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Uptake of nicotine and extracellular space markers by isolated rat ganglia in relation to receptor activation

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Summary

1. Uptake of ^{3}H-nicotine by isolated rat superior cervical sympathetic (SCG) and nodose (NG) ganglia was measured *in vitro*. Depolarization of the ganglia by nicotine was measured electrically.

2. Nicotine depolarized the SCG but not the NG. The mean ED50 for depolarization was $5{\cdot}3 \times 10^{-6}$M.

3. Both ganglia accumulated nicotine when incubated in $3{\cdot}1 \times 10^{-5}$M ^{3}H-nicotine: after 30 min incubation the ratios of tissue to medium concentrations were (mean $\pm$ S.E. of mean): SCG, $3{\cdot}49 \pm 0{\cdot}13$; NG, $2{\cdot}50 \pm 0{\cdot}09$.

4. Total water contents, estimated by drying to constant weight, were: SCG, $83{\cdot}8 \pm 0{\cdot}12\%$; NG, $80{\cdot}1 \pm 0{\cdot}21\%$. Extracellular spaces, measured as ^{3}H-mannitol space, were: SCG, $38{\cdot}8 \pm 1{\cdot}3$; NG, $40{\cdot}3 \pm 0{\cdot}8\%$ wet weight. These values were not significantly altered by nicotine.

5. Correction for tissue fluid spaces indicated that the ratio of the mean intracellular fluid concentration to the extracellular fluid concentration for ^{3}H-nicotine at $3{\cdot}1 \times 10^{-5}$M were: SCG, 7·4; NG, 5·6. The ratios were not altered in any consistent manner on varying the nicotine concentration between $3{\cdot}1 \times 10^{-7}$ and $1{\cdot}6 \times 10^{-4}$M.

6. When the nicotine concentration was sufficiently great ($6{\cdot}2 \times 10^{-6}$M or more) to evoke large SCG depolarizations, hexamethonium ($2{\cdot}5 \times 10^{-3}$M) reduced ^{3}H-nicotine uptake by the SCG by up to 19% without affecting uptake by the NG, and thereby reduced the uptake difference between the two ganglia. With nicotine concentrations $< 6{\cdot}2 \times 10^{-6}$M, hexamethonium did not modify uptake by either ganglion.

7. It was concluded that nicotine may be concentrated within neurones, and that such intracellular accumulation may be augmented during depolarization induced by nicotine.

Introduction

Previous experiments have shown that radioactively labelled nicotine readily enters the neurones of the cat superior cervical ganglion after close-arterial injection *in vivo*, and that the overall concentration of nicotine in the superior cervical ganglion tends to exceed that in the adjacent nodose ganglion (Appelgren, Hansson & Schmiterlow, 1963; Brown, Hoffmann & Roth, 1969b). In view of the insensitivity

of the nodose ganglion (a sensory ganglion) to nicotine, it was suggested that the differential retention of nicotine in the two ganglia might indicate some relation between neural uptake of nicotine and neural response (Brown *et al.*, 1969b).

Further exploration of this possibility by *in vivo* experiments was hindered by the variations in ganglionic nicotine concentrations—probably reflecting differences in blood flow. For this reason, some more experiments have been attempted using isolated rat ganglia incubated in nicotine solution. The results of these experiments form the subject of the present paper. In essence they confirm the suggestion made previously that nicotine is concentrated in nerve cells, and that the degree of concentration may be increased when the membrane is depolarized by nicotine.

Some of these observations have been briefly described to the British Pharmacological Society (Brown, Halliwell & Scholfield, 1969a).

Methods

Superior cervical and nodose ganglia were isolated from rats of approximately 200 g weight, of either sex, and anaesthetized with urethane. The outer connective tissue sheath was removed and the ganglia placed in Krebs solution equilibrated with a gas mixture of 95% oxygen to 5% carbon dioxide. The pH was 7·4. All experiments were performed at room temperature (19–24° C).

Depolarization

Drug-produced depolarization of the superior cervical ganglion was recorded by the moving fluid electrode technique described previously (Brown, 1966a). Attempts to measure depolarization of the nodose ganglion were also made using this method, the ganglion potential being recorded with respect to the descending vagus nerve.

Uptake measurements

Ganglia were incubated in Krebs solution in baths of 3 or 5 ml capacity capable of holding four or eight ganglia respectively. The radioactively labelled substance to be studied was added to the bath in a volume of saline not exceeding 0·2 ml. After incubation the ganglia were removed from the bath, dipped once in saline to wash off surface radioactivity, blotted and rapidly frozen in liquid nitrogen.

The ganglia were weighed at room temperature to within 5 μg (0·5–1%) using an electro-torsion balance (R.I.I.C., model EMB-1). During weighing the ganglia thawed and gradually lost weight. The absolute weight loss determined in preliminary experiments was linear over the first 2–3 min after thawing, and was fairly constant during each experiment, regardless of the initial weight of the ganglion (except that weight losses of sympathetic ganglia were about twice those of nodose ganglia). Thus, a fairly reliable estimate of the fresh wet weight could be obtained by taking readings at 30 s intervals for the first 2 min after withdrawing the ganglion from the liquid nitrogen, and extrapolating the weights to zero time. The ganglia were then dried to constant weight by heating at 85°C for 1 h and reweighed to determine their total water content; there was no loss of radioactivity from pieces of filter paper loaded with the radioactive materials during this procedure. The mean wet and dry weights of fifty-six ganglia are shown in Table 1.

After weighing, the ganglia were disintegrated and radioactivity extracted by stirring for 1 h in 0·5 or 1 ml N KOH in a mixture of 75% methanol to 25% water

at 50–60°C. Extraction of radioactivity was fairly complete: recoveries of radioactive compounds added with unlabelled ganglia to the extraction mixture were as follows (% of added material, means ± S.E. of means): ^{3}H-nicotine, 100·5 ± 1·14% (n=4); ^{3}H-inulin, 101·2 ± 0·95% (n=4); Na_2 $^{35}SO_4$, 102·0 ± 0·70% (n=6); ^{14}C-sucrose, 101·2 ± 0·70% (n=8); ^{3}H-mannitol, 95·8 ± 1·06% (n=8). No corrections for recovery were made. Instead, aliquots of the bath fluid were diluted 1:100 in samples of extraction mixture to measure bath fluid radioactivity.

Triplicate samples (0·1 or 0·2 ml) of the ganglion extracts or of bath fluid dilutions were added to glass vials containing 20 ml scintillation fluid, and radioactivity was counted in a liquid scintillation spectrometer (Packard) to at least 10,000 counts. The scintillation fluid had the following composition: (per litre): toluene, 800 ml; methanol, 200 ml; 2,5-diphenyloxazole (PPO), 5 g; 1,4-bis-2(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl-POPOP), 0·25 g; silica powder (' Aerosil 200 ', Degussa, Frankfurt/M), 35 g. (The silica powder was added to prevent a slow decline in count rate with time experienced with some of the radioactive compounds, presumably due to surface adsorption.) Mean counting efficiency was determined by adding ^{3}H- or ^{14}C-toluene internal standards, and variations in quenching monitored by the channels ratios method. Normally efficiencies were within the ranges 16–20% (^{3}H) and 65–70% (^{14}C).

To check for the formation of metabolites of nicotine by the isolated ganglia, in some experiments the tritium in ganglia incubated in ^{3}H-nicotine solution was extracted as described above or by leaching in distilled water, ethanol or chloroform. The extract was then subjected to thin layer chromatographic separation using the technique of Hansson, Hoffmann & Schmiterlow (1964). The zonal distribution of tritium on the plates (see below) was associated with authentic nicotine in the same proportion (88 to 95%) as that of the original ^{3}H-nicotine solution carried through the same extraction and separation procedure. Thus, isolated ganglia did not appear to metabolize nicotine. This accords with previous findings using other *in vitro* nervous tissue (Hansson *et al.*, 1964), and with ganglia *in vivo* (Brown *et al.*, 1969b).

Radioactive compounds

The specifications of the radioactive compounds were as follows: ^{3}H-nicotine (Radiochemical Centre, Amersham; TRA 73, batch number 4); pure liquid, randomly labelled, stated specific activity, 259 mCi/mmol (1·6 mCi/mg); radiochemical purity >97%. ^{3}H-methoxy-inulin (New England Nuclear Corp., Boston, Mass.; NET-086-batch numbers 297-12 & 334-164); solid, randomly labelled, mol. wt., 5,000–5,500; stated specific activity, 164 and 151 mCi/g; radiochemical purity >99%. ^{14}C-D-sucrose (Radiochemical Centre; CFB 4, batch number 36); freeze-dried solid, randomly labelled; stated specific activity 10·6 mCi/mmol (30·9 μCi/

TABLE 1. *Weights of isolated rat superior cervical sympathetic and nodose ganglia incubated in Krebs solution at room temperature for periods up to 2 hours*

Ganglion	Wet weight (mg)	Dry weight (mg)	Water content (%)
Sympathetic	1·244 ± 0·022	0·199 ± 0·006	83·8 ± 0·12
Nodose	0·539 ± 0·009	0·107 ± 0·002	80·1 ± 0·21

(Mean ± S.E. of mean, n=56).

mg; radiochemical purity 98–99%). ^{3}H-(−)-D-mannitol (Radiochemical Centre, TRA 255, batch 4); supplied in aqueous solution; stated specific activity 500 mCl/mmol (2·75 mCi/mg, 1 mCi/ml); radiochemical purity>98%. Sodium ^{35}S-sulphate (Radiochemical Centre, SJS 1); supplied carrier-free in aqueous solution; stated radiochemical purity>99% (carrier sulphate was added before use to 1 mg/ml).

The compounds were dissolved in saline, unless supplied in aqueous solution, and were stored at +4°C. The radiochemical purities of the aqueous solutions were redetermined before and after use by thin layer chromatography on silica coated glass plates. Radioactivity was located by autoradiography using Kodak X-ray film, and the autoradiogram compared with the location of authentic unlabelled material on the plate. The distribution of radioactivity on the plate was measured by removing the silica, suspending it in vials containing the liquid scintillation fluid with added silica powder as described above, and counting in the liquid scintillation spectrometer. Details of solvent systems and results for individual compounds are described below.

^{3}H-Nicotine. The solvent system was that described by Hansson *et al.* (1964), namely, ethanol/acetone/benzene/conc. NH_4OH in proportions by volume of 1:8:10:1. Nicotine spots were located under ultraviolet light. The main locus of radioactivity coincided with that for authentic nicotine at R_F 0·4, and accounted for 88–95% of total radioactivity. Two very small secondary loci at R_F 0·24 and 0·19 accounted for a further 3–4% of radioactivity. As described above, this distribution was identical with that of extracts of ganglia incubated with ^{3}H-nicotine. For this reason, it was considered unnecessary to make any correction for apparent 'impurities' in the nicotine solution, or to attempt further purification. The chemical purity and biological activity of the ^{3}H-nicotine were also checked using gas-liquid chromatography and bioassay on the frog rectus abdominis preparation. The specific activity so determined was between 230 and 260 mCi/mmol, in reasonable agreement with that specified by the suppliers.

^{14}C-Sucrose. The solvent system was methyl-ethyl-ketone/acetic acid/water, in proportion 3:1:1, spots being visualized by anisaldehyde spray reagent (Stahl, 1965). This solvent system was chosen because it separated sucrose (R_F 0·33) from possible contaminants thought most likely to interfere with its use as an extracellular space marker, by entering the cells, such as its constituent hexose sugars fructose (R_F 0·42) and glucose (R_F 0·39), and other lower molecular weight sugars and sugar alcohols, which ran further ahead of the sucrose (for example, glycerol R_F 0·62). There was no evidence of any significant contamination (<1%) by such lower molecular weight compounds.

^{3}H-Mannitol. The solvent system for this compound was that used previously (Brown, Stumpf & Roth, 1969c). The results indicated that the radiochemical purity was maintained at >95% throughout the experiments.

Unlabelled compounds

The following non-radioactive compounds were used: nicotine liquid (B.D.H.), carbachol chloride (Koch-Light), hexamethonium bromide (Koch-Light), inulin (B.D.H.), D-(+)-sucrose and D-mannitol (Koch-Light). Doses refer to concentration of salt in the bath fluid.

Results

Ganglion depolarization by nicotine

In order to interpret the results of uptake studies in terms of ganglionic response, measurements of the depolarizing action of nicotine on twenty isolated rat superior cervical ganglia and three isolated nodose ganglia were obtained. Some of the special features of nicotine-induced depolarization are described elsewhere (Brown, 1966b; Brown & Scholfield, 1970). In this paper only those points which are of particular significance to the design and interpretation of the uptake studies are described.

Sensitivity. The depolarization after exposure for 4 min to varying concentrations of nicotine was measured in eight ganglia, and a mean dose-response curve constructed. The mean ED50 determined from this was $5{\cdot}3 \times 10^{-6}$M. Nicotine concentrations covering a wide range of depolarization from 'threshold' ($3{\cdot}1 \times 10^{-7}$M) to 'maximal' ($1{\cdot}6 \times 10^{-4}$M) were selected from this data for uptake studies: the average depolarizations, expressed as % of maximum, are indicated in Table 3. (The lower limit of nicotine concentration for uptake experiments was set by the specific activity of the ^{3}H-nicotine).

Duration. Maximal uptake of ^{3}H-nicotine is not attained until 30 min or more of incubation (Fig. 2). Depolarization measured over such times was maximal after about 2–4 min, and thereafter declined to a fairly steady submaximal level of between 50 and 80% of maximum. (Inset in Fig. 2.)

Antagonism. Nicotine depolarization was progressively reduced by adding increasing concentrations of hexamethonium within the range $2{\cdot}5 \times 10^{-7}$ to $2{\cdot}5 \times 10^{-3}$M. With $2{\cdot}5 \times 10^{-3}$M hexamethonium, depolarization produced by the highest concentration of nicotine used in uptake studies ($1{\cdot}6 \times 10^{-4}$M applied for 30 min) was reduced by >90%.

Selectivity. To obtain an indication of nicotine uptake by neurones in the absence of depolarization two procedures have been adopted in the present experiments—addition of hexamethonium and measurement of uptake by nodose ganglia. The use of the latter is based on the assumption that, being a sensory ganglion, the neurones are unlikely to be receptive to nicotine.

This seemed reasonable since Langley & Dickinson (1899) observed no effect of nicotine when painted on the nodose ganglion, and Brown *et al.* (1969b) found that intra-arterial injection of nicotine in doses up to $6{\cdot}2 \times 10^{-3}$M to the cat superior cervical and nodose ganglia blocked the sympathetic ganglion without modifying reflex responses to afferent vagal stimulation. On the other hand, relatively high concentrations of nicotine ($1{\cdot}9 \times 10^{-3}$M) impair conduction in rabbit vagal C-fibres *in vitro* (Armett & Ritchie, 1961); and recently Hancock & Volle (1969) have reported that very low doses of nicotine (down to 3 μmol) injected intra-arterially blocked conduction in cat vagal C-fibres recorded electrically. (The apparent discrepancy between these findings and those of Brown *et al.* (1969b) may be due to the fact that the reflex responses recorded by the latter investigators—vasodepression and respiratory inhibition—might have been elicited by stimulating myelinated vagal afferents (cf. Douglas & Schaumann, 1956; Douglas & Ritchie, 1956)).

In view of these observations, and of possible species differences, it was thought necessary to find out whether nicotine could depolarize nodose ganglion cells when

applied *in vitro* in the concentrations used in our uptake studies. This we checked by recording the potential difference between the nodose ganglion and the cut end of the ascending vagus nerve in a manner comparable to that used for recording depolarization of sympathetic ganglia. In concentrations up to $1{\cdot}6 \times 10^{-4}$M nicotine did not affect the nodose ganglion potential. Such negative results could occur if the reference electrode led from undamaged nerve, and nicotine produced an equal and simultaneous depolarization of both ganglion cells and afferent fibres: this, however, seems unlikely, especially since K^+ ions (150 mM) produced large changes in potential difference of up to 10 mV. We conclude that nicotine had no depolarizing action on nodose ganglion cells *in vitro* at these concentrations.

Fluid spaces in ganglia

Calculation of intracellular nicotine concentrations from uptake measurements requires some knowledge of the relative intra- and extra-cellular fluid volumes. These were estimated from the total water content (see Table 1) and the uptake of labelled extracellular space markers—^{3}H-inulin, ^{14}C-sucrose, ^{3}H-mannitol and Na_2 $^{35}SO_4$.

Uptake kinetics for these marker substances are shown in Fig. 1. There was a rapid phase of tracer uptake which was largely completed within 15–30 min (more rapidly with sulphate than with inulin), and which probably corresponded to uptake into extra-cellular space. Thereafter, there appeared to be a further slow uptake at a rate of 0·5–3% of wet weight/hour. During the entire incubation period the total water content remained constant at about 84% of total weight.

It is also apparent from Fig. 1 that the volume of distribution of the markers varied quite appreciably. Thus, after incubation for 30 min, the respective volumes (mean ± S.E. of mean) were: ^{3}H-inulin, 32·0 ± 0·57% by weight (38·1 ± 0·78% of total fluid volume); ^{14}C-sucrose, 39·1 ± 1·37% (46·6 ± 1·83%); ^{3}H-mannitol, 38·8 ± 1·27% (46·2 ± 1·42%); ^{35}S-sulphate, 44·0 ± 0·46% (52·7 ± 0·57%). This order of increasing volume of distribution is that of decreasing molecular weight: inulin (mol. wt. 5,000–5,500)<sucrose (360)≈mannitol (180) <sulphate (96).

An inverse relationship between the distribution volume and molecular dimensions of 'extracellular' markers has been reported for muscle (Law & Phelps, 1966; Goodford & Leach, 1966): therein, this has been interpreted in terms of steric hindrance by extracellular mucopolysaccharides. An additional factor may be some degree of intracellular penetration, evidence for which has been derived from autoradiographic studies (Nicholls & Wolfe, 1967; Brown *et al.* 1969c). Present data is insufficient to allow firm conclusions concerning this point, but the slow increase in volume of distribution might be due to slow intracellular penetration.

The differing volumes of distribution of the four markers raises practical problems concerning the choice of marker. In our investigation the principal requirement was for a marker which would delineate the extracellular space available to nicotine. Of those used, mannitol was considered the most reasonable since its molecular weight most closely approximated to that of nicotine, and subsequent calculations of intra- and extra-cellular nicotine concentrations are based on the mannitol space.

Effect of nicotine upon fluid spaces. A further point of importance to the interpretation of nicotine uptake measurements was whether nicotine depolarization might modify the fluid spaces, by (for instance) cell swelling. To test this, pairs of

ganglia were incubated for 60 min in Krebs solution containing the approximate marker (to obtain full equilibration) and then for a further 30 min with or without the addition of $3{\cdot}1 \times 10^{-5}$M unlabelled nicotine. The total water content and volume of distribution of the marker were then compared in the control and nicotine treated ganglia. In these tests two markers were used—^{3}H-inulin or ^{3}H-mannitol—since there seemed a possibility that nicotine might increase membrane permeability to

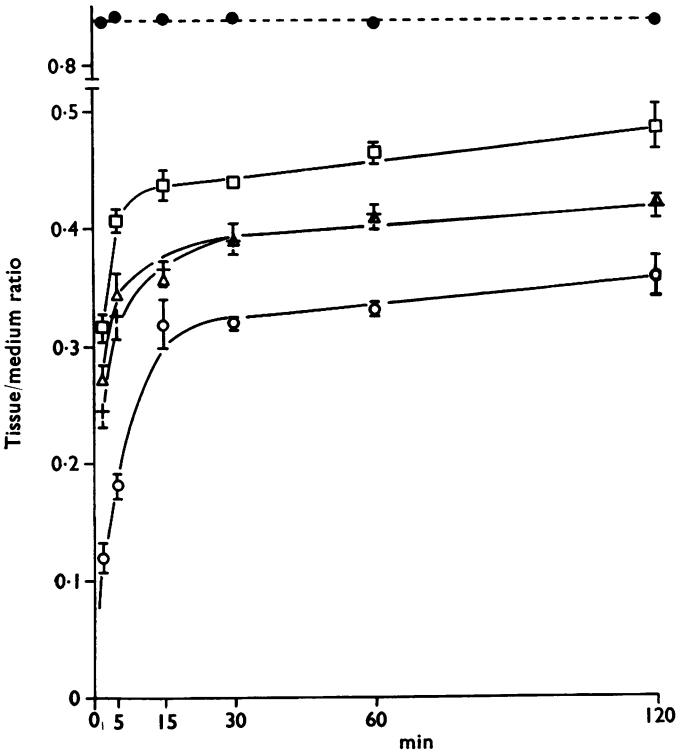

FIG. 1. Uptake of ^{3}H-inulin (○), ^{14}C-sucrose (+), ^{3}H-mannitol (△), and ^{35}S-sulphate (□) by isolated rat superior cervical ganglia. Ordinates, tissue/medium ratio ((d.p.m./mg ganglion, wet weight)/(d.p.m./μl bath fluid)). Abscissa, time after adding tracer. Each point gives the means of four–six determinations: bars show standard errors of means. Filled circles (●) indicate the mean fractional water content of the ganglia, measured by drying to constant weight.

TABLE 2. *Effect of incubation for* 30 *min in* $3{\cdot}1 \times 10^{-5}$M *nicotine solution on the inulin and mannitol spaces and total water contents of isolated rat superior cervical sympathetic and nodose ganglia, expressed as % wet weight ((d.p.m./mg ganglion)/(d.p.m./μl bath fluid)) ×100%*

Ganglion	Solution	Inulin (n=10)	Mannitol (n=8)	Water (n=24)
Sympathetic	Control	36·8±0·61	42·8±0·44	83·5±0·20
	Nicotine	37·5±0·90	43·3±0·56	83·5±0·15
	Control / Nicotine	1·025± 0·025	1·012± 0·018	1·000± 0·007
Nodose	Control	38·3±0·67	43·2±0·81	80·1±0·24
	Nicotine	37·2±0·95	45·2±0·99	79·9±0·25
	Control / Nicotine	0·975± 0·030	1·045± 0·024	0·997± 0·003

Mean±S.E. of mean.

low molecular weight compounds, in a manner comparable to its presumed effect on membrane permeability to small ions: if so, then mannitol space might show a greater increase than inulin space.

In practice, nicotine did not alter either inulin or mannitol space, nor did it modify total water content (Table 2). Thus, the cell volume was unaffected by nicotine: hence changes in nicotine uptake during depolarization (as described below) could be ascribed to true changes in intracellular concentration.

Nicotine uptake

The time course for the uptake of ^{3}H-nicotine by isolated ganglia incubated in $3{\cdot}1 \times 10^{-5}$M nicotine solution is shown in Fig. 2. The general form of the curve resembles that for the uptake of mannitol, with an initial rapid uptake phase essentially completed within 30 min, and a subsequent slow linear uptake to at least 2 hours. Measurement of nicotine uptake at short incubation times was subject to considerable inaccuracy, so compartmental analysis of uptake kinetics have not been attempted. However, certain general points can be made. First, when expressed as a fraction of the 30 min uptake value, the initial rate of nicotine uptake was rather slower than that of mannitol. On the other hand, in terms of total tissue uptake, nicotine uptake was much more rapid than that of mannitol: a ratio of tissue to medium concentrations equivalent to the 30 min mannitol space (0·38) was achieved within 1 min, and by 30 min the ratios of tissue to medium concentrations had reached 3·5 in the superior cervical ganglion and 2·5 in the nodose ganglion. This suggests first that considerable penetration and concentrative accumulation of nicotine in the intracellular compartments of the tissues occurred—in accordance with previous autoradiographic observations (Brown *et al.*, 1969b);

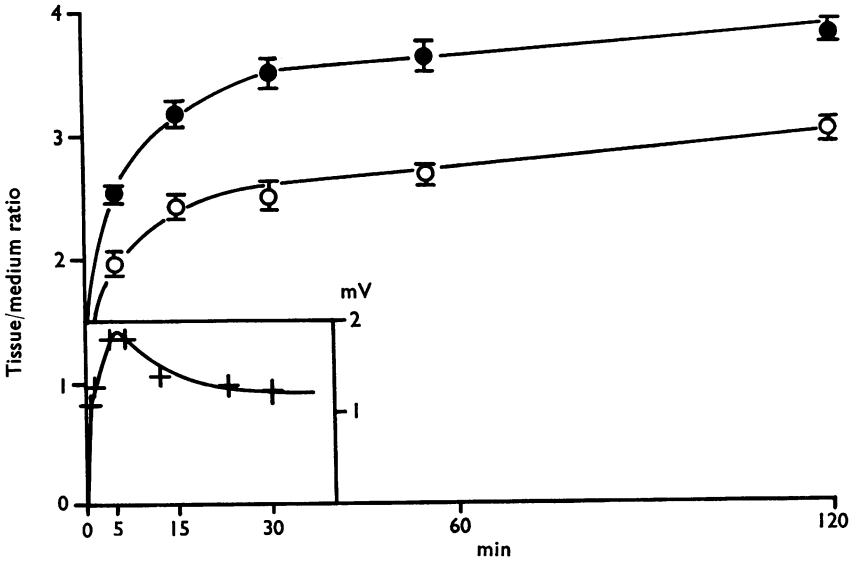

FIG. 2. Uptake of ^{3}H-nicotine ($3{\cdot}1 \times 10^{-5}$M, 8 μCi/ml) by isolated rat superior cervical sympathetic (●) and nodose (afferent vagal) (○) ganglia. Ordinate, tissue/medium ratio ((d.p.m./mg ganglion)/(d.p.m./μl bath fluid)). Abscissa, time after adding nicotine (min). Each point gives the mean of three–five determinations: bars show standard errors of means. Inset: (+) time-course of a sympathetic ganglion depolarization (measured as mV increase in the potential difference between the ganglion surface and the cut end of the postganglionic nerve trunk).

and second, that such intracellular penetration was extremely rapid. On the other hand, the time course for nicotine uptake was slow compared with that of ganglion depolarization, the latter being maximal within 5 minutes.

The greater uptake of nicotine into the sympathetic ganglion apparent from Fig. 2 accords with previous *in vivo* observations (Brown *et al.*, 1969b): then, it was surmized that it might be related to the selective response of sympathetic ganglion cells to nicotine. If so, previous addition of hexamethonium to the bathing fluid in a concentration sufficient to block the depolarization should result in a selective reduction of nicotine uptake by the sympathetic ganglion, and a consequent diminution of the difference in uptake between the two ganglia. Further, any such effect of hexamethonium would only be apparent if the initial concentration of nicotine were sufficient to produce depolarization—that is, reduction by hexamethonium would vary with the nicotine concentration.

To test these predictions, a series of experiments was undertaken, using a constant incubation time in ^{3}H-nicotine of 30 min, in which contralateral pairs of ganglia from the same rat were incubated simultaneously in two 3 ml baths. To one of the baths ^{3}H-nicotine alone was added, while to the other ^{3}H-nicotine was added 15 min after addition of $2{\cdot}4 \times 10^{-3}$M hexamethonium (previously noted to reduce the nicotine depolarization by >90%). The uptake of nicotine by the two pairs of ganglia was then compared: preliminary experiments showed that there was rather less variation in weight and uptake between ganglia from the same rat than from different rats. Five groups of such experiments were performed, using five different nicotine concentrations, ranging from $3{\cdot}1 \times 10^{-7}$ to $1{\cdot}6 \times 10^{-4}$M. (The experimental design was such as to provide a rather better analysis of the effect of hexamethonium at each dose than of the effect of nicotine dose on total uptake). Using the data for total water space and mannitol space obtained previously, the results were expressed in terms of the ratio of the overall concentration in intracellular fluid to the concentration in the bath (extracellular) fluid. They are summarized in Table 3.

TABLE 3. *Intracellular nicotine concentrations in isolated rat superior cervical (S) and nodose (N) ganglia after incubation for* 30 *min in* ^{3}*H-nicotine solution: in the absence (column* 1, *control) or in the presence (column* 2, *+C6) of* $2{\cdot}5 \times 10^{-3}$ M *hexamethonium. Column* 3: *% change of uptake produced by hexamethonium*

Nicotine concentration (M)	*n*	Mean S ganglion depolarization (% maximum)	Ganglion	Ratios of intracellular concentrations to extracellular concentrations of nicotine (mean±S.E. of mean) Control (1)	+C6 (2)	% change (3)
$3{\cdot}1 \times 10^{-7}$	10	<4%	S	7·59±0·22	7·27±0·23	−2·94±2·15
			N	6·44±0·30	5·96±0·22	−5·66±4·18
			S/N	1·19±0·04	1·24±0·03	
$1{\cdot}2 \times 10^{-6}$	6	11%	S	7·62±0·33	7·13±0·47	−6·45±4·48
			N	6·25±0·54	5·84±0·56	−5·29±5·87
			S/N	1·23±0·04	1·22±0·04	
$6{\cdot}2 \times 10^{-6}$	6	52%	S	8·58±0·46	7·58±0·21	−11·02±2·68*
			N	6·85±0·50	7·10±0·41	+5·95±6·50
			S/N	1·27±0·07	1·08±0·06	
$3{\cdot}1 \times 10^{-5}$	8	81%	S	7·80±0·22	6·59±0·20	−15·17±1·05*
			N	6·00±0·16	6·12±0·13	+1·26±4·36
			S/N	1·30±0·04	1·09±0·03	
$1{\cdot}6 \times 10^{-4}$	6	90%	S	7·02±0·06	5·68±0·19	−19·16±2·70*
			N	5·60±0·13	5·59±0·16	−0·43±4·10
			S/N	1·26±0·03	1·01±0·02	

* Significant reduction. ($P<0{\cdot}05$ by Student's *t*-test).

From this table the following points emerge: (1) The calculated mean intracellular concentration was up to eight times that in the extracellular fluid, and, under similar conditions, did not vary in any consistent or marked way with the concentration of nicotine. (2) In the absence of hexamethonium (column 1 in Table 3) the nicotine concentration in the sympathetic ganglion cells was between 19 and 30% greater than that in the nodose ganglion (that is the concentration ratios were 1·19 to 1·30). (3) When the concentration of nicotine in the bath was $6{\cdot}2 \times 10^{-6}$M or more, hexamethonium significantly reduced the uptake of nicotine by the sympathetic ganglion (by up to 19%), but did not affect uptake into the nodose ganglion (column 3). As a result, the concentration difference between the two ganglia was substantially reduced by hexamethonium, and, at $1{\cdot}6 \times 10^{-4}$M nicotine, was abolished (column 2, S/N). (4) On the other hand, at low concentrations of nicotine ($<6{\cdot}2 \times 10^{-6}$M) the uptake of nicotine and the ratios of the concentrations in sympathetic ganglia to those in nodose ganglia were not significantly affected by hexamethonium.

In so far that these results fulfil the predicted effects of hexamethonium, they indicate that the uptake of nicotine by the superior cervical ganglion cells is in-

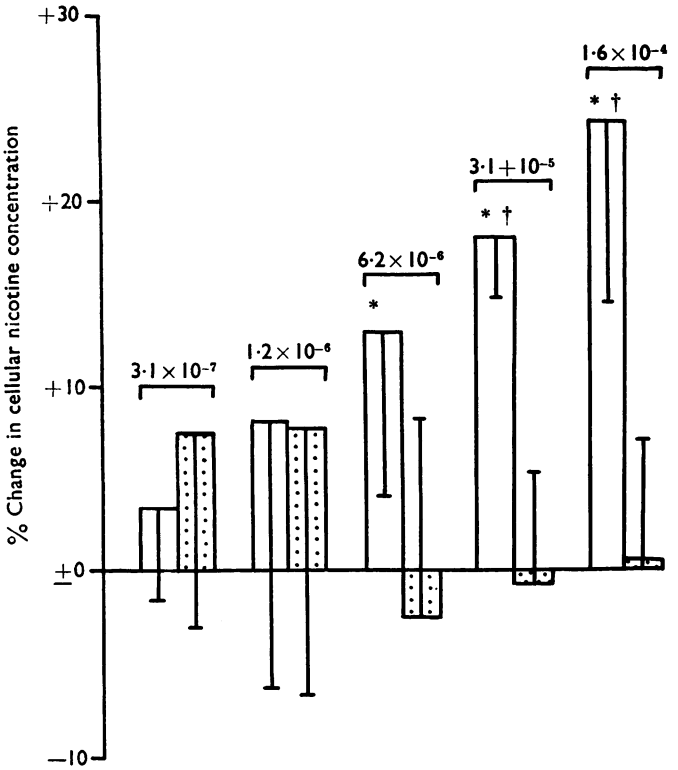

FIG. 3. Percentage change of intracellular nicotine concentrations in isolated superior cervical ganglia (clear blocks) and nodose ganglia (stippled blocks) produced by omitting hexamethonium from the bathing fluid—that is, by permitting receptor activation. Each column shows the mean change in nicotine uptake, calculated from the uptake difference between paired ganglia incubated for 30 min in ^{3}H-nicotine in the presence and absence of $2{\cdot}4 \times 10^{-3}$M hexamethonium. Figures above the columns indicate the total concentration of nicotine (M) in the incubation fluid. Vertical bars show 95% confidence limits of means (n=six–ten pairs for each column). * Significant increase of intracellular nicotine uptake on omitting hexamethonium. † Significant difference between effects of hexamethonium-omission on uptake by sympathetic and nodose ganglia. ($P<0{\cdot}02$ by Student's t-test.)

creased during nicotine depolarization. The magnitude of this increased intracellular uptake, calculated from the extra uptake observed on omitting hexamethonium from the bath fluid, is shown diagrammatically in Fig. 3: with the highest concentration of nicotine used ($1{\cdot}6 \times 10^{-4}$M), it amounted to a 24% increase in mean intracellular concentration.

Discussion

The results of this investigation indicate that nicotine is concentrated in ganglion cells to a level far exceeding that in the extracellular fluid ; that the concentration in sympathetic ganglion cells exceeded that in nodose ganglion cells ; and that this difference reflects in part an increased uptake of nicotine into sympathetic ganglion cells during nicotine-induced depolarization.

The evidence that nicotine uptake is enhanced when sympathetic neurones are depolarized by nicotine rests on the observations that hexamethonium reduced nicotine uptake by the sympathetic ganglion but not by the nodose ganglion ; and that the effect of hexamethonium increased with increasing concentrations of nicotine, correlating with increasing levels of depolarization.

On the other hand, total uptake of nicotine by the sympathetic ganglion did not show a progressive increase with increasing concentrations of nicotine. This appears to be due to the fact that the hexamethonium sensitive fraction of uptake is a minor component of total uptake (about 20%), and that the increased hexamethonium sensitive uptake was offset by a relatively small decrease in the much larger hexamethonium insensitive uptake fraction as the concentration of nicotine was raised above $6{\cdot}2 \times 10^{-6}$M. Such apparent changes in total uptake with nicotine dosage might have been an experimental artifact, in the sense that the experimental design was orientated toward detection of hexamethonium sensitivity, rather than to providing an accurate comparison of the effects of varying the nicotine dosage. However, there is no reason as yet for assuming that both hexamethonium-sensitive and insensitive uptake involve a common mechanism (see below), and hence for excluding independent and contrary variations in the two. Irrespective of total uptake, the effect of hexamethonium indicates that, at any given dose of nicotine, the uptake of nicotine is augmented during concurrent depolarization and by an amount approximately proportionate to the degree of depolarization.

One cause of an apparent increase in nicotine uptake during depolarization might be cell swelling, leading to an increased intracellular volume. Thus, with a ratio of intracellular nicotine concentration to extracellular nicotine concentration in the non-depolarized state of around 6, a 25% increase in intracellular volume could account for these observations without postulating any change in intracellular concentration. However, this is not so, for nicotine did not modify either total water content or extracellular to intracellular fluid distribution as measured by inulin or mannitol space.

Consequently, the enhanced uptake of nicotine during depolarization may be attributed to a true increase in intracellular concentration, of about 25% overall with the highest nicotine concentration used ($1{\cdot}6 \times 10^{-4}$M). It seems reasonable to assume that this concentration increase is limited to the cells on which nicotine directly exerts its depolarizing action—the neurone soma and its attendant dendrites —and does not occur in fibres and glial cells. The quantitative contribution of

these various structures to total intracellular volume is difficult to determine. In the cat superior cervical ganglion neurone somata may occupy only about 4% of the total volume of the ganglion (Brown *et al.*, 1969c). Assuming the dendrites to occupy the same volume, and also assuming that the rat is not very different from the cat in this respect, it would appear that the volume of the nicotine responsive components is less than 10% of the total volume, that is, <25% of the total intracellular compartment. If so, then the increased nicotine uptake into the somata and dendrites would be very much greater than the overall figure of 24%—perhaps up to 100%. Thus, the effect of depolarization on nicotine uptake may be not inconsiderable.

The view has been taken that the high tissue concentrations of nicotine are due to intracellular accumulation rather than to surface 'binding' on the basis of arguments presented previously (Brown *et al.*, 1969b)—namely, that uptake does not appear to be dose saturable, that intracellular nicotine can be detected autoradiographically (Appelgren *et al.*, 1963 ; Brown *et al.*, 1969b), and that the amount of nicotine accumulated is too great to be explained by binding. It could be conjectured that the 'resting' (that is hexamethonium-insensitive) uptake might be intracellular, but that the hexamethonium-sensitive fraction might represent 'receptor-bound' nicotine. However, even this fraction is quantitatively too great for such a conclusion, amounting (at a bath concentration of $1{\cdot}6 \times 10^{-4}$M) to ~300 nmol/ganglion. Assuming $\sim 10^4$ neurones/ganglion at an average diameter of 20 μm, the total somatic surface area is $\sim 1{\cdot}3 \times 10^{13}$ Å^2—with dendrites, perhaps 10^{14} Å^2, to accommodate around 6×10^{13} molecules of nicotine.

Thus, our general conclusion from these experiments is that there exists a relatively rapid concentrating process for nicotine in cells of the sympathetic ganglion, resulting in a substantial intracellular-extracellular concentration gradient ; and that this uptake process has essentially two components: (*a*) a component observed in the absence of depolarization by nicotine which leads to a mean intracellular concentration about six times that in the extracellular fluid, and which may be termed the 'resting uptake' ; and (*b*) an additional uptake linked in some manner with the effect of nicotine upon the receptors, serving to increase the overall intracellular concentration by a further 25% (and perhaps by considerably more in the responsive cells themselves), which may be designated 'activation uptake'. Neither concentrating process appears to be active, in so far that they were not clearly dose saturable within the range of nicotine concentrations used ($3{\cdot}1 \times 10^{-7}$ to $1{\cdot}6 \times 10^{-4}$M). Beyond that our experiments do not provide any clear lead concerning the mechanism of either resting or activation uptake, but some speculation may be in order.

One hypothesis might be that nicotine, being largely protonated at pH 7·4 (about 80% ionized at 25° C: Barlow & Hamilton, 1962), distributes across the cell membrane in accordance with the transmembrane potential, in a manner comparable to that of K^+ ions. This type of hypothesis has been advanced to explain the concentrative accumulation of carbachol by rat cerebral cortical slices (Creese & Taylor, 1967) and of decamethonium in skeletal muscle fibres (Creese & England, 1970). The resting membrane potential of mammalian sympathetic neurones is about −65 to −70 mV (Pascoe, 1955 ; Eccles 1955 ; Woodward, Bianchi, & Erulkar, 1969). According to the Nernst equation this would result in an equilibrium concentration gradient of protonated nicotine of about 20 to 1. Even allowing for the presence

of cellular elements with lesser transmembrane potentials (for example, connective tissue cells, the membrane potential of satellite cells is probably high: cf. Blackman & Purves, 1969), this would seem adequate to account for the experimentally observed gradients of 6:1 to 8:1. However, such a hypothesis does present distinct problems. First, it seems unlikely that the membrane is highly permeable to protonated nicotine (Weiss, 1966). Creese & Taylor (1967) interpreted the cellular uptake of carbachol as a carrier mediated process, susceptible to apparently competitive inhibition by tubocurarine. No kinetic studies on nicotine uptake appropriate to such transport mechanisms have been so far undertaken, but the available evidence does not suggest such a phenomenon (witness the absence of any clear dose saturation of uptake). However, there may really be no need for a carrier mechanism for nicotine permeation, for nicotine might penetrate the cell membrane as the more lipoid-soluble free base (Weiss, 1966). This could explain the relatively rapid accumulation, and the final concentration gradient may then be determined by the electrochemical gradient for the protonated cation.

A second, and rather more serious objection to this hypothesis is that depolarization of the neurones should reduce the cellular uptake of nicotine, whereas our experiments indicate an increased uptake during depolarization. A more crucial test might be to measure nicotine uptake in ganglia depolarized by K^+ ion. This reduces uptake of carbachol by cerebral cortical slices (Creese & Taylor, 1967) or of decamethonium by striated muscle (Creese & England, 1970). However, preliminary experiments (J. V. Halliwell, unpublished observations) suggest that substitution of NaCl by K_2SO_4 does not materially reduce nicotine uptake into ganglia. Hence the electrochemical gradient hypothesis is probably untenable in this context.

An alternative hypothesis might be that nicotine is distributed across the cell membrane in accordance with the relative pH of the intra- and extracellular fluid, as suggested by Weiss (1968) in respect of nicotine distribution in skeletal muscle. This presupposes that the cell membrane is permeable to the free base and essentially impermeable to protonated nicotine, so that the final distribution ratio depends upon the degrees of ionization in the two phases. Preferential concentration of nicotine within the cell would occur if the intracellular pH were lower than that of the surrounding fluid (a not unreasonable suggestion: cf. Waddell & Bates, 1969). Augmentation of the nicotine gradient during receptor activation would then require an increase in the pH gradient. Experiments are in progress to test these hypotheses.

Irrespective of the mechanism of uptake, the high intracellular concentrations of nicotine may have some pharmacological consequences. For example, one might envisage that it could form a 'reservoir' of surplus nicotine, sufficiently slowly removed to maintain extracellular concentrations on washing out extracellular nicotine and thereby delaying recovery. Such a process may partly explain the persistent residual depolarizing effect of nicotine on isolated ganglia following its removal from the bath fluid (Brown & Scholfield, 1970). It may also have some bearing on the pronounced tachyphylaxis to nicotine.

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