metabolic oxidation of 5-MCR gave 5-hydroxymethylchrysene (5-HCR) as a major metabolite, dihydrodiols, and phenols in mouse skin *in vivo* [3] and in the livers of mice and rats *in vitro* [3, 4]. 5-HCR had as high carcinogenicity to mouse skin as 5-MCR, suggesting it to be one of proximate metabolites in the mouse skin [5]. The carcinogen 5-HCR was demonstrated to be activated to a potent mutagenic metabolite, 5-HCR sulphate, by rat liver cytosolic sulphotransferase in the presence of a 3′-phosphoadenosine 5′-phosphosulphate (PAPS)-generating system [6]. The active sulphate bound covalently to calf thymus DNA at pH 7.4 through its 5-methylene carbon with loss of a sulphate anion and was inactivated to a non-mutagenic glutathione (GSH) conjugate by rat liver cytosolic GSH transferase in the presence of GSH [7].

However, nothing is known of whether 5-HCR is biotransformed to the active sulphate in the skin. Little information is available as yet for the metabolic conjugation of hydrophobic alcohols to the corresponding sulphates in the skin. To our knowledge, dehydroepiandrosterone (DHA) sulphate formation from DHA in the human skin was the first instance for these informations [8]. Sulphation of steroid alcohols has recently been demonstrated with

DHA and cholesterol to be mediated in the presence of PAPS by a partially purified hydroxysteroid sulphotransferase preparation from the newborn mouse skin [9]. It is of interest that this enzyme preparation also catalysed the transformation of 4-nitrophenol (4-NP) to 4-NP sulphate [9]. 7-Hydroxycoumarin was also reported to be sulphated to 7-sulphoxycoumarin by hairless rat and mouse skin strips [10]. The present communication deals with the PAPS-dependent biotransformation of 5-HCR to active 5-HCR sulphate in the target tissue, mouse skin, for this carcinogen.

Sulphation of 5-HCR was carried out by the incubation of radioactive [methylene-^{3}H]5-HCR ([^{3}H]5-HCR) with mouse skin cytosols fortified with PAPS. [^{3}H]5-HCR was synthesized from 5-formylchrysene, which was prepared as previously reported [11], by reduction with [^{3}H]sodium borohydride in aqueous methanol containing sodium hydroxide in the usual manner. The skin cytosols used were prepared at 0–4° as follows: male and female ddy mice, purchased from Tokyo Laboratory Animals Science Co. Ltd., Tokyo (12 animals each), weighing 25–30 g (7 weeks of age), were decapitated, and the skin (6 cm^2) was removed from the supracaudal region of the back of each mouse, chilled and washed in cold isotonic KCl to remove blood, extended on a cold glass plate, shaved with a Point razor blade (Shiseido Co. Ltd., Tokyo), and cut into strips with scissors. The pooled skin strips (12 g) from the 12 mice were homogenized in 2 vol. of isotonic KCl with a Polytron homogenizer (Kinematica, Switzerland). The homogenate was squeezed through a cheesecloth and rehomogenized with a Teflon-pestled Potter–Elvehjem-type homogenizer. The skin cytosolic fraction was obtained by centrifugation (105,000 *g*, 60 min) of a post-mitochondrial fraction of the homogenate, followed by dialysis of the separated soluble supernatant fraction at 1° for 24 hr against 2500 vol. of 0.1 M phosphate buffer, pH 7.4. Protein contents (6–6.5 mg/ml) in the supernatant fractions were determined by the method of Lowry *et al.* [12].

[^{3}H]5-HCR (20 μCi, 0.1 mM) dissolved in dimethyl sulphoxide (DMSO, 0.05 ml) was incubated at 37° for 60 min in 0.1 M phosphate buffer (0.95 ml), pH 7.4, with the dialysed soluble supernatant fractions (S105s) from the male and female mouse skins in the presence of PAPS (0.6 mM). After unlabelled sodium 5-HCR sulphate (50 nmol) dissolved in methanol (0.05 ml) was added to the incubation mixture (1 ml) as a carrier for the subsequent isotope dilution method, most of unreacted [^{3}H]5-HCR was removed from the mixture by repeated extractions with ether–ethyl acetate (2:1, v/v, 2 ml each). The residual aqueous phase was, then, shaken three times with ethyl acetate (2 ml each) in the presence of tetra-*n*-butylammonium (TBA) bromide (5 mM) to extract metabolically formed radioactive 5-HCR sulphate, diluted with the unlabelled sulphate, as a hydrophobic ion-pair complex with a TBA cation arising from the bromide. The ethyl acetate extract, concentrated to dryness *in vacuo* at 30° and redissolved in methanol (0.1 ml), was subjected to high pressure liquid chromatography (HPLC) carried out on an octadecylsilica column in aqueous methanol containing 2 mM tetra-*n*-propylammonium (TProA) iodide (purification step I) (Fig. 1). Radioactive 5-HCR sulphate was eluted together with the unlabelled sulphate as an ion-pair complex with a TProA cation from the reverse partition chromatogram monitored by UV absorptiometry and then successively rechromatographed for further purification on the octadecylsilica column as ion-pair complexes with the tetra-*n*-alkylammonium (TAA) cations, TBA (purification step II) and TProA (purification step III) in the aqueous methanol.

At purification step I, the 5-HCR sulphate-TProA complex, eluted from the column at a retention time of 11.3 min, was completely separated from less polar radioactive 5-HCR which was eluted at a retention time of 30 min, and at purification steps II and III, specific radioactivities of the HPLC fractions of the 5-HCR sulphate-TAA complexes became constant within an experimental error (Fig. 1). No detectable amount of the radioactivity was found in the isolated 5-HCR sulphate when the skin S105s were boiled or when PAPS was omitted from the incubation mixtures. Thus, 5-HCR proved to be activated by sulphotransferase to the potent, intrinsic mutagen, 5-HCR sulphate, in the male and female mouse skins which had been demonstrated to be one of the most representative target organs for carcinogenesis induced by the carcinogen when topically applied [5].

Formation of the sulphate ester from 5-HCR in male and female mouse skin S105s was compared with that of sulphate esters from 4-NP and DHA (Table 1), which are typical substrates for phenol and hydroxysteroid sulphotransferases in mouse liver, respectively [13, 14]. The male and female mouse skin sulphotransferase activities showed little sex difference towards 5-HCR and DHA, but showed a little difference towards 4-NP, although marked sex differences were observed with the hepatic sul-

Table 1. Sulphotransferase activities of mouse skin and liver cytosols towards 5-HCR, DHA, and 4-NP

	Sulphate formed in skin cytosols (pmol/mg protein/min)			Sulphate formed in liver cytosols (pmol/mg protein/min)		
Substrate	Male	Female	Male/female	Male	Female	Male/female
5-HCR*	0.14	0.13	1.1	4.0	17.0	0.2
DHA†	4.7	4.4	1.1	0.12	0.48	0.3
4-NP‡	13.1	9.8	1.3	1130	1370	0.8

* Incubation of mouse liver cytosols (2 mg protein/ml) with [^{3}H]5-HCR was carried out under the same conditions as described in Fig. 1.

† DHA sulphate formation was assayed by using [1,2,6,7-^{3}H]DHA as previously reported [22].

‡ 4-NP (0.1 μmol) dissolved in DMSO (0.05 ml) was incubated with mouse skin and liver S105s (4.5 and 0.5 mg protein/ml, respectively) in the presence of PAPS (0.6 μmol) for 30 min under the same conditions as used for 5-HCR. 4-NP sulphate formed in the cytosols was extracted from the incubation mixtures with ethyl acetate as an ion-pair complex with TBA under the same conditions as used for 5-HCR sulphate after removal of 4-NP by the extraction with the organic solvent mixture and then measured by HPLC carried out on an octadecyl silica column (Finepak SIL C_{18}, 5 μm in particle size, 4 × 250 mm) in methanol–water (35:75, v/v, 0.8 ml/min) containing 2 mM TBA bromide. 4-Nitrobenzyl alcohol used as an internal reference was eluted at 8 min before the peak of the 4-NP sulphate–TBA complex appearing at 12 min.

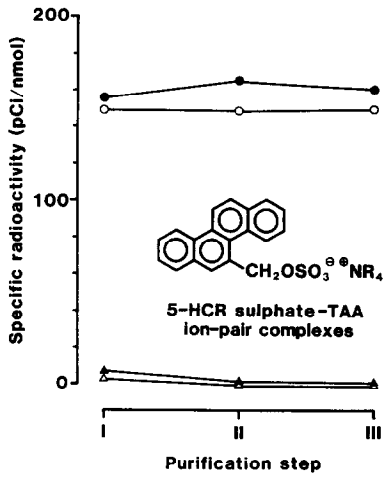

Fig. 1. Identification of radioactive 5-HCR sulphate formed from [^{3}H]5-HCR in male and female mouse skin cytosols as ion-pair complexes with tetra-*n*-alkylammonium (TAA) cations. [^{3}H]5-HCR (20 μCi, 0.1 μmol) dissolved in DMSO (0.05 ml) was incubated at 37° for 60 min with male (closed circles) or female (open circles) S105 (4.5 mg protein) in a final volume of 1 ml of 0.1 M Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, containing PAPS (0.6 μmol). Incubations were also carried out under the aforementioned conditions by using boiled male and female mouse skin S105s (closed triangles) instead of the untreated S105s and by omitting PAPS (open triangles) from the mixtures containing untreated S105s. Radioactive 5-HCR sulphate formed in the incubation mixtures was diluted with unlabelled 5-HCR sulphate and extracted with ethyl acetate as a hydrophobic ion-pair complex with TBA as described in the text. Purification steps I and III: 5-HCR sulphate was eluted at a retention time of 11.3 min from an octadecylsilica column (Finepak SIL C_{18}, 5 μm in particle size, 4 × 250 mm) with methanol–water (7:3, v/v, 0.8 ml/min) containing 2 mM TProA iodide. Purification step II: 5-HCR sulphate was eluted at a retention time of 15 min from the octadecylsilica column with methanol-water (7:3, v/v, 0.8 ml/min) containing 2 mM TBA bromide. Under these chromatographic conditions, radioactive 5-HCR was eluted at 30 min. Specific radioactivities of chromatographically isolated 5-HCR sulphate were obtained by the scintillation counting method and absorptiometry at 269 nm. Data are expressed as arithmetic mean values from at least four experiments. The specific radioactivities showed maximum deviations from the illustrated mean values as follows: ±14% for the triangles and ±3% for the circles.

photransferase activities towards these substrates. In contrast with the skin sulphotransferase activities, the hepatic enzyme activity towards 5-HCR was 4 times higher in the female than in the male.

Female mouse skin S105 was examined for the effect of various sulphotransferase inhibitors on sulphation of 5-HCR, because female mice had been preferably used for the studies of skin carcinogenesis by 5-MCR and 5-HCR. Addition of the enzyme inhibitors (0.1 mM) to the incubation mixture inhibited the 5-HCR sulphate formation by 69% for 3′-phosphoadenosine 5′-phosphate, a non-specific inhibitor to sulphotransferases, by 62% each for pentachlorophenol and 2,6-dichloro-4-nitrophenol, both phenol sulphotransferase inhibitors, and by 23% for DHA sulphate, a hydroxysteroid sulphotransferase inhibitor. The data on the enzymic inhibition may suggest that phenol sulphotransferase(s) would catalyse the sulphation of 5-HCR in the female mouse skin, though sulphate conjugation of the carcinogen 7,12-dihydroxymethylbenz[*a*]anthracene (DHBA) to the highly reactive mutagen, DHBA 7-sulphate, had been indicated to be mediated by an enzyme other than phenol sulphotransferases in rat liver [15].

Hydroxymethyl-polycyclic aromatic hydrocarbons (-PAHs) such as 7-hydroxymethyl-benz[*a*]anthracene (-BA), 7-hydroxymethyl-12-methyl-BA, 12-hydroxymethyl-7-methyl-BA, and DHBA, all of which have carcinogenicity towards mouse skin [16, 17] and are oxidative metabolites of the carcinogens 7-methyl-BA and 7,12-dimethyl-BA, have been demonstrated to be activated to their potently mutagenic sulphate esters by rat liver cytosol [15, 18–21]. In connection with this, evidence provided by Cavalieri *et al.* is of importance that synthetic 6-hydroxymethylbenzo[*a*]pyrene (6-HBP) sulphate was a more potent carcinogen towards mouse skin than 6-HBP [16]. This evidence suggests that the sulphate ester of 6-HBP could be a potential ultimate form of 6-HBP, though no investigation has been made yet on the biotransformation of 6-HBP to the sulphate. In this communication, the first direct evidence was obtained for the formation of the active sulphate ester of the carcinogenic hydroxymethyl-PAH by sulphotransferase in the skin.

It is of interest that Hecht *et al.* reported that an arylsulphatase treatment of water-soluble metabolites of 5-MCR in mouse skin *in vivo* gave not only dihydrodiols and phenols as major products but also 5-HCR as the minor [3]. Their result suggests that the skin would activate 5-MCR to 5-HCR sulphate *via* 5-HCR *in vivo*. Isolation and identification of 5-HCR sulphate formed in mouse skin and covalent binding of the sulphate to skin DNA *in vivo* are now in progress in our laboratory.

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[^{3}H]Quinuclidinyl benzilate binding to the human lung muscarinic receptor*

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The parasympathetic (cholinergic) nervous system is an important regulator of airway tone [1] and plays a major role in the diseases of hyperreactive airways. The human lung receives its parasympathetic innervation from the vagi which supply the bronchial tree to the level of the respiratory bronchioles. Impulses from the postganglionic fibers are transmitted by acetylcholine which stimulates muscarinic receptors located on a variety of target cells [1]. Stimulation of muscarinic receptors has been shown to increase total lung cyclic GMP levels [2], increase the immunologic release of mast cell mediators [2], increase mucus release from cultured human airways [3], and contract human airway smooth muscle [4]. Target cell response begins with the number and affinity of the surface receptors for their neurotransmitter. Therefore, it is important to characterize the human lung muscarinic receptor system in order to understand the role of these receptors in peripheral lung responses and to ascertain whether binding parameters differ among patient populations. Using a computer modeling program which allowed us to combine and analyze radioligand binding data from multiple experiments, we determined that human peripheral lung contains a relatively low concentration of single-site, high-affinity muscarinic receptors.

Materials and methods

Reagents. Bovine serum albumin (BSA†), scopolamine, atropine, oxotremorine, methacholine chloride, carbachol, histamine diphosphate and D,L-isoproterenol were purchased from Sigma, St. Louis, MO; sucrose and magnesium chloride were purchased from the Fisher Scientific Co., Fair Lawn, NJ; Tris was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN; Dulbecco's medium was purchased from Hazleton, Denver, PA; quinuclidinyl benzilate (QNB) was a gift from Hoffmann-La Roche, Nutley, NJ; and [^{3}H]QNB (39.4 Ci/mmol) was purchased from New England Nuclear, Boston, MA.

Preparation of human lung tissue. Human lung tissue was obtained at the time of resection for lung cancer. Macroscopically normal areas of peripheral lung tissue were dissected free of pleura, large bronchi (>3–5 mm) and large blood vessels, and were washed in Dulbecco's medium. Tissue was frozen at −70° until use.

Plasma membrane preparation. To determine the optimal lung plasma membrane preparation for the radioligand binding assays, the specific binding of [^{3}H]QNB to five different plasma membrane preparations was compared (Fig. 1). Specific binding was calculated as the difference in binding when 4 nM [^{3}H]QNB was incubated at 37° for 60 min in the absence or presence of 1 μM unlabeled QNB. The lung was minced, placed in ice-cold 10 mM Tris (pH 7.4) containing 0.25 M sucrose and 0.5% BSA (10 ml/g tissue), homogenized, gauze filtered, and centrifuged slowly. The resultant supernatant fraction was tested directly for specific binding or was centrifuged further at either 27,000 *g* for 20 min or 40,000 *g* for 45 min. The pellets and supernatant fractions were then resuspended in the buffer used for the radioligand binding assays (50 mM Tris with 25 mM $MgCl_2$, pH 7.2), and analyzed for specific binding. As shown in Fig. 1, the lowest specific [^{3}H]QNB binding occurred in the supernatant fractions (<7 fmol/mg protein), whereas the highest specific [^{3}H]QNB binding occurred in pellet 3 (27.8 fmol/mg protein). The specific binding measured in pellet 3 was 88% of the total binding. Therefore, all subsequent radioligand binding studies used the plasma membrane isolation procedure outlined for pellet 3 (Fig. 1). The protein content [5] of this plasma membrane preparation was adjusted to 1–3 mg/ml with Tris $MgCl_2$ buffer.

Radioligand binding assays. Radioligand binding assays were done as described previously [6]. Aliquots (100 μl) of the plasma membrane preparations suspended in the Tris $MgCl_2$ buffer were used in the binding assays in a final volume of 160 μl. The assay mixtures were incubated at 37° for 60 min with either increasing concentrations of the radioligand, [^{3}H]QNB, or a fixed concentration of radioligand and different concentrations of various agonists and antagonists. Incubations were terminated by adding 4 ml of ice-cold incubation buffer followed by rapid vacuum filtration through Whatman GF/C Glass Microfibre Filters. The filters were washed immediately thereafter with 20 ml of ice-cold buffer, dried, and then assayed in a liquid scintillation system using a Beckman LS-7000 counter. All samples were run in duplicate to triplicate, and replicates differed from each other by less than 10%.

Modeling and statistical evaluation of receptor binding. The binding data were analyzed using a weighted, nonlinear, least-squares curve fitting as provided in the computer program LIGAND [7]. The binding curves were first re-expressed in terms of bound [^{3}H]QNB concentration versus total concentration added, considering both the labeled and unlabeled ligand. Contrary to common custom for binding studies, nonspecific binding (*N*) was not measured for each individual concentration by use of a 100-fold excess unlabeled ligand concentration. Rather, *N* was modeled directly as an extra, very low-affinity, nonsaturable class of

* Supported in part by a VA Merit Review Award.

† Abbreviations include: BSA, bovine serum albumin; K_d, dissociation constant; *N*, ratio of nonspecifically bound to free ligand; QNB, quinuclidinyl benzilate; *R*, binding capacities; and *RMS*, root-mean square.