Biochemical Pharmacology, Vol. 26, pp. 1631–1635. Pergamon Press, 1977. Printed in Great Britain.

FACTORS AFFECTING THE TISSUE BINDING OF NICOTINE IN VARIOUS SPECIES

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(*Received* 13 *December* 1976; *accepted* 7 *March* 1977)

Abstract—The binding of [^{14}C]nicotine to various tissue fractions has been studied in the cat, pigeon and rat using the technique of equilibrium dialysis. Liver homogenates from all three species bound the drug to varying extents though the distribution of radioactivity showed species differences. Cat and rat liver binding appeared to reside predominantly in the microsomal fraction whereas in the pigeon the activity was essentially present in the cytosol. Lung tissue from all three species showed only a small binding capability which was not associated with the microsomes or soluble cell components. Brain homogenates in cat and rat showed a similar small degree of binding but there was a significant degree of binding in pigeon brain homogenate. Cat and pigeon kidney homogenates showed a small degree of tissue binding which appeared to be uniformly distributed in the cell fractions. Rat kidney, however, exhibited an extremely large *in vitro* binding capability which was apparently associated with the cytosol. The percentage binding was increased with increasing buffer pH over range pH 6–8 corresponding to an increase in the proportion of free base. A Scatchard plot over the concentration range 62–1540 nM gave a linear response, calculations from which indicated a low affinity, high capacity, binding capability for nicotine. *In vivo* distribution studies in the rat after subcutaneous administration of 0.4 mg/kg [^{14}C]nicotine revealed a high degree of localisation of radioactivity in the kidney relative to other tissues and subsequent tissue fractionation confirmed the *in vitro* observations. Plasma from all three species showed no significant binding properties. The nature of the binding entity is not yet known though it can be concentrated by chromatography on Sephadex G-100 and is associated with a fraction of relatively low molecular weight containing little, if any, lipid.

Pharmacokinetic studies of nicotine and investigations on its metabolic fate have been frequently described over recent years in a variety of animal species including man [1–3] and a number of metabolites of the drug have been characterised [4, 5]. Much is known regarding the pharmacological effects of this compound in relation to tobacco smoking [6] and effects of nicotine on the central nervous system have been correlated with pharmacokinetic data [7, 8]. It is known from autoradiographic evidence that radioactivity accumulates in certain areas of the brain after administration of [^{14}C]nicotine [9] it has been shown that the hypothalamus [8] and other regions [10] of the brain preferentially concentrate the drug. After administration of [^{14}C]nicotine to animals [7, 11] or man [3] it is often found that less than quantitative excretion/elimination of radioactivity occurs over 4–7 day study periods and long term retention of the drug and/or its metabolites has been detected autoradiographically in the respiratory tract and urinary bladder of rodents [12]. Pharmacokinetic studies on nicotine in rats [11] and man [3] indicate that the drug has a high apparent volume of distribution and it is known that the tissue to plasma concentration ratios for nicotine are often large [7, 13]. The binding of this important smoke constituent in tissues from three species using the technique of equilibrium dialysis has therefore been investigated.

MATERIALS AND METHODS

Male Hooded Lister rats, bred at these laboratories, weighing between 250–350 g were used. They were fed on a standard laboratory diet (41B, Oxoid Ltd) with free access to water.

Male cats weighing between 2.5 to 3 kg bred in these laboratories were also used.

Female White Carneau Pigeons, obtained from the Palmetto Pigeon Plant, Sumter, NC, weighing between 500–600 g were fed a pellet diet with free access to water.

[2′-^{14}C]Nicotine hydrogen tartrate at a specific activity of 20 mCi/m-mole was synthesised in these laboratories by Dr. T. H. Houseman using the method of Decker [14]. Unlabelled nicotine hydrogen tartrate and all other reagents were obtained from the British Drug Houses, Poole, Dorset.

In vitro *studies.* Rats and pigeons were sacrificed by cervical dislocation. Cats were anaesthetised by intraperitoneal administration of Nembutal (Abbot Laboratories, Queenborough, Kent) at a dose of 60 mg/kg.

With the animal or bird on a cold surface tissues were excised, dissected free of connective tissue, weighed and chopped finely. Homogenates (20% (w/v)) were made in ice cold 0.1 M phosphate buffer, pH 7.4 using a Potter–Elvehjem glass/teflon homogeniser. A portion of each homogenate was centrifuged at 10,000 *g* for 30 min at 0° and the supernatant removed. A portion of this supernatant was further centrifuged for 1 hr at 100,000 *g* and the clear

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Table 1. Percentage binding of [^{14}C]nicotine fractions in different species expressed as mean ± S.E.M.

Species	Fraction	Tissue: Liver	Lung	Brain	Kidney
Rat (6)	Homogenate	39.6 ± 4.7	4.5 ± 1.1	7.2 ± 0.8	263.9 ± 39.6
	$10^4 \times g$ supernatant	28.2 ± 6.9	nil	nil	258.4 ± 46.2
	$10^5 \times g$ supernatant	7.1 ± 1.4	nil	nil	285.0 ± 39.9
Cat (4)	Homogenate	19.7 ± 2.0	4.4 ± 1.0	7.7 ± 1.1	8.1 ± 0.8
	$10^4 \times g$ supernatant	18.1 ± 1.5	nil	nil	6.4 ± 0.8
	$10^5 \times g$ supernatant	9.2 ± 1.5	nil	nil	4.4 ± 0.9
Pigeon (4)	Homogenate	34.3 ± 4.6	6.6 ± 2.5	22.7 ± 1.9	5.3 ± 1.9
	$10^4 \times g$ supernatant	26.6 ± 2.9	nil	3.4 ± 0.5	8.1 ± 0.5
	$10^5 \times g$ supernatant	24.4 ± 3.7	nil	4.4 ± 1.0	9.8 ± 0.8

Figures in parentheses indicate the number of animals. Original nicotine conc. (Co) = 154 nM.

supernatant thus produced, was separated. Heparinised plasma was also used from blood obtained by aortic puncture.

Equilibrium dialysis. Equilibrium dialysis was performed on tissue extracts in the following manner: A portion of each extract (1 ml) was pipetted into a Visking cellulose membrane bag (The Scientific Instrument Centre, London) of 0.025 mm thickness, which was then sealed and dialysed on a roller mixer for 12 hr at 4° against a larger volume (40 ml) of 0.1 M phosphate buffer containing 0.56 μCi [^{14}C]nicotine hydrogen tartrate. At the end of this time the Visking bag was carefully removed, blotted and the contents sampled (200 μl) after brief inversion to ensure homogeneity. A portion (200 μl) of the buffer solution was also counted. On each occasion a buffer blank was similarly dialysed to act as control.

In vivo *studies.* Three male rats were each given a subcutaneous injection of [^{14}C]nicotine hydrogen tartrate (2 μCi) in physiological saline at a dose of 0.4 mg/kg (0.4 mg/ml). Twenty min later each animal was killed by cervical dislocation and the brain, lungs, kidneys and liver removed. Each tissue was homogenised, centrifuged to separate the 10,000 *g* and 100,000 *g* fractions, and portions of each fraction counted.

Radioactivity measurements. Clear aqueous samples were counted directly by adding to a dioxan based Scintillator (15 ml) [7]. All other samples containing tissue debris were solubilised with Soluene-350 (Packard Instrument Ltd.), in vial, to which was then added the scintillator. Radioactivity was measured on a Packard Model 3375 liquid scintillation spectrometer (Packard Instrument Ltd.). All samples were counted for 10,000 counts.

Bound drug concentration (C_B) was obtained from the difference between the tissue fraction concentration (C_T) and the final buffer concentration (C_F). The percentage activity bound is therefore $(C_T - C_F/C_F) \times 100$.

RESULTS

In vitro *dialysis studies.* Analysis of tissue blanks, where the contents of the bag were replaced by buffer solution, indicated, after dialysis, that the expected equilibrium concentration had been attained. There was no evidence of drug binding to the Visking membrane.

The results of equilibrium dialysis on subcellular fractions of four tissues from the rat, cat and pigeon are shown in Table 1. In all three species there was evidence of significant tissue binding to liver which in the cat and rat occurred predominantly in microsome containing supernatant. Removal of the microsomes by ultracentrifugation, reduced binding significantly in rat and cat liver fractions but not in the pigeon liver.

Lung tissue in all three species showed a small degree of binding, but microsomal and cytosol preparations were without activity. Plasma proteins from all species exhibited no significant binding of radioactivity.

Brain homogenates from the rat and cat showed a small degree of binding, but as with lung, the microsomal and cytosol preparations derived from the homogenates were, in this context, inactive. Pigeon brain homogenates however, showed a considerable degree of binding though the $10^4 \times g$ and $10^5 \times g$ supernatants possessed a much reduced capability.

The most striking species difference occurred in the kidney. Cat and pigeon kidney homogenates showed relatively low degrees of binding which appeared to be uniformly distributed thoughout the subcellular fractions. Rat kidney homogenates, in contrast, exhibited a very large degree of binding which, on subcellular fractionation, appeared to reside exclusively in the cytosol.

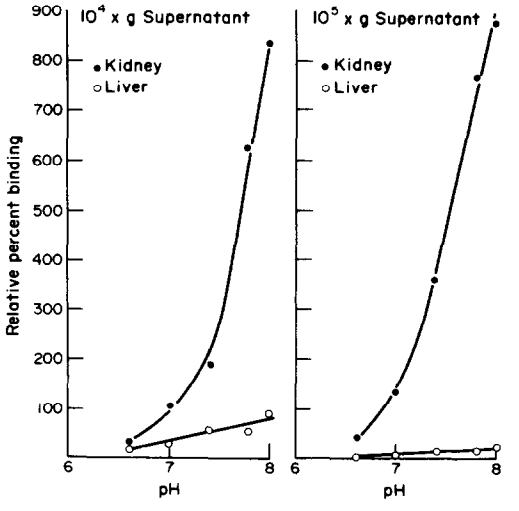

Fig. 1. The effect of variation in pH on the binding of [^{14}C]nicotine to rat tissue fractions. Co = 154 nM.

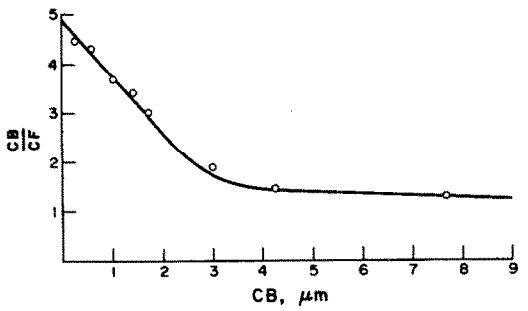

Fig. 2. Modified Scatchard plot of the binding of [^{14}C]-nicotine to rat kidney cytosol over the concentration range 62 to 6200 nM.

Effect of pH on tissue binding of nicotine to rat liver and kidney fractions. The effect of pH of the dialysis buffer and tissue homogenate, on the binding of [^{14}C]nicotine was investigated and the data are illustrated graphically in Fig. 1. It may be seen that, in both tissues examined the degree of binding increased with pH over the range studied. The effect of pH on the degree of binding in kidney was particularly marked.

Effect of nicotine concentration on tissue binding. The degree of binding to rat kidney cytosol was estimated over a range of [^{14}C]nicotine concentrations (62–5200 nM). The resultant data were used to construct a modified Scatchard plot [15] which, as shown in Fig. 2, resulted in a curve the shape of which indicated the possibility of two types of binding site. Calculations and extrapolation of the initial linear response to increasing nicotine concentration, over the range 62–1540 nM, enabled an apparent association coefficient of $1.1 \times 10^3\ M^{-1}$ and a binding capacity of 322 nmoles per mg protein to be derived.

In vivo *tissue distribution studies.* Tissues obtained from rats after subcutaneous administration of [^{144}C]nicotine were fractionated and the radioactivity measured in each fraction. Table 2 shows the results where the data are calculated relative to the activity in each homogenate. In lung and brain the radioactivity, at 20 min after dosing was uniformly distributed throughout the cells. The same phenomenon occurred in rat kidney. In liver, however, the distribution data suggested a relative concentration in the microsomes and perhaps other cell organelles. The table also shows the total individual tissue homogenate activity distribution expressed relative to that in the liver. When expressed this way, brain and lung contained relatively less activity than liver but kidney contained some five times as much activity as hepatic tissue.

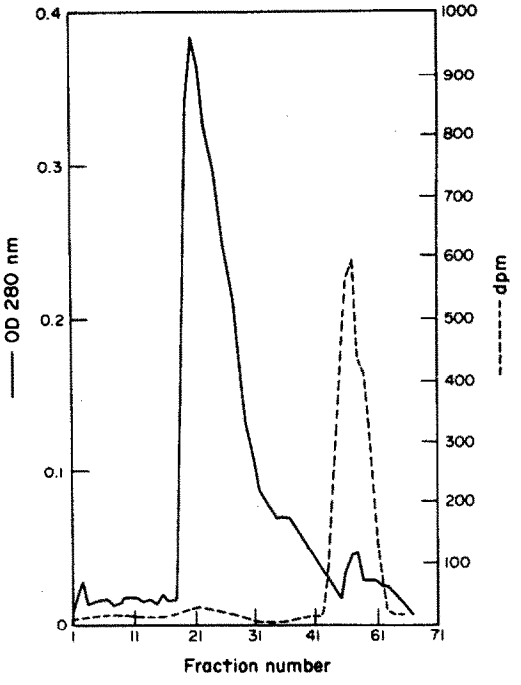

Fig. 3. Chromatography of a rat kidney $10^5 \times g$ supernatant on a sephadex G 100 column.

Chromatographic studies of the tissue binding. A portion (1 ml) of the dialysed rat kidney $10^5 \times g$ supernatant at pH 7.4, was applied to a 60 × 1.1 cm Sephadex G-100 column and activity eluted with 0.1 M phosphate buffer pH 7.4 containing 0.02% sodium azide. Figure 3 shows the elution profile. Elution of a single peak of radioactivity was associated with a relatively small protein containing peak (measured at 280 nm) approximately one void volume after the main fraction. On the same column [^{14}C]nicotine was eluted between fractions 59–62.

DISCUSSION

The results indicate that [^{14}C]nicotine will bind to tissues in certain species—particularly the rat. Such tissue binding is consistent with the apparently large volumes of distribution [11] and tissue to plasma levels [13] of the drug which have been reported in that species. It seems clear that in livers of all three species the principal binding occurs in microsomes which is consistent with their metabolic involvement. However, it is known that in the metabolism of nicotine the cytosol is also required, at least for cotinine

Table 2. Distribution of radioactivity in subcellular fractions of rat tissues 20 min after subcutaneous administration of 0.4 mg/kg [^{14}C]nicotine

Percentage dpm/ml in cellular fractions relative to homogenate	Liver	Lung	Brain	Kidney
$10^4 \times g$ supernatant	80.4 ± 4.4	96.4 ± 7.3	94.9 ± 2.7	98.7 ± 6.6
$10^5 \times g$ supernatant	40.7 ± 4.3	97.0 ± 15.0	95.6 ± 9.9	100.1 ± 4.2
Percentage dpm/gm tissue relative to liver	100	32 ± 3	39 ± 3	538 ± 55

production [16] and so the findings of residual binding in that fraction is not surprising. *In vitro* studies [17] using the method of Hucker *et al.* [16] have indicated a much greater rate of hepatic nicotine metabolism in pigeon as compared to rat and it is known that the LD_{50} for nicotine is much less in this bird than that observed in other species [18]. These earlier and the present observations may well be consistent with important differences in avian drug metabolism.

Both the lung and brain of the rat and cat showed little tendency to bind nicotine suggesting that the observed binding in other tissues may not be related purely to lipid solubility. Luddon *et al.* [19] have reported that lung, and liver slices, when incubated with [^{14}C]nicotine under anaerobic conditions both bind the drug significantly. Yamamoto *et al.* [13] however, observed only a relatively low degree of binding in lung tissue using a similar technique.

Perfused lung studies indicate that nicotine itself does not bind significantly *in vivo* to dog lung although metabolites of nicotine such as the *N*-oxide appear to do so [20]. Nicotine is actively metabolised, *in vitro*, in liver preparations at 37° and both kidney and lung are also capable of much lower degree of metabolism. Brain tissue appears to be without metabolic activity [9]. Under the conditions of our experiments we consider it unlikely, however, that significant metabolism occurred and examination of a liver homogenate, after dialysis, by solvent extraction revealed only [^{14}C]nicotine.

Pigeon brain was unusual in that the homogenate bound much larger proportions of the drug than did the tissue from the other two species. Few comparative studies have been performed on this species with nicotine or other basic drugs and so it is difficult to speculate on the reason for this difference. Pigeon brain, however, appears to be relatively more sensitive to enzyme inhibition by nicotine than is rat brain [21].

The most striking observation, however, relates to rat kidney, the cytosol of which showed a considerable capability to bind [^{14}C]nicotine.

The *in vivo* radioactivity distribution and the evidence from earlier tissue slice studies [12] were also consistent with this finding. Though the solid content of both 10^4 and $10^5 \times g$ supernatants would vary from tissue to tissue the homogenates were made up on the basis of identical wet weight to buffer ratios and so the relative increase in radioactivity, provided a valid means for comparison. Rat brain and lung activity, in the centrifuged fractions, were not significantly lower than the homogenate which was consistent with the *in vitro* observations. The same relationship in the rat kidney was also not surprising since the *in vitro* binding appeared exclusively in the $10^5 \times g$ supernatant which was common to all three fractions. The *in vivo* distribution in rat liver however, complemented the *in vitro* data indicating microsomal binding. In the *in vivo* studies it is certain that some metabolism would have occurred which could account for the relatively greater concentrations of [^{14}C] activity in the lung and brain homogenates. However, the time of sampling was arranged to coincide with the peak blood level [22] and it is known that metabolism of [^{14}C]nicotine in the rat is relatively slow [22].

The nature of the binding is unusual since it appears to increase with increasing pH which for nicotine means increasing proportions of the free base. This would suggest a non covalent lipid containing fraction in the kidney extract. Such binding would not be particularly strong or specific and the Scatchard plot data indicate binding sites which possess a low affinity though with high capacity. The evidence of at least one other type of other binding site is of only academic importance in relation to the nicotine concentrations likely to be achieved in tobacco smoking related experiments.

The lack of plasma protein binding at physiological pH is consistent with the relatively uniform distribution between red cells and plasma in a number of species [23].

The nature of the entities responsible for binding the nicotine in rat kidney is not known though the chromatographic studies including separation on Sephadex G-100 indicate a component with a relatively low molecular weight.

Cat and chicken kidney cortex have been reported to bind organic bases such as hexamethonium [24] and active uptake processes are involved. Yamamoto *et al.* [13], however, reported that the uptake of nicotine by rat kidney was a passive process, but was not dependent wholly on lipid solubility.

The relevance of these results to the human smoker are unknown since the nicotine bindings properties in human tissues have not been studied. The observations are clearly of importance however in relation to the disposition and pharmacodynamics of other basic chemicals the moreso in relation to the widespread use of the rat as a model for toxicological screening of drugs.

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