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DRUG-INDUCED DEPLETION OF ACETYLCHOLINE IN THE RABBIT CORNEAL EPITHELIUM

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Abstract—The possibility that the high level of acetylcholine (ACh) in the corneal epithelium is associated with sensory mediation was investigated using the drugs hemicholinium-3 (HC-3), triethylcholine (TECh) and *trans*-4-(1-naphthylvinyl) pyridine hydrochloride (NVP). None of these drugs when topically administered to the rabbit cornea affected the corneal ACh content, as measured by bioassay. Administered by intraocular injection into the aqueous humour, HC-3 produced no ACh depletion, despite a 60 per cent reduction in ^{14}C-choline uptake from the aqueous humour. Intraocularly injected TECh produced a transient depletion of corneal ACh, while NVP caused a reduction to 38 per cent of the control level. In no case did these drugs cause abolition of the corneal reflex. Experiments using topical application of neostigmine in conjunction with ACh depletion by NVP suggest the possible existence of more than one pool of ACh in the rabbit corneal epithelium.

ACETYLCHOLINE (ACh) has been implicated as a possible sensory mediator in the chemoreceptors of the carotid body[1] and many other sensory receptors such as thermal and mechano-receptors.[2] Nevertheless, no substance has been definitely identified as a mediator at sensory endings.

The outermost layer of the mammalian cornea, the epithelium, is not only very rich in sensory nerve endings but it contains one of the highest concentrations of ACh of any mammalian tissue[3,4] even exceeding that found in sympathetic ganglion or brain.[5] The possibility that ACh in the corneal epithelium might be associated with sensory mediation was advanced by Fitzgerald and Cooper,[6] since they found that, in the rabbit, 40–60 per cent depletion of epithelial ACh by hemicholinium-3 (HC-3) was accompanied by loss of the corneal reflex. Since the mechanism of ACh depletion by HC-3 is uncertain,[7] we have investigated this postulated association using the drugs *trans*-4-(1-naphthylvinyl) pyridine hydrochloride (NVP), a specific choline acetyltransferase (ChAc) inhibitor,[8] and triethylcholine (TECh), a drug known to cause ACh depletion.[9] The effect of HC-3 was also re-investigated. Part of this work has already been briefly reported.[10]

METHODS

All experiments on the cornea were carried out on Dutch rabbits (minimum age 3 months) using one eye as test and the other eye as control. Drugs were administered either by intraocular injection into the anterior chamber or applied topically to the cornea. Intraocular injections of a constant 10 μl volume of test or control solution were made through the choroid (approximately 1 mm from the limbus) of the proptosed eye while the animal was under halothane–N_2O–O_2 anaesthesia. All concentrations of drugs injected intraocularly are expressed as final concentrations in

the anterior chamber, assuming that the volume of the anterior chamber in the adult rabbit eye is 250 μl.[11] This allowed a more direct comparison of drug concentrations with those used *in vitro*. A few minutes before completion of drug treatment (topical or injected) the corneal reflex was tested (1) by touching the surface of the cornea with a whisker (tactile) and (2) by exposing the cornea to a metered puff of ammonia vapour (painful). The animal was then anaesthetized and the corneal epithelium scraped off, homogenized and bioassayed for ACh on the dorsal muscle of the leech as previously described.[12]

Experiments using NVP were performed in a darkroom under dim red light, since the active *trans*-isomer of NVP photoisomerizes readily to the less active *cis*-isomer.[13]

Inhibition of ChAc and cholinesterase (ChE) activities in homogenates of rabbit corneal epithelium *in vitro* was determined using concentrations of NVP from 10^{-7} to 10^{-3} M. ChAc activity was measured by a modification[14] of the radioisotopic assay described by Schrier and Shuster[15] and ChE activity by the photometric assay of Ellman *et al.*[16]

Concentrations of NVP (in saline) sufficient to produce maximum *in vitro* inhibition of ChAc were applied topically to the cornea *in vivo* for various times up to 60 min to determine the effect of ACh levels in the corneal epithelium. Similar experiments were performed using NVP dissolved in 100% dimethylsulphoxide (DMSO) or 0·1 M EDTA Na_2, pH 7·4 (in saline) in an effort to increase the permeability of the epithelium to topical NVP.

By measuring ACh levels in the corneal epithelium at various intervals after intraocular injections of 3×10^{-4} M and $2{\cdot}4 \times 10^{-3}$ M NVP the optimal time of action of NVP was determined. (In all cases intraocular injections of 10 mM acid phosphate buffer of appropriate pH were administered to the contralateral control eye). ACh levels in the corneal epithelium were then determined 60 min after intraocular injections of various concentrations of NVP. The effect of repeated injections of NVP after different time intervals was also investigated.

Intraocular injections of NVP, producing concentrations of $1{\cdot}2 \times 10^{-3}$ M and $2{\cdot}4 \times 10^{-3}$ M in the aqueous humour, were administered to one rabbit eye. The corneal epithelium was removed after 60 min and the *in vitro* ChAc activity measured in the test and contralateral control epithelia after homogenizing in 3 and 6 vol. of buffer, respectively. By estimating the percentage inhibition of ChAc in the test eye relative to the control, the amount of NVP which had reached the epithelium from the aqueous humour was estimated.

In another series of experiments 3% neostigmine was applied to the corneal surface of the test eye immediately before removal of the epithelium and extraction of the ACh from both eyes. The effect of this application on the amount of ACh extracted in untreated animals and in animals in which both eyes had been treated 60 min previously with NVP was determined.

TECh and HC-3. The degree of acetylation *in vitro* of TECh and HC-3 relative to choline acetylation by corneal epithelium homogenates was found using the ChAc assay. The properties of the acetylated products were investigated on the leech muscle.

The effect of intraocular HC-3 on radioactive choline uptake from the aqueous humour to the corneal epithelium of a rabbit eye was estimated by injecting HC-3

($2{\cdot}78 \times 10^{-3}$ M) into the test eye and saline into the contralateral control eye, followed 5 min later by 10 μl ^{14}C-choline (0·0158 μmoles; 0·951 μCi) injected into both eyes. After 60 min the epithelium of each eye was removed, suspended in 10 ml Instagel® and the total radioactivity counted in a scintillation spectrometer.

HC-3, producing a concentration of $2{\cdot}78 \times 10^{-3}$ M in the aqueous humour, was injected into the anterior chamber of one rabbit eye. After 60 min the corneal epithelia from both eyes (treated and control) were removed and homogenized in 0·01 N HCl. The homogenate was extracted and the fluorescence determined at the optimum wavelength for HC-3.[17]

The native fluorescence from the epithelium of an untreated rabbit's eye was also determined, to serve as blank. The quantification of HC-3 was confirmed by means of internal standards. An approximation of the amount of endogenous choline in the corneal epithelium was determined by using the reaction mixture for the ChAc assay in the absence of exogenous choline.

RESULTS

In vitro inhibition of ChAc and ChE by NVP is shown in Fig. 1 and indicates I_{50} values at 37° of $3{\cdot}4 \times 10^{-6}$ M and approximately $1{\cdot}3 \times 10^{-3}$ M, respectively.

NVP applied topically failed to reduce ACh levels in the corneal epithelium below control levels. 0·1 M EDTA was found to be more effective than 100% DMSO in increasing the permeability of the rabbit corneal epithelium *in vivo* to 0·5% fluorescein solution. This apparently reflected their ability to increase the permeability of the epithelium to NVP since EDTA plus the topical application of NVP (10^{-3} M) in EDTA reduced ACh levels to 66·2 per cent whereas NVP (10^{-3} M) in DMSO reduced ACh levels only to 80·2 per cent of control values.

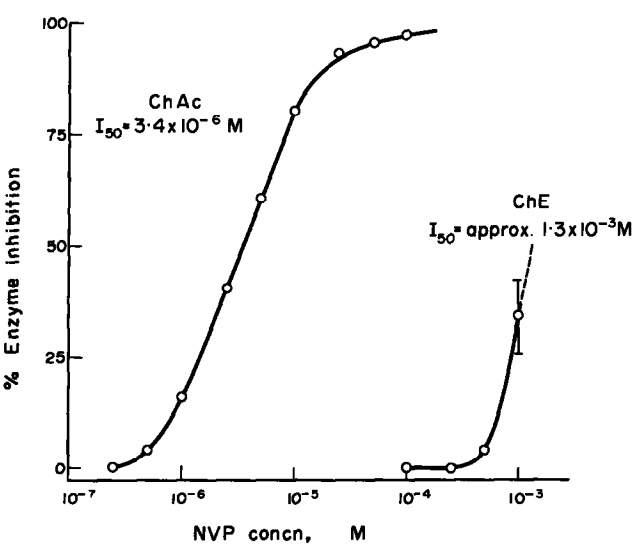

FIG. 1. Inhibition by NVP *in vitro* of choline acetyltransferase and cholinesterase from rabbit corneal epithelium.

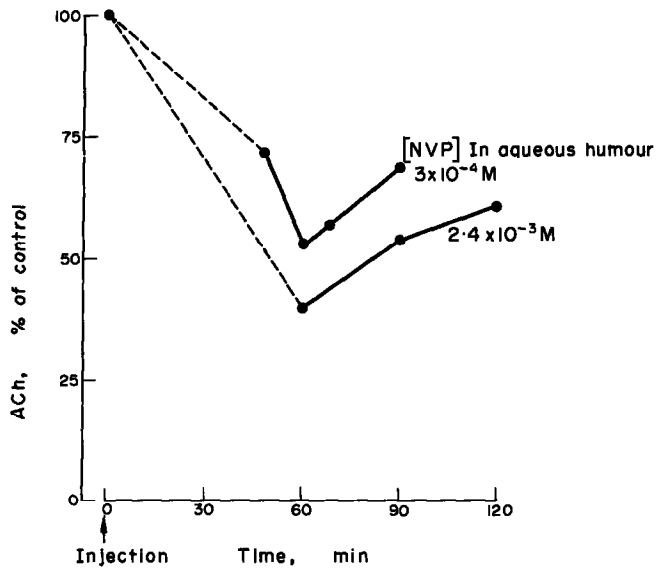

FIG. 2. Time course of ACh depletion in rabbit corneal epithelium due to intraocular injection of NVP.

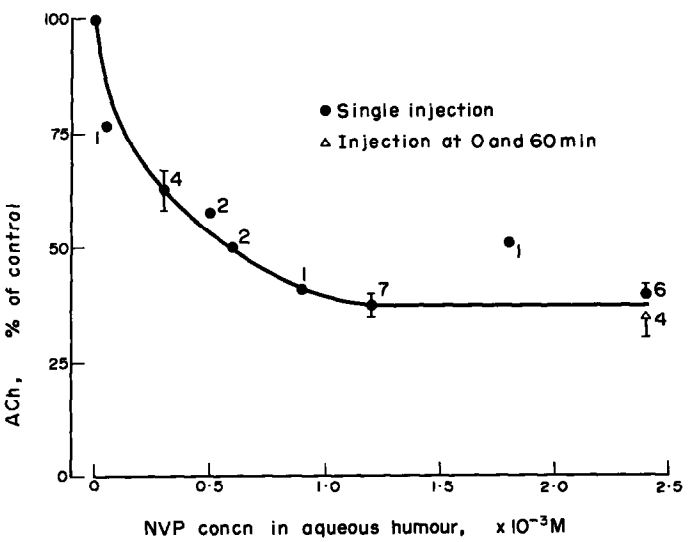

FIG. 3. Dose-dependence of ACh depletion in corneal epithelium by intraocular NVP.

TABLE 1. EFFECT OF SINGLE AND DOUBLE INJECTIONS OF NVP ON ACh LEVELS IN RABBIT CORNEAL EPITHELIUM

	ACh (% of control)		
NVP (final concn of each injection)	After 60 min Single injection of NVP	After 120 min Injections of NVP at 0 and 60 min	After 90 min Injections of NVP at 0 and 30 min
$1{\cdot}2 \times 10^{-3}$ M	37·7 ± 2·3 (7)	42·6, 48·0	42·1, 54·3
$2{\cdot}4 \times 10^{-3}$ M	40·0 ± 2·2 (6)	35·1 ± 4·3 (4)	

When NVP was injected into the anterior chamber of the eye its optimal time of action in reducing ACh levels in the corneal epithelium was 60 min (Fig. 2). The effect of increasing the dose of NVP injected into the anterior chamber was investigated starting with 5×10^{-5} M (final concn) which was the concentration shown to produce 90 per cent inhibition of ChAc *in vitro* (Fig. 1). In fact, intraocular injection of NVP producing concentrations up to $1{\cdot}2 \times 10^{-3}$ M resulted, 60 min later, in dose-dependent depletion of ACh in the corneal epithelium (Fig. 3). Neither doubling this dose nor repeating the injection of NVP 30 min or 60 min later reduced ACh levels significantly further (Table 1). The concentration of NVP resulting in the corneal epithelium from intraocular injections of NVP ($1{\cdot}2 \times 10^{-3}$ M and $2{\cdot}4 \times 10^{-3}$ M) was found to be $1{\cdot}65 \times 10^{-4}$ M (3) and $1{\cdot}71 \times 10^{-4}$ M (2), respectively.

In all cases tested, rabbits whose epithelial ACh content had been depleted by injecting NVP possessed a corneal reflex to both painful and tactile stimuli.

Application of 3% neostigmine to the corneal surface of an untreated eye immediately before removal of the epithelium resulted in $24{\cdot}9 \pm 6{\cdot}2$ per cent more ACh being extracted. This increase was significant at $P = 0{\cdot}001$ (Fig. 4—Control). However, when the total ACh content of the corneal epithelium was reduced by previous intraocular injection of NVP, only $5{\cdot}0 \pm 3{\cdot}7$ per cent more ACh could be extracted. This increase was not significant (Fig. 4—NVP). The relative proportions of the neostigmine-protected (i.e. ChE-labile) ACh and the stable ACh (not susceptible to ChE under these conditions) in NVP-treated corneae were significantly different from those in normal corneae.

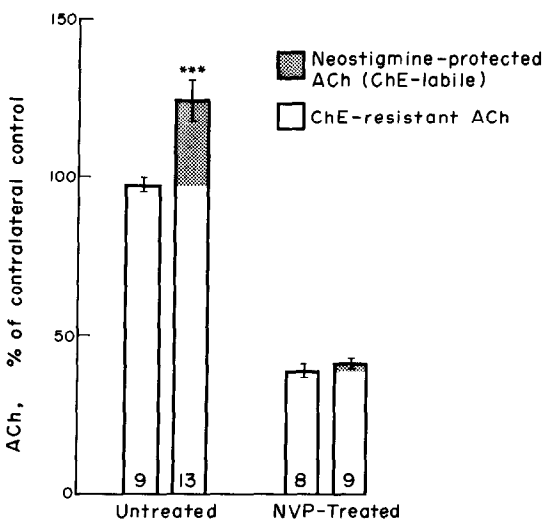

FIG. 4. Effect of neostigmine application before removal of corneal epithelium on the ACh content of control and NVP-treated corneae.

TECh and HC-3. Neither topical application of TECh (0·055 M and 1·1 M) nor of HC-3 ($1{\cdot}74 \times 10^{-2}$ M) reduced ACh levels in the corneal epithelium below control levels over periods up to 60 min. Intraocular injection of TECh, but not of HC-3, did lower the ACh content. The optimal time of action of intraocular injections of TECh ($4{\cdot}4 \times 10^{-2}$ M) was found to be 10 min (Fig. 5). However, TECh even at these

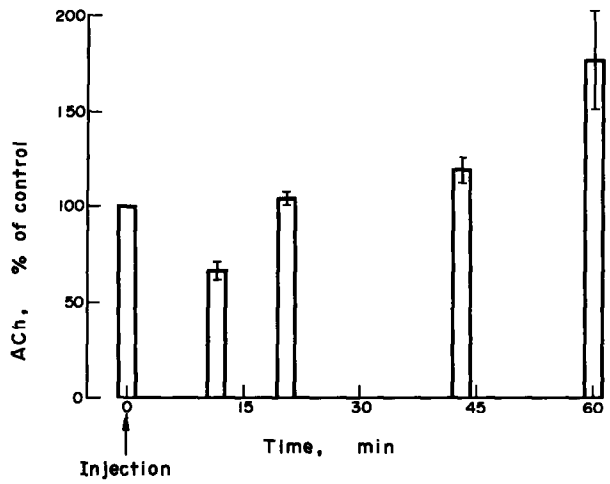

FIG. 5. Time course of the effect of intraocular TECh on ACh levels in the corneal epithelium.

high concentrations failed to reduce ACh in the corneal epithelium to less than 65·8 ± 4·2 per cent of control levels. It should be noted that after longer periods of action an increase in ACh-like activity became evident. Intraocular injections of HC-3 failed to reduce ACh levels in the corneal epithelium below that of control levels (Table 2).

TECh ($2{\cdot}5 \times 10^{-3}$ M) was acetylated *in vitro* at 16·7 per cent the rate of choline acetylation by the same enzyme preparation. This acetyl-TECh was shown to have an agonist effect on the dorsal muscle of the leech that was 21 per cent as potent as ACh on a weight basis.

The relative rates of acetylation *in vitro* of different concentrations of choline and HC-3 were determined as in Fig. 6. It can be seen that the rate of acetylation depends on substrate concentration and that choline has a higher affinity than HC-3 for ChAc from rabbit corneal epithelium. HC-3 reduced the uptake of ^{14}C-choline from the aqueous humour to the corneal epithelium to 40·4 ± 3·5 per cent (3) of the control rate over a period of 60 min.

The percentage recovery of a known quantity of HC-3 from the corneal epithelial homogenate using the spectrofluorimetric assay was found to be 70·9 ± 2·1 per cent

TABLE 2. EFFECT OF HC-3 ON EPITHELIAL ACh LEVELS 60 min AFTER ADMINISTRATION

HC-3	Route of administration	ACh in epithelium (% of control)
$1{\cdot}39 \times 10^{-3}$ M (in aqueous humour)	anterior chamber	94·2; 112·4
$2{\cdot}78 \times 10^{-3}$ M (in aqueous humour)	anterior chamber	104·8 ± 6·6 (5)
$6{\cdot}96 \times 10^{-2}$ M	subconjunctival	86·1; 114·5
HC-3 + NVP		
$1{\cdot}39 \times 10^{-3}$ M $1{\cdot}2 \times 10^{-3}$ M (in aqueous humour)	anterior chamber	98·5 ± 8·2 (3)

* Injected into anterior chamber 1 min after HC-3.

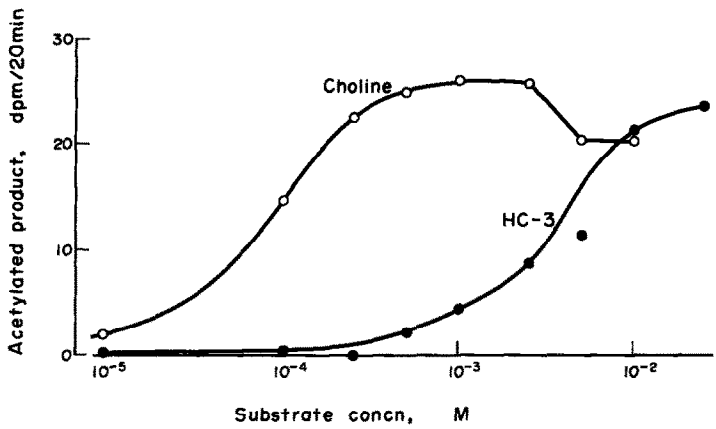

FIG. 6. Acetylation *in vitro* of choline and HC-3 by homogenates of rabbit corneal epithelium.

(5). From this the amount of HC-3 penetrating the test cornea after injecting HC-3 ($2{\cdot}78 \times 10^{-3}$ M in the aqueous humour) was determined as $0{\cdot}73 \pm 0{\cdot}13$ μg (6). No HC-3 was detected in the epithelium of the contralateral control eye. Therefore, assuming the volume of the epithelium to be 10 μl, the concentration of HC-3 in the corneal epithelium was about $1{\cdot}3 \times 10^{-4}$ M (cp. endogenous ACh at $3{\cdot}2 \times 10^{-4}$ M).

The mean concentration of endogenous choline in the rabbit corneal epithelium was estimated to be 0·4 μmoles/mg protein (3). This represents an epithelial concentration of approximately 4×10^{-5} M.

Rabbits treated with TECh and HC-3 had a positive corneal reflex to both tactile and painful stimuli in all cases tested.

DISCUSSION

Experiments *in vitro* showed that in rabbit epithelial homogenates NVP has an almost 400-fold greater inhibitory potency against ChAc than against ChE. Of the many estimates which have been made of *in vitro* ChAc inhibition by NVP[8,18–22] only that of Aquilonius *et al.*,[20] who studied bovine caudate nucleus, suggests an inhibitory potency as high as the present value for rabbit corneal epithelium. All reports of the inhibition of ChE by NVP suggest an I_{50} in excess of 10^{-3} M, as was found in our study. However, when NVP in saline, at these concentrations which produced over 90 per cent inhibition of ChAc but with no detectable inhibition of ChE, was applied topically to the corneae of conscious rabbits, it failed to reduce the ACh level in the epithelium of the test cornea below that of the contralateral control. It should be noted that ACh levels in the corneal epithelium from left and right eyes of the same rabbit are not significantly different (G. G. Fitzgerald and J. R. Cooper, personal communication). While topical application of NVP in DMSO or EDTA produced a small effect, intraocular injection of NVP was more efficient in reducing ACh levels in the corneal epithelium. This finding is surprising in view of the expected lipid solubility of NVP,[20] and highlights the ability of the exterior surface of the corneal epithelium to act as a barrier against foreign molecules. On the other hand, access to the epithelium via its interior surface is relatively easy, presumably allowing penetration of nutrients essential for maintenance of the epithelium.

Despite the difficulties which have been encountered in demonstrating *in vivo* inhibition of ChAc in other tissues,[20,22] corneal ACh levels do fall when synthesis is inhibited. ACh in the rabbit corneal epithelium is depleted *in vivo* to 37·9 per cent of control levels after intraocular injections of NVP producing a concentration of $1{\cdot}2 \times 10^{-3}$ M in the aqueous humour. Neither doubling the dose of NVP nor repeating the injection 30 or 60 min later reduced ACh levels significantly further. In all cases tested, rabbits, whose epithelial ACh content had been depleted in this way, possessed a corneal reflex to both painful and tactile stimuli. Thus, it seems that depletion of ACh levels in the epithelium below 40 per cent does not *per se* abolish the corneal reflex.

The tenacity with which this remaining 30–40% ACh persists could be explained in any of three ways:

(1) ChE inhibition when NVP reaches high concentrations in the epithelium;

(2) existence of a ChAc pool inaccessible to NVP;

(3) ACh pool with a slow turnover rate.

Of these three explanations, as yet only the first can be excluded experimentally. At the concentrations of NVP in the aqueous humour which are optimal for ACh depletion we have estimated the resulting concentration of NVP in the epithelium to be $1{\cdot}71 \times 10^{-4}$ M. At this concentration, NVP would not inhibit epithelial ChE.

While we have no proof of a separate ACh pool with a slow turnover rate, the experiments using neostigmine suggest that more than one pool of ACh could exist. When neostigmine was applied topically to an untreated eye immediately before removal of the corneal epithelium 25 per cent more ACh could be extracted. This ChE-labile fraction of ACh may be an artifact or could represent a functionally distinct pool of ACh. However, when the total ACh content of the corneal epithelium was reduced by previous intraocular injection of NVP, there was no significant increase in the ACh extracted using neostigmine. Thus, the ACh which is subject to depletion by NVP is also susceptible to hydrolysis by ChE, while the ACh which resists depletion by NVP is not susceptible to ChE. From this data it would appear that these two ACh fractions may represent more than one pool of ACh in the corneal epithelium. The possible existence of more than one pool of ACh in the corneal epithelium finds some support in the survival of only half of the original ACh in rabbit corneal explants cultured *in vitro* for 6–13 days.[23]

Neither TECh nor HC-3 produced any great reduction in ACh levels in the rabbit corneal epithelium. TECh was found unsuitable for blocking ACh synthesis in the corneal epithelium since it is itself acetylated to a compound possessing ACh-like activity. The ability of TECh to increase total ACh-like activity in the epithelium after 60 min may indicate synthesis of large amounts of acetyl-TECh or may be a reflection of the stimulated choline transport which Hemsworth and co-workers[24] have postulated.

HC-3 neither decreased nor increased ACh levels in the epithelium. Chemical purity of the sample used was checked and its pharmacological profile confirmed: mice were completely protected against a lethal dose (0·075 mg/kg, i.p.) by simultaneous injection of choline (100 mg/kg). Depletion of ACh in the densely innervated portion of the rat hemidiaphragm was demonstrated *in vitro* after supramaximal stimulation of the phrenic nerve in the presence of HC-3. Our failure to reduce epithelial ACh using HC-3 cannot be explained, as with TECh, by formation of acetyl-HC-3 since

the concentration of $1{\cdot}3 \times 10^{-4}$ M HC-3 reaching the epithelium is not likely to be acetylated to any great extent by epithelial ChAc (see Fig. 6). The estimated concentration of endogenous choline of 4×10^{-5} M would probably be acetylated faster than the 3-fold higher concentration of HC-3.

While HC-3 did reduce by 60 per cent uptake of labelled choline from the aqueous humour to the corneal epithelium, it did not reduce ACh levels in this tissue. It appears, therefore, that choline uptake from the aqueous humour may not be rate limiting for the formation of ACh in the corneal epithelium. Furthermore, the presence of HC-3 in the epithelium itself seems to have no effect on ACh synthesis. It is thus surprising, that Fitzgerald and Cooper[6] reported loss of the corneal reflex after administration of HC-3 subconjunctivally and into the anterior chamber of the eye. One explanation of this could be that HC-3 may have penetrated to the efferent side of the corneal reflex arc resulting in blockade of synaptic transmission at the eyelid.

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