J. Physiol. (1977) **264**, *pp.* 489–509
With 10 *text-figures*
Printed in Great Britain

THE EFFECT OF PREGANGLIONIC NERVE STIMULATION ON THE ACCUMULATION OF CERTAIN ANALOGUES OF CHOLINE BY A SYMPATHETIC GANGLION

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(*Received 7 June 1976*)

SUMMARY

1. Cat superior cervical ganglia were perfused with a Krebs solution containing 10^{-6} M [^{3}H]homocholine (2-hydroxypropyl-trimethylammonium) or 10^{-5} M [^{14}C]triethylcholine (2-hydroxyethyl-triethylammonium). Preganglionic nerve stimulation (20 Hz) increased the accumulation of homocholine (3·2-fold) and of triethylcholine (2·1-fold). This increased accumulation during stimulation was not the result of increased metabolism.

2. The increased accumulation of homocholine or triethylcholine induced by preganglionic nerve stimulation was not reduced by tubocurarine or by atropine, but it was blocked by choline and by hemicholinium. These results suggested that preganglionic nerve stimulation increased choline analogue accumulation into cholinergic nerve terminals.

3. The increased accumulation of homocholine or of triethylcholine induced by preganglionic nerve stimulation was reduced when the Ca^{2+} concentration was reduced and was abolished in the absence of Ca^{2+}. However, changes in the Mg^{2+} concentration which depressed acetylcholine (ACh) release by amounts comparable to those induced by altered Ca^{2+} concentrations did not alter the uptake of homocholine or triethylcholine. It is concluded that the uptake of choline analogues is not regulated by transmitter release but that stimulation increases the uptake of the choline analogues by a Ca^{2+}-dependent mechanism.

4. The accumulation of ACh by ganglia perfused with a Krebs solution containing choline and high $MgSO_4$ (18 mM) was measured. The ACh content of these ganglia did not increase, although choline transport presumably exceeded that necessary for ACh synthesis to replace released ACh. It is concluded that choline transport does not limit ACh synthesis in ganglia.

INTRODUCTION

Cholinergic nerves cannot synthesize choline (Bremer & Greenberg, 1961; Ansell & Spanner, 1967, 1971; Browning & Schulman, 1968), the immediate precursor of the neurotransmitter acetylcholine (ACh), and they are dependent upon extracellular choline for ACh synthesis. Studies on isolated synaptosomes prepared from brain have suggested that choline for ACh synthesis is transported into cholinergic nerve terminals by a high affinity uptake mechanism (Haga, 1971; Yamamura & Snyder, 1972, 1973; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973*a*; Haga & Noda, 1973; Kuhar, Sethy, Roth & Aghajanian, 1973; Sorimachi & Kataoka, 1974; Carroll & Buterbaugh, 1975; Guyenet, Lefresne, Beaujouan & Glowinski, 1975). Based on indirect evidence from experiments on the cat's superior cervical ganglion, Collier & Katz (1974) suggested that preganglionic nerve stimulation accelerates choline uptake into cholinergic nerve terminals.

The first objective of the present experiments was to test more directly the suggestion that nerve stimulation accelerates choline uptake, and to determine whether the increased accumulation induced by stimulation was a consequence of ACh release or of nerve terminal depolarization. To measure choline transport without the complication of choline acetylation, we used two analogues of choline, γ-homocholine (2-hydroxypropyl-trimethylammonium) and triethylcholine (2-hydroxyethyl-triethyl-ammonium). These compounds appear to be transported by the choline transport system (Potter, 1968; Hemsworth & Bosmann, 1971; Acara, Kowalski, Rennick & Hemsworth, 1975; B. Collier, S. Lovat, D. Ilson, L. A. Barker & T. W. Mittag, in preparation), but they are poor substrates for choline acetyltransferase (Burgen, Burke & Desbarates-Schonbaum, 1956; Dauterman & Mehrotra, 1963; Hemsworth & Morris, 1964; Hemsworth & Smith, 1970; Currier & Mautner, 1974; Barker & Mittag, 1975; Mann & Hebb, 1975).

The second objective of the present studies was to determine if the rate of choline transport determines the rate of ACh synthesis. The processes of ACh release and ACh synthesis are closely linked (Mann, Tennenbaum & Quastel, 1938; Birks & MacIntosh, 1961; Browning & Schulman, 1968; Collier & MacIntosh, 1969; Sharkawi & Schulman, 1969; Potter, 1970; Grewaal & Quastel, 1973); at rest transmitter release and ACh synthesis are slow and both increase during activity such that tissue ACh levels are maintained fairly constant. The mechanism by which ACh synthesis is controlled is not known with certainty, but it has been suggested that choline transport might be the rate-limiting process in ACh synthesis (Whittaker, Dowdall & Boyne, 1972; Yamamura & Snyder, 1972; Guyenet

et al. 1973*a*; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973*b*; Mulder, Yamamura, Kuhar & Snyder, 1974; Barker & Mittag, 1975; Kuhar, DeHaven, Yamamura, Rommelspacher & Simon, 1975; Simon & Kuhar, 1975; Whittaker & Dowdall, 1975). The present experiments measured the ACh content of ganglia that had been exposed to conditions under which the amount of choline accumulated by nerve terminals should have exceeded that necessary to replenish released ACh. The results indicate that choline transport may not be the rate-limiting process in ACh synthesis in the cat superior cervical ganglion.

A preliminary account of some of the results was presented at a meeting (Collier & Ilson 1976).

METHODS

Ganglion perfusion

Cats (1·6–3·0 kg) of either sex were used; anaesthesia was induced by ethyl chloride followed by ether and was maintained by chloralose (I.V., 80 mg/kg). Superior cervical ganglia were prepared for perfusion by Kibjakow's (1933) procedure as modified by Feldberg & Gaddum (1934). Perfusion was with Krebs solution (composition in mM: NaCl 120, KCl 4·6, $CaCl_2$ 2·4, KH_2PO_4 1·2, $MgSO_4.7H_2O$ 1·2, glucose 9·9, $NaHCO_3$ 25), which was equilibrated with 5 % CO_2 in O_2 so that the pH was approximately 7·4 at 37° C. In some experiments the Krebs solution contained low Ca^{2+} (0·5 mM-$CaCl_2$), high Mg^{2+} (18 mM-$MgSO_4.7H_2O$), both low Ca^{2+} and high Mg^{2+}, or was Ca^{2+}-free (no added $CaCl_2$ and containing 10^{-4} M [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA; Eastman)). When the accumulation of choline analogues was measured, the perfusion fluid contained either [^{3}H]homocholine iodide (N-methyl labelled; 900 mci/m-mole; 10^{-6} M; generously provided by Drs A. L. Barker and T. W. Mittag, Mount Sinai Medical School, New York) or [^{14}C]triethylcholine iodide (N-ethyl labelled; 8·6 mci/m-mole; 10^{-5} M; Amersham-Searle). Other drugs added to the perfusion fluid in some experiments were: tubocurarine (3×10^{-5} M, Calbiochem), choline chloride (5×10^{-6} M, 10^{-5} M, or 5×10^{-5} M, British Drug Houses), hemicholinium-3 dibromide (5×10^{-6} M or 5×10^{-5} M, provided by the late Dr V. B. Haarstad), eserine sulphate ($3{\cdot}5 \times 10^{-5}$ M, Nutritional Biochemicals Corp.), atropine sulphate (10^{-6} M, Nutritional Biochemicals Corp.).

The preganglionic sympathetic trunk was cut low in the neck in all experiments. When necessary the nerve was stimulated with supramaximal rectangular pulses (8–10 V, 0·3 msec, 10 or 20 Hz). During stimulation, the electrode was moved a few mm proximally along the nerve every 5 min.

Preparation of ganglion extracts and identification of radioactive products

At the end of each experiment, the ganglia were removed, minced finely in 2 ml. ice-cold 10 % (w/v) trichloroacetic acid and allowed to stand (0° C) for 60 min; the trichloroacetic acid was removed from the extracts by shaking them with water-saturated ether. Separate aliquots of the aqueous extracts (pH 4–6) were used for liquid scintillation counting and for the separation of unchanged homocholine or unchanged triethylcholine from their metabolic products. Radioactivity incorporated into phospholipids was extracted from the tissue residue by overnight treatment with 1 ml. of a mixture of chloroform and methanol (2:1) containing hydrochloric acid (HCl, 0·25 %).

The amount of phosphorylated homocholine or phosphorylated triethylcholine in

the aqueous extracts of ganglia was determined by shaking aliquots of the extract with an equal volume of tetraphenylboron in heptanone (10 mg/ml.); > 99 % of the phosphorylated compounds remained in the aqueous phase and > 90 % of the non-phosphorylated compounds complexed with the tetraphenylboron and was recovered with the organic phase. The distribution of radioactivity between the two phases indicated the amount of phosphorylated homocholine or triethylcholine formed by the ganglion. Standards of phosphorylated homocholine or triethylcholine were prepared by incubating the bases with choline kinase (EC 2.7.1.32), followed by purification by thin-layer chromatography as described below.

Acetylhomocholine in the extracts of ganglia was separated from unchanged homocholine by thin-layer chromatography. Both compounds were extracted into heptanone containing tetraphenylboron, they were then recovered from the organic phase by shaking it with HCl (1 N; 2 vol. organic: 1 vol. acid) and the HCl was removed by evaporation in a vacuum centrifuge. The residue was dissolved in 10 μl. H_2O and applied to a thin-layer chromatography plate (Polygram CEL 300 PEI, Macherey-Nagel, Düren, Germany) which was developed for 2–3 hr in butanol: ethanol:acetic acid:water (8:2:1:3). The plates were dried, cut into 0·3 cm pieces and each piece was placed into a scintillation vial containing 10 ml. of the dioxane-containing mixture described previously (Collier & Lang, 1969). Radioactivity of each segment was determined by liquid scintillation spectrometry with an efficiency of approx. 20 %. This procedure separated acetylhomocholine (R_F about 0·7) from unchanged homocholine (R_F about 0·5) and this was confirmed in each experiment by simultaneous chromatography of standards processed in the same way as the test samples. For this purpose, [^{3}H]acetylhomocholine was prepared by reacting [^{3}H]homocholine with acetylchloride.

To separate acetyltriethylcholine in the extracts of ganglia from unchanged triethylcholine, both compounds were extracted into heptanone containing tetraphenylboron, then into HCl and dried (as described above). The unchanged triethylcholine was converted to phosphoryltriethylcholine by incubation (30 min at 30° C) with choline kinase (prepared from yeast; see Kato, Collier, Ilson & Wright, 1975) in 100 μl. of a reaction medium (pH 8) that contained ATP (4 mM), $MgCl_2$ (12·5 mM), glycylglycine (83 mM) and choline kinase (0·025 units; one unit phosphorylated 1 μmole of choline/min under these conditions). The phosphoryltriethylcholine so formed was separated from unchanged acetyltriethylcholine by shaking the aqueous incubation mixture with an equal volume of tetraphenylboron in heptanone; phosphoryltriethylcholine was recovered with the aqueous phase, and acetyltriethylcholine was recovered with the organic phase. In each experiment, standard amounts of [^{14}C]triethylcholine and [^{14}C]acetyltriethylcholine were treated in the same way as were the samples; for this [^{14}C]acetyltriethylcholine was prepared by reacting [^{14}C]triethylcholine with acetylchloride.

During the procedures used to separate homocholine from acetylhomocholine, and triethylcholine from acetyltriethylcholine, the break-down of the acetylated derivatives was 10–15 %.

Assay of ACh

The ACh content of ganglia or of the effluent collected from ganglia was determined by the radio-enzymic assay of Goldberg & McCaman (1973) as described previously (Kato *et al.* 1975). ACh was extracted from aqueous ganglion extracts or from ganglion effluents by tetraphenylboron dissolved in heptanone (10 mg/ml.); ACh was then recovered from the organic phase by shaking it with HCl (1 N) and the acid phase was evaporated to dryness in a vacuum centrifuge. Each of the dried samples was dissolved in 30 μl. of a reaction medium (pH 8) that contained ATP (0·8 mM),

$MgCl_2$ (12·5 mM), glycylglycine (83 mM) and choline kinase (0·005 units; see above) and the samples were incubated at 30° C for 15 min to phosphorylate all endogenous choline in the samples. Five μl. of a solution containing acetylcholinesterase (EC 3.1.1.7; 1 μg protein containing 1·4 units; Type V; Sigma) and [^{32}P]ATP (about 0·4 μCi; 20–40 Ci/m-mole; New England Nuclear) was then added to each sample which was incubated at 30° C for 10 min. During this second incubation the sample's ACh was hydrolysed and the choline formed was quantitatively phosphorylated to [^{32}P]phosphorylcholine. The reaction was stopped by adding 100 μl. ice-cold water to each sample and the radioactive phosphorylcholine generated from the sample's ACh was separated from unchanged radioactive ATP by ion exchange chromatography on a 50 × 5 mm column of Dowex AG 1-X8 (formate form). Phosphorylcholine was eluted by 1·8 ml. ammonium formate (75 mM), and unchanged ATP was left at the top of the column. The radioactivity collected in the phosphorylcholine fraction from the Dowex column was determined by a liquid scintillation spectrometer using Hydromix (Canatech) as the solvent system. In each experiment, standard amounts of acetylcholine chloride (0–200 p-mole) in water (for assay of ganglion contents) or in Krebs solution (for assay of ganglion effluents) were extracted and assayed at the same time as the test samples; standards in Krebs solutions with the altered Ca^{2+} or Mg^{2+} content used were not significantly different from those using normal Krebs solution. The value obtained for the blank sample (no added ACh) was subtracted from that for all other samples and the radioactivity generated from the ACh standards was used to calculate the amount of ACh in the test samples. In some experiments a blank was prepared by incubating a test sample with acetylcholinesterase before it was assayed; these blanks did not differ from those prepared from Krebs solution alone, showing that the first incubation with choline kinase removed all endogenous choline in the test samples.

In a few experiments, choline kinase (purchased from Sigma) was used in this ACh assay. This enzyme required the presence of dithiothreitol (4×10^{-3} M) in the incubation medium for activity.

Determination of radioactivity

Radioactivity was determined by liquid scintillation spectrometry (Intertechnique) using Hydromix as the solvent system, except where noted for the thin-layer plates. Samples were corrected for quench by adding internal standards; [^{32}P] was determined with close to 100 % efficiency, [^{14}C] with about 87 % efficiency and [^{3}H] with about 39 % efficiency.

RESULTS

1. *Accumulation of choline analogues by stimulated and non-stimulated ganglia*

To test whether preganglionic nerve stimulation increased the accumulation of homocholine or triethylcholine by ganglia, both left and right ganglia were perfused for 20 min with a Krebs solution containing [^{14}C]triethylcholine (10^{-5} M) or [^{3}H]homocholine (10^{-6} M); in each animal, one ganglion was stimulated (20 Hz) continuously and the contralateral one was not. At the end of the experiment, the ganglia were removed and the accumulation of unchanged choline analogue and its metabolic products was determined. Stimulation clearly increased the amount of radioactivity accumulated by ganglia (Fig. 1). The increase in accumulated radioactivity

during stimulation was due to an increased uptake of unchanged homocholine or triethylcholine and was not due to accelerated metabolism of the analogues. In all experiments, the only metabolite of either analogue that was detected under the conditions of the experiments was phosphorylated product. The amount of acetylated homocholine or triethylcholine formed was below the level of detection of the methods used which would have

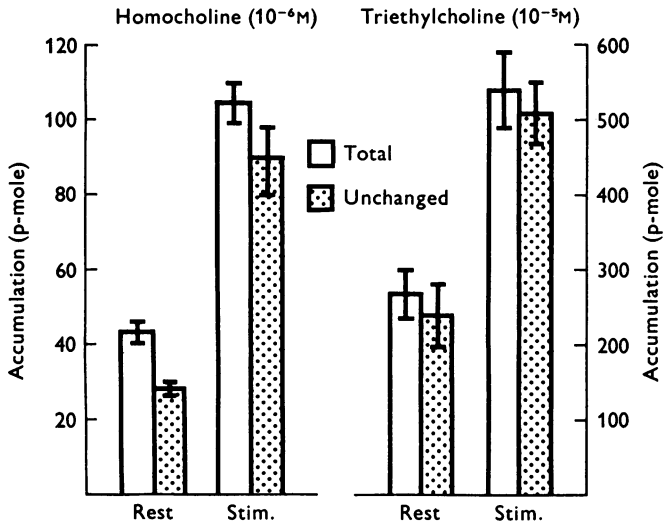

Fig. 1. Effect of stimulation on the accumulation of choline analogues by ganglia. The accumulation (total and unchanged (i.e. non-metabolized)) of [^{3}H]homocholine and [^{14}C]triethylcholine by cat superior cervical ganglia was measured; ganglia were perfused either at rest or during preganglionic nerve stimulation (20 Hz) for 20 min with Krebs solution containing either [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M). Each column represents the mean ± S.E. of at least four experiments.

detected acetylated products if they represented > 5% of the total radioactivity accumulated. The amount of phosphorylhomocholine or phosphoryltriethylcholine formed by stimulated and rested ganglia was similar; there was a considerable variation between experiments in the amount of analogue phosphorylated, but, in any one experiment, the variation between the left and the right ganglion was < 15%. In all experiments, the amount of radioactivity incorporated into phospholipids was < 4% of the amount of radioactivity extracted by trichloroacetic acid. The amount of unchanged choline analogue accumulated by ganglia was used as the measure of choline analogue uptake for the rest of the results presented in this paper.

The accumulation of unchanged choline analogue by rested ganglia

represented clearance of approx. 28 μl. perfusion fluid in the experiments that used homocholine, and approx. 24 μl. in the experiments that used triethylcholine; therefore, the accumulation of the choline analogues by the ganglia represents more than trapped extracellular fluid (the ganglion extracellular space is approx. 4 μl. (Collier & MacIntosh, 1969)).

2. *Lack of effect of tubocurarine or atropine on choline analogue accumulation by stimulated ganglia*

The enhanced accumulation of the choline analogues during preganglionic nerve stimulation might represent enhanced uptake into preganglionic nerve terminals or into structures depolarized by the released ACh.

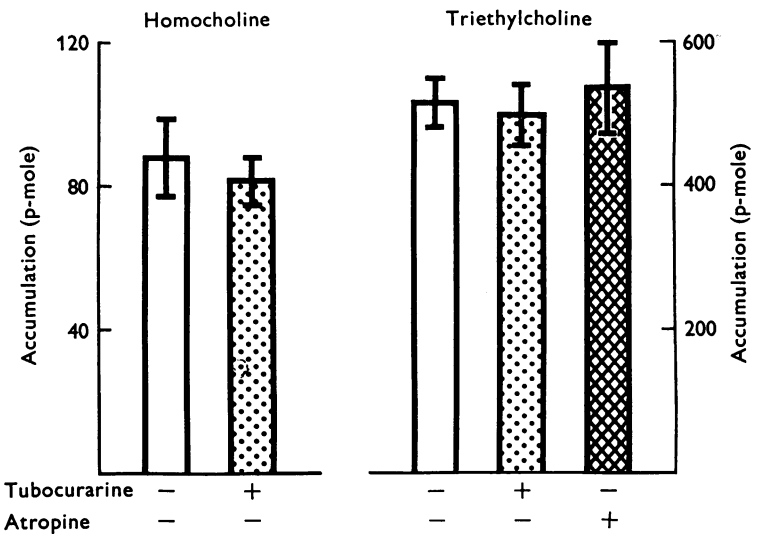

Fig. 2. Lack of effect of ACh blocking agents on the accumulation of choline analogues by ganglia. Cat superior cervical ganglia were perfused for 20 min with a Krebs solution containing [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M) with or without (as indicated) atropine (10^{-6} M) or tubocurarine (3×10^{-5} M); the preganglionic nerve was stimulated throughout (20 Hz). Each column represents the mean ± S.E. of four experiments.

To distinguish between these two possibilities, stimulated ganglia were perfused for 20 min with Krebs solution containing [^{3}H]homocholine or [^{14}C]triethylcholine together with a ganglion-blocking concentration (3×10^{-5} M) of tubocurarine to block nicotinic receptors, or with 10^{-6} M atropine to block muscarinic receptors. The accumulation of homocholine or triethylcholine was not reduced by tubocurarine and the accumulation of triethylcholine was not reduced by atropine (Fig. 2; limited quantities

of homocholine precluded testing the effects of atropine on homocholine accumulation). Thus, the increased accumulation of choline analogues during stimulation appeared not to be the result of an action of released ACh on post-ganglionic cell bodies or on other structures.

3. *Inhibition of choline analogue accumulation by choline or by hemicholinium*

If the choline analogues were accumulated within the preganglionic nerve terminals by the choline transport system, their accumulation should be inhibited by choline or by hemicholinium. To test this, ganglia at rest were first perfused for 15 min with a Krebs solution containing choline (5×10^{-6} M for experiments with homocholine; 5×10^{-5} M for experiments with triethylcholine) or hemicholinium (same concentrations as for choline), and then for 20 min with the same solution containing either [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M). One ganglion was rested and the other was stimulated (20 Hz) during exposure to the choline

TABLE 1. The effect of hemicholinium (HC-3) or choline on the accumulation of [^{3}H]homocholine or of [^{14}C]triethylcholine by stimulated (20 Hz) or rested ganglia

	Accumulation of [^{3}H]homocholine (p-mole)			Accumulation of [^{14}C]triethylcholine (p-mole)	
	Stim.	Rest		Stim.	Rest
Control	90 ± 8	28 ± 3	Control	509 ± 43	241 ± 38
HC-3 (5×10^{-6} M)	36 ± 4	33 ± 4	HC-3 (5×10^{-5} M)	216 ± 23	220 ± 8
Choline (5×10^{-6} M)	42 ± 4	34 ± 3	Choline (5×10^{-5} M)	261 ± 32	263 ± 36

The results are given as mean ± S.E. of four experiments.

analogue, and the accumulation of homocholine or triethylcholine was compared to that measured in the absence of choline or hemicholinium (Table 1). The accumulation of choline analogues by resting ganglia was not measurably reduced by choline or by hemicholinium but both of these compounds completely blocked the enhanced accumulation of homocholine or of triethylcholine induced by preganglionic nerve stimulation.

4. *Effects of Ca^{2+} and Mg^{2+} on choline analogue accumulation and on ACh release*

These experiments were designed to test whether the enhanced uptake of choline analogues by ganglia during preganglionic nerve stimulation was the result of increased ACh release or was a consequence *per se* of nerve terminal depolarization. One way to vary the release of ACh while maintaining a constant impulse flow was to reduce the Ca^{2+} concentration in the perfusion medium. First, the effect of altered Ca^{2+} on the amount of ACh released during preganglionic nerve stimulation (20 Hz for 2 min) was

tested. In these experiments perfusion was started with normal (2·4 mM) $CaCl_2$-Krebs solution containing eserine ($3{\cdot}5 \times 10^{-5}$ M) to preserve the released ACh, and release was tested during two separate 2 min periods of preganglionic nerve stimulation; perfusion was then switched to an eserine-Krebs solution containing lowered (0·5 mM) or zero $CaCl_2$, and 15 and 45 min later the release of ACh was again tested (20 Hz for 2 min); finally perfusion was returned to eserine-normal Ca^{2+}-Krebs solution and control ACh release was tested again. The amount of ACh released during

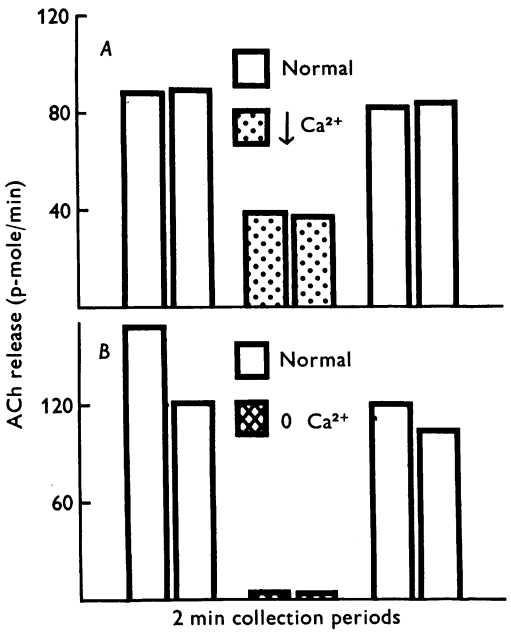

Fig. 3. Release of ACh from ganglia perfused with Krebs solution containing eserine. Each column represents the amount of ACh released from a cat's superior cervical ganglion by preganglionic nerve stimulation (20 Hz for 2 min). The open columns represent release during perfusion with medium containing normal (2·4 mM) $CaCl_2$; in *A* the middle columns represent release in the presence of 0·5 mM-$CaCl_2$, and in *B* the middle columns represent release in the absence of Ca^{2+}.

each period of stimulation was measured. Fig. 3 shows the results of two typical experiments, one testing a 0·5 mM-$CaCl_2$ (Fig. 3*A*) and one testing a Ca^{2+}-free Krebs solution (Fig. 3*B*); ACh release was reduced to about 40% by the low Ca^{2+} medium and was undetectable in the experiments with Ca^{2+}-free medium. In four such experiments, the control ACh release was calculated as the mean of the four tests in the presence of normal Ca^{2+} and the ACh release during perfusion with Krebs solution of altered Ca^{2+}

concentration was compared to this control value. Release in 0·5 mM-$CaCl_2$ was 41 ± 4% (mean ± S.E.) of the control release and release in the absence of Ca^{2+} was below the sensitivity of the assay used (< 5% of control).

The effect of altered Ca^{2+} on the accumulation of homocholine or triethylcholine was then measured. In these experiments, ganglia (not stimulated) were perfused for 15 min with medium containing the altered Ca^{2+} concentration and then for 20 min with the same solution containing

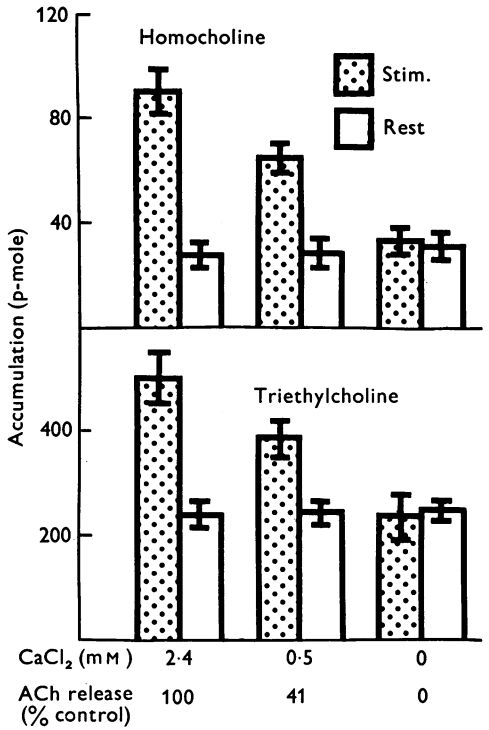

Fig. 4. Effect of altered Ca^{2+} on the accumulation of choline analogues by ganglia. Cat superior cervical ganglia were perfused for 20 min with Krebs solution containing varied Ca^{2+} ($CaCl_2$ concentration indicated) and either [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M); the preganglionic nerve was either stimulated throughout (20 Hz) or was rested. Each column represents the mean ± S.E. of at least four experiments. ACh release was calculated (see text) from the release measured in other experiments; release is expressed as % of the release in Krebs solution containing normal (2·4 mM) $CaCl_2$.

either [^{3}H]homocholine or [^{14}C]triethylcholine during which time the preganglionic nerve was either rested or stimulated. Fig. 4 summarizes the results of these experiments. In the absence of Ca^{2+}, stimulated ganglia accumulated no more homocholine or triethylcholine than did rested

ganglia; in the presence of 0·5 mM-$CaCl_2$, the increased accumulation of homocholine and triethylcholine induced by stimulation was reduced to about 56% and 54% (respectively) of that in the normal Ca^{2+} medium. These changes paralleled the alterations of ACh release that resulted from the changes in Ca^{2+} concentration. The accumulation of homocholine and triethylcholine by rested ganglia was not appreciably altered by the decrease or the complete removal of Ca^{2+} from the perfusion fluid.

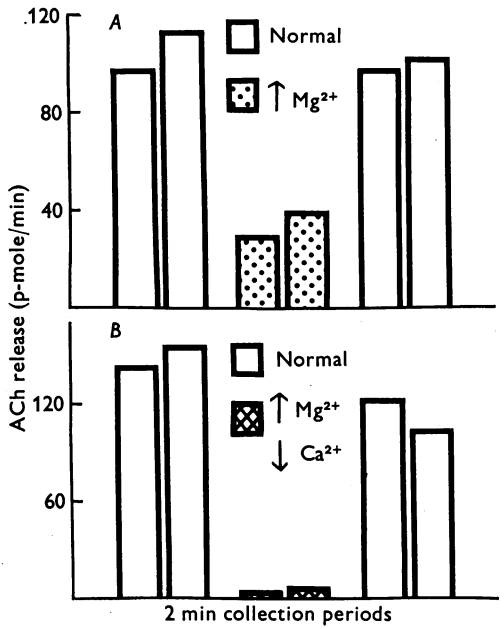

Fig. 5. Release of ACh from ganglia perfused with Krebs solution containing eserine. Each column represents the amount of ACh released from cat's superior cervical ganglion by preganglionic nerve stimulation (20 Hz for 2 min). The open columns represent release during perfusion with medium containing normal (1·2 mM) $MgSO_4$-normal (2·4 mM) $CaCl_2$; in *A* the middle columns represent release in the presence of 18 mM-$MgSO_4$, and in *B* the middle columns represent release in the presence of 18 mM-$MgSO_4$ 0·5 mM-$CaCl_2$.

The decreased accumulation of homocholine and triethylcholine by stimulated ganglia in the presence of lowered Ca^{2+} could indicate that the choline transport mechanism is regulated by the release of ACh, or it could indicate that choline transport is regulated by nerve impulses but that the mechanism is Ca^{2+}-dependent. To differentiate between these two possibilities, the effects of raised $MgSO_4$ (18 mM) on ACh release and choline analogue accumulation were tested. The protocol of these experiments was

the same as for those described above using medium of altered Ca^{2+} content. Fig. 5*A* shows the results of a typical experiment that measured ACh release; in the presence of high Mg^{2+}, ACh release was reduced to about 40% of the control releases. In four such experiments, ACh release was reduced to $37 \pm 3\%$ (mean ± S.E.) of the control release by high Mg^{2+}. However, in contrast to the experiments with decreased Ca^{2+}, where changes in choline analogue accumulation paralleled changes in ACh

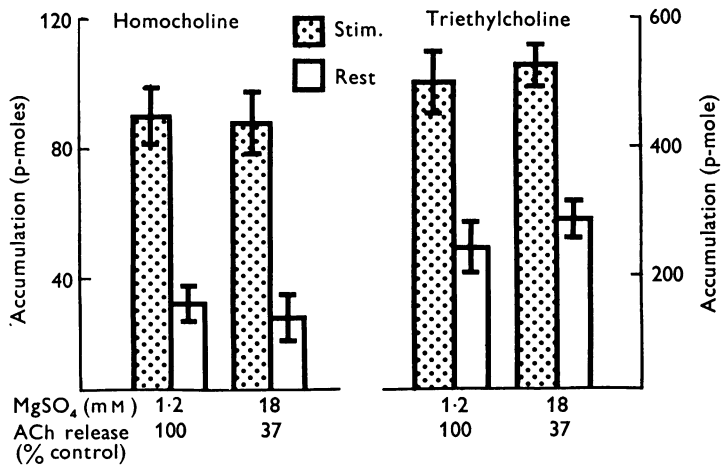

Fig. 6. Lack of effect of high Mg^{2+} on the accumulation of choline analogues by ganglia. Cat superior cervical ganglia were perfused for 20 min with Krebs solution containing varied Mg^{2+} ($MgSO_4$ concentration indicated) and either [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M); the preganglionic nerve was either stimulated throughout (20 Hz) or was rested. Each column represents the mean ± S.E. of at least four experiments. ACh release was calculated (see text) from the release measured in other experiments; release is expressed as % of the release in Krebs solution containing normal (1·2 mM) $MgSO_4$.

release, the accumulation of homocholine and triethylcholine by stimulated ganglia was not significantly altered by increasing the Mg^{2+} concentration in the perfusion medium ($P > 0{\cdot}3$ for both comparisons; Fig. 6). This result was confirmed by other experiments which measured the accumulation of [^{3}H]homocholine or [^{14}C]triethylcholine by stimulated ganglia in the presence of eserine. These experiments were done because, in the absence of an anticholinesterase agent, changes in choline analogue uptake induced by altered ACh release might have been masked by an altered concentration of choline, derived from hydrolysed ACh, in the synaptic cleft. Choline produced by the hydrolysis of released ACh has been shown to be recaptured by ganglia (Collier & Katz, 1974). To test if the

accumulation of homocholine or triethylcholine in the presence of a high Mg^{2+} medium was reduced (compared to the accumulation in normal Mg^{2+} medium) when released ACh was preserved, ganglia were perfused for 20 min with Krebs solution containing [^{3}H]homocholine or [^{14}C]triethylcholine in the presence of eserine ($3{\cdot}5 \times 10^{-5}$ M). In each experiment, both the left and the right ganglia were stimulated throughout the 20 min exposure to the choline analogue, one ganglion was perfused with normal $MgSO_4$ Krebs (1·2 mM) and the other with high $MgSO_4$ (18 mM) Krebs solution. Under these conditions, the accumulation of homocholine or triethylcholine was not reduced by raised Mg^{2+} (Fig. 7).

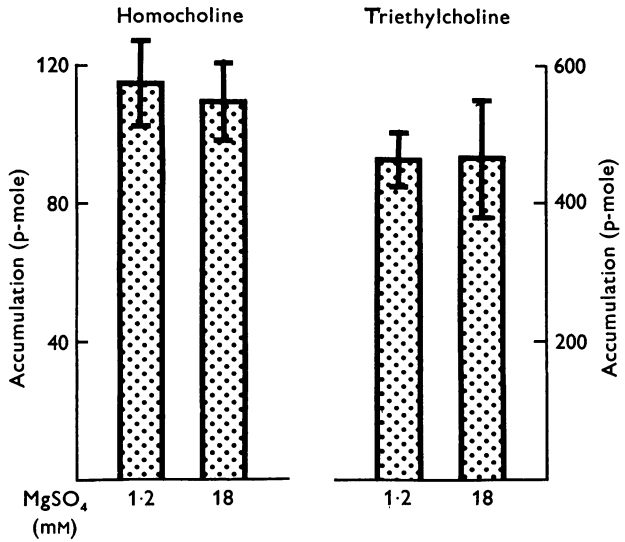

Fig. 7. Lack of effect of high Mg^{2+} on the accumulation of choline analogues by ganglia exposed to eserine. Cat superior cervical ganglia were perfused for 20 min with Krebs solution containing eserine ($3{\cdot}5 \times 10^{-5}$ M), varied Mg^{2+} ($MgSO_4$ concentration indicated) and either [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine 10^{-5} M; the preganglionic nerve was stimulated (20 Hz) throughout. Each column represents the mean ± S.E. of at least four experiments.

To further test the relationship of ACh release and choline analogue accumulation, ganglia were perfused with a medium containing 0·5 mM-$CaCl_2$ and 18 mM-$MgSO_4$. ACh release during preganglionic nerve stimulation was abolished in this medium (Fig. 5*B*), but the amount of homocholine or of triethylcholine accumulated by stimulated and rested ganglia was not less than the amount that was accumulated in lowered Ca^{2+} medium (Fig. 8).

5. *Effect of frequency of preganglionic nerve stimulation on choline analogue accumulation*

To test if the accumulation of choline analogues is regulated by the frequency of preganglionic nerve stimulation, ganglia were stimulated at either 20 or 10 Hz throughout the 20 min period of exposure to [^{3}H]homocholine or [^{14}C]triethylcholine. Fig. 9 summarizes the results: reducing the frequency of stimulation by 50 % decreased the net (stimulated minus rested) accumulation of homocholine by approx. 53 % and of triethylcholine by approx. 58 %.

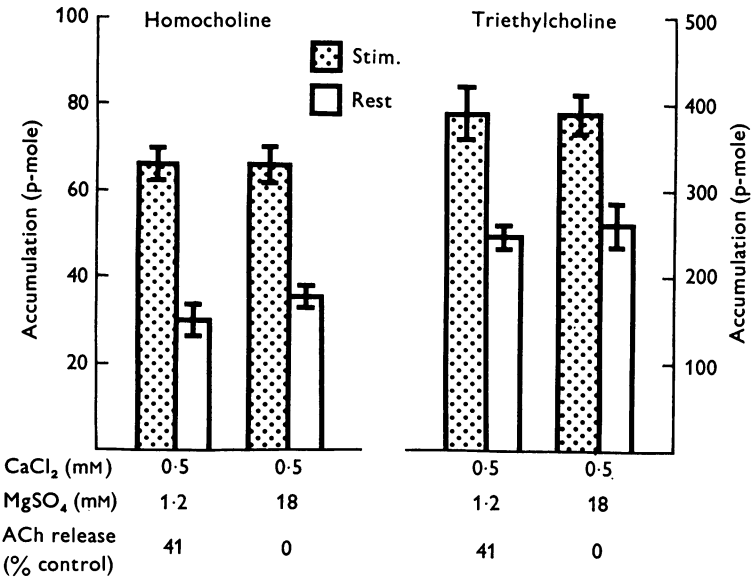

Fig. 8. Dissociation of ACh release and increased choline analogue accumulation by stimulated ganglia. Cat superior cervical ganglia were perfused for 20 min with Krebs solution containing varied Ca^{2+} and Mg^{2+} (concentrations as indicated) and either [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M); the preganglionic nerve was either stimulated throughout (20 Hz) or was rested. Each column represents the mean ± S.E. of at least four experiments. ACh release was calculated (see text) from the release measured in other experiments; release is expressed as % of the release in Krebs solution containing normal (2·4 mM) $CaCl_2$ and normal (1·2 mM) $MgSO_4$.

6. *Relationship between choline accumulation and ACh synthesis*

If, as has been suggested (see Introduction), ACh synthesis is regulated by choline availability, and if, as the above results suggest, enhanced choline transport during stimulation is independent of ACh release, the ACh content of ganglia stimulated in the presence of high Mg^{2+} and choline

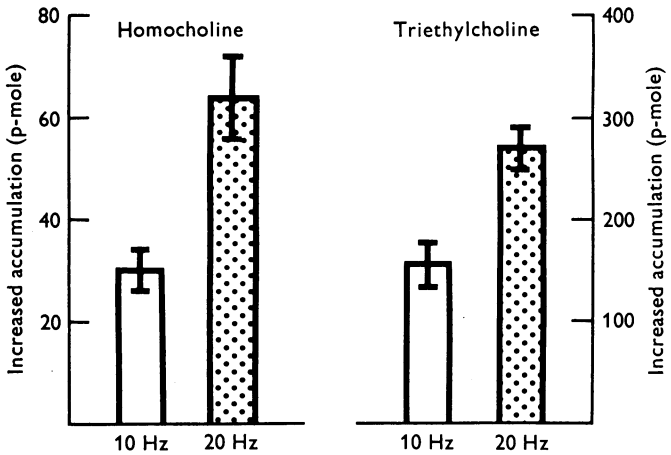

Fig. 9. The accumulation of choline analogues by ganglia stimulated at 10 or 20 Hz. Cat superior cervical ganglia were perfused for 20 min with Krebs solution containing [^{3}H]homocholine (10^{-6} M) or [^{14}C]triethylcholine (10^{-5} M); the preganglionic nerve was stimulated throughout at 10 or 20 Hz. Each experiment determined the difference in the amount of choline analogue accumulated by stimulated and by rested ganglia and each column represents the mean ± S.E. of at least four experiments.

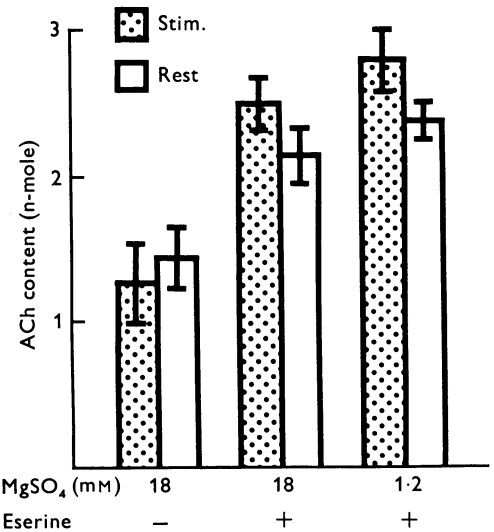

Fig. 10. Lack of effect of high Mg^{2+} on ACh content of ganglia. Cat superior cervical ganglia were perfused for 20 min with Krebs solution containing choline (10^{-5} M) and either high $MgSO_4$ (18 mM), high Mg^{2+} and eserine ($3{\cdot}5 \times 10^{-5}$ M), or normal $MgSO_4$ (1·2 mM) and eserine; the preganglionic nerve was either stimulated throughout (20 Hz) or was rested. Each column represents the mean ± S.E. of at least four experiments.

should increase. These conditions would be such that the rate of choline transport would exceed that necessary for synthesis to replace released ACh. Thus the ACh content of ganglia perfused with Krebs solution containing 10^{-5} M choline and 18 mM-$MgSO_4$ was measured; one ganglion was stimulated and the other was not. The ACh content of the stimulated ganglia was no greater than that of the rested ganglia (Fig. 10). Similar experiments in the presence of eserine gave a similar result (Fig. 10): both ganglia increased their ACh content due to the accumulation of surplus ACh (Birks & MacIntosh, 1961; Collier & Katz, 1971), but the ACh content was not significantly greater in the stimulated ganglia than in the rested ones ($P > 0{\cdot}8$). Furthermore, the ACh content of ganglia stimulated in the presence of high Mg^{2+} and eserine was not significantly different ($P > 0{\cdot}2$) from that of ganglia stimulated or rested in the presence of normal Mg^{2+} and eserine (Fig. 10).

DISCUSSION

The present experiments used the choline analogues, homocholine and triethylcholine, to make inferences about choline transport into the cholinergic nerve terminals of the cat's superior cervical ganglion during preganglionic nerve stimulation. The accumulation of the two analogues appeared to be by the choline transport mechanism because the uptake of homocholine and of triethylcholine was blocked by choline and by hemicholinium, a drug that is considered to selectively inhibit the choline transport mechanism of cholinergic nerve terminals (MacIntosh, Birks & Sastry, 1956; Birks & MacIntosh, 1961; Gardiner, 1961; Schuberth, Sundwall, Sörbo & Lindell, 1966; Marchbanks, 1968; Collier & MacIntosh, 1969; Diamond & Milfay, 1972; Guyenet *et al.* 1973*a*, *b*; Yamamura & Snyder, 1973; Holden, Rossier, Beaujouan, Guyenet & Glowinski, 1975).

Under the conditions of the experiments described in this paper, accumulated homocholine or triethylcholine was not acetylated at a measurable rate, and accumulation, therefore, appears to represent transport; however, with more prolonged exposure of the ganglion to either of the two analogues, the formation of acetylated products can be demonstrated (Ilson & Collier, 1975; B. Collier, S. Lovat, D. Ilson, L. A. Barker & T. W. Mittag, in preparation). The two choline analogues were used to test whether choline transport by cholinergic nerve terminals is enhanced during nerve stimulation. Previous attempts to demonstrate this have used radiolabelled choline which is rapidly acetylated by cholinergic nerve endings, and the results have, therefore, been unclear. Thus in ganglia, preganglionic nerve stimulation appeared to increase [^{3}H]choline accumulation, but this effect was small compared to the increase in [^{3}H]ACh synthesis (Collier & MacIntosh, 1969). Indirect tests by Collier & Katz (1974)

indicated that preganglionic nerve stimulation accelerated choline uptake into cholinergic nerve terminals because most of the choline recaptured from hydrolysed transmitter was used for ACh synthesis. Experiments on isolated synaptosomes suggested that stimulation accelerates choline transport (Atweh, Simon & Kuhar, 1975; Simon & Kuhar, 1975; Barker, 1976; Simon, Atweh & Kuhar, 1976), but these results were not entirely definitive because the test of choline uptake was made after, not during, stimulation; furthermore, stimulation likely altered endogenous ACh levels, and most of the choline accumulated was rapidly acetylated. The present experiments provided a clear answer: preganglionic nerve stimulation increased the accumulation of homocholine or of triethylcholine by ganglia and this appeared to be into cholinergic nerve terminals because it was not blocked by agents that prevent the action of released transmitter. The increased accumulation of both choline analogues was determined by the frequency of nerve stimulation, for doubling the rate of stimulation doubled the amount accumulated. Cholinergic nerve terminals are dependent upon choline transport for ACh synthesis and enhanced transport during stimulation presumably functions under physiological conditions to provide the extra choline required for increased ACh synthesis that is necessary to replenish released transmitter.

It has been suggested that choline transport might be regulated by the intracellular ACh concentration (Whittaker *et al.* 1972; Whittaker & Dowdall, 1975), or by nerve impulse activity *per se* (Simon & Kuhar, 1975; Atweh *et al.* 1975; Simon *et al.* 1976). In the first case, the release of transmitter would transiently lower the intracellular ACh concentration and thus stimulate choline uptake; in the second case, nerve terminal depolarization would accelerate choline transport in anticipation of increased transmitter synthesis. To distinguish between these possibilities, the present experiments measured the enhanced accumulation of homocholine or triethylcholine under conditions that altered ACh release. When transmitter release was altered by reducing the concentration of Ca^{2+}, the accumulation of choline analogues appeared to parallel the changes in transmitter release, but when ACh release was altered to a similar extent by raising the concentration of Mg^{2+}, it was clear that the accumulation of choline analogues was independent of the changes in transmitter release. The simplest explanation of these results is that increased choline uptake during stimulation is a Ca^{2+}-dependent process controlled by nerve terminal depolarization and not controlled by transmitter release. The uptake of choline by synaptosomes isolated from brain appears not to be Ca^{2+}-dependent (Yamamura & Snyder, 1972, 1973; Haga & Noda, 1973), but the high affinity uptake of choline into cholinergic nerve endings in the guinea-pig myenteric plexus is Ca^{2+}-dependent (Pert & Snyder, 1974).

Thus central and peripheral cholinergic nerve terminals appear to differ in in this respect, and the results of studies of the mechanism of choline transport on any one tissue preparation should be extrapolated to other tissues with some reservations.

The present experiments that measured homocholine and triethylcholine accumulation by stimulated ganglia exposed to media containing high Mg^{2+} suggested that under this condition choline transport should exceed that required for synthesis to replace released transmitter. It has been suggested that choline transport might be rate-limiting for ACh synthesis (see Introduction), and in brain there is some evidence to support this. Thus, altered plasma choline levels appear to alter the ACh content of brain (Haubrich, Wedeking & Wang, 1974; Cohen & Wurtman, 1975, 1976). The results of the present experiments do not support the hypothesis that choline transport limits ACh synthesis in ganglia: increased choline uptake in the presence of high Mg^{2+} did not enhance ganglionic ACh content. This result agrees with that of Birks & MacIntosh (1961) who measured ACh release from ganglia perfused with plasma containing an increased choline concentration; a 100-fold increase in choline concentration did not increase the amount of ACh released. It is not yet certain that this difference between experimental results from studies on brain and on ganglia indicates a fundamental difference in the mechanism by which ACh synthesis is controlled in the two tissues, but it is clear that further studies on this important point are necessary.

We are grateful to Gisèle Johnson for technical assistance, and to the Medical Research Council of Canada for financial support. D. Ilson was supported by studentships from the Medical Research Council and the Conseil de la Recherche en Santé du Québec.

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