(seawater side negative) which was close to the mean p.d. of 31.1 ± 0.9 mV (seawater side negative), suggesting that Na^+ was at equilibrium across these epithelia.

The predicted and observed flux ratios for the experiments listed in Table 1 are presented in Table 2. Although further studies are required to establish statistical significance, the present data suggested that Cl^- was not at equilibrium across these epithelia and that the Cl^- efflux was active and the Cl^- influx was passive. The data suggested that Na^+ was at equilibrium and that the unidirectional Na^+ fluxes

TABLE 2

Cl^- and Na^+ flux ratios across isolated opercular epithelia of seawater-adapted *F. heteroclitus*

	Cl^- Flux Ratios	
	Predicted	Observed
Control (5)	10.11 ± 1.21	1.42 ± 0.34
Isoproterenol (5) 10^{-5} M, serosa	11.48 ± 1.27	1.36 ± 0.43
Arterenol (5) 10^{-5} M, serosa	6.59 ± 0.79	2.77 ± 0.29
	Na^+ Flux Ratios	
	Predicted	Observed
Control (7)	0.91 ± 0.03	1.52 ± 0.43
Ouabain (7) 10^{-6} M, serosa	1.78 ± 0.10	2.14 ± 0.32

Mean ± S.E.M.; Number of paired experiments in parentheses.

across these epithelia were passive processes. These results were in good agreement with those obtained with several intact SW-adapted teleosts (see data compiled by Maetz and Bornancin, Fortschr. Zool. 23: 322-362, 1975). Relatively large fluxes of both Na^+ and Cl^- across the gills in both directions were observed. Cl^- was secreted against an electrochemical gradient, suggesting active transport, and the measured gill potentials were similar to the calculated Na^+ equilibrium potential. The presence of α- and β-adrenergic receptors in the gill are known (Pic et al. Comparative Physiology, pp. 293-321, North Holland Press, 1973). Stimulation of the α-receptors inhibits branchial Cl^- secretion while stimulation of the β-receptors reportedly alters the branchial hydraulic conductivity. These results with opercular epithelia suggest that α-adrenergic inhibition of gill Cl^- secretion does not result from changes in the branchial hemodynamics. No observations that β-adrenergic stimulation increases gill Cl^- secretion have been reported. These studies were supported by NIH grants EY 01340 (JAZ) and EY 05059 (KJD).

EVALUATION OF THE RENAL HANDLING OF 2,4-D BY THE WINTER FLOUNDER

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Among the most widely used pesticides today are 2,4-D (2,4-dichlorophenoxyacetic acid) and related herbicides. These compounds are generally thought to be relatively non-toxic to mammals, at least in part due to their rapid renal excretion via the organic acid transport system (Toxicol. Appl. Pharmacol.

26: 559-570, 1973; Fd. Cosmet. Toxicol. 12:209-217, 1974). However, the mechanisms governing 2,4-D excretion in aquatic organisms have not been evaluated and even in mammals renal clearance studies have not been used to assess the significance of the organic acid system in 2,4-D excretion.

In the experiments reported below we have used both the isolated teased tubule preparation in vitro (Science, 108: 65-67, 1948) and renal clearance techniques in vivo (Am. J. Physiol. 233:F126-F132, 1977) to evaluate the parameters controlling 2,4-D excretion. Protein binding of organic acids used in this study was determined by ultrafiltration (Cancer Chemotherapy Rpt. 57:125-140, 1973).

Results of incubation of teased flounder tubules with 1 μM ^{14}C-2,4-D for 60 min at 15°C are summarized in Table 1. Tissue-to-medium ratios (T/M) reached 30 or more and this uptake was reduced severely (T/M of only 1.8) by cyanide, which blocks metabolism. The organic acids, probenecid (PROB) and chlorophenol red (CPR), significantly depressed ($P < 0.05$), 2,4-D uptake when present at 10 μM or higher concentrations; whereas p-aminohippuric acid (PAH) did not cause significant inhibition until its concentration was raised to 100 M. Addition of flounder plasma to the incubation medium also produced significant inhibition of

TABLE 1

Inhibition of the renal uptake of 1 μM ^{14}C-2,4-D by organic acids and cyanide in vitro[1]

Inhibitor (μM)	Probenecid	CPR	PAH	CN^-
0	32.8 ± 6.5	35.5 ± 2.6	28.8 ± 0.8	29.0 ± 1.9
0.1	34.1 ± 7.0	30.7 ± 2.9		
1	34.4 ± 7.0	29.9 ± 2.3	29.4 ± 0.5	
10	17.4 ± 3.0	18.8 ± 3.0	25.6 ± 1.0	
100	7.2 ± 1.3	5.5 ± 0.9	21.4 ± 1.1	
1000			7.7 ± 0.3	1.8 ± 0.1

[1]Results expressed as T/M ratio. Each value is the mean of determinations in at least 4 fish. CPR = chlorophenol red; PAH = p-aminohippuric acid.

2,4-D uptake (Figure 1). The uptake of another organic acid pesticide, DDA (2,2-bis[p-chlorophenyl] acetic acid), was even more markedly reduced by plasma. Measurement of the binding of these two organic acids to plasma macromolecules demonstrated that the difference in inhibition reflected the much greater

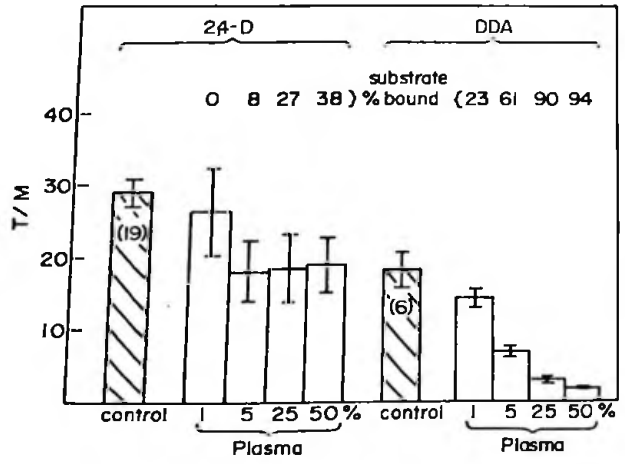

Figure 1. Influence of plasma binding on the ability of isolated flounder tubules to accumulate 2,4-D. The tissue to medium ratios (T/M) resulting after 60 min accumulation of 10 μM ^{14}C-2,4-D or ^{14}C-DDA with isolated tubules at 15°C are shown for controls and media containing the stated concentration of flounder media. Also shown are the per cent of substrate bound to plasma macromolecules at each plasma concentration.

binding of DDA. Subsequent experiments using both plasma and bovine serum albumin demonstrated an excellent correlation (0.89) between the binding of these organic acids and the inhibition of their uptake by the tubules (Figure 2). When the binding was equal, the per cent inhibition of uptake was equivalent.

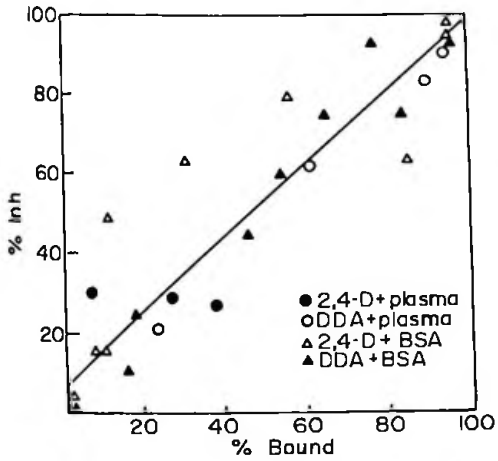

Figure 2. The percent inhibition of 2,4-D and DDA uptake by isolated flounder tubules is plotted against the percent of the substrate bound to macromolecules (either flounder plasma proteins or bovine serum albumin). The regression line over all 30 points is shown. Its correlation coefficient was 0.89.

To assess the affinity of the organic acid carrier for 2,4-D we followed the kinetics of its uptake by the teased tubules according to methods previously described (Bull. MDIB 16:55-58, 1976). Over a range of substrate concentrations from 20 to 400 µM, the rate of accumulation followed Michaelis-Menton kinetics. Kinetic parameters were estimated by plotting V (velocity) versus V/S (V ÷ substrate concentration), i.e., the Hofstee plot, since this analysis is less subject to systematic bias than Lineweaver-Burk (1/V vs. 1/S) (J. Biol. Chem. 240:863-869, 1965). The K_m for 2,4-D uptake was 78.8 µM and the V_{max} was 3.9 µmol/g/hr. Additional experiments using ^{14}C-PAH as substrate and 2,4-D as inhibitor gave a K_m of 178.4 µM for PAH alone and an apparent K_m of 379.4 (± 54.0) in the presence of 50 µM 2,4-D. The calculated K_i for 2,4-D was thus 44.4 µM, similar to the K_m determined directly.

It has been reported by Koschier and Berndt (Biochem. Pharmacol. 26:1709-1713, 1977) that 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) causes inhibition of organic base transport in mammalian kidney slices at doses which do not inhibit respiration. We have observed similar results using DDA (Pritchard and Cauthen, unpublished). However, as shown in Table 2, 2,4-D did not inhibit tetraethylammonium (TEA) uptake by flounder tubules.

Measurement of the binding of 2,4-D to whole plasma confirmed that it is far less avidly bound than DDA. Whereas 97% of the plasma DDA was bound (Am. J. Physiol. 233:F126-F132, 1977), only 67 ± 2% (at 10 µM) and 71 ± 1% (at 1 µM) of the available 2,4-D was bound. Thus, at equal plasma concentrations free 2,4-D (∿ 30%) would be 10 times the free DDA concentration (3%). This fact is reflected in the renal clearance of 2,4-D (Table 3). Under control conditions (2.5 µmol/kg,i.m.) the mean clearance of 2,4-D was nearly 500 times the GFR, while DDA clearance showed maximum values of ∿ 50 times the GFR (Am. J. Physiol. 233:F126-F132, 1977). Increasing the dose of 2,4-D to 25 µmol/kg indicated that a transport maximum (T_{max}) had been reached, for the clearance of 2,4-D fell to less than 40% of control values. CPR and PROB were effective inhibitors of 2,4-D transport in vivo, as they were in vitro. On the other hand, PAH was not inhibitory at the dost tested (25 µmol/kg, which results in ∿ 10-50 µM PAH in plasma). Since significant inhibition of 2,4-D uptake was not achieved in vitro (Table 1) until the PAH concentration was 100 µM, this result is not surprising. Additional experiments were run to quantitate the T_{max} for 2,4-D (Figure 3). Over the range of plasma concentrations giving reduced 2,4-D clearance (10-60 µM), mean tubular transport in these fish was 847 ± 133 nmol/hr/g kidney. If we assume that tubules comprise 25-50% of the

TABLE 2

Uptake of TEA by isolated flounder tubules in vitro[1]

[TEA] (μM)	Control	+ 2,4-D[2] (100 μM)	
1	17.5 ± 1.4	16.4	3.8
10	13.4 ± 1.1		
100	6.4 ± 0.7		
1000	2.1 ± 0.1		

[1]Results expressed as the mean T/M (± S.E.) of 4 fish. TEA = tetraethylammonium (as the chloride).

[2]Lower concentrations were also tested and produced similar lack of inhibition.

TABLE 3

Summary of Renal Clearance of 2,4-D by the Winter Flounder[a]

	CONTROL (2.5 μmol/kg)	HIGH DOSE (25 μmol/kg)	+ CPR	+ PROB	+ PAH
Fish wt. (kg)	0.282 ± 0.017	0.256 ± 0.023	0.318 ± 0.033	0.304 ± 0.007	0.243 ± 0.044
Urine Flow (ml/hr)	0.310 ± 0.070	0.242 ± 0.092	0.367 ± 0.110	0.250 ± 0.013	0.247 ± 0.048
Plasma PEG (mg/ml)	0.766 ± 0.066	1.102 ± 0.236	0.879 ± 0.076	0.893 ± 0.287	0.807 ± 0.161
Urine PEG (μg/ml)	1.471 ± 0.295	1.727 ± 0.492	0.960 ± 0.290	1.182 ± 0.784	0.710 ± 0.078
U/P PEG	1.947 ± 0.336	1.614 ± 0.421	1.062 ± 0.253	1.160 ± 0.503	0.969 ± 0.351
Clearance PEG (ml/hr)	0.498 ± 0.073	0.324 ± 0.078	0.422 ± 0.22	0.294 ± 0.139	0.263 ± 0.138
Plasma 2,4-D (nmol/ml)	1.140 ± 0.073	29.18 ± 11.41	1.856 ± 1.108	1.625 ± 0.519	0.716 ± 0.227
Urine 2,4-D (nmol/ml)	565.9 ± 64.4	3672 ± 565	74.68 ± 30.48	145.11 ± 93.35	265.02 ± 0.09
U/P 2,4-D	605.1 ± 129.4	177.9 ± 47.9	90.48 ± 41.59	78.72 ± 31.98	469.52 ± 170.30
Clearance 2,4-D (ml/hr)	175.4 ± 39.6	51.4 ± 27.2	22.59 ± 8.74	19.97 ± 8.90	126.11 ± 65.55
Clearance Ratio	487.0 ± 102.2	179.2 ± 65.6	118.9 ± 59.7	69.90 ± 3.01	496.2 ± 3.8
T/P PEG	0.290 ± 0.033	0.336 ± 0.013	0.277 ± 0.018	0.279 ± 0.006	0.210 ± 0.059
T/P 2,4-D	4.998 ± 0.755	11.23 ± 3.45	3.64 ± 2.06	1.865 ± 0.345	5.40 ± 0.32
N[b]	11	3	3	2	2

[a]Abbreviations are 2,4-dichlorophenoxyacetic acid (2,4-D); chlorophenol red (CPR); probenecid (PROB); p-aminohippuric acid (PAH); and polyethylene glycol (PEG). CPR, PROB, and PAH were given i.v. at doses of 25 μmol/kg after 4 control clearance periods.

[b]A total of 76 clearance periods in 11 fish for control; 26 in 3 for high dose; 15 in 3 fish for CPR; 14 in 2 fish for PROB; 13 in 2 fish for PAH.

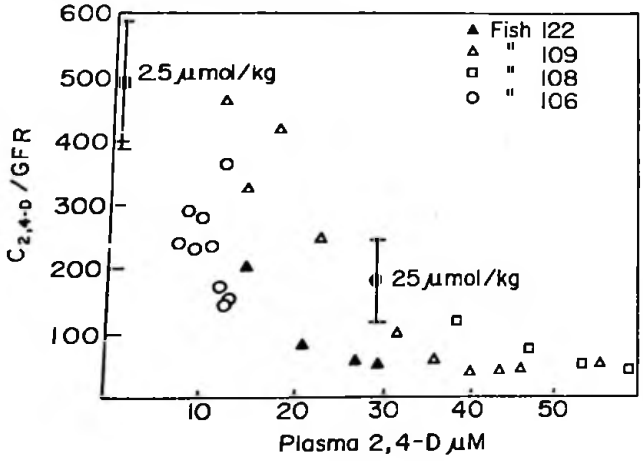

Figure 3. The clearance ratio (i.e., clearance of 2,4-D ÷ the clearance of the glomerular marker, PEG) is plotted against the plasma concentration of 2,4-D. Individual points from 4 fish are shown. In addition, the mean values ± SE for fish given 2.5 μmol 2,4-D/kg and 25 μmol/kg are shown.

kidney wet weight, the T_{max} would then be 1694 (50% tubules) to 3388 (25% tubules) nmol/hr/g tubules. These values are quite close to the V_{max} predicted by the kinetic experiments discussed above, 3900 nmol/hr/g tubules.

Finally, we tested the effectiveness of 2,4-D as an inhibitor of PAH transport in vivo. It was inhibitory, producing 75% inhibition at a dose of 25 µmol/kg (10 x the substrate dose), much less than CPR, PROB, or DDA (Bull. MDIBL 16:55-58, 1976). This result was predicted by the kinetic studies since the K_i for 2,4-D (44 µM) was much higher than the K_i for the others (5-15 µM).

In conclusion, 2,4-D is actively transported on the renal organic acid system in fish as it is in mammals. Its plasma binding is less extensive than DDA and its renal transport is consequently more effective in vitro and in vivo, reflecting its greater availability at the transport site. Its transport in vitro and in vivo is inhibited by other organic acids, and it is an inhibitor of transport of other organic acids. However, due to its lower affinity for the carrier, it is a less effective inhibitor than CPR, PROB, or DDA. In vivo 2,4-D shows a T_{max} of ∿ 1000 nmol/hr/g kidney, a value which correlates well with the predictions of in vitro kinetic measurements.

DDT IN THE ROCK CRAB: TISSUE DISTRIBUTION, METABOLISM AND INHIBITION OF GILL Na,K-ATPase

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The Na,K-ATPase of many aquatic organisms has been shown to be sensitive to DDT inhibition in vitro (Nature 233:148-149, 1971; Comp. Biochem. Physiol. 40B:823-827, 1971). However, in only a few studies with teleosts has it been possible to show both Na,K-ATPase inhibition and osmoregulatory deficits following in vivo exposure to DDT (Environ. Health Perspect. 1:169-173, 1972; Comp. Biochem. Physiol. 49A: 197-205, 1974). While several crustaceans were shown to be highly sensitive to organochlorine pecticides, sublethal doses had little effect upon their ability to hyperosmoregulate in a dilute environment (Environ. Pollut. 8:283-300, 1975; Pollution and Physiology of Marine Organisms, F. John Vernberg and Winona B. Vernberg, Eds. Acad. Press, 1974, pp. 427-443).

The objectives of this study were to determine the in vitro and in vivo effects of DDT on gill Na,K-ATPase from the rock crab, *Cancer irroratus*. Secondary objectives were to determine the tissue distribution and metabolism of DDT after intravascular injection.

Hemolymph samples were obtained by puncturing the membrane at the base of one of the walking legs; osmolarity was then measured by vapor pressure osmometry (Wescor, Inc.). Na,K-ATPase was assayed essentially as described by Miller et al. (Amer. J. Physiol. 231:370-376, 1976). The final assay medium contained the following concentrations: 50 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 2 mM disodium ATP, 0.67 mM EDTA, 92 mM Tris (pH 7.4). The distribution and metabolism of ^{14}C-DDT were determined as described by Guarino et al. (Toxicol. Appl. Pharmacol. 29:277-288, 1974).

Although many of the decapod crustacea hyperosmoregulate very effectively in water of low salinity, the results shown in Figure 1 illustrate that *Cancer irroratus* osmoconforms over the range tested. Furthermore, there was no significant increase in gill Na,K-ATPase activity when crabs were adapted to 50% seawater (7-10 days) as occurs in hyperosmoregulators such as the blue crab (Amer. Zool. 16:223, 1976; J. Exp. Zool. 196:315-321, 1976). The specific activity of the enzyme remained about 2.7 µmoles P_i/mg protein/hr when assayed at 30°C. These data do not agree with those of Cantelmo et al. (Comp. Biochem. Physiol. 51A:537-542, 1975), who concluded that the rock crab was able to hyperosmoregulate in seawater below 75%.

Both in vitro and in vivo exposure of the gill Na,K-ATPase to DDT resulted in significant inhibition of the enzyme (Figures 2 and 3). DDT at 0.5 ppm (1.43 µM) produced significant inhibition in vitro with