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$$K_{ad} = \frac{F_T}{2}\left\{1 + \frac{\xi - 1}{[(\xi - 1)^2 + 4\xi n^{-2}]^{1/2}}\right\} \quad (1)$$

where K_{ad} is cellular potassium in (wet weight) micromoles per gram, F_T is the maximum level of potassium plus sodium in the cell in (wet weight) micromoles per gram, ξ is defined as $K^{00}_{Na\rightarrow K}$ (K_{ex}/Na_{ex}), $K^{00}_{Na\rightarrow K}$ is the selectivity ratio of potassium over sodium, and $n = e^{-\gamma/2RT}$, where $-\gamma/2$ is the energy of nearest-neighbor interactions. The cell sodium and potassium contents are related by

$$Na_{ad} = F_T - K_{ad} \quad (2)$$

8. It is assumed that $-RT \ln K^{00}_{Na\rightarrow K} = \Delta F^{00} = \Delta H^{00} - T\Delta S^{00}$. Here ΔF^{00}, ΔH^{00}, and ΔS^{00} are intrinsic free energy change, intrinsic enthalpy change, and intrinsic entropy change, respectively, for the exchange of sodium by potassium on a site. An expression was developed earlier (*4*) for the transition temperature (T_c) at which the cell potassium has decreased by one-half:

$$T_c = \frac{\Delta H^{00}/R}{\left(\ln \frac{K_{ex}}{Na_{ex}} + \frac{\Delta S^{00}}{R}\right)} \quad (3)$$

The behavior of taenia coli was quantitatively described by this equation at two different values of K_{ex}. Qualitatively, the behavior of frog muscle at different temperatures is also consistent with this equation.
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DDT: Inhibition of Sodium Chloride Tolerance by the Blue-Green Alga Anacystis nidulans

Abstract. Anacystis nidulans, *a freshwater blue-green alga, has been found to tolerate sodium chloride (1 percent by weight) and DDT [1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane] (800 parts per billion) separately, but growth was inhibited in the presence of both compounds. This inhibition was reversed by an increased calcium concentration. It is possible that inhibition of (Na+,K+)-activated adenosine triphosphatase) by DDT causes this species to lose the ability to tolerate sodium chloride.*

The direct toxicities of DDT and other chlorinated hydrocarbon insecticides on algae have been studied by several research groups (*1*). They have found, however, that these chemicals are toxic to algae at relatively higher concentrations than one normally finds in nature. One important aspect of ecological studies is to discover conditions which enhance the toxicity of pollutants so as to be able to predict situations in which such chemicals can become a threat to the environment.

The role of adenosine triphosphatase in ionic transport across membranes has been well established. Recently, it has been suggested that a (Na^+,K^+)-activated, Mg^{2+}-dependent adenosine triphosphatase may be involved in the mechanism of NaCl tolerance in yeasts (*2*) and blue-green algae (*3*). Recent reports of DDT-inhibited (Na^+,K^+)-activated Mg^{2+}-dependent adenosine triphosphatase (*4–7*) and polychlorinated biphenyl inhibition of fish adenosine triphosphatase (*8*) have led us to investigate the influence of DDT on the NaCl tolerance of *Anacystis nidulans* (strain TX20), a coccoid blue-green alga obtained from the Laboratory of Algal Physiology of the University of Texas at Austin.

Algal cultures were grown in medium Cg10 (*9*) with NaCl and DDT added as needed. The growth of the test-tube cultures was followed colorimetrically according to the method of Kratz and Myers (*10*). The growth rate of the algae is expressed by the equation

$$kt = \log (N_t/N_0)$$

where k is the growth-rate constant, t is 24 hours, N_t is the cell number at time t, and N_0 is the cell number at time 0. The growth-rate value reported herein is the specific growth-rate constant, k, in logarithmic units to the base 10 per 24 hours. When $k = 0.301$, the generation time is 24 hours. The purity of the algal cultures was checked by inoculating nutrient broth (Difco) tubes with fractions of experimental algal cultures and incubating in darkness at 30°C and at room temperature. Bacterial contamination was not a problem during the course of these experiments.

The DDT-inhibition of the NaCl tolerance of *A. nidulans* was investigated by growth-rate studies. The growth-rate values in Table 1 show that *A. nidulans* was unable to grow in regular medium Cg10 + 1 percent NaCl (by weight) + DDT [800 parts per billion (ppb)]. One such culture remained colorless and optically clear after 11 days of incubation under optimum growth conditions. Three fractions of this culture were back-transferred to equal volumes of fresh media to make Cg10 + 0.5 percent NaCl + 800 ppb DDT, Cg10 + 1 percent NaCl + 400 ppb DDT, and Cg10 + 1 percent NaCl + 800 ppb DDT. The resulting growth rates of these cultures were $k = 1.24$, 1.52, and 0.0, respectively, after 3 days of incubation. Further back-transfers were made with fractions of the culture containing 1 percent NaCl + 400 ppb DDT to Cg10 and Cg10 + 1 percent NaCl. After 35 hours of incubation the growth rates of duplicate cultures in Cg10 returned to $k = 2.25$ and 2.29. Growth rates of duplicate cultures in Cg10 + 1 percent NaCl were $k = 1.58$ and 1.51. These values compared favorably with the growth rates of control cultures shown in Table 1. This result indicated that, although the growth of *A. nidulans* was suppressed at the highest combined concentrations of DDT and NaCl tested, this suppression was

Table 1. Growth rates of *A. nidulans* showing the reversal of NaCl + DDT growth inhibition when the $Ca(NO_3)_2 \cdot 4H_2O$ concentration is increased fivefold. All values represent the mean of five or six replicates ± the standard deviation of the *k* values. The initial cell inoculation was 120,000 cells per milliliter; cultures were incubated at 39°C under F20T12/CWX fluorescent lamps (7000 lumen/m²) and were continually gassed with a mixture of 2 percent CO_2 in air. Ethanol cultures contained the same amount of ethanol solvent (45 μl/20 ml) as the DDT-treated cultures. The symbol (a) denotes a significant difference between NaCl-treated and untreated cultures at 99 percent confidence; the symbol (b) denotes no significant difference.

Culture	Medium Cg10 + 25 mg of $Ca(NO_3)_2$ per liter		Medium Cg10 + 125 mg of $Ca(NO_3)_2$ per liter	
	No NaCl	+1% NaCl	No NaCl	+1% NaCl
Plus DDT (800 ppb)	1.95 ± 0.10(a)	0.0(a)	1.80 ± 0.07(a)	1.53 ± 0.10(a)
No DDT	2.01 ± 0.12(a)	1.41 ± 0.13(a)	1.89 ± 0.08(b)	1.84 ± 0.05(b)
Ethanol	2.13 ± 0.02(a)	1.40 ± 0.09(a)	2.14 ± 0.25(a)	1.76 ± 0.10(a)

relieved upon dilution of either DDT or NaCl. The growth rates approached that of DDT-free controls upon back-transfer to Cg10 ± NaCl. Growth was not observed in Cg10 + 1 percent NaCl + DDT (800 ppb) after 3 weeks of incubation. Thus *A. nidulans* tolerated separately either DDT or NaCl; however, in terms of growth the combined stress of DDT and NaCl was algistatic.

In one series of growth studies NaCl was replaced by KCl. No growth was observed in Cg10 + 1 percent KCl + DDT (885 ppb). Growth did occur in cultures with lower concentrations of either KCl or DDT.

Additional growth experiments were carried out with glycerol in order to determine the role of osmotic pressure. Cultures in Cg10 + glycerol (15, 30, or 60 g/liter) + DDT (885 ppb) showed no growth inhibition as compared to control cultures without DDT. This indicated that the NaCl + DDT inhibition of the algae was probably an ionic effect, and not due to osmotic stress.

It has been reported that Ca^{2+} alleviates NaCl stress (*11*) and DDT stress (*12*). Results in Table 1 show that increased concentrations of $Ca(NO_3)_2 \cdot 4H_2O$ (125 mg/liter, five times the normal concentration) not only allowed higher growth rates of the alga in Cg10 + 1 percent NaCl, but also allowed growth to resume in Cg10 + 1 percent NaCl + DDT (800 ppb). It is known that Ca^{2+} decreases the permeability of the plasma membrane of *Laminaria* to water and other substances, whereas Na^+ has the opposite effect (*13*). Possibly a similar situation prevails here; if the permeability of *A. nidulans* cells to NaCl is reduced by Ca^{2+}, so is NaCl stress reduced. Under these conditions the postulated growth-inhibiting interaction of DDT with the cell's Na-pump would be less effective.

To prepare cells for adenosine triphosphatase assays, fresh algal cultures were washed once (0.25*M* sucrose, 0.1 m*M* of the disodium salt of ethylenediaminetetraacetic acid) and harvested by centrifugation. The cell pellet was resuspended in tris buffer [tris(hydroxymethyl)aminomethane] (*p*H 7.0) containing 1.0 m*M* $MgCl_2$ and ultrasonicated for 60 to 90 seconds at 15-second intervals. After centrifugation and decantation, the cell material was resuspended in the same buffer. For the measurement of adenosine triphosphatase activities, a 0.1-ml fraction of cell preparation was pipetted into a 10-ml test tube containing 1.7 ml of assay buffer (1.0 m*M* $MgCl_2$, 100 m*M* NaCl, 20 m*M* KCl) at *p*H 7.0. Comparative assays were made without the added NaCl and KCl. Assays of adenosine triphosphatase activity were adapted after the procedure followed by Matsumura and Narahashi (*5*). Ouabain ($1 \times 10^{-2}M$ aqueous solution) and DDT ($1 \times 10^{-3}M$ ethanol solution) were added (20 μl) to the enzyme mixture by means of a Hamilton's microsyringe, and the system was held at 24°C for 10 minutes. The reaction was initiated by the addition of 1 μmole of adenosine triphosphate (disodium salt) with 0.2 ml of distilled water. The temperature was maintained at 37°C throughout the assay period (30 minutes).

As previously reported (*3*), the portion of adenosine triphosphatase activity that can be attributed to (Na^+,K^+)-activated adenosine triphosphatase was found to be relatively small. Results in Table 2 show that DDT-sensitive adenosine triphosphatases are present in the cells, and that the degree of inhibition by DDT exceeds that of inhibition by ouabain. Thus it appears that DDT inhibited a certain portion of Mg^{2+}-dependent adenosine triphosphatase activity as well as that due to ouabain-sensitive, (Na^+,K^+)-adenosine triphosphatase. The proportion of (Na^+,K^+)-adenosine triphosphatase activity in relation to the total adenosine triphosphatases present in the algal cells was not as much as that reported for eel intestinal mucosa (*6*) and rat brain (*4*).

It is important to stress here that a decrease in the NaCl tolerance in some algae could lead to serious consequences. In estuarine and marine species, for instance, the effects of DDT may become much more pronounced whenever these algae encounter water with higher NaCl concentrations. Although further evidence is needed to substantiate our suggestion that DDT inhibition of (Na^+,K^+)-adenosine triphosphatase is the cause of the loss of NaCl tolerance in *A. nidulans*, the finding that the toxicity of DDT increases at high NaCl concentrations warrants special attention in view of the importance of blue-green algae as primary producers.

Table 2. Inhibition of two types of adenosine triphosphatases in *A. nidulans* by ouabain and DDT. Data are expressed in terms of the number of micromoles of adenosine triphosphate hydrolyzed per milligram of dry cells per 30 minutes. Values are the averages of duplicate assays with upper and lower values shown by ±. The concentrations of ions were 1.0 m*M* $MgCl_2$ for Mg^{2+}-dependent adenosine triphosphatase and 1.0 m*M* Mg^{2+}, 100 m*M* NaCl, and 20 m*M* KCl for (Na^+,K^+)-activated adenosine triphosphatase. Final inhibitor concentrations were $1 \times 10^{-5}M$ and $1 \times 10^{-4}M$ for DDT and ouabain, respectively.

System	Mg^{2+}		Na^+,K^+	
	Activity	Inhibition (%)	Activity	Inhibition (%)
Control	0.587 ± 0.003	0	0.150 ± 0.013	0
Ouabain	.580 ± .010	1.4	.110 ± .015	26.7
DDT	.510 ± .010	13.2	.110 ± .005	26.7
DDT + ouabain	.510 ± .010	13.2	.110 ± .008	26.7

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Morphine-3-Succinyl–Bovine Serum Albumin: An Immunogenic Hapten-Protein Conjugate

Abstract. *Morphine-3-hemisuccinate was synthesized by reaction of morphine with succinic anhydride. This compound was conjugated to bovine serum albumin by the mixed anhydride method, and the degree of conjugation was determined by base hydrolysis of the conjugate, extraction, and measurement of free morphine. An average of 6.5 molecules of morphine were conjugated to each molecule of protein. Eleven rabbits immunized with varying doses of the conjugate were producing antibody 8 weeks later, as determined by a modification of the ammonium sulfate method, which measures primary binding of antigen by antibody.*

Antibodies specific for physiologically active haptens can have specific antagonistic effects in vivo (*1*). In immunologic assay systems for the detection of small quantities of morphine and related opium alkaloids in biological fluids (*2–4*), the 3-*O*-carboxymethyl derivative of morphine coupled to bovine serum albumin (BSA) has been used as antigen. Since the presence or absence of antagonism in vivo may hinge on either differences in antibody affinity or on the ability of antibody to recognize critical regions in the hapten structure, it seems important to study the antibody response to different types of morphine-protein conjugates.

We have prepared a succinic acid ester of morphine which conjugates to BSA forming stable, easily definable hapten-protein conjugates. Evidence for the antigenicity of morphine-3-hemisuccinate (M-3-HS) conjugated to BSA is presented.

Morphine-3-hemisuccinate (*5*) was prepared by heating morphine with three equivalents of succinic anhydride in pyridine for 4 hours (Fig. 1). The pyridine was evaporated at reduced pressure, the residue was washed five times with hot ethanol, and the residue was recrystallized twice from 60 percent ethanol, yielding crystals melting at 237° to 239°C (with decomposition). Thin-layer chromatography (TLC) was performed in a solvent system of ethyl acetate, methanol, and ammonium hydroxide (85 : 10 : 5). The crystalline product would not migrate in this solvent system, while morphine migrated with an R_F of 0.3. A ferric chloride color test for free phenol was negative. The infrared spectrum of the product showed bands at 1730 cm^{-1} (ester carbonyl) and between 1550 to 1660 cm^{-1} (carboxylate). The mass spectrum showed: m/e, 385(M^+); 341 (M-CO_2); 285 (M-$COCH_2CH_2CO_2$); 268 (M-$COCH_2CH_2CO_2$-OH). Elemental analysis showed C, 65.17; H, 6.19; and N, 3.52 percent; calculated values for M-3-HS ($C_{21}H_{23}O_6N$) are C, 65.44; H, 6.02; and N, 3.63 percent.

The M-3-HS was conjugated to BSA by the mixed anhydride method (*6*). To 25 ml of dioxane we added 0.300 g (0.744 mmole) of M-3-HS, 0.097 ml (0.744 mmole) of isobutyl chloroformate, and 0.178 ml (0.744 mmole) of tributylamine. The mixture was stirred for 30 minutes with the temperature maintained below 20°C (Fig. 1). Crystalline BSA (Pentex) (0.5210 g; 0.0074 mmole) was dissolved in 100 ml of water, and 100 ml of dioxane (spectral grade) was added slowly with stirring. The solution of BSA in water and dioxane was initially *p*H 5, which was adjusted to *p*H 8.5 with 1*N* NaOH. The solution became cloudy at approximately *p*H 6 and then cleared above *p*H 7. The BSA solution was cooled to 5°C, and at the end of 30 minutes the mixture of M-3-HS and isobutyl chloroformate was added. An additional 25 ml of water was added

Table 1. The effect of different doses of M-3-HS-BSA on serum antigen-binding capacity and relative antibody affinity. The antigen was emulsified in complete Freund's adjuvant and injected subcutaneously into rabbits on day 0 and day 49. The antigen-binding capacity (ABC) is expressed as the number of picomoles of morphine bound per milliliter of undiluted antiserum when 88.0 pmole of [^{14}C]morphine was used. The effect of dilution (ED), a measure of relative antibody affinity (*9*), is (ABC at 8.8 pmole [^{14}C]morphine/ABC at 88.0 pmole [^{14}C]morphine) × 100; Tr, trace; 0, no detectable binding.

Conjugate immunizing dose (mg)	5 weeks		8 weeks		13 weeks	
	ABC	ED	ABC	ED	ABC	ED
0.5	556	18.0	3733	48.1	5064	65.7
	1088	29.8	5556	63.8	2402	48.8
	1186	8.2	2199	50.0	506	51.7
	Tr		1186	61.0	1013	65.7
Mean	708	18.7	3806	55.7	2246	58.0
5.0	535	21.9	4138	53.1	1187	83.7
	Tr		2373	69.8	1302	88.9
	1273	39.3	13892	61.5	10998	51.9
	0		660	66.7	1070	66.2
Mean	452	30.6	5266	62.8	3639	72.5
50.0	0		Tr		0	
	0		318	61.8	0	
	Tr		1447	54.0	Tr	
Mean	0		588	57.9	0	