J. Physiol. (1979), **297**, *pp.* 597–620
With 7 text-figures
Printed in Great Britain

INTRACELLULAR pH AND THE DISTRIBUTION OF WEAK ACIDS AND BASES IN ISOLATED RAT SUPERIOR CERVICAL GANGLIA

BY D. A. BROWN AND J. GARTHWAITE*

From the Department of Pharmacology, School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX

(*Received 11 August 1978*)

SUMMARY

1. The steady-state intracellular/extracellular concentration ratios (C_i/C_o) of a number of radiolabelled weak bases in isolated rat superior cervical ganglia were measured.

2. Observed values for C_i/C_o (mean ± S.E. of mean) were [^{3}H]nicotine, 6·17 ± 0·12; [^{14}C]morphine, 6·08 ± 0·14; [^{3}H]atropine, 7·10 ± 0·16; [^{14}C]trimethylamine, 6·73 ± 0·13; [^{14}C]procaine, 10·13 ± 0·26. If C_i/C_o were determined by the transmembrane pH gradient, the intracellular pH (pH_i) appropriate to these concentration gradients lay between 6·4 and 6·6 at an extracellular pH (pH_o) of 7·4.

3. The steady-state value of C_i/C_o for the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) was 0·87 ± 0·007. The appropriate pH_i was 7·31 ± 0·003.

4. The difference between the values of pH_i calculated from the distribution of the weak bases and of DMO could not be attributed to (i) experimental error, (ii) partial permeation of protonated base, (iii) intracellular binding or carrier-mediated transport of base, (iv) lipid uptake of base or (v) different pK'_a inside and outside cells.

5. The difference between the measurements of pH_i made with DMO and nicotine (pH_{DMO}-pH_{nic}) was reduced or abolished by uncoupling agents, which act as transmembrane proton carriers. This effect was not reproduced by respiratory inhibitors or by exposure to lactate.

6. pH_{DMO}-pH_{nic} was small (< 0·1 units) in human erythrocytes, which contain no intracellular organelles, and was exaggerated (1·0 unit) in slices of lipid-depleted brown adipose tissue which contained an abundance of mitochondria.

7. It is concluded that the different values of pH_i determined using weak acids and bases arise from the presence of membrane-bound intracellular compartments of differing pH, and that where the use of pH-sensitive micro-electrodes is impracticable, it is desirable to measure pH_i with both a weak acid and a weak base unless these can be shown equal over a wide range of pH_i values.

INTRODUCTION

Where direct measurement with pH-sensitive electrodes is impracticable, intracellular pH may be estimated from the steady-state distribution of weak acids or

* Present address: M.R.C. Developmental Neurobiology Unit, Institute of Neurology 33 John's Mews, London WC1N 2NS.

0022-3751/79/6220-0680 $01.50

bases between the intracellular and extracellular fluids. The indicator usually used is the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) introduced by Waddell & Butler (1959).

In previous experiments on isolated sympathetic ganglia (Brown & Halliwell, 1972), the DMO method indicated an intracellular pH (pH_i) of 7·3 at an extracellular pH (pH_o) of 7·4. The weak base nicotine, however, distributed as though the effective pH_i was 6·6. It was suggested that the concentration gradients for DMO and nicotine might both be compatible with a pH-dependent distribution if the cell interior was not homogeneous with respect to pH. In this case, DMO would selectively concentrate in the more alkaline compartments, whereas nicotine would concentrate in the more acidic compartments, the respective pH_i values being biassed accordingly (see Caldwell, 1956; Robson, Bone & Lambie, 1968). Adler (1972) proposed the same interpretation of differences in pH_i yielded using nicotine and DMO in rat muscle.

In the present experiments, we have subjected this hypothesis to some further experimental tests. First, the distribution of several other weak bases in sympathetic ganglia has been measured in order to eliminate influences peculiar to the distribution of nicotine. Secondly, on the assumption that some of the heterogeneity of intracellular pH might reside in membrane-bound intracellular organelles (Robson *et al.* 1968), the pH_i values registered using DMO and nicotine (pH_{DMO} and pH_{nic}) were compared (*a*) in sympathetic ganglia under conditions where pH gradients across intracellular membranes should be reduced and (*b*) in other cells with different degrees of subcellular anatomical compartmentation. For the latter experiments we used human erythrocytes, devoid of major intracellular organelles, and brown adipose tissue depleted of lipid, in which mitochondria occupy an unusually large fraction (30%) of the total intracellular volume (Lindgren & Barnard, 1972).

METHODS

In principle, a weak acid (ionizing as $AH \rightleftharpoons H^+ + A$) or base ($B + H^+ \rightleftharpoons BH^+$) which permeates a membrane only in its unionized form will reach equilibrium when $[AH]_i = [AH]_o$ or $[B]_i = [B]_o$. (Subscripts i and o refer to internal and external media.) If pH_i does not equal pH_o, $[A^-]_i \neq [A^-]_o$ and $[BH^+]_i \neq [BH^+]_o$, and so the total internal concentration (C_i) of acid ($C_i = [A^-]_i + [AH_i]$) or base ($C_i = [BH^+]_i + [B]_i$) will then differ from that outside (C_o). pH_i can be calculated from C_i/C_o (measured from the total tissue uptake after correcting for the extracellular and total water spaces), pH_o and the pK'_a of the compound, according to the equations (Waddell & Butler, 1959; Irvine, Saunders, Milne & Crawford, 1960) for an acid:

$$pH_i = pK'_a + \log\left(\frac{C_i}{C_o}(1 + 10^{(pH_o - pK'_a)}) - 1\right) \quad (1)$$

and for the base:

$$pH_i = pK'_a - \log\left(\frac{C_i}{C_o}(1 + 10^{(pK'_a - pH_o)}) - 1\right). \quad (2)$$

Experimental procedures

1. *Sympathetic ganglia*

Superior cervical ganglia (usually twelve to sixteen per experiment) were dissected from male Wistar rats (180–260 g body weight) anaesthetized with urethane (1·5 g/kg I.P.). The connective tissue sheaths were removed and the ganglia placed into Krebs solution continuously bubbled

with a gas mixture of 95% O_2, 5% CO_2 at room temperature (18–25 °C). After 1–2 hr under these conditions ganglia were incubated in small Perspex baths (1–5 ml. capacity) containing Krebs solution, radioactively labelled weak acids or bases and/or an extracellular space marker. The incubation temperature was 25 °C unless otherwise stated. The Krebs solution contained (m-equiv. $l.^{-1}$): Na^+ (143), K^+ (5·9), Mg^{2+} (1·2), Ca^{2+} (2·5), Cl^- (128), HCO_3^- (25), SO_4^{2-} (1·2), $H_2PO_4^-$ (1·2) and glucose (11). The pH of the incubation medium (about 7·4) was monitored continuously where possible but otherwise at the end of each incubation, using a conventional pH electrode (Activion) coupled to an E.I.L. Vibret pH meter. In some experiments a HEPES-buffered Krebs solution, in which 20 mM-HEPES was substituted for $NaHCO_3$, was used. The pH was adjusted as required using NaOH, and Na isethionate was added to give a total of 143 m-equiv $l.^{-1}$ Na^+.

The experimental routine for measuring uptake of labelled markers was essentially as previously described (Brown, Halliwell & Scholfield, 1971; Brown & Halliwell, 1972) except that in preparation for scintillation counting, the tissue was dissolved in 'Soluene' (Packard) and 'Instagel' (Packard) was used as scintillant. When the uptake of [^{14}C]trimethylamine was measured, however, some modification was necessary as the free base form of trimethylamine is volatile (b.p. 3 °C) and, although there was no detectable loss of radioactivity during incubation, the use of 'Soluene' (a quaternary ammonium base in which the free base form of trimethylamine would predominate) to dissolve the tissue resulted in a substantial loss and so a falsely low uptake of this compound was calculated. (These spurious initial results were included in a published abstract: Garthwaite, 1976). [^{14}C]trimethylamine was subsequently extracted by immersing the ganglia in distilled water, freezing and thawing, and leaching overnight at 4 °C. There was no loss of radioactivity during this procedure and, after dissolving the leached tissue in nitric acid, there was no measurable radioactivity remaining.

Extracellular space. The volume of distribution of different markers for the extracellular space of ganglia and other tissues has been shown to be inversely related to their molecular weights. Thus, in the ganglion, after 30 min incubation, the measured fractional extracellular space ranged from 0·32 ml. g^{-1} using inulin (molecular weight 5000–5500) to 0·44 ml. g^{-1} using sulphate (molecular weight 96) (Brown *et al.* 1971). Since the molecular weights of the compounds to be tested were in general between 100 and 300, mannitol (182) or sucrose (342) were considered most suitable for delineating the extracellular space available to the pH indicators. Some initial experiments, however, showed the volume of distribution of [^{3}H]mannitol to be larger and more variable that that of [^{14}C]mannitol such that the use of the former might produce significant errors in calculating intracellular concentrations. This discrepancy was apparently caused by a transfer of tritium to other species (Garthwaite, 1977). A similar, though less marked, deviation was found between the ^{3}H- and ^{14}C-labelled sucrose spaces. [^{14}C]mannitol was considered a suitable tracer for use with the tritiated compounds since it showed a low 'second phase' uptake (Fig. 1*A*) and no significant metabolism (Garthwaite, 1977). For ^{14}C-labelled weak bases and phenobarbitone mean [^{14}C]mannitol spaces (data from Fig. 1*A*) were used. This is unlikely to produce significant errors since in practice the [^{14}C]mannitol space varies little between different ganglia under normal conditions: in a group of eight ganglia incubated for 30 min with [^{3}H]nicotine, there was no significant difference between the ratio C_i/C_o calculated using the simultaneously measured [^{14}C]mannitol spaces ($C_i/C_o = 6{\cdot}13 \pm 0{\cdot}13$; mean ± S.E. of mean) and the mean 30 min space given in Fig. 1*A* ($C_i/C_o = 6{\cdot}21 \pm 0{\cdot}20$), or between the values of pH_i calculated therefrom. [^{3}H]inulin was used with [^{14}C]DMO; if sulphate was used instead, an error of only 0·01 units in the normal DMO-derived pH_i, would result. For calculating the distribution of [^{14}C]trimethylamine (molecular weight 59), the sulphate spaces, taken from the data of Brown *et al.* (1971), were used as being more representative of the extracellular space available to small molecules or ions (Brown *et al.* 1971; Brown & Scholfield, 1974*a*).

Compounds. To enable rapid screening, the weak acids and bases were chosen from those available as radioactively labelled derivatives. Those tested were the weak bases, [^{3}H]nicotine, [^{14}C]morphine, [^{3}H]atropine, [^{14}C]procaine and [^{14}C]trimethylamine, and the acids [^{14}C]DMO and [^{14}C]phenobarbitone.

Metabolism. Metabolism of the compounds by ganglia was assessed by thin layer chromatography. The solvents chosen were usually not those recommended by the suppliers since these on occasion did not allow sufficient separation from likely contaminants or breakdown products. It has previously been established that neither nicotine nor DMO are significantly metabolized

in isolated ganglia (Brown *et al.* 1971; Brown & Halliwell, 1972). To check for metabolism of the other compounds, ganglia (usually four) were incubated for 1 or 2 hr at 25 °C in the presence of the test compound. The ganglia were then pooled and homogenized in distilled water at 0 °C. As controls, an equal number of unincubated ganglia (the contralateral ganglia of the tests) was homogenized as above and the labelled compound was added to the homogenate. Duplicate aliquots of these homogenates, bath fluid samples, labelled and unlabelled standards were then analysed using thin layer chromatography (Table 1). In extracts of ganglia incubated in the

TABLE 1. Metabolism of weak acids and bases by ganglia

Compound	Incubation time (hr)	Concentration (μM)	Solvent*	Purity (%)	Metabolism (%)
[^{14}C]morphine	—	—	1	95†	—
	2	44	2	95†	2
[^{3}H]atropine	2	360	3	94	2
	—	—	4	95	—
[^{14}C]procaine	1	400	5	97	16
	1‡	400	5	95	2
[^{14}C]trimethylamine	1	500	6	97	2
[^{14}C]phenobarbitone	1	200	7	96	2
	1	200	8	98	2
[^{3}H]nicotine	—	—	9	96	—

* The solvents were: 1, methanol; 2, chloroform (40), methanol (30), *n*-butanol (30), 5 N ammonia (4); 3, chloroform (75), methanol (20), acetic acid (5); 4, chloroform (90), diethylamine (10); 5, methanol (99), ammonia (1); 6, ether (40), methanol (40), HCl (4), H_2O(12); 7, chloroform (90), acetone (10); 8, isopropanol (45), chloroform (45), ammonia (10); 9, benzene (50), acetone (40), ethanol (5), ammonia (5). Adsorbents were silica gel except with [^{14}C]trimethylamine when cellulose was used. Locating agents for the cold, authentic, compounds were taken from Stahl (1965) or Smith (1969). Labelled spots were located using X-ray plates.

† The purity of [^{14}C]morphine depended on the length of time that the samples had been exposed to light (fluorescent laboratory lighting) before and following application to the t.l.c. plates. Experiments in which ganglion extracts and bath fluid samples were subjected to measured exposures to light showed an exponential decline in purity. Values shown were derived by extrapolating back to zero time. There was no apparent breakdown during incubation.

‡ Ganglia were pre-incubated for 1 hr in the presence of eserine (30 μM) before incubation with [^{14}C]procaine + eserine. Metabolism (final column) is expressed as % difference from 'controls' for which the labelled compound was added to a ganglion homogenate at 0 °C before plating. The purity in the bath fluid samples following incubation was similar to that of 'controls' and standards.

presence of [^{14}C]morphine, [^{3}H]atropine, [^{14}C]trimethylamine or [^{14}C]phenobarbitone, the radioactivity was associated with the authentic compound in similar proportions (94–98 %) to those of controls, bath fluid samples and stock solutions, indicating that they are not metabolized significantly by ganglia or hydrolysed in solution. In ganglia incubated with [^{14}C]procaine (400 μM) for 1 hr, however, 16 % of the radioactivity was not associated with the parent compound, but formed a discrete spot on the plates. Inclusion of eserine (30 μM) in the incubation medium (60 min pre-incubation) prevented this breakdown (presumably to *p*-aminobenzoic acid by a cholinesterase).

Pharmacological activity. Although many of the compounds have well known pharmacological actions, these were controlled either by addition of antagonists (e.g. hexamethonium with nicotine; see Brown & Halliwell, 1972), by using concentrations below those with pharmacological activity (tested by electrophysiological recordings: cf. Brown *et al.* 1971), or when an effect on pH_i was suggested, by testing their effects on the distribution of nicotine or DMO.

pK'_a. The pK'_a assigned to nicotine was 0·81 (Barlow & Hamilton, 1962). Values for the other weak bases were taken from Perrin (1965) and for the weak acids from Kortüm, Vogel & Andrussens (1961). When necessary, temperature corrections were made using the method of Albert & Serjeant (1962).

2. *Experiments on erythrocytes*

Fresh human blood (about 5 ml.) was drawn into heparinized syringes, centrifuged, and the plasma and buffy layer removed. The cells were washed five times in three volumes of a suspending medium containing (mM): NaCl (129), KCl (5), $CaCl_2$ (1), $MgSO_4$ (2), NaH_2PO_4 (1), glucose (5), and HEPES (20). The pH of this medium was adjusted to about 7·3 using NaOH giving a final concentration of Na^+ of about 140 m equiv. $l.^{-1}$. Aliquots (0·5 ml.) of a 50% suspension of the washed erythrocytes were added to tubes containing 4·5 ml. of medium, [^{14}C]DMO (10 μM) and [^{3}H]nicotine (1 μM) at 37 °C. The tubes were inverted several times to mix and incubated in a shaking water bath at 37 °C. In some experiments the pH of the incubation medium was adjusted to 8·0. At the end of the incubation, duplicate 1 ml. samples were taken and centrifuged for 10 min at 2000 *g* and 37 °C. The supernatant was withdrawn for measurement of radioactivity and pH. The surface of the packed cells was blotted and duplicate aliquots (10 μl.) were taken for determination of extracellular water (using the haematocrit method), total water (by a gravimetric method similar to that used for ganglia) and radioactivity (using the method of Mahin & Lofberg, 1966). Initially, [^{3}H]- and [^{14}C]mannitol were used as extracellular space markers but the haematocrit method, while yielding essentially the same values (about 10% of total water), allowed the simultaneous measurement of [^{14}C]DMO and [^{3}H]-nicotine in the same samples.

3. *Experiments on brown adipose tissue*

In contrast to white adipose tissue which acts as a storage depot, the role of brown adipose tissue is to produce heat, and is important in mammals during early post-natal life, exposure to cold and arousal from hibernation (see reviews by Joel, 1965; Smith & Horwitz, 1969; Flatmark & Pederson, 1975). The cells normally contain many triglyceride droplets, most of the remaining cytoplasm being packed with mitochondria (Suter, 1969). When animals are exposed to a cold environment, the lipid stores are rapidly depleted leaving a virtually lipid-free cytoplasm, abundant in mitochondria and almost devoid of other membranous components (Suter, 1969). The tissue then resembles liver both grossly and histologically (Hull & Segall, 1966; Cardiasis, Blanc & Sinclair, 1972).

Rats (about 200 g) were exposed to temperature of 4 °C, without food (to ensure good depletion of lipid; Joel, 1965) but with free access to water, for 24 hr. This treatment could be expected to result in over 90% of the lipid of brown adipose tissue being discharged, the remainder being mainly structural (Joel, 1965). Conventional histology showed very few lipid droplets remaining after such an exposure. The rats were then anaesthetized with urethane and the interscapular brown adipose tissue was dissected out on both sides and freed from muscle, white adipose tissue and facia. The tissue was sliced freehand into pieces weighing 1–2 mg (Joel, 1966) which were then placed in oxygenated Krebs solution for about 2 hr before use. Subsequent treatment of the slices was the same as that used for ganglia.

Metabolic inhibitors

In experiments using ganglia and brown adipose tissue slices, inhibitors of respiration (cyanide, rotenone, antimycin A and amylobarbitone) and of the mitochondrial ATPase (oligomycin), and uncouplers of oxidative phosphorylation (2,4-dinitrophenol, DNP and *m*-chloro-carbonylcyanidephenylhydrazone, Cl-CCP) were used. The effective concentrations of some of these inhibitors in ganglia were determined by their ability to abolish the hyperpolarization of the ganglion following removal of nicotinic depolarizing agents, which is due to an electrogenic extrusion of Na^+ from the cells and depends on aerobic metabolism (Brown, Brownstein & Scholfield, 1972; Brown & Scholfield, 1974*a*, *b*). The ganglionic hyperpolarization after removal of carbachol (200 μM; 2 min application) was monitored using the method of Brown & Marsh (1975). Cyanide (2 mM), DNP (0·2–1 mM) and amylobarbitone (0·6 mM) have previously been found to be effective in rat ganglia in either reducing the after-hyperpolarization (Brown *et al.* 1972) or inhibiting respiration (Brauser, Bücher & Dolivo, 1970). Antimycin A (1·8 μM), rotenone (5μM) and Cl-CCP (5 μM) abolished the hyperpolarization, although the time taken for complete inhibition by these concentrations of antimycin and rotenone was quite long (about 90 min) as was recovery (cf. Greengard & Straub, 1962). Since oligomycin can also

inhibit the Na^+-K^+ATPase directly (Whittam & Chipperfield, 1975) it could not be tested in this way but low concentrations (about 1 μg/ml.) are effective using isolated mitochondria (e.g. Nicholls, 1974).

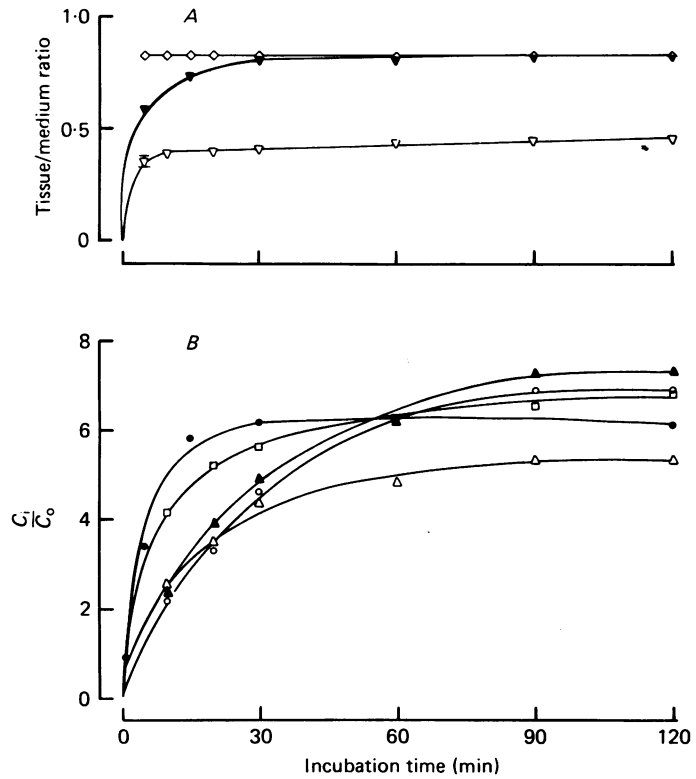

Fig. 1. *A*, uptake of [^{14}C]urea (▼) and [^{14}C]mannitol (▽) by isolated superior cervical ganglia and mean fractional water content (◇) measured by drying to constant weight. Ordinate: tissue/medium ratio (ml. g^{-1}). *B*, uptake of [^{3}H]nicotine (●; 10 μM), [^{14}C]-morphine (▲; 9 μM), [^{3}H]atropine (○; 9 μM), [^{14}C]procaine (□; 200 μM) and [^{14}C]trimethylamine (△; 90 μM) into ganglia. Ordinate: intracellular/extracellular concentration ratio (C_i/C_o). Abscissae: time of incubation (min). In *A* and *B* each symbol represents the mean of at least four ganglia. Standard errors in *A* mostly fall within the dimensions of the symbols and are excluded in *B* for clarity, but were similar to those shown in Figs. 2 and 4. Hexamethonium (2·5 mM) was included in the incubation medium with [^{3}H]nicotine and [^{14}C]trimethylamine, and eserine (30 μM) with [^{14}C]procaine.

RESULTS

1. Experiments on sympathetic ganglia

Water spaces

The mean total water content of ganglia, measured by drying to constant weight, was $0{\cdot}83 \pm 0{\cdot}001$ ml. g^{-1} (mean $\pm$ S.E. of mean; $n = 81$). The [^{14}C]urea space, after 30–60 min incubation, was essentially identical to this value (Fig. 1*A*): in all subsequent experiments, the former (gravimetric) method for measuring water content was used.

[^{14}C]mannitol showed initial rapid uptake into the tissue, but after this phase was complete (20–30 min) a further small, slow uptake, corresponding to about 0·025 ml. g^{-1} hr^{-1} occurred, as discussed previously (Brown *et al.* 1971). Mean values for the 30 min [^{14}C]mannitol and [^{3}H]inulin spaces were (ml. g^{-1} ± S.E. of mean): 0·394 ± 0·003 (n = 81) and 0·308 ± 0·004 (n = 47) respectively.

Uptake of weak bases

All of the weak bases ([^{3}H]nicotine, [^{3}H]atropine, [^{14}C]morphine, [^{14}C]trimethylamine and [^{14}C]procaine) accumulated in ganglia to reach a steady-state concentration within 2 hr (Fig. 1*B*). At this time the intracellular concentrations were between five and eight times those in the incubation medium.

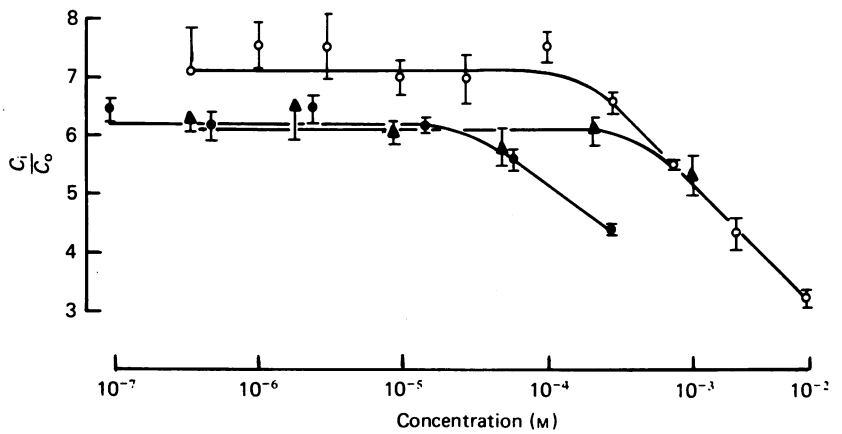

Fig. 2. Concentration dependence of the steady-state C_i/C_o attained by [^{3}H]nicotine (●; 30 min incubation), [^{14}C]morphine (▲; 90 min), and [^{3}H]atropine (○; 90 min) in ganglia. Points represent means of four to six ganglia; error bars are S.E. of mean. Bath fluid pH was 7·36–7·39.

[^{3}H]nicotine, [^{3}H]atropine and [^{14}C]morphine. The ratio C_i/C_o for [^{3}H]nicotine and [^{14}C]morphine, both of pK'_a of about 8, was maintained at about 6 across a large range of bath fluid concentrations (Fig. 2). The distribution of [^{3}H]atropine followed the same pattern: the ratio C_i/C_o was higher (about 7), but remained constant with concentrations within the range 0·3–100 μM. The pH_i values calculated from the distribution of these three bases over the 'plateau' concentration ranges were similar: [^{3}H]nicotine, 6·49, [^{14}C]morphine, 6·51 and [^{3}H]atropine, 6·54.

Intracellular alkalinization. At high values of C_o, the ratio C_i/C_o decreased. One explanation for this might be that cellular accumulation of base makes the cell interior more alkaline. If so, a high concentration of one base should produce a comparable increase in the apparent pH_i registered by both its own uptake and by the uptake of a low concentration of another base. To test this, the influence of unlabelled atropine on the uptake of [^{3}H]nicotine was assessed. Ganglia were incubated for 90 min with [^{3}H]nicotine (corresponding to the incubation time with [^{3}H]atropine) in the presence of 9 μM or 6 mM unlabelled atropine. The high concentration of atropine reduced the value of C_i/C_o for [^{3}H]nicotine from 6·96 to 2·76, corresponding

to a rise in pH_i from 6·49 to 6·86 (Table 2). This coincided very closely with the values for pH_i calculated from the concentration gradient for [^{3}H]atropine itself at the two external concentrations.

TABLE 2. Effect of atropine on the distribution of [^{3}H]nicotine and [^{3}H]atropine

Marker	[Atropine] (μM)	n	Mean pH_o	C_i/C_o	Calculated pH_i
[^{3}H]nicotine	0	8	7·40	6·96 ± 0·13	6·49 ± 0·006
	9	4	7·36	6·64 ± 0·08	6·46 ± 0·005
	6000	4	7·38	2·76 ± 0·02	6·86 ± 0·003
[^{3}H]atropine	9	8	7·33	6·99 ± 0·30	6·49 ± 0·020
	6000	4	7·31	3·20 ± 0·14	6·80 ± 0·050

Using [^{3}H]nicotine as a pH_i marker, ganglia were incubated for 90 min in the presence or absence of 9 μM or 6 mM unlabelled atropine. [^{3}H]nicotine (3 μM) was included for the final 30 min. All solutions contained hexamethonium (2·5 mM). Values for the distribution of [^{3}H]atropine are taken from the data illustrated in Fig. 2. Data given as mean ± S.E. of mean.

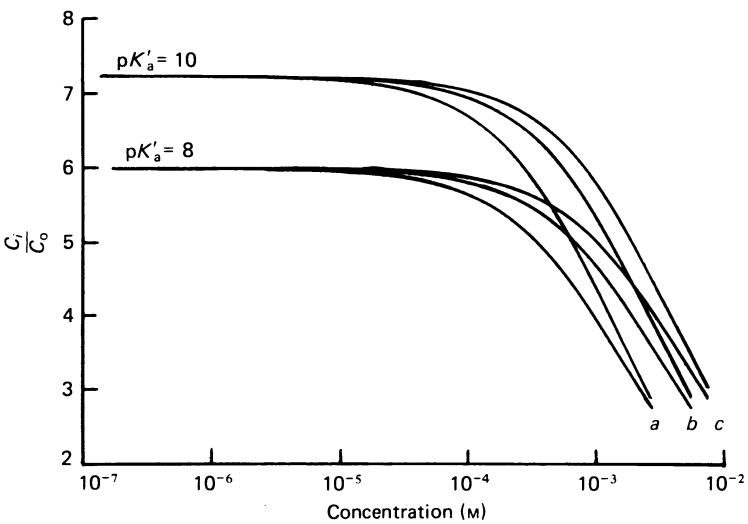

Fig. 3. Theoretical intracellular/extracellular concentration ratios (C_i/C_o) of weak bases in relation to extracellular concentration. It is assumed that the bases penetrate the cells predominantly in their unionized form. The extent by which the bases will alkalinize the cells and thus reduce their own uptake will depend on the concentration, pK'_a, and on the apparent intracellular buffering power. Curves *a*, *b*, and *c* are constructed with values for the latter of 20, 40 and 60 slykes (m-equiv. l.$^{-1}$ pH unit^{-1}) respectively, for bases of $pK'_a = 10$ (upper curves) and 8 (lower curves). pH_i and pH_o are assumed to be 6·54 and 7·40 respectively.

Fig. 3 shows some calculated effects expected from alkalinization by bases of $pK'_a = 8$ and 10 (like nicotine and atropine respectively) penetrating the cells only in their unionized form. The three curves at each pK'_a correspond to three nominal values for intracellular buffering power, covering the usual range of reported estimates: 20, 40 and 60 slykes. (1 slyke is equivalent to 1 m-equiv/l. of H^+ or OH^- per pH unit.)

These curves accord quite well with the experimental observations, indicating that a reduction in C_i/C_o at an external concentration above 30–100 μM is likely to occur as a result of direct

alkalinization. The earlier fall-off with nicotine (Fig. 2) might be related to the shorter incubation time: with longer incubation times, effective buffering power may be reinforced by processes other than physicochemical buffering (Thomas, 1974, 1976).

[^{14}C]trimethylamine and [^{14}C]procaine. With these two bases, the relationships between the ratio C_i/C_o and C_o were more complicated than those indicated in Figs. 2 and 3 (see Fig. 4). The specific activity of [^{14}C]trimethylamine precluded measurement of its uptake at bath concentrations lower than 4·5 μM but the values of C_i/C_o at this concentration and at 9 μM (about 6·7) were not significantly different and yielded a value for pH_i of 6·58, in reasonable agreement with values obtained using the other bases above. At concentrations higher than 9 μM, C_i/C_o for [^{14}C]trimethylamine fell steadily to reach about 5 at C_o = 700 μM. The effect of trimethylamine at concentrations of 9 and 700 μM on the distribution of [^{3}H]nicotine was

TABLE 3. Effect of trimethylamine (TMA) on the distribution of [^{3}H]nicotine and [^{14}C]trimethylamine

Marker	[TMA] (μM)	n	Mean pH_o	C_i/C_o	Calculated pH_i
[^{3}H]nicotine	0	8	7·400	6·87 ± 0·14	6·48 ± 0·015
	9	4	7·405	6·35 ± 0·10	6·52 ± 0·007
	700	4	7·360	4·56 ± 0·19	6·63 ± 0·018
[^{14}C]trimethylamine	4·5–9	8	7·410	6·73 ± 0·13	6·57 ± 0·011
	700	4	7·410	5·04 ± 0·02	6·71 ± 0·028

Where [^{3}H]nicotine was used as a pH_i marker, ganglia were incubated for 120 min in Krebs solution in the absence or presence of unlabelled TMA (9 μM or 0·7 mM). [^{3}H]nicotine was included for the final 30 min. All solutions contained hexamethonium (2·5 mM). Values for the distribution of [^{14}C]TMA are taken from the data illustrated in Fig. 4. Values are mean ± S.E. of mean.

examined (Table 3). At 9 μM, trimethylamine was without effect on the uptake of [^{3}H]nicotine but at 700 μM a significant fall occurred. The increase in pH_i was similar whether calculated from the distribution of [^{3}H]nicotine (0·15 units) or [^{14}C]trimethylamine (0·14 units) indicating that the reduced uptake of [^{14}C]trimethylamine at the higher concentrations was indeed due to an alkalinization. However, the pattern of this reduction in uptake appears different from that observed with the other bases since it begins at much lower concentrations than expected and the slope of the curve is shallower than it should be for a direct alkalinization of the cell interior (cf. Fig. 3).

The concentration curve for [^{14}C]procaine was sigmoidal for the range of concentrations tested (Fig. 4). When C_o was 2–20 μM, C_i/C_o was about 10, which would require a pH_i somewhat lower (6·40) than those calculated from the distributions of the other bases. At higher concentrations, C_i/C_o fell to reach about 5 when C_o was 0·5–2 mM (pH_i = 6·64). Using [^{3}H]nicotine, such high concentrations induced a substantial increase in the pH_i from 6·56 ± 0·01 to 7·00 ± 0·01 (S.E. of mean; n = 4). Calculation of pH_i from the distribution of 2 mM-[^{14}C]procaine, however, yielded a value of 6·67, corresponding to an increase of pH_i by 0·27 units compared to that calculated from the distribution of [^{14}C]procaine at the lower concentrations. This is 0·17 units lower than the increase measured using [^{3}H]nicotine as a marker.

Assuming the measurements made with [^{3}H]nicotine to be correct, the appropriate values for C_i/C_o for [^{14}C]procaine should have been 7·10 and 2·47 at 20 μM and 2 mM respectively.

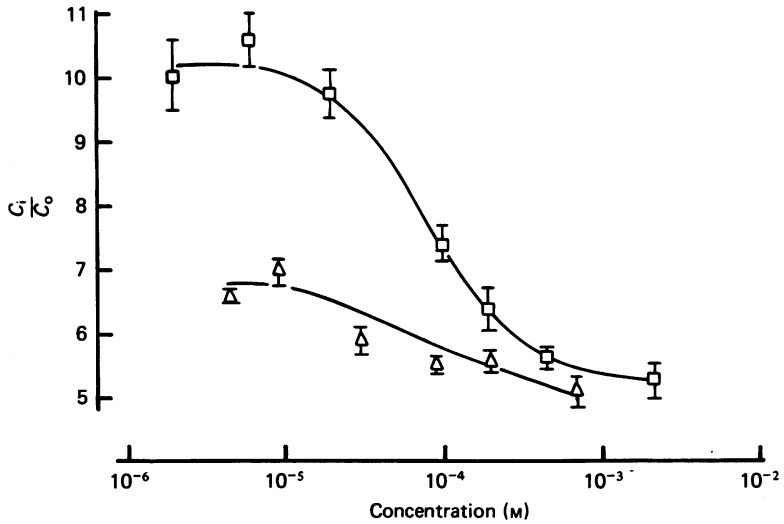

Fig. 4. Concentration-dependence of the steady-state uptake (C_i/C_o) for [^{14}C] trimethylamine (△; 120 min uptake) and [^{14}C]procaine (□; 60 min uptake). Points are the mean of four to six ganglia. Error bars are S.E. of mean. Mean bath fluid pH was 7·405.

Permeation of ionized base. Except under the special case where the proton permeability is sufficiently high for protons to distribute across the membrane in accordance with the membrane potential (see Roos, 1965), the distribution of a weak base will only accurately reflect the transmembrane pH gradient if the permeability of the membrane to the ionized base (BH^+) is very much less than that to the unionized base (B). If, on the other hand, there is appreciable permeation of ionized base, the value of C_i/C_o for total base will exceed that expected from pH_i, so giving an erroneously low estimate of pH_i. Thus, if the membrane is predominantly permeable to BH^+, the transmembrane BH^+ concentration gradient will be determined primarily by the membrane potential and approach that of any cation obeying the Nernst equation – about 25 for ^{86}Rb or ^{42}K distribution in this tissue (Brown & Scholfield, 1974*b*). Hence, partial permeation of BH^+ might contribute to the high values of C_i/C_o observed with the weak bases. If the membrane is permeable to both BH^+ and B then the equation derived by Boron & Roos (1976) can be used:

$$\frac{[B]_i}{[B]_o} = \left(\frac{P_B}{P_{BH^+}} - \frac{E_m F[H^+]_o}{RT(1-\epsilon)K'_a}\right) \Big/ \left(\frac{P_B}{P_{BH^+}} - \frac{E_m F\epsilon[H^+]_i}{RT(1-\epsilon)K'_a}\right), \quad (3)$$

where $\epsilon = \exp(E_m F/RT)$. C_i/C_o is given by:

$$\frac{C_i}{C_o} = \frac{[B]_i}{[B]_o}\frac{(K'_a+[H^+]_i)}{(K'_a+[H^+]_o)}. \quad (4)$$

From eqns. (3) and (4) it can be calculated for nicotine that if the true value of pH_i was about 7, and the membrane potential -70 mV (Adams & Brown, 1975), a value of 6 for C_i/C_o would require that the permeability of ionized nicotine be about a quarter to a third of that to the free base (i.e. $P_B/P_{BH^+} = 3$–4). In this case C_i/C_o would be reduced on depolarizing the cells (to about 2 at -20 mV) but would be effectively *independent* of extracellular pH as P_B/P_{BH^+} then numerically approaches $-E_mF/RT(1-\epsilon)$ so that eqn (4) approximates to:

$$\frac{C_i}{C_o} = \frac{K'_a + [H^+]_i}{K'_a + [H^+]_i \epsilon}. \quad (5)$$

These two predictions regarding the distribution of nicotine have been tested experimentally.

(i) To test the effects of changing extracellular pH, ganglia were pre-incubated for 60 min in HEPES-buffered Krebs solution at pH = 7·4, then incubated in HEPES-buffered Krebs solution of adjusted pH, containing [^{3}H]nicotine, for a further

TABLE 4. Effect of high [K^+] on the distribution of [H]nicotine

	H_2O (%)	'Extracellular space' (μl./μl. tissue water)	C_i/C_o	pH_i
Control (6 mM K^+)	82·8 ± 0·1	0·462 ± 0·007	5·45 ± 0·18	6·59 ± 0·01
124 mM-K^+	81·1 ± 0·3	0·524 ± 0·011	5·37 ± 0·28	6·58 ± 0·02

Values are the mean ± S.E. of mean of seven ganglia.

Ganglia were incubated for 30 min in Krebs solution with or without added KCl (118 mM), containing [^{3}H]nicotine (3 μM), hexamethonium (2·5 mM) and the extracellular space marker [^{14}C]mannitol. Total water was measured by drying to constant weight.

30 min. Mean values (± S.E. of mean) for C_i/C_o were 2·57 ± 0·09 at $pH_o = 6·89$, 8·12 ± 0·18 at $pH_o = 7·80$ and 15·20 ± 1·20 at $pH_o = 7·95$ ($n = 4$). These values correspond quite closely to those predicted by eqns. (3) and (4) on the assumption that $P_B \gg P_{BH^+}$ and $pH_i \approx pH_{nic} \approx 6·5$ rather than $P_B/P_{BH^+} = 3$–4.

(ii) To test the effect of membrane depolarization, ganglia were incubated in a medium containing 124 mM-K^+. C_i/C_o for [^{3}H]nicotine was unchanged (Table 4), again indicating a low permeability to ionized nicotine.

In the light of these results therefore it can be concluded that ionized base BH^+ does not permeate the membrane to any significant extent compared with non-ionized base B.

Uptake of weak acids

In agreement with previous observations (Brown & Halliwell, 1972), 30 min incubation in [^{14}C]DMO (30 μM) yielded a value of 0·87 ± 0·007 for C_i/C_o ($n = 46$), with a corresponding pH_i of 7·31 ± 0·003.

[^{14}C]phenobarbitone ($pK'_a = 7·45$) equilibrated with the tissue in 20–30 min to give a C_i/C_o of 2·66 ± 0·05 ($pH_i \approx 8·0$) sustained throughout the concentration range 8 μM-3 mM. Phenobarbitone (100 μM) was without effect on pH_i measured using DMO or nicotine.

Effect of temperature, DMO and nicotine on pH_{nic} and pH_{DMO}

All the above experiments were carried out at 25 °C; ganglionic ion contents, transmission, responses to, and recovery from, nicotinic depolarizing agents are well maintained at this temperature (Brown *et al.* 1972; Brown & Scholfield, 1974*a b*). Increasing the incubation temperature to 37 °C was without effect on the water spaces or on the pH_i measured using DMO or nicotine (Table 5).

DMO (30 μM) caused no change in pH_{nic} and nicotine (3 μM) did not affect pH_{DMO}.

TABLE 5. Effect of temperature on water spaces and pH_i

Temperature (°C)	Total water (% wet weight)	I.c.w. (% total water)	pH_i (nicotine)	pH_i (DMO)
25	83·1 ± 0·2 (7)	55·0 ± 1·8 (4)	6·53 ± 0·010 (4)	7·32 ± 0·010 (4)
37	83·3 ± 0·3 (7)	53·3 ± 0·6 (4)	6·55 ± 0·015 (5)	7·33 ± 0·009 (5)

Ganglia were incubated for 30 min in the presence of [^{3}H]nicotine (3 μM) + hexamethonium (2·5 mM) or [^{14}C]DMO (30 μM). Values for the intracellular water space (i.c.w.) were obtained using [^{14}C]mannitol as an extracellular space marker and are expressed as a percentage of the total tissue water. Data are given as mean ± S.E. of mean; numbers of ganglia used indicated in parentheses.

Effect of uncoupling agents

If the different values for pH_i measured with bases and DMO result from different pH values within the membrane-bound organelles of the cells (see Discussion), uncouplers might be expected to reduce the difference between pH_{DMO} and pH_{base} since they catalyse the equilibration of protons across membranes (see Mitchell, 1976). Fig 5*A* shows the effects of Cl-CCP, one of the more potent uncouplers (Heytler & Pritchard, 1962), on pH_{nic} and pH_{DMO}.

Two points emerge. *First*, at low concentrations (5 and 50 μM) Cl-CCP induced opposite changes in pH_{nic} and pH_{DMO}. Thus, at a concentration of 5 μM, pH_{DMO} decreased and pH_{nic} increased by similar amounts (about 0·1 unit); at 15 μM there was little further increase in pH_{nic} but pH_{DMO} declined by an additional 0·14 units, so that $pH_{DMO} - pH_{nic}$ was reduced by half. *Secondly*, at the higher concentrations of Cl-CCP (50 and 150 μM), both pH_{DMO} and pH_{nic} fell but the reduction in pH_{DMO} predominated, so that at 150 μM both pH_{DMO} and pH_{nic} became approximately equal at 6·5.

The effect of another (less potent) uncoupler, DNP, is shown in Fig. 5*B*. The resulting changes in pH_{DMO} and pH_{nic} were similar to those observed with Cl-CCP, although higher concentrations were required as expected: $pH_{DMO} - pH_{nic}$ was reduced to 0·2 units or less at a concentration of 3 mM.

The reduction of pH_{DMO} from 7·3 to 6·5 represents a reduction of the intracellular/extracellular concentration ratio of DMO from 0·9 to 0·2. At this level, appreciable error in measuring the intracellular concentration of DMO might arise from errors in measuring its volume of distribution in the extracellular space. One source of such error is the higher molecular weight of inulin (the extracellular space 'marker') compared to DMO (5000–5500 against 129), since the apparent extracellular space in this tissue varies with the molecular weight of the marker

(Brown *et al.* 1971). The small difference between pH_{DMO} and pH_{nic} at and above 50 μM-Cl-CCP or at 3 mM-DNP could be fully accounted for by correcting for the molecular weight of the marker using the data of Brown *et al.* (1971).

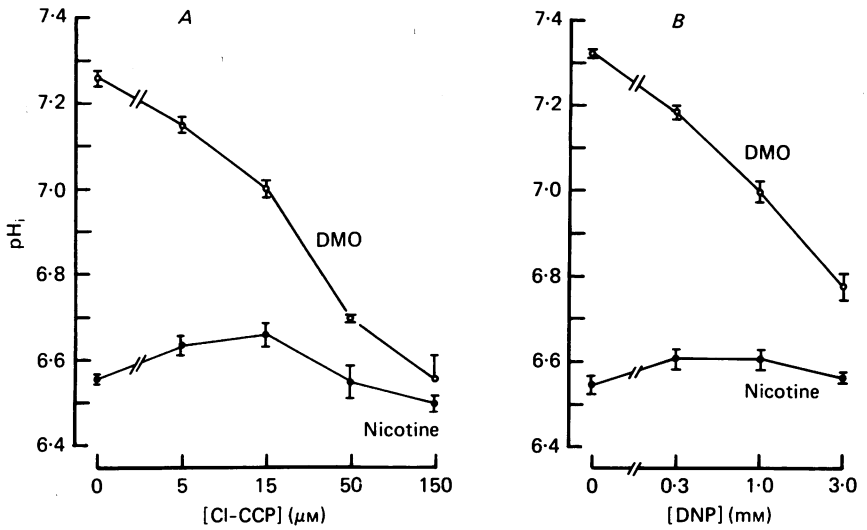

Fig. 5. Effect of *A* Cl-CCP and *B*, DNP on pH_{DMO} (○) and pH_{nic} (●). Contralateral ganglia from the same rats were incubated for 30 min in Krebs solution containing either [^{14}C]DMO (30 μM) or [^{3}H]nicotine (3 μM) in the absence or presence of Cl-CCP or DNP (concentrations on abscissae). Each point represents the mean of four to six ganglia; vertical bars represent S.E. of mean.

Metabolic inhibition. Changes in pH_i following addition of uncoupling agents might be secondary to metabolic readjustments. To check this, the effects of some respiratory inhibitors (antimycin A, rotenone, cyanide and amylobarbitone) and an inhibitor of the mitochondrial ATPase (oligomycin) were tested using concentrations sufficient to inhibit electrogenic Na^+ extrusion from the ganglion (see Methods). All of the respiratory inhibitors increased pH_{nic} by 0·07–0·13 units, but (unlike uncouplers) did not reduce pH_{DMO} (Table 6). Oligomycin did not alter either pH_{nic} or pH_{DMO}. Combinations of inhibitors (e.g. 1·8 μM-antimycin plus 5 μM-rotenone) or prolonged exposure times (to 2 hr) failed to affect pH_{DMO}. Further, if antimycin and rotenone were included with an uncoupler the effects of the uncoupler were unaltered.

Effect of lactate. Respiratory inhibitors and uncouplers might be expected to produce an accumulation of metabolic acid in the cells. Though this could not, in itself, explain the rise in pH_{nic} noted above, it might complicate measurement of changes in pH_i. To test the effects of acidosis, ganglia were subjected to an acid load in the form of the permeant weak acid, lactate.

Exposure to 20 mM-lactate (Fig. 6) reduced both pH_{nic} and pH_{DMO}, but the pattern and magnitude of the measured changes differed with the two markers. A small but significant fall in pH_{nic} of about 0·05 units occurred after 60 min but thereafter it increased to reach a value not significantly different from that of control ganglia at 90 and 120 min. The fall in pH_{DMO} on the other hand was larger (0·1 unit after 60 min) and was sustained throughout the rest of the incubation period. These results

TABLE 6. Effect of respiratory inhibitors on pH_i measured using DMO (pH_{DMO}) and nicotine (pH_{nic})

Inhibitor	n	pH_{nic}	ΔpH_{nic}	pH_{DMO}	ΔpH_{DMO}
None	7	6·52 ± 0·012	—	7·32 ± 0·004	—
Antimycin A (1·8 μM)	7	6·60 ± 0·014*	+0·08	7·34 ± 0·011	+0·02
Antimycin A (18 μM)	3	6·62 ± 0·011*	+0·10	7·30 ± 0·014	−0·02
None	7	6·55 ± 0·006	—	7·35 ± 0·008	—
Rotenone (5 μM)	7	6·62 ± 0·011*	+0·07	7·38 ± 0·011	+0·02
Rotenone (50 μM)	4	6·64 ± 0·010*	+0·09	7·33 ± 0·025	−0·02
None	7	6·57 ± 0·022	—	7·29 ± 0·009	—
Sodium cyanide (2 mM)	4	6·70 ± 0·009*	+0·13	7·31 ± 0·009	+0·02
None	8	6·56 ± 0·010	–	n.t.	—
Sodium amylobarbitone (0·6 mM)	8	6·63 ± 0·005*	+0·07	n.t.	—

Ganglia were incubated for 45 min in the absence or presence of the inhibitor, with [^{3}H]nicotine or [^{14}C]DMO present for the final 30 min. Hexamethonium (2·5 mM) was present throughout. Values show means ± S.E. of calculated pH_i.

* Significant difference from no inhibitor ($P < 0·005$); n.t. = not tested.

suggest that intracellular acidosis would not explain the selective fall in pH_{DMO} produced by uncouplers, and the changes in pH_{DMO} and pH_{nic} produced by acidosis are too small to interfere seriously with previous measurements.

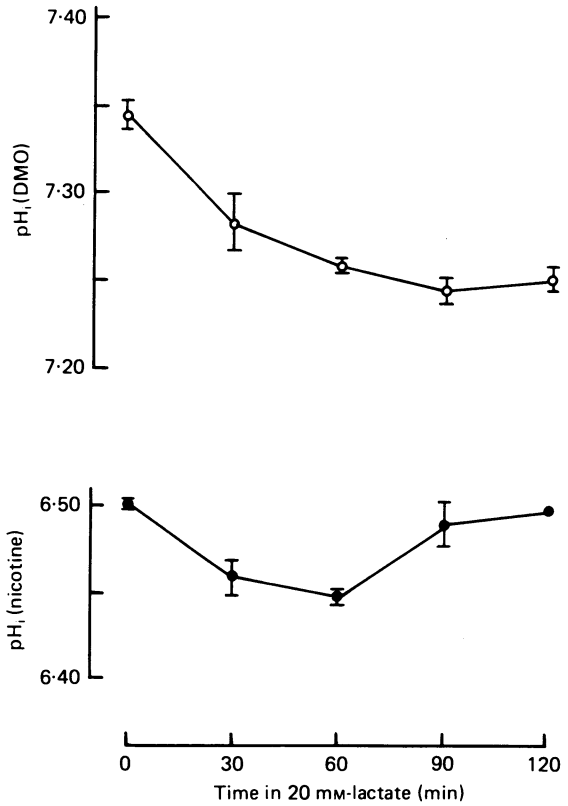

Fig. 6. Effect of lactate on pH_i. Ganglia were incubated in the presence of sodium lactate (20 mM) for times up to 2 hr. Ordinates: pH_i measured using DMO (pH_{DMO}, ○) or nicotine (pH_{nic}, ●). Abscissa: time of incubation in lactate (min). Each point represents the mean of four ganglia; error bars are S.E. of mean.

Effect of uncouplers on the effective conductance of the cell membrane to protons. Although the resting proton conductance of the cell membrane seems to be very low, to judge from the effects of changing the membrane potential (E_m) and extracellular pH on pH_{nic} and pH_{DMO} (see above), uncouplers might increase the proton conductance of the cell membrane sufficiently to affect the pH_i. This possibility was tested by measuring the pH_i of ganglia incubated in media containing additional K^+ (to depolarize the cells) in the presence or absence of 50 and 150 μM-Cl-CCP (Fig. 7).

In the absence of Cl-CCP, pH_{nic} and pH_{DMO} were little altered even at a K^+ concentration of 124 mM. In the presence of 15 and 150 μM-Cl-CCP, however, both pH_{nic} and pH_{DMO} progressively increased with increasing K^+ concentration.

The dashed lines in Fig. 7 show the changes expected if, in the presence of uncouplers, the cell membrane was sufficiently permeant to protons for the pH_i to be governed by the membrane potential, i.e. $E_m = 59\ (pH_i\text{-}pH_o)$ at 25 °C. The value of

E_m at different K^+ concentrations has been estimated from the constant-field equation assuming a resting value of -70 mV at $[K^+]_o = 6$ mM (see Adams & Brown, 1975) and values for $[Na^+]_i$, $[K^+]_i$ and P_{Na}/P_K from Brown & Scholfield (1974*a*). The observed changes in pH_i were clearly smaller; even at 150 μM-Cl-CCP these were a third or less than those expected.

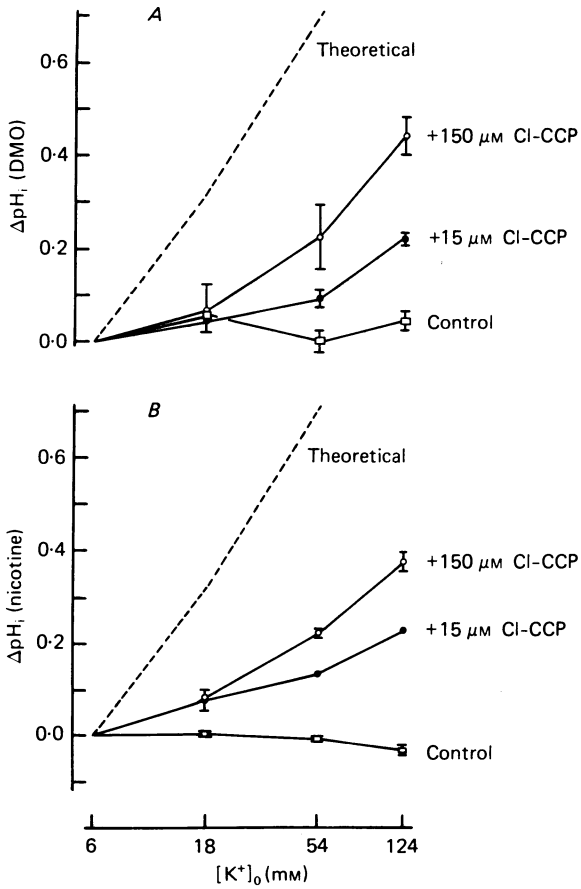

Fig. 7. Changes in *A*, pH_{DMO} and *B*, pH_{nic} induced by increasing the K^+ concentration in the bathing medium (by addition of KCl), in the absence (□) or presence of 15 μM (●) or 150 μM (○) Cl-CCP. The incubation time was 30 min. The dashed line indicates changes in pH_i with $[K^+]$ expected if the cell membrane was freely permeable to protons (see text). Changes in pH_i (= Δ pH_i) were calculated as the differences in pH_{DMO} or pH_{nic} from the mean respective values of pH_i in 6 mM-K^+ Krebs solution. Each point is the mean of three to five ganglia. Bars are S.E. of the mean, when this exceeds the dimensions of the symbol.

2. *Experiments on erythrocytes*

If the difference in pH_i values denoted by nicotine and DMO arose from an anatomical heterogeneity of intracellular pH, no such difference would be expected in human erythrocytes. Table 7 shows that this was the case. At two different values

for pH_o, 7·255 and 8·000, the values for pH_{DMO}-pH_{nic} were 0·07 and 0·08 respectively, as against 0·8 for ganglia (see Table 6).

3. pH_i of brown adipose tissue depleted of lipid

Values for the water spaces of slices of brown adipose tissue incubated in Krebs solution were (ml./g ± S.E. of mean): total water, 0·777 ± 0·003 (n = 60; 30–120 min incubation); [^{14}C]mannitol space (120 min incubation), 0·502 ± 0·013 (n = 11); and [^{3}H]inulin space (30–120 min incubation), 0·409 ± 0·011 (n = 21). The relatively large extracellular space probably reflects the histological changes occurring during lipid depletion (Suter, 1969).

TABLE 7. pH_i of washed erythrocytes

Mean pH_o	n	C_i/C_o (DMO)	C_i/C_o (nic)	pH_{DMO}	pH_{nic}	$pH_{DMO} - pH_{nic}$
7·255	4	0·91 ± 0·013	1·40 ± 0·021	7·15 ± 0·007	7·08 ± 0·007	0·07 ± 0·000
8·000	4	0·52 ± 0·025	1·66 ± 0·114	7·71 ± 0·022	7·63 ± 0·042	0·08 ± 0·052

TABLE 8. The uptake of [^{14}C]DMO and [^{3}H]nicotine into brown adipose tissue slices

pH_i marker	Incubation time (min)	n	C_i/C_o	pH_i
[^{3}H]nicotine	120	9	8·56 ± 0·051	6·39 ± 0·028
[^{14}C]DMO	30–120	20	0·97 ± 0·020	7·39 ± 0·010

[^{14}C]DMO equilibrated with the tissue slices in about 30 min but the uptake of [^{3}H]nicotine was more variable and usually required longer; 120 min were allowed for equilibration routinely. Values for the distribution of [^{14}C]DMO and [^{3}H]nicotine and the pH_i calculated from these are given in Table 8.

Both markers were more concentrated in the brown adipose tissue slices than they were in ganglia, the intracellular/extracellular concentration ratios (C_i/C_o) being 8·56 for [^{3}H]nicotine and 0·97 for [^{14}C]DMO. The pH_i calculated from these distributions were 6·39 (nicotine) and 7·39 (DMO). pH_{DMO}-pH_{nic} was therefore greater than normally observed in ganglia (1 pH unit compared to 0·8). Extending the time that the rats were exposed to cold (for lipid depletion) to 36 hr did not affect these results. In one experiment (five slices), the distribution of [^{3}H]atropine was found to be similar to that of [^{3}H]nicotine (C_i/C_o = 8·77 ± 0·48; pH_i = 6·47 ± 0·03).

Increasing the concentration of nicotine to 100 μM or including hexamethonium (2·5 mM) in the bathing medium caused no significant change in pH_{nic} after 2 hr incubation.

The effect of DNP at a concentration (1 mM) which reduced pH_{DMO}-pH_{nic} by half in the ganglion was also tested (Table 9). One hour was allowed for equilibration of the markers in this case. DNP reduced pH_{DMO}-pH_{nic} from 1·09 to 0·44. The reduction in pH_{DMO} was similar to that observed in ganglia at that concentration (0·32 units), but the increase in pH_{nic} was greater (0·33 units compared to 0·06 in the ganglion).

TABLE 9. Effect of DNP on pH_i of brown adipose tissue slices

Conditions	n	pH_{nic}	ΔpH_{nic}	pH_{DMO}	ΔpH_{DMO}	$pH_{DMO} - pH_{nic}$
Control	4	$6{\cdot}25 \pm 0{\cdot}027$	—	$7{\cdot}34 \pm 0{\cdot}009$	—	1·09
+DNP	4	$6{\cdot}58 \pm 0{\cdot}009$*	+0·33	$7{\cdot}02 \pm 0{\cdot}029$*	−0·32	0·44

Tissue slices were incubated for 60 min in Krebs solution containing either [^{3}H]nicotine or [^{14}C]DMO in the absence or presence of 1 mM-2,4-dinitrophenol (DNP).

* Indicates significant difference from control ($P < 0{\cdot}001$).

DISCUSSION

The principle results of the experiments on the uptake of weak bases are summarized in Table 10. These confirm, for a wider range of bases than hitherto used, previous observations (Brown & Halliwell, 1972) that, in isolated sympathetic ganglia base-indicated intracellular pH (pH_{base} = 6·4–6·6) is about 0·8 units lower than that indicated by the weak acid DMO (pH_{DMO} = 7·3). In other words, bases in general show a much higher steady-state intracellular concentration than expected were the true value of pH_i throughout the intracellular space equal to that indicated by DMO.

The distributions of the bases trimethylamine and procaine and the acid phenobarbitone, however, showed more complex behaviour than that of other bases or DMO. There seems to be some mechanism whereby procaine is held in the tissue to give a C_i/C_o of about 3 above that expected from the distribution of the other bases and as a consequence a lower value for pH_i (= 6·4) is obtained. The value for pH_i given by phenobarbitone (about 8·0) is unrealistically high, suggesting some excess uptake through other causes such as binding.

High concentrations and C_i/C_o values of bases have also been noted in other tissues. For example, high intracellular concentrations of nicotine have been observed in skeletal muscle (Adler, 1972), salivary glands (Putney & Borzelleca, 1971) and lung (Effros & Chinard, 1969). Paton & Rang (1965) reported a value of 6–7 for C_i/C_o for atropine in smooth muscle (pH_{base} = 6·6) and a similar value for pH_{base} may be deduced from the distribution of procaine in smooth muscle (Hudgins & Putney, 1972). Finally, the uptake of atropine (Heilbronn, 1969) and morphine (Teller, De Guzman & Lajtha, 1974) into brain cortex slices accords with a value of 6·5–6·6 for pH_{base}.

There are various possible reasons why an individual base might become concentrated in cells to a level exceeding that expected from the intracellular pH alone: for example intracellular binding, carrier-mediated uptake, dissolution in lipids, or, if the membrane is substantially permeable to the protonated base, BH^+, through a potential-dependent Donnan equilibrium. However, as Table 10 shows, the common feature of the distribution of the bases is not the *concentration gradient* but the requisite value of pH_i. This means that the capacity of the binding or carrier sites or, for a permeant BH^+ species, the permeability ratio P_B/P_{BH+}, would have to vary for each base by an amount which fortuitously gives the same apparent value for pH_i, which is unlikely. Penetration as BH^+ rather than B is also shown to be unlikely by the fact that reduction of the membrane potential with increasing external K^+ did not reduce C_i/C_o whereas changing pH_o did.

It is equally clear from Table 10 that the uptake is not correlated with lipid solubility: for example atropine and trimethylamine yield comparable values for C_i/C_o yet differ by sixfold in the logarithm of their octanol–water partition coefficients. Differences in ionic strength on the two sides of the membrane is unlikely to be an important factor (Boron & Roos, 1976).

TABLE 10. Summary of the distribution of weak bases and calculated pH_i of ganglia

Base	pK'_a	log P_{oct}*	Concentration range (μM)	n	C_i/C_o	Calculated pH_i
[^{3}H]nicotine	8·01	1·17	0·10–14	20	6·17 ± 0·12	6·49 ± 0·009
[^{14}C]nicotine	7·94	0·73	0·35–210	36	6·08 ± 0·14	6·51 ± 0·011
[^{3}H]atropine	9·71	1·81	0·34–100	21	7·10 ± 0·16	6·54 ± 0·013
[^{14}C]trimethylamine	9·81	0·27	4·5–9·0	8	6·73 ± 0·13	6·58 ± 0·011
[^{14}C]procaine	8·91	1·90	2·0–20	12	10·13 ± 0·26	6·40 ± 0·012

Values are given as mean ± S.E. of mean.

* Logarithm of the octanol–water partition coefficient (from Leo, Hansch & Elkins, 1971).

Heterogeneity of pH

One factor which might give rise to substantial differences between pH_{acid} and pH_{base} is that intracellular pH is not homogeneous. Weak acids, for example, would become concentrated in alkaline organelles and relatively excluded from acidic organelles and vice-versa for weak bases. pH_i heterogeneity might also arise from multiple Donnan-type equilibria between the bulk aqueous phases of the cell interior and the charged surfaces of proteins or membranes (see Caldwell, 1956; Robson *et al.* 1968). The extent to which regional differences in pH would influence the over-all distribution of acids and bases will depend on their relative volumes and pH values (see below) but the net result would be that pH_{acid} will be greater than pH_{base}. pH_i heterogeneity therefore provides the simplest explanation for our observations.

Three lines of experimental evidence suggest that it is mainly membrane-bound compartments which are responsible.

(i) The difference between pH_{acid} and pH_{base} in isolated ganglia (as represented by pH_{DMO}-pH_{nic}) was reduced or abolished by uncoupling agents, known to increase the effective permeability of membranes to protons (see Mitchell, 1976). This may be attributed to a primary effect on proton gradients rather than a secondary response to metabolic disturbance, as the effect of uncouplers was not replicated by other types of metabolic inhibition or by exposure to lactate.

(ii) pH_{DMO}-pH_{nic} was very small in human erythrocytes which contain no membrane-bound intracellular organelles. The absolute values for pH_i so determined, especially for pH_{nic}, agree very closely with those previously measured by electrometric methods (Fitzsimons & Sendroy, 1961; Hilpert, Fleischmann, Kemp & Bartels, 1963; Funder & Wieth, 1966). Bone, Verth & Lambie (1976) have also reported that DMO and ammonia yielded similar values for pH_i in human erythrocytes, whereas in nucleated avian erythrocytes, $pH_{ammonia}$ was consistently below pH_{DMO} by an amount corresponding to an internal pH gradient of 0·5 units.

(iii) In contrast, pH_{DMO}-pH_{nic} was substantial (about 1 unit) in brown adipose

tissue which contained an abundance of intracellular organelles in the form of numerous mitochondria. As in ganglia, the difference was reduced by uncoupling agents.

Anatomical basis for pH_i heterogeneity

Robson *et al.* (1968) pointed out that for n pH compartments of volumes V_1, $V_2 \ldots V_n$, a base indicates the *arithmetic* mean H^+ concentration:

$$\overline{[H^+]}_{base} = \frac{V_1[H^+]_1 + V_2[H^+]_2 + \ldots + V_n[H^+]_n}{V_1 + V_2 + \ldots + V_n} \quad (6)$$

whereas an acid indicates the *harmonic* mean concentration:

$$\overline{[H^+]}_{acid} = \frac{V_1 + V_2 + \ldots + V_n}{\frac{V_1}{[H^+]_1} + \frac{V_2}{[H^+]_2} + \ldots + \frac{V_n}{[H^+]_n}}. \quad (7)$$

It is clearly possible to generate several anatomical models for a complex tissue such as the sympathetic ganglion. For example, one might subdivide the tissue into its various gross compartments: neurones, fibres, glial cells, etc. However, since the aim of pH_i measurements is usually to estimate the cytoplasmic pH, it seems more helpful to treat the cell population as relatively uniform (in the absence of contrary evidence), and then simply divide the total intracellular space into two pH compartments: the cytoplasm, which in the tissue as a whole comprises the bulk (88%) of the intracellular volume (Brown & Halliwell, 1972), and the residual space occupied by membrane-bound intracellular organelles. Taking the relative volumes of these two compartments (V_{cyt} = 88%, V_{res} = 12%) and setting $pH_{acid} = pH_{DMO} = 7{\cdot}3$ and $pH_{base} = 6{\cdot}55$, eqns. (6) and (7) yield two solutions (Bone *et al.* 1976):

(*a*) $pH_{cyt} = 6{\cdot}50$, $pH_{res} = 8{\cdot}15$, or

(*b*) $pH_{cyt} = 7{\cdot}36$, $pH_{res} = 5.69$.

In the first case, pH_{base} (6·55) corresponds closely to that of the cytoplasm, whereas pH_{DMO} reads some intermediate value; in the second case, pH_{DMO} (7·30) approximates to pH_{cyt} whereas pH_{base} records an intermediate value.

Of these two alternatives, the latter, a more alkaline cytoplasm with $pH_{DMO} \approx pH_{cyt}$, offers the advantage that pH_{cyt} conforms broadly with many values reported in nerve and muscle cells from the use of intracellular pH-sensitive micro-electrodes (e.g. Caldwell, 1954, 1958; Thomas, 1974; Aickin & Thomas, 1977). Further, Boron & Roos (1976) and Hinke & Menard (1976) have reported good agreement between micro-electrode measurements and pH_{DMO} in barnacle muscle fibres. However, the significance of this is offset by the fact that, in these fibres, the base methylamine also gave similar values (Boron & Roos, 1976), the small discrepancy (0·1–0·2 units) being best explained in this case by penetration of ionized methylamine. Likewise, Carter (1972) obtained equivalent values for pH_i in barnacle muscle fibres using DMO and nicotine. (As the latter author points out, this does not, of itself, negate the possibility of intracellular pH heterogeneity, though it does imply different compartmental parameters from those in sympathetic ganglia.) In rat muscle fibres, where $pH_{nic} \neq pH_{DMO}$ (Adler, 1972), there have been differing reports that pH_i measured

using micro-electrodes reflects either pH_{nic} (6·7, Paillard, 1972) or pH_{DMO} (7·1, Aickin & Thomas, 1977).

In our view, a plausible argument may be also made for the first option – an acid cytoplasm, with $pH_{base} \approx pH_{cyt}$, and pH_{DMO} biassed by alkaline organelles – along the following lines.

(i) There is direct autoradiographic evidence for a high cytoplasmic concentration of labelled nicotine in ganglion cells, with apparent exclusion from the nucleus (Applegren, Hansson & Schmiterlöw, 1963; Brown, Hoffman & Roth, 1969), suggesting the nucleus to be effectively more alkaline. This would accord with indicator measurements by Chambers & Chambers (1961) in various cells, showing a nuclear pH (7·6–7·8) about 1 unit above that of the cytoplasm (6·6–6·8). Further, isolated respiring mitochondria have been shown to generate a substantial pH gradient across their inner membranes, the inside being alkaline (e.g. Mitchell & Moyle, 1969; Nicholls, 1974). Although the intramitochondrial pH under normal conditions *in vivo* is uncertain, the maintenance of a proton gradient across the mitochondrial membrane is to be anticipated from current theories of energy transduction (Mitchell, 1961, 1976). Thus it seems likely that these two compartments, the nucleus and mitochondria, which comprise most of the residual cell volume (Brown & Halliwell, 1972), are effectively alkaline.

On the other hand, some intracellular organelles, such as lysosomes, are likely to be more acidic than the cytoplasm (Reijngoud & Tager, 1977). However, from inspection of electron micrographs, it seems very doubtful whether the proportion of the total ganglionic cell volume occupied by lysosomes ($< 0{\cdot}1\ \%$) is sufficiently great to take up a significant proportion of total intracellular base.

(ii) The relative constancy of pH_{nic} accompanying the large reduction of pH_{DMO} induced by uncouplers in ganglia may be explained most readily by a dissipation of pH gradients across the membranes of relatively small alkaline regions which had selectively accumulated DMO. The effect of DNP on brown adipose tissue differed slightly from that on ganglia in that the reduction of pH_{DMO} was accompanied by a near-equal elevation of pH_{nic}. On the basis of the proposed scheme, this may be explained as the result of a redistribution of H^+ between two subcellular compartments which are more nearly equal in size in the adipose tissue than in the ganglion. If on the other hand pH_{DMO} approximated to pH_{cyt} this would imply that the primary effect of the uncoupler was to increase the proton flux across the cell membrane such that H^+ distributed in accordance with the membrane potential. Under these conditions, depolarization of the cell membrane by (e.g.) K^+ should elevate pH_{DMO}: in practice, the changes in pH_{DMO} produced by depolarization in the presence of uncoupler are far less than those predicted by the requisite high transmembrane permeability (Fig. 7).

(iii) The transient effect of lactate on pH_{nic} (Fig. 6) accords with the effects of acid on (presumably) cytoplasmic pH measured with pH micro-electrodes (Boron & DeWeer, 1976; Thomas, 1976, 1977). Further, the buffering power calculated from the peak fall in pH_{nic} assuming $pH_{cyt} = 6{\cdot}5$ (50 slykes) also accords with that expected from micro-electrode measurements in other cells (see Thomas, 1974, 1976; Aickin & Thomas, 1975, 1977). If $pH_{cyt} = 7{\cdot}36$, an extraordinary buffering power of 182 slykes would be required to accommodate the sustained reduction (0·1 unit) in pH_{DMO}.

If this interpretation is correct, namely that $pH_{cyt} \approx pH_{base}$ and $pH_{cyt} < pH_{acid}$, then a weak acid alone becomes of limited use as an indicator either of resting cytoplasmic pH or of changes in cytoplasmic pH. For example, suppose, within the terms of our model, pH_{cyt} were raised by 0·5 units to pH 7·0, then a weak acid would register an increase of only 0·1 unit, from 7·3 to 7·4, whereas pH_{base} would rise from 6·55 to 7·05.

Although the validity of this particular model for a rather complex tissue is as yet uncertain, the present experiments do show that where direct measurement is impracticable, it is best to measure both pH_{base} and pH_{acid}; where these diverge the latter may not necessarily be the more appropriate measure of cytoplasmic pH.

We are grateful to S. Marsh for assistance in some of these experiments and to Monica Barton for preparing the manuscript. This investigation was aided by a grant from the American Medical Association Education and Research Foundation.

REFERENCES

ADAMS, P. R. & BROWN, D. A. (1975). Actions of γ-aminobutyric acid on sympathetic ganglion cells. *J. Physiol.* **250**, 85–120.

ADLER, S. (1972). The simultaneous determination of muscle cell pH using a weak acid and weak base. *J. clin. Invest.* **51**, 256–265.

AICKIN, C. C. & THOMAS, R. C. (1975). Micro-electrode measurement of the internal pH of crab muscle fibres. *J. Physiol.* **252**, 803–815.

AICKIN, C. C. & THOMAS, R. C. (1977). Micro-electrode measurement of the intracellular pH and buffering power of mouse soleus muscle fibres. *J. Physiol.* **267**, 791–810.

ALBERT, A. & SERJEANT, E. P. (1962). *Ionization Constants of Acids and Bases.* London: Methuen.

APPLEGREN, L-G., HANSSON, E. & Schmiterlöw, C. G. (1963). Localization of radioactivity in the superior cervical ganglion of cats following injection of C^{14}-labelled nicotine. *Acta physiol. scand.* **59**, 330–336.

BARLOW, R. B. & HAMILTON, J. T. (1962). Effect of pH on the activity of nicotine and nicotine monomethiodide on the rat diaphragm preparation. *Br. J. Pharmac. Chemother.* **18**, 543–549.

BONE, J. M., VERTH, A. & LAMBIE, A. T. (1976). Intracellular acid-base heterogeneity in nucleated avian erythrocytes. *Clin. Sci.* **51**, 189–196.

BORON, W. F. & DEWEER, P. (1976). Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *J. gen. Physiol.* **67**, 91–112.

BORON, W. W. & ROOS, A. (1976). Comparison of microelectrode, DMO and methylamine methods for measuring intracellular pH. *Am. J. Physiol.* **231**, 799–809.

BRAUSER, B., BÜCHER, TH. & DOLIVO, M. (1970). Redox transitions of cytochrome and pyridine nucleotides upon stimulation of an isolated rat ganglion. *FEBS Lett.* **8**, 297–300.

BROWN, D. A., BROWNSTEIN, M. J. & SCHOLFIELD, C. N. (1972). Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat. *Br. J. Pharmac.* **44**, 651–671.

BROWN, D. A. & HALLIWELL, J. V. (1972). Intracellular pH in rat isolated superior cervical ganglia in relation to nicotine-depolarization and nicotine-uptake. *Br. J. Pharmac.* **45**, 349–359. **45**, 349–359.

BROWN, D. A., HALLIWELL, J. V. & SCHOLFIELD, C. N. (1971). Uptake of nicotine and extracellular space markers by isolated rat ganglia in relation to receptor activation. *Br. J. Pharmac.* **42**, 100–113.

BROWN, D. A., HOFFMAN, P. C. & ROTH, L. J. (1969). [^{3}H]nicotine in cat superior cervical and nodose ganglia after close-arterial injection *in vivo*. *Br. J. Pharmac.* **35**, 406–417.

BROWN, D. A. & MARSH, S. (1975). A very simple method for recording ganglion depolarization. *J. Physiol.* **246**, 24–26*P*.

BROWN, D. A. & SCHOLFIELD, C. N. (1974*a*). Changes of intracellular sodium and potassium ion concentrations in isolated rat superior cervical ganglia induced by depolarizing agents. *J. Physiol.* **242**, 307–319.

BROWN, D. A. & SCHOLFIELD, C. N. (1974*b*). Movements of labelled sodium ions in isolated rat superior cervical ganglia. *J. Physiol.* **242**, 321–351.

CALDWELL, P. C. (1954). An investigation of the intracellular pH of crab muscle fibres by means of micro-glass and micro-tungsten electrodes. *J. Physiol.* **126**, 169–180.

CALDWELL, P. C. (1956). Intracellular pH. *Int. Rev. Cytol.* **5**, 229–277.

CALDWELL, P. C. (1958). Studies on the internal pH of large muscle and nerve fibres. *J. Physiol.* **142**, 22–62.

CARDIASIS, C. A., BLANC, W. A. & SINCLAIR, J. C. (1972). The effects of ambient temperature on the fasted newborn rabbit. II. Gross and microscopic changes in cervical and interscapular brown adipose tissue. *Biologia Neonat.* **21**, 347–360.

CARTER, N. W. (1972). Intracellular pH. *Kidney Int.* **1**, 341–346.

CHAMBERS, R. & CHAMBERS, E. L. (1961). *Exploration into the Nature of the Living Cell*, chaps. 5 and 6. Cambridge, Mass.: Harvard University Press.

EFFROS, R. M. & CHINARD, F. P. (1969). The *in vivo* pH of the extravascular space of the lung. *J. clin. Invest.* **48**, 1983–1996.

FITZSIMONS, E. J. & SENDROY, J. JR. (1961). Distribution of electrolytes in human blood. *J. biol. Chem.* **236**, 1595–1601.

FLATMARK, T. & PEDERSON, J. J. (1975). Brown adipose tissue mitochondria. *Biochim. biophys. Acta* **416**, 53–103.

FUNDER, J. & WIETH, J. W. (1966). Chloride and hydrogen ion distribution between human red cells and plasma. *Acta physiol. scand.* **68**, 234–245.

GARTHWAITE, J. (1976). The uptake of weak acids and bases into isolated rat superior cervical ganglia in relation to intracellular pH. *Br. J. Pharmac.* **56**, 353*P*.

GARTHWAITE, J. (1977). Drugs and intracellular pH of sympathetic ganglia. Ph.D. thesis University of London.

GREENGARD, P. & STRAUB, R. W. (1962). Metabolic studies on the hyperpolarization following activity in mammalian non-myelinated nerve fibres. *J. Physiol.* **161**, 414–423.

HEILBRONN, E. (1969). The effect of phospholipases on the uptake of atropine and acetylcholine by slices of mouse brain cortex. *J. Neurochem.* **16**, 621–635.

HEYTLER, P. G. & PRICHARD, W. W. (1962). A new class of uncoupling agents – carbonyl cyanide phenylhydrazones. *Biochem. biophys. Res. Commun.* **7**, 272–275.

HILPERT, P., FLEISCHMANN, R. G., KEMP, D. & BARTELS, H. (1963). The Bohr effect related to blood and erythrocyte pH. *Am. J. Physiol.* **205**, 337–340.

HINKE, J. A. M. & MENARD, M. R. (1976). Intracellular pH of single crustacean muscle fibres by the DMO and electrode methods during acid and alkaline conditions. *J. Physiol.* **262**, 533–552.

HUDGINS, P. M & PUTNEY, J. W. (1972). Distribution of local anaesthetics and the intracellular pH in vascular smooth muscle. *J. Pharmac. exp. Ther.* **181**, 538–546.

HULL, D. & SEGALL, M. M. (1966). Distinction of brown from white adipose tissue. *Nature, Lond.* **212**, 469–472.

IRVINE, R. O. H., SAUNDERS, S. J., MILNE, M. D. & CRAWFORD, M. A. (1960). Gradients of potassium and hydrogen ions in potassium-deficient voluntary muscle. *Clin. Sci.* **20**, 1–18.

JOEL, C. D. (1965). The physiological role of brown adipose tissue. In *Handbook of Physiology*, section 5, ed. RENOLD, A. E. & CAHILL, G. F. JR., chap. 9, pp. 59–83. Washington: Am. Physiol. Soc.

JOEL, C. D. (1966). Stimulation of metabolism of rat brown adipose tissue by addition of lipolytic hormones *in vitro*. *J. biol. Chem.* **241**, 814–821.

KORTÜM, G., VOGEL, W. & ANDRUSSOW, K. (1961). *Dissoziations Konstanten Organischer Säuren in Wässeriger Losung.* London: Butterworths.

LEO, A., HANSCH, C. & ELKINS, D. (1971). Partition coefficients and their uses. *Chem. Rev.* **71**, 525–616.

LINDGREN, G. & BARNARD, T. (1972). Changes in interscapular brown adipose tissue of rat. during perinatal and early postnatal development and after cold acclimation. *Expl Cell Res.* **70**, 81–90.

MAHIN, D. T. & LOFTBERG, R. T. (1966). A simplified method of sample preparation for determination of tritium, carbon-14, or sulphur-35 in blood or tissue by liquid scintillation counting. *Analyt. Biochem.* **16**, 500–509.

MITCHELL, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature, Lond.* **191**, 144–148.

MITCHELL, P. (1976). Vectorial chemistry and the molecular mechanics of chemiosmotic coupling: power transmission by proticity. *Biochem. Soc. Trans.* **4**, 399–430.

MITCHELL, P. & MOYLE, J. (1969). Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. *Eur. Jl Biochem.* **7**, 471–484.

NICHOLLS, D. G. (1974). The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution. *Eur. Jl Biochem.* **50**, 305–315.

PAILLARD, M. (1972). Direct intracellular pH measurement in rat and crab muscle. *J. Physiol.* **223**, 297–319.

PATON, W. D. M. & RANG, H. P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc.* B **163**, 1–44.

PERRIN, D. D. (1965). *Dissociation Constants of Organic Bases in Aqueous Solution.* London: Butterworths.

PUTNEY, J. W. & BORZELLECA, J. F. (1971). On the mechanisms of [^{14}C]nicotine distribution in the rat submaxillary gland *in vitro*. *J. Pharmac. exp. Ther.* **178**, 180–191.

REIJNGOUD, D.-J. & TAGER, J. M. (1977). The permeability properties of the lysosomal membrane. *Biochim. biophys. Acta* **472**, 419–449.

ROBSON, J. S., BONE, J. M. & LAMBIE, A. T. (1968). Intracellular pH. *Adv. clin. Chem.* **11**, 213–275.

ROOS, A. (1965). Intracellular pH and intracellular buffering power of the cat brain. *Am. J. Physiol.* **209**, 1233–1246.

SMITH, I. (1969). *Chromatographic and Electrophoretic Techniques*, vol. 1, 3rd ed. London: Heinemann.

SMITH, R. E. & HORWITZ, B. A. (1969). Brown fat and thermogenesis. *Physiol. Rev.* **49**, 330–425.

STAHL, E. (Ed.) (1965). *Thin Layer Chromatography.* Berlin: Springer.

SUTER, E. R. (1969). The fine structure of brown adipose tissue. I. Cold-induced changes in the rat. *J. Ultrastruct. Res.* **26**, 216–241.

TELLER, D. N., DE GUZMAN, T. & LAJTHA, A. (1974). The mode of morphine uptake into brain slices. *Brain Res.* **77**, 125–126.

THOMAS, R. C. (1974). Intracellular pH of snail neurones measured with a new pH-sensitive glass micro-electrode. *J. Physiol.* **238**, 159–180.

THOMAS, R. C. (1976). The effect of carbon dioxide on the intracellular pH and buffering power of snail neurones. *J. Physiol.* **255**, 715–735.

THOMAS, R. C. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *J. Physiol.* **273**, 317–338.

WADDELL, W. J. & BUTLER, T. C. (1959). Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog. *J. clin. Invest.* **38**, 720–729.

WHITTAM, R. & CHIPPERFIELD, A. R. (1975). The reaction mechanism of the sodium pump. pump. *Biochim. biophys. Acta* **415**, 149–171.