Effect of acetylcholine on melanophores of *Rana tigrina*

Drugs (mg/kg)	*n*	Melanophore size index (mean ± SEM)		
		Before	After (peak effect)	Difference
Acetylcholine (0.1)	20	55.0 ± 1.68	17.45 ± 1.06 $P_1 < 0.001$	37.55 ± 1.42
Atropine (5) + Acetylcholine (0.1)	10	52.7 ± 2.51	20.3 ± 1.58	32.4 ± 2.71 $P_2 > 0.05$
Pentolinium (5) + Acetylcholine (0.1)	10	53.6 ± 2.28	17.1 ± 1.38	36.5 ± 1.77 $P_2 > 0.05$
Procaine (2) + Acetylcholine (0.1)	10	56.1 ± 2.19	49.9 ± 1.93	6.3 ± 0.78 $P_2 < 0.001$

P_1, Statistical significance between pre and peak post drug response.
P_2, Statistical significance between differences in blocker pretreated and control acetylcholine groups.

pretreated with a cholinesterase inhibitor (physostigmine, 0.1 mg/kg), the effect of acetylcholine appeared in 3–5 min, reached a peak effect by 20–30 min and lasted for 45–60 min. This dose of physostigmine did not have any significant effect of its own. Pretreatment with either atropine (5 mg/kg) or pentolinium (5 mg/kg) failed to inhibit significantly the effect of acetylcholine. Procaine (2 mg/kg) markedly inhibited the skin colour blanching and melanin aggregating effects of acetylcholine. All blocking agents were administered 15 min prior to acetylcholine and none of them had any significant effect per se on the test parameters.

The effect of acetylcholine on melanophores of *R. tigrina* was probably not mediated through cholinergic receptors, since atropine (*m*-cholinolytic) and pentolinium (*n*-cholinolytic) both failed to antagonize its effects. The marked and highly significant antagonism produced by procaine can be explained on the basis of the membrane stabilizing effect[3] of the local anaesthetic, which would prevent acetylcholine induced ion influx into the melanophores. It has been suggested[4] that sodium is involved in the melanin aggregating and dispersing effects of acetylcholine and melanocyte stimulating hormone (MSH), respectively. Acetylcholine induced inhibition of the melanin dispersal effect of MSH[5,6] has been suggested to be due to a mechanism involving membrane polarity and ion transport across the melanophore membrane[4].

It is interesting to note that, embryologically, melanophores are derived from the neural crest[7,8]. Procaine, which blocks nerve transmission, including cholinergic neurotransmission, has been shown to inhibit cholinergic melanophore response as well, the underlying mechanism probably being similar in either case.

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Action of Stannous and Stannic Chlorides on Bacteria

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Summary. Stannous or stannic chlorides reduced the growth rate of *K. aerogenes*, *Ps. reptilovora* and an unidentified bacterium in a minimal liquid medium and on agar plates. The greatest effect was observed with *K. aerogenes* and was accompanied by a decreased viability, but 100% survival occurred with the other strains. The metal was loosely bound to the cells and there was no direct correlation between the amount adsorbed and the biological response.

This work forms part of a general study of the actions of metal ions on bacteria and their concentration by the organisms during growth. Initial experiments showed that stannous or stannic chloride at concentrations between 0.25 and 2.0 m*M* reduced the growth rate of organisms in liquid culture and accordingly the investigation reported here was undertaken. Other workers have commented on the innocuousness of soluble inorganic tin compounds towards bacteria[2] and fungi[3].

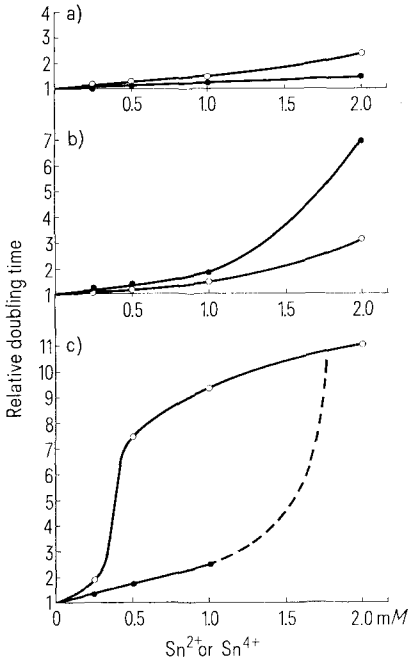
Materials and methods. Klebsiella aerogenes NCIB 418, *Pseudomonas reptilovora* NRRL B 334 and strain X, an organism, as yet unidentified, which was isolated by exposing medium containing stannous chloride to the atmosphere, were used. They were grown aerobically at 28°C in the minimal medium described by CARTER and DEAN[4] supplemented with KCl to increase the K^+ concentration from the normal level of 9 m*M* to 64 m*M*. This is a glucose-inorganic salts medium (pH 7.1) in which exhaustion of the glucose limits growth and the added KCl, besides increasing the stability of the tin chlorides, enabled strain X to grow at its maximum rate. Its growth rate in the presence of 9 m*M* KCl was 66% of that ob-

tained with 64 m*M* KCl but the additional KCl had no effect on the growth rate of the other two strains. Agar medium was prepared by solidifying minimal medium, as modified above, with 1.25% Difco Agar-Noble.

Results and discussion. Increasing the concentration of either of the tin salts in minimal liquid medium led to a progressive increase in the doubling time (reciprocal of the growth rate) of strain X but Sn^{2+} was more inhibiting than Sn^{4+} (Figure a)[5]. Both $Sn^{2+}$and Sn^{4+} had a greater action on *Ps. reptilovora* but the order of their activities was reversed (Figure b). For example, the doubling time of this organism was increased 7-fold by 2 m*M* Sn^{4+} and 3-fold by 2 m*M* Sn^{2+} compared to the increases of only 50% and 140% respectively obtained with strain X, and this difference was also in evidence at the lower concentrations tested. In sheer magnitude of effect the response of *K. aerogenes* to Sn^{2+} was the most striking. Even at relatively low concentrations the doubling time increased sharply culminating in an 11-fold increase at 2 m*M* Sn^{2+}. Low concentrations of Sn^{4+} produced less dramatic responses but nevertheless these were greater than observed with the other strains and at 2 m*M* Sn^{4+} no growth occurred within 30 h. This is indicated by the broken line in Figure c. These experiments were carried out in duplicate on successive days and the doubling times were reproducible to within ± 5%.

The uptake of Sn^{2+} by *K. aerogenes*, *Ps. reptilovora* and strain X was 0.76, 0.70 and 0.44% respectively of the dry weight of the organisms, which might suggest a correspondence between uptake and the degree of inhibition, but the differences are not as great as might be expected from the responses in the Figure. Also, changing the carbon source in the growth medium from glucose to citrate (0.1 *M*) increased the uptake of Sn^{2+} by strain X from 0.44% to 7.9%, but this increased uptake was not associated with a similarly augmented doubling time. Indeed the latter was only increased 2.2-fold relative to the control, which is very similar to the inhibition obtained when the uptake was only 0.44% (Figure a). The absence of any direct correlation between uptake and biological response was also apparent with Sn^{4+}. The uptake in glucose-salts medium was 0.52, 0.42 and 0.61% respectively for *K. aerogenes*, *Ps. reptilovora* and strain X. Samples withdrawn at the end of the growth cycle in medium containing 2 m*M* Sn^{2+} or Sn^{4+} were used in these metal-binding experiments and the metal was easily removed from the organisms by washing with saline (8.5 mg NaCl ml^{-1}).

Viable count determinations showed that the survival of *K. aerogenes* was 14% on minimal agar containing 2 m*M* $SnCl_2$ and 12% on agar containing 2 m*M* $SnCl_4$, which contrasts with earlier reports[6] that inorganic tin salts are not bactericidal, but 100% survival was obtained in corresponding experiments with strain X and *Ps. reptilovora*. Nevertheless, even when 100% survival occurred, the colonies grew slowly and moreover the number appearing on the plates increased over a period of 6 days in the presence of both tin chlorides compared to 3 days in their absence. This process was even more protracted in the *K. aerogenes* experiments. All the colonies on the control plates appeared within 2 days of incubation at 28°C but 8 days were necessary in the presence of Sn^{4+} and 10 days in the presence of Sn^{2+}. In suitable conditions colony growth rate can be obtained by measuring colony diameter during the phase of colony development in which this parameter increases linearly with time[7] but a comparison of values so obtained with those found in liquid culture would not be meaningful since precipitation of tin compounds, thereby rendering the concentration of Sn^{2+} or Sn^{4+} uncertain, occurred progressively as incubation of the plates proceeded. This did not occur in the liquid cultures.



Effect of Sn^{2+} or Sn^{4+} on the relative doubling time of a) strain X, b) *Ps. reptilovora* and c) *K. aerogenes*. The relative doubling time is the ratio of the doubling time obtained in the presence of the appropriate tin salt to that obtained in its absence.
The values obtained for the latter were 2.4, 1.7 and 0.9 h respectively for strain X, *Ps. reptilovora* and *K. aerogenes*. ○, Sn^{2+}; △, Sn^{4+}.

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