The Toxic Influeuce of Heavy Metal Salts upon Mosquito Larvae[1]

By

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(With 5 Text-figures and 6 Tables)

With respect to the influence of heavy metal salts upon aquatic animals, a great number of studies have been carried out hitherto in connection with the problems of anti-fouling paint and of the disposal of industrial wastes. In consequence, the toxic effects of heavy metal salts on animals have been found to vary according to both the physico-chemical nature of the compounds and the specificity of the animals. Earlier workers encountered serious difficulties in deducing a general mechanism underlying the diverse action of heavy metals, and some of them abandoned such attempt. The views proposed hitherto concerning the action mode of heavy metal salts are broadly classified into two theories, viz., the "contact theory" and the "penetration theory": the former assumes that the salts act externally and cause abnormal vital phenomena such as enormous secretion of mucus (Carpenter, 1930; Jones, 1935, 1937, 1938, 1941, 1948), while the latter postulates the penetration of the salts into the animal body with resultant destruction of such vital functions as respiration and excretion (Ludwig, 1927; Pyefinch and Mott, 1948; Corner and Sparrow, 1956, 1957). Thanks to the improvement of experimental apparatus and other technical progress, evidence to support the penetration theory has been gradually accumulated. Recently Holm-Jensen (1948), and Corner and Rigler (1958) by using radioactive isotopes have gained direct evidence of the penetration of heavy metal salts into animals.

Resulting from his study of various action of salts, acids and alkalis upon mosquito larvae, Wigglesworth (1933 a, b) concluded that the body surface is almost impermeable to water, and salts diffuse into the animals through anal gills which take up water by osmosis. Along this line numerous works have been undertaken, with respect of the mechanism of uptake of sodium and potassium (Ramsay, 1950, 1951, 1953; Treherne, 1954), and of chloride ion (Koch, 1938; Wigglesworth, 1938). However, the effects of heavy metal salts upon mosquito larvae have attracted scarcely any attention. So far as the writer is aware, Koch and Krogh (1936) alone demonstrated that anal gills of dipterous larvae absorb silver ions selectively. While studying the effects of various salts upon mosquito larvae, the writer had an oppotunity to observe the pecuriar action of heavy metal salts in relation with their penetration into the anal gills. The present paper deals with the relationship between toxicity of salts and their mode of penetration.

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Material and method

Material: Fourth (final) stage larvae of a common house-mosquito, *Culex pipiens pallens* Coquillett, reared in rice-straw infusion with yeast products in the laboratory, were used through the experiments. But some experiments were executed with animals collected under natural conditions. Judging from the fairly satisfactory results obtained

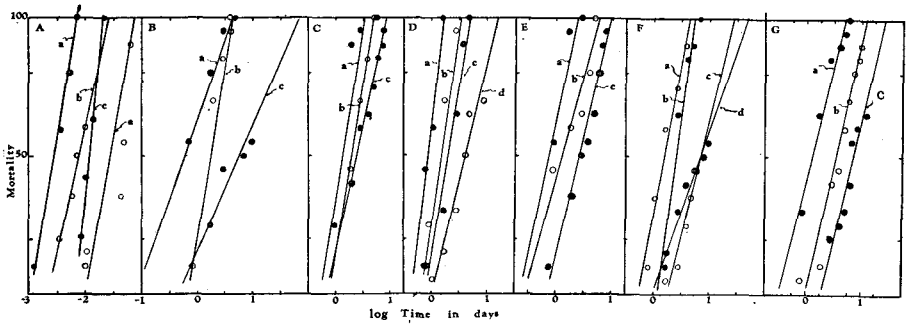

Fig. 1. Mortality of final instar larvae in the solutions of heavy metal salts (Silver nitrate, normal conc. : others, % conc.). A, $AgNO_3$: a, 10^{-2} b, 10^{-3} c, 10^{-4} d, 10^{-5} ; B, $HgCl_2$: a, 10^{-2} b, 10^{-3} c, 10^{-4} : C, $CoCl_2$: a, 10^{-1} b, 10^{-2} c, 10^{-3} ; D, $CdCl_2$: a, 10^{-1} b, 10^{-2} c, 10^{-3} d, 10^{-4}, E, $CuCl_2$: a, 1 b, 10^{-1} c, 10^{-2} ; F, $BaCl_2$: a, 1 b, 10^{-1} c, 10^{-2} d, 10^{-3} ; G, $CuSO_4$: a, 10^{-1} b, 10^{-2} c, 10^{-3}.

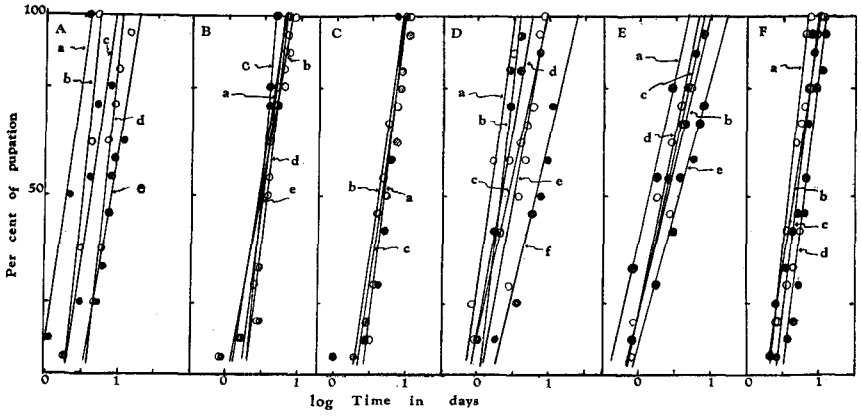

Fig. 2. Pupation in the solutions of haevy metal salts (Silver nitrate, normal conc. : others, % conc.). A, $AgNO_3$: a, dist. water b, 10^{-9} c, 10^{-8} d, 10^{-7} e, 10^{-6} ; B, $HgCl_2$: a, dist. water b, 10^{-8} c, 10^{-7} d, 10^{-6} e, 10^{-5} ; C, $CoCl_2$: a, 10^{-6} b, 10^{-5} c, 10^{-4} ; D, $CuCl_2$: a, dist. water b, 10^{-7} c, 10^{-6} d, 10^{-5} e, 10^{-4} f, 10^{-3} ; E, $CuSO_4$: a, dist. water b, 10^{-7} c, 10^{-6} d, 10^{-5} e, 10^{-4} ; F, $MnCl_2$: a, 10^{-6} b, 10^{-4} c, 10^{-3} d, 10^{-2}.

from both reared and wild animals, it may be said that no serious discrepancy arose by using two populations of different origins. Prior to the experiments, animals were transferred to glass-distilled water and kept in this medium for 24 hours without food.

Reagents: The heavy metal salts tested were chlorides of cadmium, nickel, cobalt, manganese, strontium, mercury, copper, barium, and sulphates of copper, zinc and silver nitrate. Glass-distilled water was employed to dissolve these salts. All experiments were carried out with water at temperatures ranging from 16°C to 20°C.

Measurement of toxicity: Twenty larvae were transferred to 100 ml of the toxic solution containing a given concentration of heavy metal salt. Inspection was made at suitable intervals (2–4 hours) within a day. In order to eliminate the influence of decomposing food in the course of the experiments, the remnants of food (yeast products) were removed with a spuit from each toxic solution at the last inspection of every day and small amounts of the food were newly added to the medium at the first inspection of the next day. Larvae transferred to the toxic solution furiously swing their body and settle down on the bottom of the container: subsequently, larvae left in this state do not show any signs of motion in reaction to picking with a needle. Under microscopical examination, some of such intoxicated larvae were occasionally observed to continue a faint pulsation of heart. But it was proved that the pulsation stopped within a few minutes when the larvae were returned to the toxic solution. Therefore, all the animals which showed no signs of motion were regarded as dead; their number was recorded at the time of each inspection. By plotting the cumulative mortality of larvae against the logarithm of time in days, a linear relation was obtained in each test of every salt. Some representative examples are shown in Figure 1. The equation corresponding to the linear relation, $T=K\times 10^{np}$ or $\log T=\log K\times np$ was obtained by calculating the regression, where P is cumulative mortality; T is time in days and K, n, constants. The time of 50% mortality (TD_{50}) in each salt concentration is easily determined from the equation. In a solution of which the toxic action is not very violent, larvae grew to pupae and emerged without showing mortality higher than 50%, though the speed of growth was slower than in controls. In this case too, the cumulative percentage of pupated and emerged individuals hold a linear relation against the logarithm of time in days (Figures 2, 3). Therefore, by applying the equation, $T=K\times 10^{np}$ used for mortality as mentioned above, the time of 50% pupation (TP_{50}) and of 50% emergence (TE_{50}) could be calculated respectively.

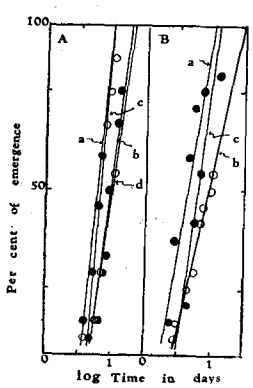

Fig. 3. Emergence in the solution of heavy metal salts (Silver nitrate, normal conc. : cadmium chloride, % conc.). A, $AgNO_3$: a, dist. water b, 10^{-9} c, 10^{-8} d, 10^{-7}; B, $CdCl_2$: a, dist. water b, 10^{-6} c, 10^{-5}.

Results

1. Relative toxicity of heavy metal salts

Fourth instar larvae: Table 1 shows data for TD_{50}, TP_{50}, and TE_{50} of

animals in solutions of the various heavy metal salts tested. It can generally be said from the table that TD_{50} increases in parallel to the decrease of salt concentration, though the effective minimum concentrations differ among different salts. Among numerous equations concerning the relation between death time of animals and concentration of toxicants, Ostwald's equation has been recognized to have an extensive suitability. It is written as follows : $TC^n=K$ or $\log T=\log K-n\log C$, where T is time of death ; C is concentration of toxicant and K, n, constants. As seen in Table 2 and Figure 4, this equation can be applied to the present results with a satisfactory fittness. Furthermore, it was found that in this regression

Table 1. The times required to reach 50% mortality of larvae (TD_{50}), 50% pupation (TD_{50}) and 50% emergence (TE_{50}) in various concentrations of heavy metal salts (Time in days).

Salt		Concentration (%)										
		1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	0
*$AgNO_3$	TD_{50}			0.003	0.007	0.012	0.031					
	TP_{50}							7.861	6.566	3.068	3.20	1.877
	TE_{50}								9.57	6.85	9.65	6.244
$HgCl_2$	TD_{50}		0.17	0.58	1.913	5.992						
	TP_{50}						4.285	3.771	3.11	3.024		2.858
	TE_{50}						5.116	5.535	4.714	5.629		4.891
$CdCl_2$	TD_{50}		0.879	1.527	1.996	4.115						
	TP_{50}						3.721	3.798	3.5	3.23		2.599
	TE_{50}						6.546	9.167	5.70	5.03		4.678
$CoCl_2$	TD_{50}		1.394	2.069	2.473							
	TP_{50}					4.638	4.213	5.087				4.358
	TE_{50}					6.454	6.217	7.117				6.316
$CuSO_4$	TD_{50}		1.277	3.655	7.232							
	TP_{50}					3.754	2.146	2.324	2.498			0.715
	TE_{50}							8.261	7.13			5.926
$CuCl_2$	TD_{50}	0.748	1.321	2.669								
	TP_{50}				6.78	3.377	2.196	2.663	2.231			1.545
	TE_{50}				7.227	6.253	7.589	7.054	6.413			5.973
$SrCl_2$	TD_{50}	0.26	1.414	8.199								
	TP_{50}				4.54	4.589	4.078	4.888				3.864
	TE_{50}				6.882	6.485	6.111	6.762				5.496
$BaCl_2$	TD_{50}	1.549	2.576	7.054	6.985							
	TP_{50}					1.382	1.037	1.468				1.211
	TE_{50}					4.886	4.03	4.657				4.518
$NiCl_2$	TD_{50}	0.709	2.594	6.072								
	TP_{50}				1.941	1.794	2.467	2.837				1.84
	TE_{50}				5.137	5.447	5.426	7.288				5.032
$MnCl_2$	TD_{50}	0.71	7.05									
	TP_{50}			6.460	5.112	4.743	4.515	4.037				3.811
	TE_{50}			9.041	7.362	7.019	6.646	6.236				5.959
$ZnSO_4$	TD_{50}	2.254	6.847	9.076	23.0							
	TP_{50}					4.24	4.267	4.574				5.808
	TE_{50}					7.314	7.457	7.967				9.546

* Normal solution

equation, log T=log K−$n$log C, n or the angular coefficient is different according to the kinds of salts. Hence, as seen in Figure 4, crossing of the lines of the equations of comparable salts may occur in some instances. In such cases, as the results of comparison of TD_{50} become inverse before and after the crossing point, it is necessary to submit the original equation to a suitable treatment for comparison of the toxicity common to every concentration used. For this reason, the mean of n of all salts tested was adopted as a representative value of the angular coefficient, and by using the mean, b, the revised equation log $T+b$ log C=log K was obtained in each salt (Table 3). The toxicity of salts is determined by the magnitude of log K of the revised quation and reduction of the value of

Table 2. Relation between log time t and log concentration c at the death of 50% larvae for heavy metal salts.

Salt	Regression equation $t+nc=k$	Original equation $TC^n=K$	Degrees of freedom	Reliability in t test (%)
$AgNO_3$	$t+0.33825c=-2.80567$	$TC^{0.33825}=0.00156$	3	99
$HgCl_2$	$t+0.37100c=-0.96787$	$TC^{0.37100}=0.1076$	3	80
$CdCl_2$	$t+0.21073c=-0.26625$	$TC^{0.21073}=0.5417$	3	80
$CoCl_2$	$t+0.12446c=0.03548$	$TC^{0.12446}=1.185$	2	99
$CuSO_4$	$t+0.37652c=-0.24356$	$TC^{0.37652}=0.5707$	2	99
$CuCl_2$	$t+0.27618c=-0.13573$	$TC^{0.27618}=0.7316$	2	95
$SrCl_2$	$t+0.74924c=-0.58938$	$TC^{0.74924}=0.2574$	2	99
$BaCl_2$	$t+0.25630c=0.14807$	$TC^{0.2563}=1.406$	3	90
$NiCl_2$	$t+0.46629c=-0.11699$	$TC^{0.46629}=0.7639$	2	90
$MnCl_2$	$t+1.01858c=-0.14907$	$TC^{1.08185}=0.7095$	1	
$ZnSO_4$	$t+0.31492c=0.40463$	$TC^{0.31492}=2.539$	3	95

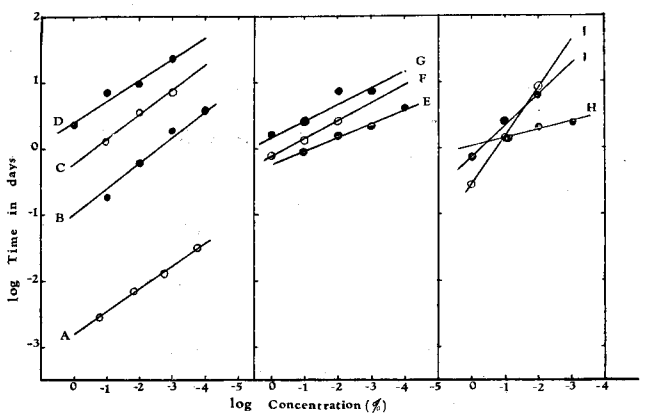

Fig. 4. Relation between log concentration (%) and log time of death of 50% larvae (TD_{50}) for heavy metal salts. A, $AgNO_3$; B, $HgCl_2$; C, $CuSO_4$; D, $ZnSO_4$; E, $CdCl_2$: F, $CuCl_2$; G, $BaCl_2$; H, $CoCl_2$; I, $SrCl_2$.

Table 3. The comparison of toxicity of heavy metal salts for the death of 50% larvae (Explanation in text).

Salt	Angular coefficient of original equation	Revised equation $\log T+b\log C=\log K$ or $t+bc=k$	K_{zn}/K	Order of toxicity
$AgNO_3$	0.33825	$t+0.40931c=-2.96694$	1694.3	1
$HgCl_2$	0.37100	$t+0.40931c=-1.06363$	21.27	2
$CdCl_2$	0.21073	$t+0.40931c=-0.76268$	10.61	3
$CoCl_2$	0.12446	$t+0.40931c=-0.53421$	6.27	4
$CuSO_4$	0.37652	$t+0.40931c=-0.30914$	3.73	5
$CuCl_2$	0.27618	$t+0.40931c=-0.26887$	3.40	6
$SrCl_2$	0.74924	$t+0.40931c=-0.24945$	3.26	7
$BaCl_2$	0.25630	$t+0.40981c=-0.08143$	2.21	8
$NiCl_2$	0.46629	$t+0.40931c=-0.06001$	2.10	9
$MnCl_2$	1.01858	$t+0.40931c=0.15557$	1.28	10
$ZnSO_4$	0.31492	$t+0.40931c=0.26305$	1.00	11
Mean b	0.40931			

log K shows increase of toxicity. Amongst salts employed, log K of zinc sulphate was the maximum and log K of silver nitrate the minimum. Relative toxicity of a given metallic salt then was computed from the difference of colog K between the salt in question and zinc sulphate adopted as the standard of comparison. For example, in the case of silver nitrate, colog $K/K_{zn}=-\log K/K_{zn}=\log K_{zn}/K$ $=-(-2.966942-0.263046)=+3.22899$, and log $3.22899=1694.3$. Thus silver nitrate is 1694 times as toxic as zinc sulphate in equi-concentration. The results of the computation yielded the following as the order of toxicity (Table 3):

Ag>Hg>Cd>Co>Cu>Sr>Ba>Ni>Mn>Zn

Pupation and emergence: As shown in Table 1, TP_{50} and TE_{50} of animals transferred to various salt solution except zinc sulphate are each greater than corresponding values for control animals reared in glass-distilled water; the range of concentration giving such growth retardation differs among salts. If the toxic effects are still kept in a concentration lower than that which is sufficient to kill more than 50 % of larvae tested, TP_{50} and TE_{50} are considered to increase according to the increase of concentration of the toxicant. For this reason, $T=KC^n$ or $\log T=\log K+n\log C$ might be expected as an equation showing the relation between TP_{50} and concentration of toxicant and between TE_{50} and concentration. Unfortunately, this was not proved with the exception of four salts, silver nitrate, chlorides of copper, cadmium and manganese. Therefore, the treatment employed in the case of mortality of larvae was not adopted for comparing toxic effects of heavy metal salts upon pupation and emergence. Relative toxicity of each salt on pupation and emergence was roughly determined by comparing the minimum within a series of concentrations in which TP_{50} and TE_{50} each were more retarded than in controls. From the results of comparison, the order of toxic action is as follows (Table 4):

For pupation, Hg Cd>Ag>Cu>Co Ni Mn Sr Ba

Table 4. The minimum concentration (%) to retard 50% pupation and 50% emergence.

Salt	Pupation	Emergence
$HgCl_2$	10^{-8}	10^{-8}
$CdCl_2$	10^{-8}	10^{-8}
$AgNO_3$	1.7×10^{-8}	1.7×10^{-7}
$CuSO_4$	10^{-7}	10^{-7}
$CuCl_2$	10^{-7}	10^{-7}
$SrCl_2$	10^{-6}	10^{-6}
$BaCl_2$	10^{-6}	10^{-6}
$NiCl_2$	10^{-6}	10^{-6}
$CoCl_2$	10^{-6}	10^{-6}
$MnCl_2$	10^{-6}	10^{-6}

For emergence, Hg Cd>Ag>Cu Ni Mn Sr Co Ba

These series are conceivable as being nearly parallel to the result obtained in respect to the mortality of larvae.

2. Influence of heavy metal salts upon anal gills of final instar larvae

Before considering the influence of various salts upon them, the structure of the anal gills is briefly described hitherwith. Anal gills consist of four thin elongate papillae; the surface is covered by delicate porous cuticle; in mature larvae, each papilla is 0.3–0.4 mm in length and about 0.15 mm in maximum median width. Flattened cells each containing a comparatively large nucleus, are arranged along the periphery of a papilla; in living state no cell boundaries are detectable. A tracheal branch runs along the median axis, giving off its branchlets to cells. Body fluids were contained in the terminal portion of the branchlets. In this state it is difficult to discriminate the tracheoles from the inner structure of a papilla. But, under the influence of salts, the fine structure often becomes clearly visible. This will be referred to subsequently further as "intoxication of tracheoles." According to Wigglesworth (1933a), the height of body fluids entering into tracheoles, changes with the osmotic pressure of the fluids. The cell layer is divided from the cavity of a papilla by a highly refractile membrane which terminal branches of the tracheoles pierce to enter into the cytoplasm. The outer membrane of the cell layer is in contact with the comparatively hard cuticle layer and vertical filaments are held between them: in living animal the membrane and the filaments are both ordinarily obscure. The description stated above fairly corresponds to the observations reported by Wigglesworth in *Aedes* (*Stegomyia*) *argenteus* Poir.

Sodium chloride: This salt was employed in order to compare its effect with that of heavy metal salts. Hypertonic NaCl evoked conspicuous changes in the anal gills. Figure 5 A shows the structural change in gills, after the intact larvae had been transferred to 1.2 % NaCl solution. Immediately after immersion, the cells swelled up from the tip of the papilla and the branches of tracheoles became filled with air. A large number of elongate vacuoles appeared between the

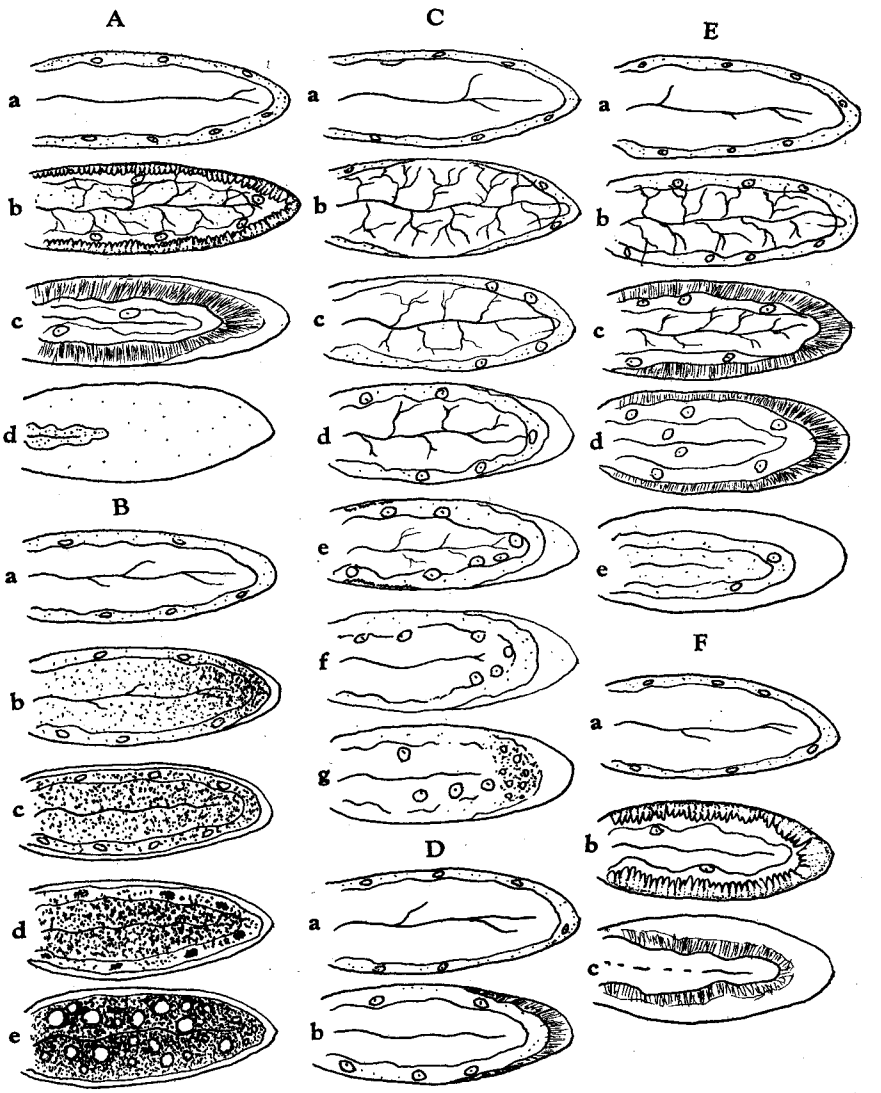

Fig. 5. Effects of salts on anal gills. A ; a, dist. water b, 3 minutes after placing larva in 1.2% NaCl c, 2 hours d, 20 hours ; B : a, dist. water b, 1 minute in 10^{-5} N $AgNO_3$ c, 3 minutes d, 5 minutes e, 1 hour ; C : a, dist. water b, 3 minutes in 0.01% $HgCl_2$ c, 6 minutes d, 14 minutes e, 30 minutes f, 54 minutes g, 1 hour ; D : a, dist. water b, 20 minutes in 0.01% $CdCl_2$; E : a, dist. water b, 8 minutes in 1% $CuCl_2$ (0.06 mol.) c, 15 minutes d, 3 hours e, 20 hours ; F : a, dist. water b, 5 minutes in 1% $CuSO_4$ (0.06 mol.) c, 3 hours.

vertical striations of the cells, consequently, the papilla took a remarkably granulated appearance. If larvae in this state are returned to distilled water, the cells shrink within about 10 minutes and the granules disappeared from the surface of the papilla : the larvae do not differ in outer appearance from normal ones and live for 24 hours or so. In the larvae left in hypertonic NaCl for a long interval, the haemolymph again entered into tracheoles and the vertical striations so extremely elongated towards inner part of the cell that the cells were finally forced to be separated from the cuticle together with the faint filaments, which connect the cells to the cuticle in normal state. Rounded nuclei were found protruding from the cells into the cavity of papilla. After 20 hours, both the inner membrane of the cells and the trachea occupying the cavity retracted as far as the base of the papilla, leaving behind granular fluids alone, in which active Brownian movement was occasionally observed. In such a case, the larvae died within 24 hours. The minimum concentration of NaCl to effect the above mentioned change to the mosquito larvae was 0.6 % or 0.103 M. The same effect was observed in 0.8 % KCl and 0.9 % $NaNO_3$ solutions. As seen in Table 5, these solutions almost coincide in the values of molar concentration (0.103–0.107 M) ; in the concentrations lower than 0.1 M, these three salts do not cause the preceding change to the anal gills after immersion of the larvae for a fairly long duration of time (3–20 hours). Hence, it is surmised that the osmotic value of haemolymph of the larvae is nearly equal to 0.1 molar concentration.

Silver nitrate : Figure 5 B shows the effect of 10^{-5} N $AgNO_3$ upon anal gills. Immediately after the larva was placed in the solution, the gill surface became light brown from the terminal part ; in parallel with this change the cells were separated from the cuticle. Neither swelling up of the cells nor intoxication of tracheoles (filling tracheoles with air) was observed. Within 3 or 5 minutes the separation of the cells from the cuticle proceeded to the base of papilla and nuclei became opaque. After 1 hour numerous white spots of variable sizes appeared throughout the dark gill surface, and the animal was found to die or at least to become moribund. The minimum concentration to evoke such an action was 10^{-6} N or 1.7×10^{-5} %.

Mercuric chloride : Figure 5 C shows the effect of 0.01% $HgCl_2$ solution. Within 3 minutes after placing larvae in the solution, the cells rapidly swelled up and the tracheole branches were extremely intoxicated. When the swelling proceeded through the half of a gill, the cells were detached from the cuticle at the tip of the anal gill and the tracheoles were again filled with haemolymph ; faint striations occurred in the cells which were relieved of the detachment of the cuticle. After 1 or 2 hours the cells evoke cytolysis at the tip of the papilla and rounded nuclei were thrown out in the cavity of the papilla. Such animals died within 15 hours.

Cadmium chloride : After immersion of larvae into 0.1% $CdCl_2$ during 2 hours, no changes were observed in outer appearance except a heavy intoxication

of tracheoles. Figure 5 D shows the effect at 20 hours after putting larvae into 0.01 % $CdCl_2$. The gill surface became granular, the cells at the tip of gills were extremely detached from the cuticle and the median trachea retreated to the base of the gills. The animal lived for about 20 hours after the appearance of such changes.

Cobalt chloride: The effect of this salt upon the anal gills differs little from that of $CdCl_2$. But the speed of detachment of the cells from the cuticle seems to be much slower than in the cases of the three salts described above. In 1% $CoCl_2$, the detachment of the cells required about 24 hours.

Cupric chloride and Cupric sulphate: Figure 5 E and Figure 5 F show the action of 1 % (0.065M) $CuCl_2$ and 1 % (0.063M) $CuSO_4$ respectively. In the anal gills of larva transferred to 1% $CuCl_2$, the trancheoles were rapidly filled with air to the end of their branches; the swelling of the cells was not very remarkable. After 15 minutes the vertical filaments extended to an enormous degree and pushed up the cell layer towards the cavity of the gill; simultaneously, the intoxication of the tracheoles was arrested. This state continued for about 3 hours. After 20 hours the vertical filaments disappeared and cytoplasm which was separated from the cuticle became faintly granulated; in the papilla, only haemolymph was left. One percent $CuSO_4$ is nearly similar in its action to 1% $CuCl_2$ but seemingly, is lightly more toxic than the latter judging from the speed and degree of elongation of the vertical filaments.

Other heavy metal salts: The action of strontium, barium, nickel, manganese and zinc are each quite alike the case of copper. The separation of cytoplasm from the cuticle was commonly observed. It was confirmed, however, that each salt has its own specific minimum concentration for toxicating the anal gills.

Discussion

In mosquito larvae immersed into hypertonic NaCl, the cells of anal gills become swollen, perhaps due to the diffusion of the salt into the cells caused by the difference of the concentration between haemolymph and external medium. NaCl can pass relatively easily through the outer membrane of the cells but faces much resistance to penetration into the inner membrane; accordingly, the concentration of NaCl in the cells rises above that in the haemolymph and water from the latter moves into the cells by osmosis. Thus the difference of osmotic pressure between body fluids and external medium is considered to be one of the main causes in evoking the swelling of cells. Another cause seems to be the elasticity of vertical filaments occurring in the cell itself. These filaments have the rôle of connecting the cells to the chitinous cuticle. If the larvae are left for a long duration of hours in hypertonic NaCl, the filaments are dissolved; consequently, the cells having lost their elasticity are naturally detached from the delicate cuticle. This mechanism has already been suggested by Wigglesworth (1933a).

As to the action of heavy metal salts except that of $AgNO_3$, all salts used

at first raise the swelling of cells of anal gills and secondarily cause the separation of the cells from the cuticle. Conforming to the explanation given in the case of hypertonic NaCl, it may be assumed that this fact suggests the penetration of heavy metal salt into the cells and the dissolution of elastic filaments. In the solution of $AgNO_3$, the cells of anal gills are separated from the chitinous cuticle, becoming coloured dark brown but never expanded. This may be interpreted as follows : both the outer membrane of the cells and the elastic filaments may be destroyed simultaneously by the contact with silver ions, then a large quantity of

Table 5. The minimum concentration to cause swelling of cells.

Salt	Conc. (%)	Conc. (Mol.)
NaCl	0.5	0.103
$NaNO_3$	0.9	0.107
KCl	0.8	0.106

Table 6.

Salt	Minimum Conc. (%) to cause separation of cells from cuticle	Molar conc.	Minimum conc. (%) to kill 50% larvae	Order of toxicity
$AgNO_3$	1.7×10^{-5}	10^{-6}	1.7×10^{-4}	1
$HgCl_2$	10^{-4}	3.7×10^{-6}	10^{-4}	2
$CdCl_2$	10^{-4}	4.4×10^{-6}	10^{-4}	3
$CoCl_2$	10^{-3}	4.2×10^{-5}	10^{-3}	4
$CuSO_4$	10^{-3}	6.3×10^{-5}	10^{-3}	5
$CuCl_2$	10^{-2}	6.5×10^{-4}	10^{-2}	6
$SrCl_2$	10^{-2}	3.8×10^{-4}	10^{-2}	7
$BaCl_2$	10^{-3}	4.1×10^{-5}	10^{-3}	8
$NiCl_2$	10^{-2}	4.2×10^{-4}	10^{-2}	9
$MnCl_2$	10^{-1}	5.0×10^{-3}	10^{-1}	10
$ZnSO_4$	10^{-1}	3.5×10^{-3}	10^{-3}	11

silver ions may penetrate into the cells. The ions taken up may be precipitated as an insoluble silver salt within the cells and reduced later to a dark brown metallic silver under the influence of light. Consequently, the cohesion within the cells may increase to such an extent that the difference in osmotic pressure between the cell contents and haemolymph may be insufficient to cause the expansion of the cells. In either case, it is almost certain that the detachment of cells from the cuticle is indication of the penetration of heavy metal salts into animals through the anal gills. Since the effects mentioned above occur even at a considerably more diluted concentration than that of NaCl as seen in Table 6, there seems to be difference between hypertonic NaCl and heavy metal salts in the mechanism of penetration. Ramsay (1953) demonstrated in *Aedes aegypti* L. that the anal gills are able to take up sodium and potassium from about 0.01% solution

and pointed out that in fairly diluted solutions, the anal gills may actively absorb the salts. Also in the case of heavy metal salts, it may be said that the salts are taken into the cells of anal gills by means of active absorption instead of by means of diffusion based upon a concentration gradient between the in- and outside of anal gills. But, as it is generally known that heavy metal salts combine easily with the cytoplasm, a different explanation is possible : the heavy metal salts are concentrated on the surface of anal gills by avidity occurring between the cells and the salts, and finally are taken up into the cells.

Animals of which the anal gills were destroyed in the solutions of heavy metal salts were certainly dead, and such animals always showed the separation of cells from the cuticle of anal gills. From these facts, it may be easily presumed that the animals were killed by the penetration of the salts. There are some evidences to support this presumption. At first, in each salt the minimum concentration to bring about the separation of anal gill cells from the cuticle nearly accords with the minimum concentration to kill 50% of larvae (Table 6). Next, in each salt, in parallel with the decrease of the concentration, both the time required to evoke the lethal injury of gills was lengthened and the time of death of the larvae was postponed. This seems to show that with the accumulation of heavy metal salts within the body, the animals suffered injuries and finally were killed. Also pupation seems to be retarded by the accumulation of the salts. This is supported by the fact that the time required for 50% pupation was lengthened with the increase of concentration of the salts (Table 1). The same is true in respect to emergence. From these results, it may be concluded that heavy metal salts are absorbed at a definite rate, specific to each and the animals are either killed or retarded in pupation and emregence according to the quantity of the salt taken up in proportion to the concentration. Therefore, difference of toxicity, i.e., difference of time required to reach 50% larval mortality at equi-concentrations may be explainable by assuming that the rate of penetration or of accumulation varies with the kinds of salts. Table 6 indirectly supports this view. It may be seen from the table that the minimum concentration sufficient to evoke the lethal change of anal gills differs by different salts and is lower in the salts which exhibit their toxic action after a short duration of time. This seems to mean that salts easily absorbed by anal gills are more effective as a toxicant. The changes in the cells of anal gills can be regarded as a symptom of toxic action of heavy metal salts upon mosquito larvae. Through further studies using this clue, the mode of action of heavy metal salts may be clarified in detail.

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Summary

1. The toxic action of heavy metal salts on the larvae of *Culex pipiens*

pallens Coquillett was studied by the use of $AgNO_3$, $CdCl_2$, $HgCl_2$, $CoCl_2$, $CuCl_2$, $CuSO_4$, $SrCl_2$, $BaCl_2$, $NiCl_2$, $MnCl_2$, and $ZnSO_4$.

2. In each concentration of these salts, the cumulative mortality of the larvae showed a linear relation against the logarithm of time in days. The same relation was found in the pupation and the emergence. From these results, the time required to kill 50% of larvae, to attain 50% pupation and to reach 50% emergence were determined respectively.

3. The relation of the time required to kill 50% of larvae to the concentration of the salt could be expressed by Ostwald's equation, $CT^n=K$. By equalizing n, the toxicity of each salt was compared from the size of K, or the time required for 50% mortality of larvae at equi-concentration. The order of toxicity, thus, was determined as follows: Ag>Hg>Cd>Co>Cu>Sr>Ba>Ni>Mn>Zn. It was proved also that toxic action on pupation and emergence conforms nearly to the above order.

4. It was found that hypertonic NaCl (above 0.6%) gives conspicuous changes (swelling of cells of anal gills and detachment of the cells from cuticle) caused apparently by its osmotic action. In heavy metal salts, such effects occurred even at the considerably lower concentrations (below 0.01%) than that of NaCl.

5. Parallelism was found between the destruction of anal gills by heavy metal salts and the death of the larvae. From the results, it was presumed that the animals are poisoned by the penetration and accumulation of the salts.

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