The accumulation of cyclic AMP is dependent on the concentration of adenosine applied. The effect is a maximum at an adenosine concentration of 500 μM; it stabilizes at 1 mM and above, with an increase of cyclic AMP to 230% of controls (Figure 2). A concentration of 100 μM adenosine was used for all further experiments.

c) *Inhibition.* The accumulation of cyclic AMP by adenosine can be inhibited by theophylline. The inhibition is partial with 1 mM theophylline and complete with 10 mM (Figure 3).

We have applied a large number of substances, which are known to inhibit the effect of different agents on adenylate cyclase in other systems. These included α-adrenergic (phentolamine, 0.1 mM), β-adrenergic (sotalol, 0.1 mM), dopaminergic (haloperidol, 0.05 mM), muscarinic (atropine, 0.05 mM), nicotinic (hexamethonium, 0.5 mM) inhibiting agents, as well as local anaesthetics (cocaine, 1 mM; tetracaine, 1 mM and tetrodotoxin, 0.003 mM). None of these substances inhibited the increase in cyclic AMP caused by adenosine.

d) *Depolarizing agents.* Since adenosine is thought to be an intermediary between depolarization and cyclic AMP accumulation in brain[12], the effect of depolarizing agents was tested in the vagus nerve. Pieces of nerve were incubated in presence of high potassium (100 mM) or veratridine (500 μM), which are known to depolarize frog nerve fibres[13] and rabbit vagus nerve[14]. Neither of these two agents produced an increase in cyclic AMP.

Discussion. Our experiments show that there is a very marked accumulation of cyclic AMP when the rabbit vagus nerve is exposed to adenosine. As in guinea-pig brain slices[15], the effect of adenosine is inhibited in the presence of a xanthine derivative, theophylline. It appears that the adenylate cyclase response to adenosine in the vagus nerve is direct and specific, since it is unaffected by agents which, in other tissues, inhibit the adenylate cyclase response to catecholamines, cholinomimetics, or depolarizing agents.

In guinea-pig brain, cyclic AMP content is increased by exposure to depolarizing agents, or to adenosine, and it is thought that adenosine is the intermediary between depolarization and cyclic AMP accumulation[12]. In bovine superior cervical ganglion, depolarizing agents, but not adenosine, produce an accumulation of cyclic AMP[16,17], and it is thought that the intermediary between depolarization and cyclic AMP accumulation is at least partially catecholamines. We have also tested rabbit superior cervical ganglions for their response to adenosine. No significant accumulation of cyclic AMP was found (121 $\pm$ 11% of unstimulated controls, $n = 4$, 100 μM adenosine).

In vagus nerve, only adenosine has an effect on cyclic AMP content. The absence of any effect of depolarization in vagus nerve reveals either the absence of an endogenous adenosine releasing system, or a difference in function of the adenosine-sensitive adenylate cyclases of nerve axons and of central nervous system. It is not known whether cyclic AMP accumulates in the axon or in supporting tissue (Schwann cells).

It will be of interest to investigate further the adenylate cyclase in vagus nerve in order to elucidate the seemingly complex functions of cyclic AMP in the nervous system.

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The Loss of Biological Activity of 5-Hydroxytryptamine Creatinine Sulphate

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Summary. 5-Hydroxytryptamine creatinine sulphate loses its biological activity when maintained at room temperature. The loss of 5-HT activity (in stimulating sodium transport across frog skin) is greater than the loss of creatinine sulphate activity (inhibition of sodium transport).

5-Hydroxytryptamine stimulates ion transport in a variety of tissues e.g. blood platelets[1], the nervous system at central and peripheral synapses[2], vertebrate nephrons[3], insect Malpighian tubules[4], and insect salivary glands[5] but inhibits transport in other tissues e.g. erythrocytes[6] and the nervous system[2]. 5-Hydroxytryptamine creatinine sulphate complex (5-HTCS) has been shown to have a biphasic effect on active sodium transport across isolated frog skin[7]. At low concentrations (3×10^{-5} M) transepithelial sodium transport is stimulated whereas at higher concentrations transport is inhibited. The stimulatory and inhibitory actions of 5-HTCS can be attributed to the separate components of the complex: 5-hydroxytryptamine being stimulatory and creatinine sulphate inhibitory. During the initial study of the dose-response characteristics of the induced changes in sodium transport major discrepancies in the magnitude of response became apparent in that smaller responses were consistently observed in experiments performed some hours after preparation of the 5-HTCS solution than in experiments performed using freshly prepared 5-HTCS. As the experimental protocol remained standard this suggested that the 5-HTCS solution may be losing activity so a series of experiments were designed to examine the relative activity of 5-HTCS – both solid and in solution – under a variety of conditions.

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Materials and methods. Frogs (*Rana temporaria*) were rapidly pithed, the ventral skin removed and stretched across a double Ussing-type chamber[8]. The skin was pre-incubated in aerated frog Ringer solution for 45 min, the Ringer renewed and after a further 15 min standard volumes of either a 3×10^{-5} *M* or 3×10^{-2} *M* 5-HTCS (obtained from Sigma Co.) in Ringer solution were added to the serosal medium of one half chamber to give a final 5-HTCS concentration of 10^{-5} *M* or 10^{-2} *M*; the other half chamber received similar volumes of Ringer solution and acted as control. Membrane potential and short circuit current were monitored over a 60 min period. To obtain the relative activity[9] of a 5-HTCS sample compared to a freshly prepared sample (unit activity) the sample of unknown activity was added to the serosal medium of one half chamber as above, and freshly prepared 5-HTCS (the solid of which was kept dehydrated at −20 °C) was added to the other half chamber and the magnitude of the responses compared – the relative activity of a sample being expressed as a fraction of unity. Sigma Co. recommend storing 5-HTCS at 0–5 °C; our normal practise is to store it in the deep freeze (−20 °C) in a container containing silical gel. In one series of experiments solid samples of 5-HTCS were kept dehydrated (i.e. in the presence of silica gel) but at room temperature (approx. 21 °C) and the relative activity of freshly prepared solutions from this solid sample monitored at 28 day intervals for 280 days. In another series of experiments 5-HTCS solutions were either prepared in Ringer at 4 °C and maintained at 4 °C or prepared and maintained at room temperature and the relative activities monitored at hourly intervals. In parallel experiments solid 5-HTCS and 5-HTCS solutions were maintained in darkness to eliminate any photo-chemical effects.

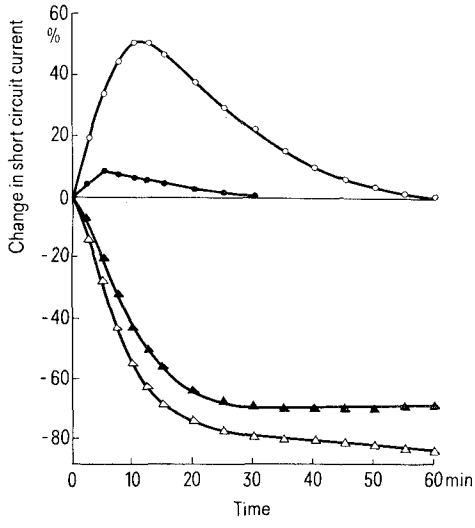

Fig. 1. The change in short circuit current (as a percentage of the control tissue) measured across frog skin in response to the addition of a freshly prepared solution (open symbols) and a solution kept at room temperature for 5 h (closed symbols) of 10^{-5} *M* (circles) and 10^{-2} *M* (triangles) 5-HTCS. Each point represents the mean of 4 experiments.

5-HTCS is acidic in solution so it was necessary to titrate the Ringer solution containing the 5-HTCS with NaOH to obtain a pH of 7.8. At the highest concentration of 5-HTCS used (10^{-2} *M*) this procedure increased the concentration of sodium in the Ringer by approximately 7 mEq/l.

Results and discussion. As shown (Figures 1 and 2) there is a loss of activity of 5-hydroxytryptamine (stimulatory response) 3 h after solution preparation if the solution is kept at room temperature; after 6 h under these conditions activity is virtually zero. If, however, the solution is maintained at 4 °C the loss of activity is substantially prevented so that even after 24 h activity has fallen by only 10%. Creatinine sulphate, which mediates the inhibitory action of high concentrations of 5-HTCS, also shows a decrease in activity if maintained at room temperature after preparation but not to such low levels as exhibited by 5-hydroxytryptamine so that

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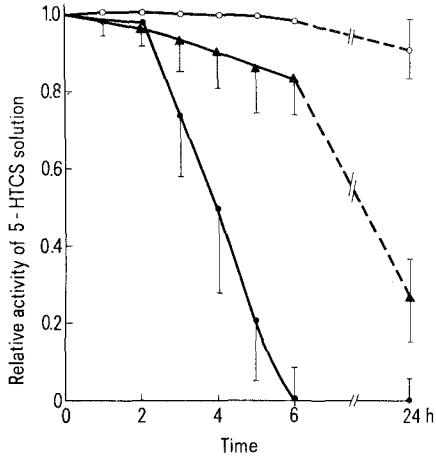

Fig. 2. The relative activity of solutions of 10^{-5} *M* (circles) and 10^{-2} *M* (triangles) 5-HTCS prepared and maintained at either room temperature (closed symbols) or 4 °C (open symbols) measured at hourly intervals from the time of preparation. Each point represents the mean of 4 solutions.

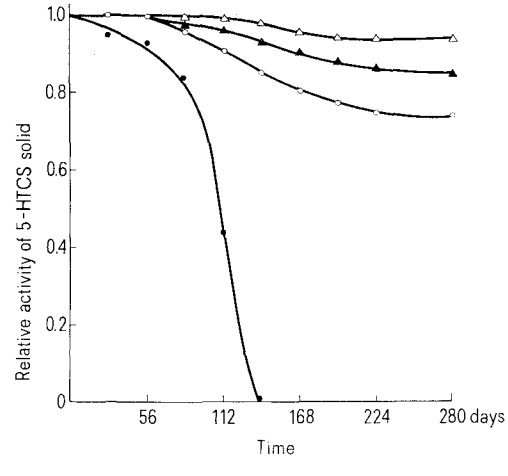

Fig. 3. The relative activities of 10^{-5} *M* (circles) and 10^{-2} *M* (triangles) 5-HTCS, the solid samples of which were maintained either at room temperature (closed symbols) or dehydrated at −20 °C (open symbols). Activity of each solid sample was measured using a freshly prepared solution at 28 day intervals. Each point represents the mean of 4 different samples.

after 6 h activity has fallen by only some 16% and after 24 h there is still some 25% residual activity. If the solution is maintained at 4 °C there is no measurable loss of activity.

The loss of activity is not restricted to 5-HTCS in solution: Figure 3 shows that if solid 5-HTCS is kept at room temperature then the stimulatory 5-hydroxytryptamine activity is markedly reduced after 84 days (3 months) and is abolished after 140 days (5 months); even if the sample is maintained dehydrated at −20 °C activity is reduced to some 75% of its original after 224 days (8 months). Creatinine sulphate activity does not appear to be seriously impaired by keeping the solid 5-HTCS at room temperature. Preliminary investigations have shown that the potency[9] of 5-HTCS is unaffected by any of the above treatments; the loss of 5-hydroxytryptamine activity in stimulating sodium transport thus probably reflects the gradual decomposition of the compound to produce an inactive derivative. This being the case the process is not photo-chemical since, as shown in the Table, maintaining 5-HTCS (either solid or in solution) in darkness does not prevent the decline in activity.

The relative activities of solutions and solid samples of 5-HTCS maintained at room temperature but under different conditions of light exposure

Solution (5 h)		Solid samples (4 month)	
Light	Dark	Light	Dark
0.21	0.26	0.44	0.51

In all cases the figures quoted are the relative activity of a 10^{-5} *M* response and represent the mean of 4 experiments.

A similar decrease in activity of 5-hydroxytryptamine has been observed by BERRIDGE (personal communication) in the stimulation of fluid secretion by the isolated salivary glands of *Calliphora erythrocephala* and by CALVERT (personal communication) in the stimulation of fluid secretion by the salivary glands of *Musca domestica*. Such changes in the activity of 5-HTCS solutions reflect either the specificity of the tissue receptor or the ability of the receptor-hormone complex to initiate the physiological response. MADDRELL et al.[10] and BERRIDGE[11] have shown that small changes in the 5-hydroxytryptamine molecule, particularly with respect to the hydroxyl group at C_5 and the quaternary nitrogen on the end of the ethylamine side chain, leads to an inactivation of the molecule but does not necessarily prevent its binding. It seems probable therefore, that the loss of 5-hydroxytryptamine activity is due not to the inability of the derivative to bind to the tissue receptor but rather to the inability of the receptor-derivative complex to initiate the physiological response. The present work thus indicates that tissues may appear to be falsely unresponsive to applied 5-hydroxytryptamine if either the solid or the prepared solution is allowed to stand at room temperature; indeed if a tissue exhibits both a stimulatory 5-hydroxytryptamine response and an inhibitory creatinine sulphate response then because of the loss of activity of 5-hydroxytryptamine but not of creatinine sulphate to the same degree the tissue could appear to be inhibited (creatinine sulphate response) by 5-hydroxytryptamine.

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Depletion of Synaptic Vesicles at the Frog (*Rana pipiens*) Neuromuscular Junctions by Tetraphenylboron

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Summary. At the frog neuromuscular junction tetraphenylboron produced a decrease in miniature endplate potential amplitude, accompanied by a decrease in the number but not the size of synaptic vesicles.

Tetraphenylborate (TPB), a highly lipid soluble anion, exhibits marked pharmacological effects at the amphibian neuromuscular junction[3,4]. Initially TPB produces a profound concentration-dependent increase in spontaneous acetylcholine release and augments evoked release. With time the high rate of spontaneous release declines and this decline is accompanied by a decrease in quantal size. Further, neuromuscular transmission is blocked by TPB, presumably by a pre-junctional mechanism, as TPB is devoid of post-junctional agonist or antagonist properties[3,4].

The present study represents an attempt to correlate the TPB-induced changes in the rate of spontaneous transmitter release and of quantal size measurable by electrophysiological techniques, with morphological changes at the frog neuromuscular junction. In particular, it was of interest to observe whether depletion of synaptic vesicles occurred and whether the reduction of quantal size was accompanied by a decrease of synaptic vesicle diameter, as such a correlation is not noted after quantal size reduction by hemicholinium[5,6].

For electrophysiological recordings, frog (*Rana pipiens*) sartorius muscle preparations were dissected in a phosphate buffered Ringer solution[7] and were subsequently

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[2] This work was supported by NIH Grants Nos. NS-07740 and NS-05707 to R. L. P. and was done during the tenure of a Research Fellowship of Muscular Dystrophy Associations of America, a Wellcome Research Travel Grant and a grant from the Carnegie Trust for the Universities of Scotland to I. G. M. The assistance of Dr. R. M. SCHNITZLER, W. G. BOLDOSSER and P. M. SPANNBAUER is gratefully acknowledged.
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