In *Lagocephalus*, heating the extract as high as 170 °C for 10 min did not detoxicate the toxic substance in the original extract of the liver, muscle and testis (Figure 2). There were indications, however, that the toxic effect, with subsequent death, would be considerably delayed. As expected, those animals injected with ovary extract showed no apparent changes. When the heating period was prolonged to 60 min instead of 10, the mice receiving muscle extract (of *Lagocephalus* and *Fugu*) injections showed no obvious changes and survived as long as 36 h, suggesting that detoxication was probably successful (although those receiving liver extract died). The fact that the muscle can be detoxicated at 120 °C while no apparent effect was observed with the liver extract provides circumstantial support to the contention that the toxin is less concentrated in the muscle than in the liver. It further suggests that it would take longer (8 times?) to detoxicate the liver when the same temperature was used. This finding on the relation of temperature and toxicity in the present study is in agreement with that in *Spheroides*[5].

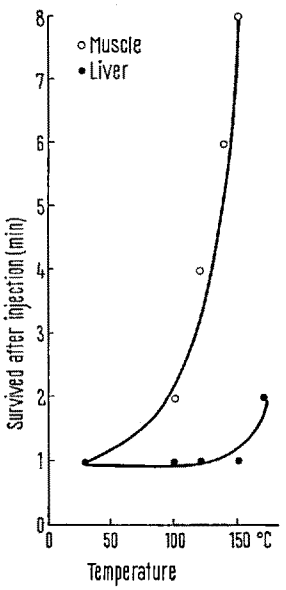

Fig. 2. Relation of temperature and time survived by mice after injection.

It has been shown that the OH^- group at C_4 and O- between C_5 and C_{10} of tetrodotoxin are probably important functional groups so that changes in these eliminate toxicity[4,9]. It is tempting to suggest that prolonged heating might dispose of the OH^- through dehydration and/or break up the oxygen link with C_5 and C_{10}. Further studies along this line are being planned.

Résumé. L'origine et la nature probable de la toxicité chez deux espèces de poissons (*Fugu* et *Lagocephalus*) a été étudiée. Les tissus énumérés dans l'ordre de toxicité décroissant sont les gonades, le foie et les muscles, avec une différence entre les deux sexes, les tissus mâle étant plus toxiques que les tissus femelles. On a éliminé la toxicité par un traitement thermique prolongé et maintenu à 120 °C.

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Sulfhydryl Group Reagents: Effect on Intestinal Smooth Muscle

Previous work in this laboratory has been aimed at obtaining more information on the area surrounding the anionic sub-site of the muscarinic receptor which might be involved in binding antagonists[1]. Our attention was drawn to the work of Karlin which indicated that a disulfide linkage and a sulfhydryl group were present on the nicotinic receptor of eel electroplax[2-4]. Karlin reported that treatment of electroplax with the reducing agent dithiothreitol (DTT) reduced sensitivity to carbachol, but mild oxidation reversed this effect by reformation of disulfide bonds. Brief exposure of the reduced preparation to N-ethylmaleimide (NEM) prevented reoxidation but had no effect on the unreduced electroplax[2]. The vastly enhanced alkylating ability of 4-(maleimido)phenyltrimethylammonium compared to NEM was considered evidence for the close proximity of the disulfide linkage to the anionic sub-site of the acetylcholine (Ach) receptor[3]. Also, bromoacetylcholine (BAC), normally a reversible agonist, became an irreversible agonist after exposure of the electroplax to DTT had freed a sulfhydryl group for reaction[4]. Karlin and Bartels[2] had also proposed that another sulfhydryl group was involved in depolarization of the electroplax, since they found that *p*-chloromercuribenzoate (PCMB) acted as an irreversible inhibitor even without prior DTT treatment. However, Župančič[5] has challenged the idea that the PCMB is active at the nicotinic receptor. We have investigated the action of some sulfhydryl-group reagents on the rat jejunum in order to determine the presence or absence of sulfhydryl or disulfide groups on the muscarinic receptor.

Methods. Jejunum from Wistar rats was suspended in 10 ml muscle baths containing Tyrode's solution at pH 7.4. Baths were aerated with 95% oxygen and 5% carbon dioxide except during incubation with DTT. Control responses to agonists were obtained and muscles were washed before exposure to DTT for 10 min. NEM or PCMB were added for an additional 10 min period in some studies. Muscles were then washed repeatedly and responses elicited. Agonists included Ach, potassium ion and BAC. Receptor protection experiments consisted

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of comparison of Ach responses before and after a 15 min incubation period with muscarinic antagonists. NEM was added for the final 10 min of this period. Tissues were then washed and challenged repeatedly to test recovery.

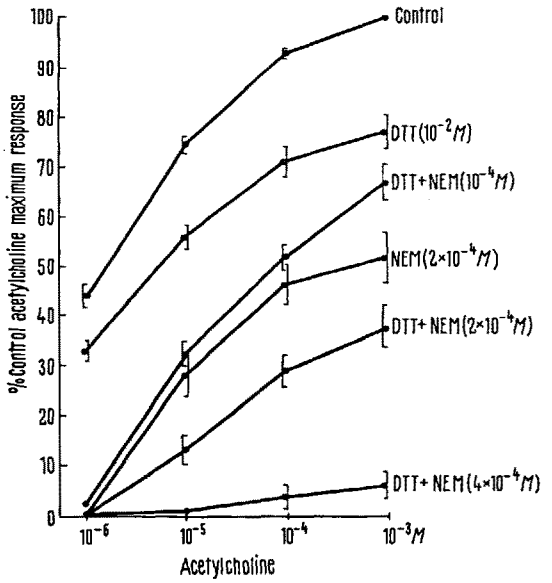
Results and discussion. The Figure shows that DTT had an inhibitory effect of its own. NEM treatment further lowered responses to Ach and this could not be reversed by washing. Increasing concentrations of NEM caused a progressive antagonism. GOODMAN and HIATT[6] reported that NEM antagonized Ach on smooth muscle; therefore we treated our preparation with NEM without prior reduction with DTT. The NEM alone caused a marked, dose dependent reduction in Ach responses that was not reversible. There does not appear to be any potentiation of NEM by the DTT treatment. Thus, NEM inhibition does not appear to depend upon alkylation of sulfhydryl groups formed by reduction of disulfide linkages.

PCMB was also tested as an antagonist for Ach. This agent was less potent than NEM, but proved to be an irreversible inhibitor. Treatment with either PCMB or NEM followed by washing, reduced maximum responses to $10^{-3}M$ Ach to $72.2 \pm 4.5\%$ and $27.3 \pm 6.4\%$ of control, respectively. Both compounds were irreversible inhibitors of potassium stimulation with a potency comparable to that against Ach. PCMB reduced maximum potassium-induced tension development to $56.0 \pm 9.9\%$, whereas NEM reduced maximum responses to $44.7 \pm 6.2\%$ of control. Both PCMB and NEM appear to form covalent bonds with sulfhydryl groups involved in production of contractions, but not necessarily involved with the Ach receptor.

Inhibition of acetylcholine ($10^{-5}M$) responses on rat jejunum following 15 min incubation with antagonist alone, with NEM ($2 \times 10^{-4}M$) for 10 min or antagonist followed by NEM

Competitive antagonist	Control acetylcholine response (%) without NEM		with NEM	
None	101.4 ± 1.9	(10)	48.5 ± 5.0	(23)
Atropine ($10^{-8}M$)	66.8 ± 8.3	(8)	21.4 ± 6.2	(9)
Lachesine ($10^{-8}M$)	68.9 ± 4.8	(8)	11.2 ± 3.0	(10)
Diphenhydramine methiodide ($10^{-5}M$)	68.3 ± 8.2	(8)	13.6 ± 3.5	(10)
3-(Dimethylamino)-1,1-diphenylpropanol methiodide ($10^{-5}M$)	72.8 ± 3.3	(8)	36.1 ± 6.9	(10)

Values are expressed as percent of initial response $\pm$ S.E.M. Number in parentheses indicates the number of muscles used for each determination.



Acetylcholine dose-response curves on rat jejunum segments. Each point represents the mean of the following number of determinations: Control ($n = 48$); DDT alone ($n = 38$); DTT + NEM ($10^{-4}M$) ($n = 18$); NEM ($2 \times 10^{-4}M$) ($n = 14$); DTT + NEM ($2 \times 10^{-4}M$) ($n = 10$); and DTT + NEM ($4 \times 10^{-4}M$) ($n = 6$). Brackets represent standard error of the mean. Drug dosages are expressed as molar concentration.

BAC has been reported by CHIOU and SASTRY[7] to be a muscarinic agonist on guinea-pig ileum. On the rat jejunum we found it to be a reversible agonist with approximately $^1/_{30}$th the activity of Ach. Dose-response measurements were repeated after DTT treatment for 10 min and were found to be unaffected. BAC was readily removed by washing; therefore, the irreversible stimulation found by KARLIN with electroplax does not occur with the muscarinic receptor.

Receptor protection was used in a final test to locate a sulfhydryl group near the muscarinic receptor. Classical, reversible, antimuscarinic agents such as atropine, lachesine, diphenhydramine methiodide, and 3-(dimethylamino)-1,1-diphenylpropanol methiodide might employ different modes of binding to the receptor and occlude different regions adjacent to the active site. Results of these experiments are presented in the Table. The inhibition by competitive antagonists proved to be reversible with repeated washing and stimulation. In the case of NEM alone, inhibition was irreversible. The muscarinic antagonist followed by NEM resulted in additive inhibition of Ach responses that failed to return to control levels. Thus, the effect of NEM on intestinal smooth muscle was not attenuated by prior treatment with reversible muscarinic antagonists. Inhibition by NEM would have been diminished if the agents had been competing for the same receptor sub-sites.

We conclude that the muscarinic receptor on rat jejunum lacks a disulfide linkage in the vicinity of the Ach receptor site. Other disulfide bonds in the cell membrane may be disrupted by DTT reduction, and this lead to a reduced sensitivity to Ach and potassium. There may also be essential sulfhydryl groups in the chain of events leading to contraction which could react irreversibly with NEM and PCMB. BAC does not affect these sulfhydryl groups, probably because penetration to an internal site in the membrane would be prevented by its cationic character[8].

Zusammenfassung. Die Acetylcholinkontraktion an Ratten-Jejunumstücken wird durch NEM gehemmt, was aber durch Vorbehandlung mit einem Reduktionsmittel nicht verstärkt wird. Sowohl NEM wie auch PCMB hemmen auch die Kaliumkontraktur, so dass die Hemmung der Acetylcholinkontraktion offenbar nicht auf den Rezeptor bezogen werden kann.

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