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THE IDENTIFICATION OF THE IMPURITY RESPONSIBLE FOR THE REPORTED PRESSOR ACTIVITY OF XYLOCHOLINE

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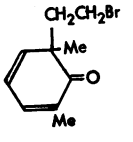
When examining a series of nuclear substituted choline phenyl ethers for nicotine-like stimulant activity Hey & Willey (1954) found that on intravenous administration 2,6-xylyl choline ether bromide (xylocholine ; TM. 10) produced a typical short-lived rise of the arterial blood pressure in the spinal cat. Subsequent doses of the same compound, however, failed to produce any rise in blood pressure. In 1959, Clark & Willey (unpublished) observed that other samples of xylocholine failed to produce a pressor response even when first administered and some attempts were made at that time to synthesize and isolate a possible impurity which might be present in xylocholine. It was thought that the impurity might be the result of carbon alkylation, instead of oxygen alkylation, and that trace quantities of this impurity might be responsible for the pressor activity.



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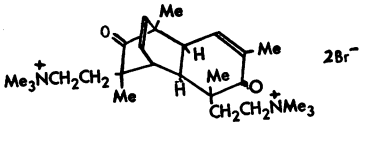


II

C-alkylation of 2,6-xylenol with ethylene dibromide would lead to the dienone I which, by analogy with 2,6,6-trimethyl-2,4-cyclohexadien-1-one, would be expected to dimerise rapidly (Brown, Curtin & Fraser, 1958). Quaternization of the resulting dibromide would yield a structure of type II. We were unable to obtain a pure bis-quaternary compound, but infra-red spectroscopy indicated the presence of ketone in the quaternary products derived from a high boiling fraction from the alkyation reaction. This material failed to produce any increase in blood pressure on administration to a spinal cat and it was concluded that the impurity responsible for the pressor response of the original xylocholine was not of this type. At this juncture we were fortunate to receive from Dr Hey a small sample of xylocholine prepared from the original sample of 2,6-xylenol using the original method of preparation. This material possessed the pressor activity described by Hey & Willey (1954) and we have investigated it by infra-red and ultra-violet spectroscopy and by chemical degradation followed by gas chromatography. The administration

of artificial mixtures of choline aryl ether bromides to spinal cats has been used to demonstrate that the results obtained by Hey & Willey (1954) may be reproduced with a suitable mixture.

METHODS

Infra-red spectra were determined on dispersions in KCl discs using 1 to 3 mg drug to 200 mg KCl, and using a Perkin-Elmer model 137 spectrophotometer.

Spectra were determined for the sample of xylocholine which exhibited pressor activity [TM. 10 (Hey)], " pure " xylocholine (i.e., a sample of 2,6-xylyl choline ether bromide, m.p. 209.5–210.5, which did not possess pressor activity), and the following aryl ethers of choline bromide: phenyl, *o*-tolyl, 2,3-xylyl, 2,4-xylyl, 2,5-xylyl, 3,4-xylyl and 3,5-xylyl.

Preparation of KCl discs. The sample to be examined was powdered in an agate mortar and sieved through a 200-mesh sieve, the required quantity was weighed and then dried at 120° under vacuum over P_2O_5 for 48 hr. The potassium chloride was similarly treated except that drying was accomplished in an oven at 500°. The materials were then mixed in a mortar under anhydrous conditions inside a glove-box. The mixture was further dried over P_2O_5 at 120° under vacuum for 24 hr and then milled in a vibrating ball mill for 2 min. The dry mixture was placed in a suitable die, the die evacuated to 0.1 mm Hg pressure and maintained at *ca* 45° for 2 hr using a stream of warm air prior to the application of 10 tons pressure for 1 min.

Ultra-violet absorption spectra were measured on aqueous solutions using a Unicam SP700 double beam recording spectrophotometer. Concentrations of 0.005 mg/ml. for the region 54,000 to 43,000 cm^{-1} and 0.05 mg/ml. for the region 43,000 to 26,000 cm^{-1} were used except for TM. 10 (Hey) and " pure " xylocholine for which concentrations of 0.5 mg/ml. were used for the lower frequency region, and for *o*-tolyl choline ether bromide for which a concentration of 0.0025 mg/ml. was used for the higher frequency region.

Chemical degradation. The sample (50 mg) was dissolved in 20% potassium hydroxide solution (5 ml.) and heated under a reflux condenser on a water bath for 4 hr. The solution was acidified to *p*H 1 using concentrated hydrochloric acid and was then further heated on the water bath under the reflux condenser for 2 hr. After cooling, the solution was extracted with ether, the ethereal solution dried ($CaSO_4$) and the ether evaporated. The residue obtained was examined by gas-liquid chromatography.

Gas-liquid chromatography. Residues from the chemical degradation of TM. 10 (Hey), " pure " xylocholine, artificial mixtures of " pure " xylocholine and *o*-tolyl choline ether bromide and mixtures of *o*-cresol and 2,6-xylenol were examined on a Pye series 104 chromatograph, model 24. 0.1 to 0.2 μl. of approximately 10% solutions of these samples in Analar benzene were injected on to columns packed with 5% xylenol phosphate deposited on 100/110 Anakrom U., using an oven temperature of 110° and a carrier gas (N_2) flow rate of 45 ml./min.

Blood pressure in the cat. The pressor activity of TM. 10 (Hey), " pure " xylocholine and mixtures of " pure " xylocholine and *o*-tolyl choline ether bromide were examined on cats. The animals were given 1 mg/kg atropine sulphate intraperitoneally, anaesthesia induced by ether, and spinal preparations made following the method of Dale as described by Burn (1952). Blood pressure was recorded from the carotid artery using a mercury manometer, and intravenous injections were made into the right femoral vein.

RESULTS

Infra-red spectroscopy. It was essential to use the tedious drying procedures described since the spectrum of xylocholine below 850 cm^{-1} alters radically if the material is hydrated. Once hydrated the compound is not readily converted to the anhydrous form and even under the conditions described weak OH stretching absorption was observed

on numerous occasions though in no case was the spectrum characteristic of the hydrated salt.

As shown in Fig. 1, the infra-red spectra of TM 10 (Hey) and " pure " xylocholine are almost identical except for the presence of a band of medium intensity at 1,124 cm^{-1} in the spectrum of TM.10 (Hey). Of the related aryl choline ethers examined, only the *o*-tolyl and 2,4-xylyl compounds show strong absorption at this frequency, the phenyl

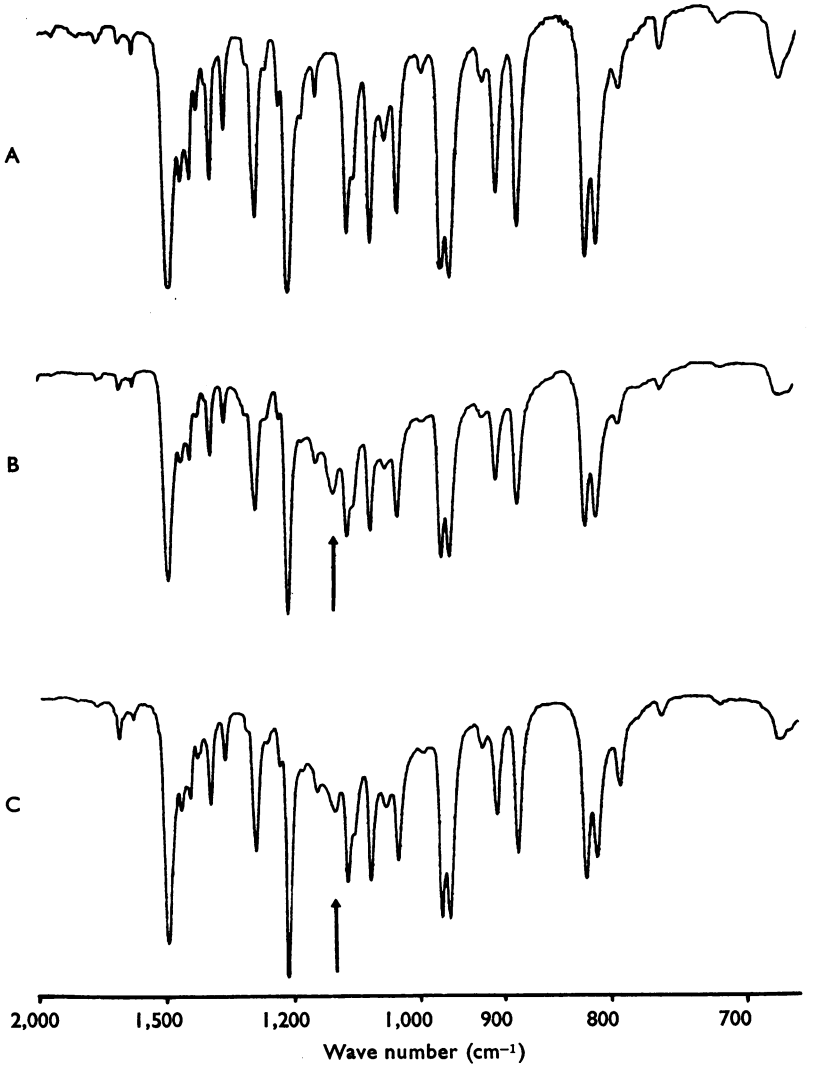


Fig. 1. Infra-red spectra of A—" pure " xylocholine, B—TM 10 (Hey), C—a mixture of 2 parts of *o*-tolyl choline ether bromide and 98 parts of " pure " xylocholine recorded between 2,000 and 670 cm^{-1}, on dispersions in KCl discs. The anomalous absorption at 1,124 cm^{-1} seen in TM. 10 (Hey) and the mixture of *o*-tolyl choline ether bromide and " pure " xylocholine is indicated by the arrows.

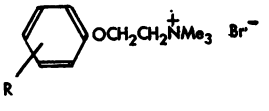
analogue shows medium intensity absorption at 1,119 cm^{-1}, and the 3,4-xylyl strong absorption at 1,129 cm^{-1}. The remaining analogues did not show selective absorption between 1,115 and 1,130 cm^{-1} or only absorbed very weakly in this region. Phenyl choline ether bromide absorbed strongly at 649 cm^{-1}, and the 2,4-xylyl analogue absorbed strongly at 869 cm^{-1}. Neither sample of xylocholine showed any selective absorption at either of these frequencies. 3,4-Xylyl choline ether bromide showed selective absorption of medium intensity at 870 and 745 cm^{-1}, strong absorption at 1,616, 1,582 and 892 cm^{-1} and very strong absorption at 1,504 cm^{-1}. Both samples of xylocholine absorbed very weakly at 1,639 and 1,595 cm^{-1}, very strongly at 1,488 cm^{-1} and strongly at 905 and 883 cm^{-1}. A mixture of " pure " xylocholine (98 parts by weight) and *o*-tolyl choline ether bromide (2 parts by weight) yielded a spectrum (Fig. 1,C) identical with that of TM. 10 (Hey) (Fig. 1,B).

Ultra-violet spectroscopy. All the compounds exhibited numerous peaks of absorption between 53,300 cm^{-1} and 50,000 cm^{-1}, maximum absorption occurring at 51,450 cm^{-1} for pure xylocholine and its *o*-tolyl analogue, and 51,500 cm^{-1} for the 2,5-xylyl compound. 2,4-Xylyl choline ether bromide exhibited a double maximum at 51,000 and 50,900 cm^{-1}, while the 2,3-xylyl analogue exhibited its maximum at 50,800, the 3,4-xylyl at 51,100 and the 3,5-xylyl at 50,300 cm^{-1}. Molecular extinction coefficients for the maxima varied between 40,000 and 92,000. The " benzenoid " ($\pi \rightarrow \pi^*$) absorptions between 38,200 and 35,600 cm^{-1} together with their $E^{1\%}_{1\ cm}$ values are given in Table 1.

TABLE 1

"BENZENOID" ($\pi \rightarrow \pi^*$) ABSORPTION OF CHOLINE ARYL ETHER BROMIDES

Spectra were determined on aqueous solutions. (s) indicates a shoulder.



R	cm^{-1}	$E^{1\%}_{1\ cm}$	cm^{-1}	$E^{1\%}_{1\ cm}$	cm^{-1}	$E^{1\%}_{1\ cm}$	cm^{-1}	$E^{1\%}_{1\ cm}$
—	37,900(s)	38·15	37,200	51·5	36,300	41·8		
2-Me			37,100	72·4	36,200	67·4		
2,3-di Me			37,200	19·16	36,200	19		
2,4-di Me					36,300	50	35,600(s)	45·6
2,5-di Me					36,600	61·6	35,900	58·6
2,6-di Me (pure)	38,200	9·86	37,400(s)	8·8				
2,6-di Me (Hey)	38,150	10·2	37,400(s)	9·56				
3,4-di Me					36,400	71	35,600(s)	65
3,5-di Me					36,800	32	36,000	32

Chemical Degradation and Gas Chromatography. The product from the degradation of " pure " xylocholine on gas chromatography yielded a single peak of retention time 34 min 15 sec whereas that from TM. 10 (Hey) yielded 2 peaks; an intense one of retention time 34 min 15 sec and a smaller peak of retention time 46 min 15 sec. Degradation of a mixture of 2 parts *o*-tolyl choline ether bromide and 98 parts pure xylocholine yielded a product which gave a gas-chromatogram identical with that from

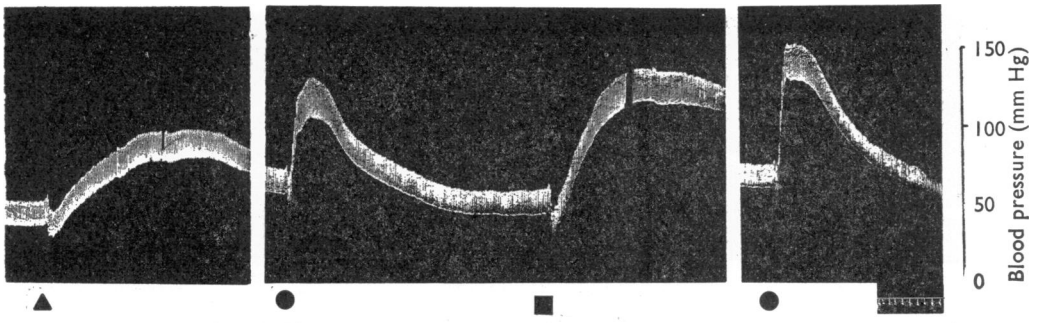


Fig. 2. Effect of *o*-tolyl choline ether bromide (0.05 mg/kg i.v. at ●) and TM. 10 (Hey) (5 mg/kg i.v. at ■) on the blood pressure of a spinal cat after the administration of " pure " xylocholine (5 mg/kg i.v.—first administration at ▲). A second dose of " pure " xylocholine was administered 18.5 min after the first dose. A similar response was observed. Time interval between first and second parts of trace—35 min and between second and third parts—10 min. Time marks, 30 sec.

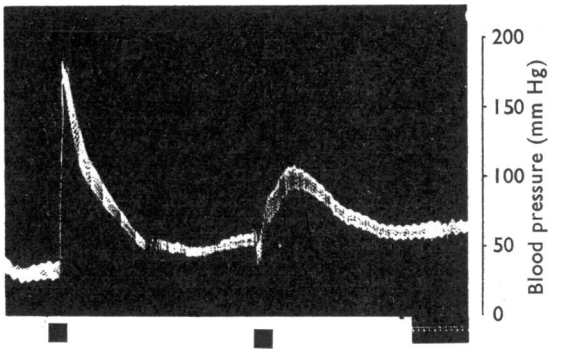


Fig. 3. Effect of TM. 10 (Hey) (5 mg/kg i.v. at ■) on the blood pressure of the spinal cat showing marked nicotine-like pressor response with first dose, and small depressor response followed by slow increase in blood pressure on second administration. Time marks, 30 sec.

TM. 10 (Hey) except that the second peak was relatively more intense. A mixture of 2,6-xylenol and *o*-cresol gave a chromatogram of two peaks of retention times 34 min 15 sec and 46 min 15 sec respectively.

Cat blood pressure. Fig. 2 shows the initial depressor effect followed by a slow rise and fall in blood pressure following the first administration of " pure " xylocholine, and Fig. 3 shows the intense pressor effect of a first dose of TM. 10 (Hey) and the initial depressor effect followed by slow rise and fall in blood pressure following a second dose. Fig. 4,A shows the similar sharp rise in blood pressure on administering a mixture of 1.5 parts of *o*-tolyl choline ether bromide and 98.5 parts of pure xylocholine. This was not seen with a mixture containing only 1% of the *o*-tolyl analogue (Fig. 4,B). Following the administration of pure xylocholine the injection of *o*-tolyl choline ether bromide alone still produces a rapid rise in blood pressure (Fig. 2).

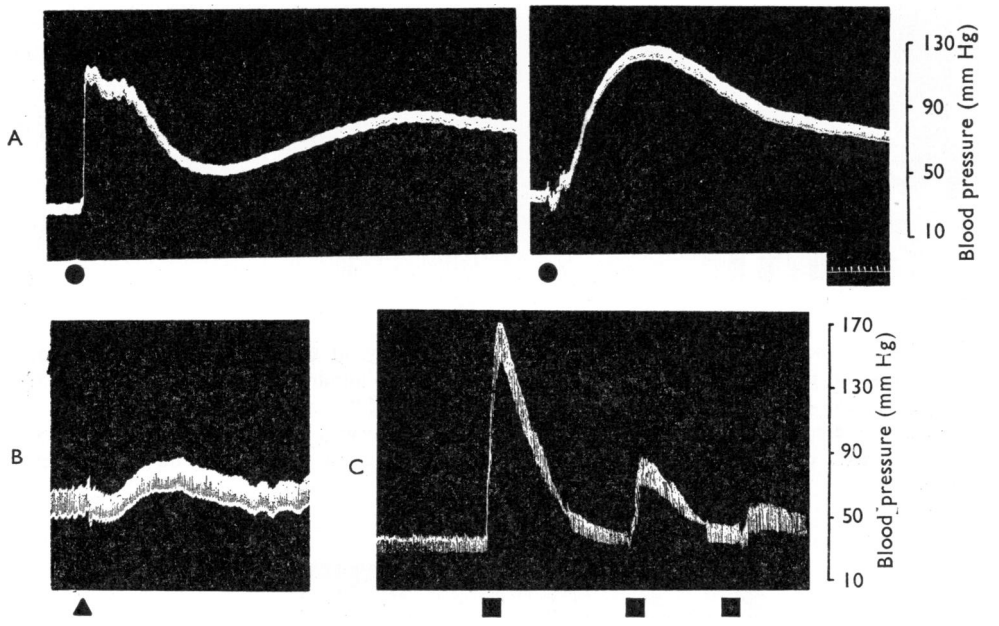


Fig. 4. Effects of first and subsequent intravenous administrations of mixtures of *o*-tolyl choline ether bromide and "pure" xylocholine on the blood pressures of spinal cats. Time marks, 30 sec. A—5 mg/kg of a mixture containing 1.5% *o*-tolyl choline ether bromide and 98.5% "pure" xylocholine administered at ●. First and second doses of mixture are shown. Time interval between first and second parts of trace—20 min. B—5 mg/kg of a mixture containing 1% *o*-tolyl choline ether bromide and 99% "pure" xylocholine administered at ▲: C—5 mg/kg of a mixture containing 2% *o*-tolyl choline ether bromide and 98% pure xylocholine administered at ■. First, second and third doses of mixture are shown.

DISCUSSION

The additional band of absorption at 1,124 cm^{-1} present in the spectrum of TM. 10 (Hey) strongly suggested the presence of either *o*-tolyl or 2,4-xylyl choline ether bromide as an impurity since both these compounds absorb strongly at this frequency. The possibility of small shifts in the frequency of absorption must not be ignored, however, and therefore consideration must be given to the possibility of either the phenyl analogue (medium intensity absorption at 1,119 cm^{-1}) or 3,4-xylyl analogue (strong absorption at 1,129 cm^{-1}) being responsible for the anomalous band. The absence of strong absorption between 1,115 and 1,130 cm^{-1} in the spectra of the other analogues enables us to be reasonably sure that they are not present. Consideration of other regions of the spectra enabled us to narrow the choice of possible contaminant still further. The absence of selective absorption in the spectrum of TM. 10 (Hey) at 694 cm^{-1} excludes phenyl choline ether bromide, and the absence of absorption at 869 cm^{-1} points to the impurity not being the 2,4-xylyl ether. The absence of any selective absorption in the spectrum of TM. 10 (Hey) at 1,616, 1,582, 870 and 745 cm^{-1} indicates the absence of the 3,4-xylyl choline ether bromide. It was concluded therefore that the contaminant present in TM. 10 (Hey) was *o*-tolyl choline ether bromide. From the biological viewpoint this could well be

the cause of the pressor effect of TM. 10 (Hey) since Hunt & Renshaw (1936) have reported that 0.1 mg *o*-tolyl choline ether bromide produced a 43 mm blood pressure rise in a spinal cat. Since the dose of TM. 10 administered by Hey & Willey (1954) was 10 mg, only 1% of the *o*-tolyl compound would be required to reach the 0.1 mg dose level. Infra-red spectroscopy is of limited value for quantitative analysis so recourse was made to the ultraviolet region. The complexity of the spectra and the rather small difference in intensity between xylocholine and its analogues in the high frequency region of the ultra-violet spectrum makes this region unsuitable for quantitative analysis. The so called " benzenoid " region ($\pi \rightarrow \pi^*$, 260–280 mμ) was more useful, the spectra being simpler and the intensity of xylocholine absorption being at slightly higher frequency and much weaker than that of its analogues. The $E^{1\%}_{1\ cm}$ values for TM. 10 (Hey) are higher than those for " pure " xylocholine reflecting the presence of the more highly absorbing impurity. Assuming that this impurity is *o*-tolyl choline ether bromide the amount of impurity present in the sample can be calculated using the absorption at 37,400 cm^{-1}. This frequency does not coincide with the frequency for maximum absorption of the *o*-tolyl compound but the difference of 300 cm^{-1} is small. The $E^{1\%}_{1\ cm}$ for *o*-tolyl choline ether bromide at 37,400 cm^{-1} is 69.4, and this value will be used for calculation purposes since it is considered that the result will be more accurate. Use of the $E^{1\%}_{1\ cm}$ value for the maximum frequency of absorption would, however, only decrease the calculated percentage of impurity by *ca* 0.05%.

If the concentrations of " pure " xylocholine and *o*-tolyl choline ether bromide in the TM. 10 (Hey) are C_1 and C_2 respectively and the optical densities contributed by each component of the mixture are D_1 and D_2 then $C_1 + C_2 = 0.05$ mg/ml. and at 37,400 cm^{-1} $D_1 + D_2 = 0.478$.

Since the cell length used was 1 cm $8.8C_1 + 69.4C_2 = 0.478$

$$\therefore 8.8(0.05 - C_2) + 69.4C_2 = 0.478$$

$$60.6C_2 = 0.038$$

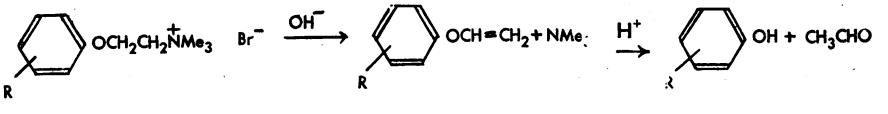
and $C_2 = 0.000627$

$$\therefore \%\ o\text{-tolylcholine ether bromide in TM. 10 (Hey)} = \frac{0.0627}{0.05} = 1.25$$

That the impurity is *o*-tolyl choline ether bromide is firmly established by chemical degradation and gas-chromatography. The reactions occurring in the degradation are shown in the flow-sheet.

The only non-volatile product is the phenol and examination of the degradation products from TM. 10 (Hey) establishes conclusively the presence of *o*-cresol in addition to 2,6-xylenol. The *o*-cresol is unlikely to have arisen from any source other than *o*-tolyl choline ether bromide.

That " pure " xylocholine contaminated with a small amount of the *o*-tolyl analogue is capable of producing the biological actions observed with TM. 10 (Hey) is well



illustrated by the administration of a mixture containing 1.5% of the contaminant to a spinal cat. The fact that a mixure containing only 1% of contaminant fails to produce a typical pressor response while a mixture containing 2% of contaminant produces a pressor response following a second administration lends further support to the spectroscopically determined value of approximately 1.25% impurity.

It is of interest to consider how the impurity arises from a commercial sample of 2,6-xylenol the infra-red spectrum of which is identical with that of the sample used to obtain the " pure " xylocholine. The reason would appear to lie in the use of ethanolic solutions for the preparation of 2-aryloxy-ethyl bromides. In our experience this reaction gives very poor yields of the 2-(2,6-xylyloxy)ethyl bromide and the greater ease of reaction with *o*-cresol leads to concentration of the impurity. The use of tert-butyl alcohol and alkylation with 2-dimethylaminoethyl chloride gives high yields of the amino-ether even with 2,6-xylenol and hence does not produce the increase in concentration of the *o*-tolyl compound as seen with ethanol.

A second point of interest is a biological one. How is it that, following a dose of xylocholine, the administration of TM. 10 (Hey), or of an artificial mixture containing " pure " xylocholine and 1.5% *o*-tolyl choline ether bromide, does not produce a rapid pressor response whereas the administration of *o*-tolyl choline ether bromide alone does cause a typical nicotine-like rise in blood pressure ? If it is assumed, as a consequence of the close similarities of the two structures, that both xylocholine and its *o*-tolyl analogue are interacting with the same receptors and that their distributions and rates of distribution in the body following intravenous injection are similar, then the drugs will be present in the biophase in the same proportional concentrations as they were in the administered solution. The initial pressor response following the first administration of the drug mixture could then be the result of a more rapid association between the *o*-tolyl choline ether and the receptors. A higher association rate constant for the *o*-tolyl compound is necessary on the basis of both Paton's rate theory (Paton, 1961) and the " occupation " theory of drug action, since by the former theory the faster rate is inherent in the stimulant action and by the occupation theory a sufficient proportion of the receptors must be occupied by the agonist to produce stimulation before blockade can develop by interaction with the more slowly associating and dissociating antagonist. Recovery of the sympathetic ganglia from the blockade produced by xylocholine appears to be virtually complete after 20 min (Exley, 1957), and this is confirmed by the observed response to the administration of *o*-tolyl choline ether bromide alone. It is clear, however, that the ganglia have not returned to normal, since if the number of receptors available for association with the two choline aryl ethers was the same as it was before the first administration of the drug mixture, a second dose would elicit the same response as the first, no alteration in the ratio of the concentrations of the two drugs in the biophase being a necessary consequence of similar distributions of the two drugs. Failure to observe ganglion stimulation on the second administration of the drug mixture may be taken to indicate that the number of receptors available for association is markedly reduced so that, either the association rate per receptor for the *o*-tolyl choline ether bromide is too low to result in stimulation, or, in terms of the " occupation " theory, the receptor occupation by xylocholine necessary to achieve blockade is reached before the proportion of receptors associating with *o*-tolyl choline ether can achieve the level necessary for stimulation.

Administration of *o*-tolyl choline ether bromide alone would, of course, alter the ratio of concentrations of the two drugs in the biophase. There would be no increase in the proportion of receptors occupied by xylocholine and the association rate per receptor for the *o*-tolyl choline ether would be higher, as also would be the proportion of the receptors occupied by this drug. The reduced number of receptors available for interaction following administration of the drug mixture must be due to a low dissociation rate constant for the breakdown of the xylocholine-receptor complex. This would be consistent, according to the "rate theory," with it being a blocking agent and would explain the more readily attained blocking occupancy, required by the "occupation" theory, on the second administration of the drug mixture. No matter which of the two theories of drug action one favours, the observations described can best be explained by taking account of the association rate constants of the two drugs and their relative concentrations in the biophase. These two parameters will reflect the ability of one drug to compete with the other for the available receptors and would explain the inability of 1% of *o*-tolyl choline ether bromide to compete successfully with xylocholine even on the first administration of the mixture (Fig. 4,B) and the ability of 2% of *o*-tolyl choline ether bromide to compete successfully with xylocholine even on a second administration of the mixture (Fig. 4,C). That the competition becomes less successful with each subsequent dose of the 2% mixture is shown by the decreasing responses to each subsequent dose.

Though the idea of competition at the ganglion receptor is attractive, it is possible that the observations might involve a peripheral action of the *o*-tolyl choline ether bromide. Peripheral action would not require the assumption, inherent in the theory involving action at the ganglion, that there exists something less than 100% adrenergic neurone blockade during the time course of the observations. We hope to investigate these ideas further.

SUMMARY

1. A sample of 2,6-xylyl choline ether bromide [TM. 10 (Hey)] which possesses nicotine-like pressor activity has been investigated by infra-red and ultra-violet spectroscopy, and by vapour phase chromatography of the products of chemical degradation.

2. Analysis of the infra-red spectra and the degradation of TM. 10 (Hey) has established that the sample contains *o*-tolyl choline ether bromide as an impurity.

3. Calculations from the absorption intensity at 37,400 cm^{-1} in the ultra-violet region indicate the presence of approximately 1.25% of the impurity.

4. Administration of mixtures of *o*-tolyl choline ether bromide and "pure" xylocholine to spinal cats produced a typical nicotine-like pressor response when the mixture contained 1.5% of the impurity but no rapid rise in blood pressure when only 1% of the impurity was present. A mixture containing 2% of the impurity produced a pressor effect, though smaller than the first, on a second administration.

5. It is concluded that the reported nicotine-like pressor response produced by 2,6-xylyl choline ether bromide is due to the presence of more than 1 and less than 2% of *o*-tolyl choline ether bromide as an impurity.

We are grateful to Dr P. Hey for the sample of xylocholine, to Professor H. N. Green, and Dr D. B. Clayson of the Department of Experimental Pathology and Cancer Research for permission to use their Unicam SP 700 spectrophotometer, and to the Smith, Kline and French Research Foundation for a grant to purchase the Pye gas chromatograph. One of use (M. de. L. S. A. J.) thanks the Portuguese Ministry of Health for leave of absence during the period of this investigation.

REFERENCES

BROWN, T. L., CURTIN, D. Y. & FRASER, R. R. (1958). Structure of 2,6,6-trimethyl-2,4-cyclohexadienone dimer. *J. Am. chem. Soc.*, **80**, 4339–4341.

BURN, J. H. (1952). Practical Pharmacology, Oxford: Blackwell.

EXLEY, K. A. (1957). The blocking action of choline 2:6-xylyl ether bromide on adrenergic nerves. *Br. J. Pharmac. Chemother.*, **12**, 297–305.

HEY, P. & WILLEY, G. L. (1954). Choline 2:6-xylyl ether bromide; an active quaternary local anaesthetic. *B. J. Pharmac. Chemother.*, **9**, 471–475.

HUNT, R. & RENSHAW, R. R. (1936). Ethers of choline and allied compounds. *J. Pharmac. exp. Ther.*, **58**, 140–154.

PATON, W. D. M. (1961). A theory of drug action based on the rate of drug-receptor combination. *Proc. R. Soc. B.*, **154**, 21–69.