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Effect of HC-3 on choline uptake by the isolated diaphragm

SIR,—It is generally accepted that the action of HC-3 reaches a maximum only at stimulation rates of 1/sec or more (Wilson & Long, 1959). This is believed to result from impairment of acetylcholine synthesis which becomes more important at higher frequencies of stimulation leading to depletion of transmitter stores. However, choline does not antagonize HC-3 blockade in the rat phrenic nerve-diaphragm preparation (Thies & Brooks, 1961), and the addition of choline to fluid bathing the diaphragm fails to increase acetylcholine output (Straughan, 1960). Thus, the supply of choline may not be a limiting factor in acetylcholine synthesis in the diaphragm as in the superior sympathetic ganglion (MacIntosh, 1963). While investigating [^{14}C]choline uptake in rabbit isolated, perfused hearts, it was of interest to examine [^{14}C]choline uptake by the rabbit diaphragm and the influence of HC-3 thereon.

The preparation consisted of the rabbit isolated, perfused phrenic nerve-diaphragm as first described by Burgen, Dickens & Zatwas (1949) and later modified for continuous perfusion by Dr. L. P. McCarty (personal communication). The diaphragms were perfused with a re-cycling system via the vena cava with oxygenated, eserinized Locke-Ringer solution, 37°, 5 ml/min, while suspended in a Locke-Ringer bath. Both phrenic nerves were placed over platinum electrodes and monophasic pulses of 0·5 msec duration and 5 V were delivered from a Grass 54B stimulator. The frequencies of stimulation ranged from 6/min to 10/sec and were administered for 5 min followed by 5 min rest. This intermittent stimulation was continued for 1 hr. The concentration of [^{14}C]choline in the perfusion fluid was 0·012 μg/ml. Extraction of [^{14}C]-labelled compounds and their subsequent paper chromatography are as previously described (Buterbaugh & Spratt, 1968).

The results obtained are shown in Fig. 1. The response to nerve stimulation was maintained throughout the 1 hr perfusion period at frequencies of 6/min and 1/sec. At 10/sec, the contraction response was vigorous at the start of each 5 min period and diminished to about 25% of the initial response by the end of each period. Higher stimulation frequencies resulted in tetany and were not used. It is evident that the highest uptake of [^{14}C]choline occurred at the lowest stimulation frequency, 6/min, and decreased to the lowest value at 10/sec.

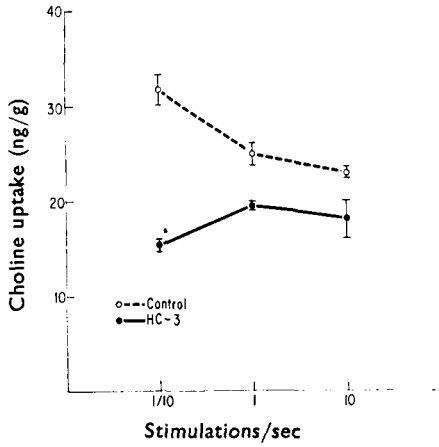

FIG. 1. [^{14}C]Choline uptake by isolated, perfused phrenic nerve-diaphragm as a function of stimulation frequency. Each point represents the mean ± s.e. for four hearts. Asterisk indicates value significantly different at $P < 0.05$ (Student's t, unpaired, two-tailed).

The significance of this observation is open to speculation. Perhaps it is evidence of a re-uptake mechanism for choline or acetylcholine at the presynaptic membrane which becomes of importance only at higher stimulation frequencies. [^{14}C]Acetylcholine could be extracted from the tissue in amounts of not more than 5% of the [^{14}C]choline uptake. However, it cannot be concluded that this [^{14}C]acetylcholine is synthesized or stored within the nerve endings since MacIntosh has concluded that over one half of the acetylcholine in leg muscle is located in non neural tissue (MacIntosh, 1963).

HC-3 added to the perfusion fluid in concentrations of 50 μg/ml had little effect on the contraction response at stimulation frequencies of 6/min and 1/sec. At 10/sec, the response was diminished to less than 25% of the initial response after the first 5 min of stimulation and did not recover. A significantly greater effect of HC-3 on [^{14}C]choline uptake was observed at the lowest stimulation frequency, 6/min, and no significant effect on choline uptake was seen at the higher stimulation frequencies of 1/sec and 10/sec. HC-3 had no effect on the level of [^{14}C]acetylcholine extracted from the tissue.

These results raise further questions directed toward cholinergic mechanisms. The action of HC-3 to produce a greater blockade of choline uptake at lower stimulation frequencies is contrary to commonly accepted facts concerning the action of HC-3 and emphasizes the uniqueness of this compound and the need for continued research on its mechanism of action.

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Modified method for the estimation of metaraminol and α-methyl-*m*-tyramine

SIR,—In recent years the catecholamine-depleting action of DL-α-methyl-*m*-tyrosine has attracted attention. Much evidence exists to suggest that the depleting agent is metaraminol, which is formed *in vivo* from methyltyrosine* via the intermediate α-methyl-*m*-tyramine (Carlsson & Lindquist, 1962). It has been postulated that metaraminol, which is taken up and stored within sympathetic nerves, may replace the noradrenaline and serve as a false transmitter (Crout, Alpers & others, 1964; Andén, 1964).

The *o*-phthalaldehyde method for the determination of metaraminol (Shore & Alpers, 1964) is specific for primary *m*-hydroxyphenylethyl amines, but will also produce fluorescent reactions with α-methyl-*m*-tyrosine and α-methyl-*m*-tyramine. It is therefore necessary to separate the three substances before condensation with *o*-phthalaldehyde. This is possible by passing the compounds through a Dowex-50 resin which allows the methyltyrosine to run through while retaining both the methyltyramine and metaraminol. Subsequent differential elution with N and 2N HCl allows a separation of the latter two amines.

We wish to report a method based on the above principles, sufficiently sensitive to allow for the analysis of single rat hearts. Columns of Dowex 50W, X-4, 450 mm by 60 mm, are prepared with sodium hydroxide, hydrochloric acid and 0·1M phosphate buffer, pH 6·5, as described by Carlsson & Lindquist (1962).

Single hearts, removed from rats treated with the methyltyrosine, are homogenized at high speed (VirTis "23" Homogenizer) in 25 ml of 0·4N perchloric acid. The supernatant is neutralized with cold 5N potassium carbonate and the ensuing precipitate removed by centrifugation (8000 rev/min for 10 min). The total amount of the supernatant remaining (approx. 25 ml) is forced through the Dowex column at a rate of 17 drops/min. The column is then washed with 10 ml of double distilled water and eluted first with 25 ml of 1N HCl and then with 25 ml of 2N HCl. The eluate is collected in 2·5 ml fractions which are then condensed with *o*-phthalaldehyde according to Shore & Alpers (1964).

The above method makes it possible to separately elute metaraminol and the methyltyramine (Fig. 1). The first curve, seen after the injection of the methyltyrosine or metaraminol, is metaraminol, and the second curve, observed only after methyltyrosine administration, is methyltyramine. Either curve can be selectively produced by the addition respectively of metaraminol or methyltyramine to extracts of hearts removed from untreated rats.

Quantitative estimation of the levels of metaraminol and methyltyramine present in the hearts is routinely obtained by summing the concentrations measured in eluates 3 to 8 inclusive, for metaraminol, and 12 to 19 inclusive for methyltyramine. We found the percentage recovery using this method to be 60·6 ± 5·3 for metaraminol and 62·8 ± 4·6 for methyltyramine.

* In this text methyltyrosine is used as an abbreviation for DL-α-methyl-*m*-tyrosine and methyltyramine for α-methyl-*m*-tyramine.