Tetrazolium Salt: Antagonism of Acetylcholine-Induced Response on Frog's Rectus

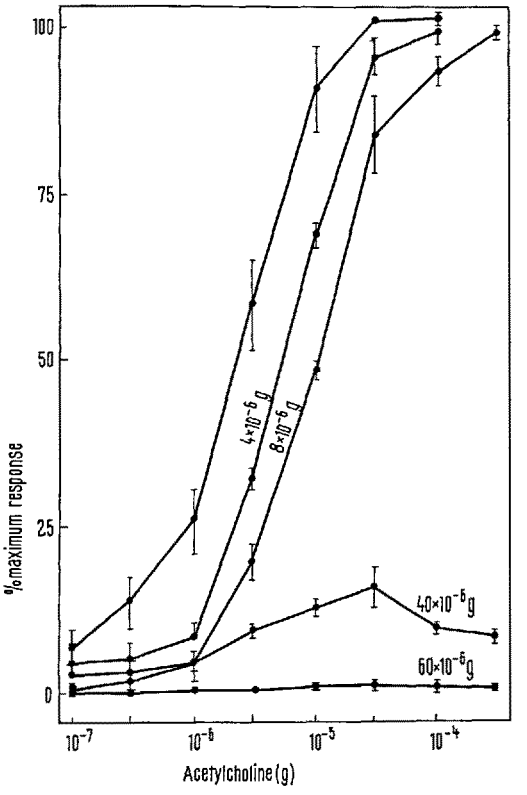
Tetrazolium salts[1] are a group of water-soluble quaternary ammonium compounds which are converted by reduction to the intensely coloured, generally water-insoluble formazans. For this property they have been extensively used as analytical reagents in histo- and cytochemical works to locate certain enzymes[2–4], screen cadaver kidneys for transplantation purposes[5,6] and for improved resolution of bacteriophage plaques[7]. These compounds have also been reported to be successfully used for assaying steroids[8,9], and various other substances[10–12], and are becoming of interest to workers from various disciplines.

However, a search of the relevant literature failed to reveal any previous report of such a major effect of 2:3:5 triphenyl tetrazolium chloride (TTC), as antagonism of acetylcholine response. In this communication, preliminary data is presented suggesting evidence for TTC behaving as an acetylcholine antagonist on frog's rectus abdominis muscle.

The rectus abdominis muscle from *Rana tigrina* was used in all the experiments. A strip of this muscle was mounted in frog-Ringer solution, through which oxygen with 5% carbon dioxide was blown. Experiments were done in an air-conditioned room at about 26 °C. Contractions were recorded with an isotonic lever on a rotating smoked drum. The load on tissue was between 0.5–1 g. In all the experiments acetylcholine was in contact with the tissue for 35 sec. It was then washed out, the preparation gently stretched, then washed again. Tissue was then left for 3 min and the next application of acetylcholine was made exactly 3 min after the previous one. In this time cycle of 3 min, a total of 3 washings was given. The recovery of the tissue after addition of drugs was complete. Contractions were recorded with graded doses of acetylcholine till a maximum response was accomplished in absence and presence of different amounts of TTC (BDH, London). TTC was always added to the bath 45 sec before applying acetylcholine.

TTC antagonized acetylcholine-induced contractions on the frog's rectus abdominis muscle; inhibition depending upon its concentration in the tissue bath solution. As can be seen in the Figure, definite antagonism of acetylcholine response could be observed at a concentration of TTC as low as 4 μg/ml of organ bath solution. In the presence of relatively lower concentration of TTC, maximum response could be restored by applying higher amounts of acetylcholine, causing a parallel shift in the dose-response curve, suggesting competitive inhibition of acetylcholine response. When the concentration of TTC was relatively higher, tissue failed to respond in a linear fashion resulting in the depression of response curve, which signifies non-competitive inhibition of acetylcholine. At a concentration of 60 μg/ml of TTC, tissue response was completely antagonized up to the doses reported in the Figure.

To our knowledge, this type of effect seems not to have been reported previously, although, besides their use as analytical reagents, tetrazolium salts are being found to possess various biochemical potentialities[12–16]. Being electron acceptors[12–14], they have very recently been utilized to intercept electron flow at known sites of the respiratory chain[13] and also have been reported to uncouple the oxidative-phosphorylation in rat liver mitochondria[15]. Other studies[16–18] have shown them to be capable of stimulating the pentose-phosphate pathway in ascites tumour cells; at relatively higher concentration TTC stimulated, and in lower concentration inhibited, the oxidation of glucose. Once the nature of antagonism of acetylcholine response



Cumulative log-concentration response curves for acetylcholine (Ach). Graph describes the effect of 2:3:5 triphenyl tetrazolium chloride (TTC) upon the Ach-induced contractions of frog's rectus muscle. The ordinate is the percentage of maximal response attainable with Ach, as TTC concentration is increased from zero. Concentrations of TTC are mentioned with each curve. Each point on each curve is the mean of at least 7 separate experiments. Note the parallel shift in the curves for Ach caused by TTC at lower doses (4×10^{-6} g/ml and 8×10^{-6} g/ml), indicating that TTC behaves as a competitive antagonist to Ach. The depression of the curves at higher concentrations signifies a non-competitive antagonism. Abscissa is log scale of Ach-concentration.

[1] F. E. SMITH, Science *113*, 751 (1951).
[2] B. SMITH, J. Histochem. Cytochem. *12*, 847 (1964).
[3] M. V. LAYCOCK, Clinica chim. acta *11*, 98 (1965).
[4] P. VESCEI, D. TANKA, M. KELLER, V. KEMANY, J. MARTON and T. GROSZTOMYI, Acta physiol. hung. *22*, 125 (1962).
[5] P. TERASKI, Transplantation *5*, 76 (1967).
[6] R. B. SMITH, J. Am. med. Ass. *201*, 160 (1967).
[7] P. A. TATTEE, J. Bact. *92*, 782 (1966).
[8] J. GREELY, W. H. HALL and T. B. MICHAELS, Ann. N.Y. Acad. Sci. *130*, 545 (1965).
[9] P. VESCEI, D. LEMMER, H. G. STEINA and H. R. WOLF, J. Chromat. *26*, 533 (1967).
[10] R. D. CAPPS, Clin. Chem. *12*, 406 (1966).
[11] K. LORENTZ, Clinica chim. Acta *13*, 660 (1966).
[12] E. G. ALLEN, J. Bact. *92*, 104 (1966).
[13] C. L. TALESORA and M. C. BLANCHAER, Can. J. Biochem. *45*, 299 (1967).
[14] K. J. OGAWA, Kumamoto med. Soc. *41*, 81 (1967).
[15] J. B. CLARK and A. L. GREENBAUM, Biochem. J. *9*, 651 (1965).
[16] S. SATO and K. SATO, J. Biochem., Tokyo *56*, 157 (1964).
[17] S. SATO and K. SATO, J. Biochem., Tokyo *58*, 470 (1965).
[18] S. SATO, Biochem. biophys. Acta *122*, 359 (1966).

on frog's rectus muscle and effect of TTC on action of different agonists on other muscle preparations is established, an opportunity would be available to search for a relationship, if any, between the pharmacological effects of TTC and its biochemical actions[12–18]. However, it should be mentioned that structurally TTC possesses 1 quaternary nitrogen in the tetrazole ring, generally an essential requirement for acetylcholine antagonists. Future investigation would disclose how far other salts of tetrazolium behave differently to TTC[19].

Zusammenfassung. Es wurde die Wirkung eines Tetrazolsalzes (2:3:5, Triphenyl-Tetrazolium-Chlorid) auf die durch Acetylcholin hervorgerufene Kontraktion des M. rectus abdominis beim Frosch geprüft. In verhältnismässig niedriger Dosierung verhält sich die Substanz wie ein kompetitiver Hemmer des Acetylcholins, während sich dieser Effekt in höherer Dosierung als nichtkompetitiv erwies.

B. Basuray and Pawan S. Chauhan[20]

St. John's Medical College, Bangalore (Mysore State) and Indian Institute of Experimental Medicine, Calcutta-32 (India), 21 June 1968.

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[20] Address for reprints: Indian Inst. of Experimental Medicine 4 Raja Subodh Mullick Road, Calcutta-32, India.

Impairment of Growth and Pancreatic Hypertrophy in Rats Fed Trypsin Inhibitor from Raw Peanuts

Raw soybean meal retards growth in young animals causing, at the same time, pancreatic hypertrophy[1–3]. In chicks impairment of growth and pancreatic hypertrophy may result from feeding of the soybean trypsin inhibitor (SBTI) but may also occur after inhibitor-free meal[1,2]. In rats more consistent changes are produced by SBTI, while heat-inactivated preparations are harmless[3]. The mode of stimulatory action is still unknown. Feeding of raw soybean meal also prolongs blood coagulation in chicks[4], but it seems unlikely that the inhibitor, being a foreign protein, could be absorbed in amounts sufficient to influence coagulation.

Another trypsin inhibitor, extracted from raw peanuts (*Arachis hypogaea*), was found to decrease the spontaneous fibrinolytic activity of blood in man[5,6] and to enhance experimentally induced arterial disease in rabbits[7]. Raw peanut meal was reported to impair growth in young pigs[8]. It was, therefore, of interest to see if trypsin inhibitor fractions from raw peanuts, like SBTI, would retard growth and produce pancreatic hypertrophy in young rats.

The trypsin-inhibitor fraction was prepared from hexane-defatted raw peanut meal[9]. Assayed against crystalline trypsin (Novo Laboratories, Copenhagen) 1 mg of the peanut fractions neutralized about 0.07 mg trypsin. Inhibitor-free preparations were obtained from solutions kept for 2 h in a boiling waterbath before precipitation with acetone. The peanut inhibitor is a stronger inhibitor of activator-induced fibrinolysis than SBTI[10]. The preparations were fed to weanling male rats (25–30 g, Sprague-Dawley strain) in a dosage of 50 mg daily/animal mixed in Purina Chow. The drinking water contained 1.0 mg ascorbic acid/animal per day. Body weights were recorded weekly for each animal. After 6 weeks, the animals were killed with ether and the pancreas carefully isolated and its wet weight recorded. All other organs were inspected morphologically. Paraffin sections were prepared from the pancreas and appropriately stained (Haematoxylin-Eosin). The results of a comparative assay with 10 animals in each group are presented in the Table.

There was a significant retardation of growth and an increase in pancreas weight in the group fed the trypsin inhibitor fraction. In both cases the p-value was less than

Body weights and pancreas weights in weanling rats fed trypsin-inhibitor fractions or heat-inactivated preparations from raw peanuts

	Inhibitor material	Heat-treated material
Original body weight (g)	27.9 ± 1.8 25–30	28.1 ± 1.4 25–30
Final body weight (g)	94.7 ± 14.5 70–126	114.5 ± 11.5 94–130
Gain in body weight (g)	66.8 ± 14.7 56.3–95.0	86.4 ± 9.2 65.4–103.3
Pancreas weight (mg)	641 ± 56 530–760	421 ± 31 380–480
Ratio of pancreas to body weight	0.0068	0.0037

10 animals in each group. Results expressed as mean ± standard deviation and with range.

[1] M. H. Pubols, H. C. Saxena and J. McGinnis, Proc. Soc. exp. Biol. Med. *117*, 713 (1964).
[2] H. C. Saxena, L. S. Jensen and J. McGinnis, Proc. Soc. exp. Biol. Med. *112*, 101 (1963).
[3] A. N. Booth, D. J. Robbins, W. E. Ribelin, F. DeEds, A. K. Smith and J. J. Rackis, Proc. Soc. exp. Biol. Med. *116*, 1067 (1964).
[4] S. L. Balloun and E. L. Johnson, Archs Biochem. *42*, 355 (1953).
[5] T. Astrup, P. Brakman, P. Ollendorff and J. Rasmussen, Thromb. Diath. haemorrh. *5*, 329 (1960).
[6] P. Brakman, K.-E. Sjølin and T. Astrup, Thromb. Diath. haemorrh. *8*, 442 (1962).
[7] H. C. Kwaan and T. Astrup, Archs Path. *78*, 474 (1964).
[8] G. E. Combs and H. D. Wallace, J. Anim. Sci. *21*, 95 (1962).
[9] T. Astrup, P. Brakman and K.-E. Sjølin, Nature *194*, 980 (1962).
[10] K. Egeblad, Thromb. Diath. haemorrh. *17*, 31 (1967).