The Accumulation of Zinc by the Mosquito

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ABSTRACT The zinc content of mosquitoes in various developmental stages was determined by spectrographic and microchemical analysis and use of $zinc^{65}$ and found to be five to ten times higher than other trace elements. Also the concentration of zinc in the mosquito was much greater than in other insects of different biological orders. Over 90 per cent of this element was localized in the Malpighian tubules at a concentration of 32 μg zinc per mg dry weight. The non-dialyzable form of zinc is loosely bound, for it was dissociated upon dialysis against ethylenediamine tetraacetate. The uptake of this trace element was correlated during larval growth with weight increase and required the presence of food particles. Furthermore, this uptake was different from that of cobalt which was not accumulated when offered as an inorganic salt or as vitamin B_{12}. Zinc was not detectable in pooled egg masses, and once embodied by the larvae, was retained under fasting conditions and at a constant level throughout the pupal stage and as long as 14 days' adult life. Supplementation of the media with EDTA caused a marked inhibition of growth that could be completely reversed by the addition of zinc or zinc plus lead. The resultant pupae, however, contained less than 5 per cent of the normal amount of zinc and were essentially zinc-free; yet their rate of growth and gross appearance were normal.

The trace element, zinc, is apparently ubiquitous in animals, and small amounts of this metal (50 to 250 μg per gm dry weight) have been found in many different organs (Mawson and Fischer, 1951, 1953). In certain parts of the human and rat male reproductive system, such as the dorsolateral prostate gland, spermatozoa, and seminal fluid (Mawson and Fischer, 1953), a relatively high concentration of zinc (2000 to 3000 μg per gm dry weight) has been reported. Furthermore the rat prostate, in contrast to other organs, has been shown to accumulate injected radiozinc (Gunn *et al.*, 1955), and a similar selective uptake occurs in the ovaries of silkworms, but not of rats (Akao,

1935; Mawson and Fischer, 1951). Evidence of the physiological significance of zinc is that testicular atrophy as well as growth failure has been demonstrated in zinc-deficient rats (Millar *et al.*, 1954). These findings suggested the possibility that zinc plays an important role in maintaining the integrity of the reproductive system.

One of the outstanding physiological characteristics of insects, and especially of the mosquito, is their fecundity or high reproductive potential. This fact combined with the above findings that zinc is associated with the gonads, formed the rationale for the present investigation. In this communication are presented the results of a study on the occurrence, distribution, and accumulation during the developmental stages, and some chemical characteristics of zinc in the mosquito.

MATERIALS AND METHODS

The mosquito strains used in this study, *Aedes aegypti* (Linn.), *Culex pipiens* (Linn.), and *Culex molestus* (Forskal), have been maintained in this laboratory for several years. Usually 200 to 300 larvae were reared in enameled pans on a diet consisting of tap water and commercial feed pellets (Lang, 1956). The rearing temperature varied from 25–28°C, and no effort was made to control the degree of illumination. For the radioisotopic studies a different procedure was followed; namely, 40 to 50 first instar larvae that emerged during a 30 minute period from eggs subjected to vacuum were placed in borosilicate crystallizing dishes (90 mm diameter by 50 mm height) containing 100 ml media. The culture medium consisted of feed pellet infusion in deionized water supplemented with radiozinc^{65} chloride. The dishes were covered with a Petri dish and incubated at 27 ± 1°C. The term, radioactive larvae, refers to mosquitoes grown under these conditions. To minimize possible zinc contamination only borosilicate or other "zinc-low" equipment and enclosures were used, and except for the adults, all samples were washed in several changes of deionized water prior to analysis.

The other insect species, *Tribolium confusum*, *Oncopeltus fasciatus*, *Lymantria dispar*, *Apis mellifera*, *Aedes aegypti*, *Culex pipiens*, and *Culex molestus*, were wild-caught or from laboratory colonies reared on crude diets.

Dissection of the mosquitoes was accomplished by traction on the terminal abdominal segments with stainless steel needles and using a borosilicate glass Petri dish as a support. The Malpighian tubules were separated completely from the adjacent mid- and hind gut.

Analytical Techniques

DRY WEIGHT Since fresh weight values are often inconstant and difficult to measure because of the rapid water loss by these small specimens, dry weight was determined instead as follows: the freshly collected samples were dried for several days at 90–100°C and then stored in a desiccator over drierite. They were then weighed on a torsion balance sensitive to 5 μg.

NITROGEN Micro-Kjeldahl nitrogen determinations were performed using a nesslerization method sensitive to less than 5 μg nitrogen (Lang, 1958).

ZINC A modification of the method of Vallee and Gibson (1948) was used. Samples were digested in a mixture of sulfuric acid and selenium oxychloride, diluted with water, and an aliquot was removed for nitrogen analysis. The remainder was neutralized with ammonium hydroxide followed by the addition of sodium potassium tartrate and thiosulfate buffer in the usual manner. This was extracted once with 5.0 ml of 1 mg per cent dithizone in carbon tetrachloride, and the optical density at 515 mμ of the zinc dithizonate was determined. The sensitivity of this method was 0.25 μg zinc, and the linear range was from 0.5 to 3.0 μg.

Radiometric determinations were carried out with a well-type, gamma ray scintil-

TABLE I

THE OCCURRENCE OF ZINC AND OTHER ELEMENTS IN INSECTS SPECTROGRAPHIC ANALYSIS

Order	Species	Zn	Zn	Fe	Cu	Mn
			Ash, *per cent*			
		μg/mg				
Coleoptera	*Tribolium confusum*	0.07	0.37	0.28	0.080	0.014
Hemiptera	*Oncopeltus fasciatus*	0.09	0.25	0.24	0.039	0.049
Lepidoptera	*Lymantria dispar*	0.11	0.17	0.08	0.039	0.022
Hymenoptera	*Apis mellifera*	0.21	0.26	0.35	0.045	0.250
Diptera	*Aedes aegypti*	0.95	1.2	0.15	0.081	0.027
	Culex pipiens	0.93	1.3	0.23	0.043	0.020
	Culex molestus	0.73	1.1	0.28	0.035	0.027

With the exception of *Tribolium confusum* and *Lymantria dispar* of which only a single sample was analyzed, the values represent the averages of two samples.

lation counter (Nuclear-Chicago Corporation) using 13 mm diameter lusteroid tubes for sample holders. The radiozinc^{65}-chloride solution had a concentration of 0.1 μg per ml and a specific activity of 1.3 millicuries per mg. With this counting system a 0.1 μg aliquot gave a net count of about 9000 CPM.

SPECTROGRAPHIC ANALYSIS Following dry ashing in platinum crucibles at 550°C for 16 to 18 hours, the samples were analyzed by a spark excitation spectrographic method using an internal standard of cobalt (Mathis, 1953). Duplicates were determined on different days and agreed within 5 per cent of each other.

RESULTS

The Occurrence of Zinc

Initially samples of seven insect species representing five biological orders were subjected to spectrographic analysis to determine the validity of the hypothesis that zinc is present in relatively high concentrations in the mosquito. Only

samples of the last developmental stages or adults were analyzed. The results shown in Table I indicated that a much higher concentration of zinc is found in various mosquito species than in other insects. Furthermore, it was apparent that this large amount of zinc is unique, for much smaller quantities of other trace elements were present in the same samples. In general, the concentration of copper and manganese was low in all samples, with the exception of the manganese content of the honeybee, *Apis mellifera*, which was five- to tenfold higher than in the other insects. The iron concentration was variable. Although only few samples were analyzed, the good agreement of duplicates gave credence to the results, which were adequate for the purpose.

Following this screening process, microchemical determinations for zinc

TABLE II

ZINC IN ADULT MOSQUITO CARCASSES
MICROCHEMICAL ANALYSIS

Species	Sex	Micrograms zinc per		
		Mosquito	Dry weight	Nitrogen
			mg	*mg*
Aedes aegypti	F	0.96	1.8	21
	M	0.57	1.6	22
Culex pipiens	F	1.3	2.0	21
	M	0.87	1.9	20
Culex molestus	F	1.8	1.7	23
	M	0.74	1.1	19

Each value represents the average of six samples of individual mosquitoes.

and for nitrogen were performed on individual adult mosquitoes of both sexes. It was noted (Table II) that the amount of zinc per female was 50 to 250 per cent greater than in the males of the same species. However, when the data were adjusted to account for the larger size of the females by expressing the zinc concentration in terms of dry weight or nitrogen, this sex difference disappeared.

Distribution of Zinc

To determine the distribution of zinc in the mosquito, individual radioactive adult males and females were dissected, and their component parts were analyzed for radioactivity. Of the total radioactivity in a mosquito (200 to 390 CPM/mosquito) average values of 90 per cent for five male samples and 88 per cent for five females were found in the Malpighian tubules. Similarly chemical analyses of twenty-five similar individual as well as pooled samples of all three mosquito species indicated that over 90 per cent of the zinc was localized in this organ. Since the average dry weight of the Malpighian tubules from a

single *A. aegypti* mosquito was about 50 μg and the zinc content, 1.6 μg, the estimated concentration of zinc in the Malpighian tubules is about 32 μg/mg. This value is about ten times greater than that found in sperm and the dorsolateral prostate gland, which have the highest zinc concentrations of all soft tissues of rat or man (Mawson and Fischer, 1953).

The Accumulation and Retention of Zinc

The accumulation of zinc in the developing mosquito was studied, and the results for *C. molestus* are presented in Table III. It will be noted that zinc was not detectable in pooled samples of eggs (106 to 164 eggs per sample) and that the rate of accumulation was proportional to the growth rate as shown

TABLE III
ZINC CONCENTRATION DURING MOSQUITO GROWTH

Stage	No. of Samples	Dry weight per mosquito	Zinc per mosquito	Zinc per mg dry weight	
				Average	Range
		μg	μg		
Egg	4	2.9	0.005	1.7	—
III Larvae	4	137	0.14	1.02	0.60–1.8
IV Larvae	6	1100	1.14	1.04	0.85–1.1
Pupae	6	990	0.84	0.85	0.65–0.90

by the relatively constant zinc to dry weight ratios. These results were confirmed using *A. aegypti* and radiozinc.

Evidence for the specificity of zinc absorption was provided by several experiments using radiocobalt^{60}-chloride and radiocobalt^{60}-vitamin B_{12}. *A. aegypti* larvae were grown under standard conditions in media supplemented with either 5 μg of $Co^{60}Cl_2$ containing 23,100 CPM or with an equal amount of Co^{60}–vitamin B_{12} with a total activity of 137,000. In addition, a control series was reared in 0.1 μg radiozinc^{65}-chloride, 8900 CPM. The resultant adults from all groups, which developed at a similar rate and appeared normal, were counted as individual and as pooled samples. Whereas the zinc group accumulated from 128 to 201 CPM per mosquito, neither the inorganic cobalt nor the cobalt-B_{12} group exhibited greater than 7 CPM above background. It was unlikely that this lack of uptake was due to an isotope dilution effect because of the high activity and relatively high concentrations of the cobalt preparations.

In an effort to elucidate the uptake of zinc by mosquito larvae, individual early fourth instar *A. aegypti* larvae, which are at the beginning of the rapid growth phase, were placed in test tubes containing isotonic saline (group 1) or media (group 2) supplemented with equal amounts of radiozinc (165 CPM).

The results in Table IV indicated that larvae accumulate more radioactivity from media than from an inorganic solution. The difference in total zinc uptake is even greater, for the media contained about 2 μg total zinc compared to 0.15 μg in the saline; thus at 72 hours, group 1 contained about 0.01 μg zinc per larva and group 2, 1 μg. A reciprocal and concomitant experiment using radioactive larvae and non-radioactive saline (group 3) or media (group 4) demonstrated the retention and lack of exchange of the incorporated zinc with the environment under fasting or growing conditions.

It was of interest to determine how long zinc remains in the mosquito following the accumulation during the larval stages. To this end *A. aegypti* larvae were reared in radiozinc media, and individual pupae of both sexes were iso-

TABLE IV

RADIOZINC UPTAKE BY EARLY FOURTH INSTAR MOSQUITO LARVAE

Group	Larvae	Medium	CPM per larva		
			0	24 hrs.	72 hrs.
1	Non-radioactive	Saline + Zn*	0	2	15
2	"	Media + Zn*	0	67	77
3	Radioactive	Saline	135	136	146
4	"	Media	157	149	151

The data represent the averages of five samples.

lated and counted at different time intervals. With this procedure a single organism could be transferred and counted repeatedly on succeeding days without damaging the organism. Young pupae, and their cast pupal skins and emergence fluids were studied as well as adults, and the results are given in Table V. No decrease in radioactivity was observed during metamorphosis, the subsequent moult, or at least as long as 14 days adult age. Thus the zinc incorporated by the developing larva is retained throughout metamorphosis and into adulthood.

Preliminary experiments to prepare zinc-deficient mosquitoes by growing them in dithizone-extracted media were unsuccessful, so another method using media supplemented with disodium ethylenediaminetetraacetic acid (EDTA) was employed. This technique has been shown to increase urinary zinc excretion and deplete certain zinc stores in the rat (Millar *et al.*, 1954). In the early experiments, EDTA-supplemented diets caused growth failure of first instar *A. aegypti* larvae which could be completely reversed by the addition of zinc. A more elaborate study using various amounts of EDTA, zinc chloride, and lead chloride was undertaken (Table VI). Lead was included because its equilibrium constant, log K, with EDTA (18.2) is greater

than that of zinc (16.2) according to Welcher (1958). Standard conditions were used, and in addition, each dish contained 0.2 μg radiozinc chloride with an activity of about 18,000 CPM; the total zinc content of the basal medium was 100 μg or 0.7 μmole, and thus the specific activity was 180 CPM per μg zinc. To obtain an estimate of growth rate and mortality, the day of first appearance and the per cent of the original forty larvae in each dish that reached the fourth larval instar and pupal stage were determined. The 11th day of growth, when about 50 per cent of the control groups 1, 2, and 3 pupated, was used as the comparison time. In addition, the radioactivity of the pupae was measured, and the equivalent zinc content calcu-

TABLE V

RADIOZINC CONTENT OF MOSQUITO PUPAE AND ADULTS

Experiment	Sample	No. of samples	CPM per mosquito	
			Average	Range
1	Pupae	10	260	127–388
	Pupal skins and emergence fluid	9	4	−10 to +13
2	Adults, day 1	4	327	301–364
	" 3	4	303	289–323
	" 5	3	318	314–326
	" 7	3	314	306–338
	" 10	2	304	290, 318
	" 14	2	314	307, 321

lated from the various specific activities. For groups 7 to 14 pooled samples of from two to four mosquitoes were also analyzed. Several points of interest are indicated by the data. First, a sixfold increase in zinc concentration of the media (group 1 *vs.* group 3) had little effect on the rate or extent of growth and only increased the zinc content of the resultant pupae by 50 per cent from 1.92 to 2.82. Second, the addition of 5 μmoles of EDTA to the basal medium (group 4) affected growth slightly and reduced the incorporation of zinc to 17 per cent of the control (group 1). Greater amounts of EDTA (groups 5 and 6) decreased the larval development and pupation rate. Third, the addition of 0.18 to 1.4 μmoles of zinc to the EDTA media reversed the inhibitory effect of this chelating agent on development, but zinc uptake was abolished (groups 7 to 10). Fourth, supplementation of media containing EDTA and zinc with 0.7 μmole of lead did not alter the results obtained with EDTA and zinc alone (groups 11 to 14).

Chemical Characteristics of Incorporated Zinc

Malpighian tubules from ten *A. aegypti* females grown in radiozinc were homogenized in 0.9 per cent saline. Measured aliquots were then placed in

cellophane bags and dialyzed against known volumes of 0.9 per cent NaCl or 0.9 per cent NaCl–0.02 M EDTA. All operations were carried out between 0–4°C. The results presented in Table VII indicate that a significant portion of zinc is non-dialyzable against saline, but is completely dialyzable against EDTA. These data, therefore, suggest that part of the zinc in Malpighian tubules is loosely bound to a non-dialyzable component.

To determine the nature of the dialyzable zinc a sample of homogenized

TABLE VI

GROWTH AND RADIOZINC UPTAKE OF MOSQUITO LARVAE

Group	Medium	Added per dish			Time of first appearance		On 11th day		CPM per pupa	Zn per pupa
		EDTA	$ZnCl_2$	$PbCl_2$	IV Larvae	Pupae	Alive	Pupae		
		μmoles	*μmoles*	*μmoles*	*day*	*day*	*per cent*	*per cent*		*μg*
1	Basal (B)	—	—	—	4	7	88	45	346	1.92
2	B + Zn	—	0.7	—	4	7	88	65	196	2.18
3	" " "	—	3.5	—	4	7	93	50	122	2.82
4	B + EDTA	5	—	—	5	8	78	40	59	0.33
5	" " "	10	—	—	8	—	88	0	—	—
6	" " "	20	—	—	11	—	63	0	—	—
7	B + EDTA + Zn	10	0.18	—	6	8	88	33	17	0.12
8	" " " " "	10	0.35	—	5	8	90	53	11	0.09
9	" " " " "	10	0.70	—	4	7	98	65	7	<0.09
10	" " " " "	10	1.40	—	4	7	93	65	5	<0.09
11	B + EDTA + Zn + Pb	10	0.18	0.70	6	9	78	23	22	0.15
12	" " " " " " "	10	0.35	0.70	4	7	93	40	11	0.09
13	" " " " " " "	10	0.70	0.70	4	7	98	58	4	<0.09
14	" " " " " " "	10	1.40	0.70	4	7	100	70	5	<0.09

radioactive Malpighian tubules was subjected to paper ionophoresis. The results indicated that all of the radioactivity migrated a distance of 1 to 2 cm in 3 hours whereas the control sample of $Zn^{65}Cl_2$ moved at 2 to 3 times that rate. These data suggest that zinc in Malpighian tubules is in a form other than a free inorganic ion.

DISCUSSION

The primary emphasis of investigations of trace elements in insects has been on the accumulation and absorption of metals, and the work of Waterhouse is exemplary. Using the blowfly, *Lucilia cuprina*, he has studied the metabolism of barium (1951), strontium (1951), iron (1940), and copper (1945). These trace elements were present in low concentrations, were lost by excretion or transovarian passage to eggs, and at least in the cases of

barium and strontium, were regarded as contaminants. The latter two elements were found in Malpighian tubular granules which contained large amounts of calcium, magnesium, and phosphorus. Formation of these granules occurred during the larval stages, and they were excreted in the meconium shortly after emergence of the adult (Waterhouse, 1950).

The metabolism of zinc in the mosquito is different in several respects. First, a high concentration of zinc in regard to other metals and other insects is accumulated and localized in a single organ. Second, the uptake during larval growth and complete retention through metamorphosis and a good portion of adult life suggest other than a fortuitous process. Although Malpighian tubules are regarded primarily as having an excretory function, the zinc deposit is apparently not a form of storage excretion. Third, the absence of zinc in the egg obviates the possibility of mother to egg transfer.

TABLE VII

DIALYZABILITY OF RADIOZINC IN MALPIGHIAN TUBULES

Dialysate	Compartment	Total radioactivity	
		Theoretical	Observed
		per cent	*per cent*
0.9 per cent NaCl	Inside bag	4.7	22.7
	Outside	95.3	77.3
0.9 per cent NaCl–EDTA·2Na	Inside bag	4.2	6.8
	Outside	95.8	93.2

The theoretical distribution was calculated on the basis of a completely dialyzable solute at equilibrium under isoosmotic conditions.

Finally, zinc-free mosquitoes can be grown at a normal growth rate. Although minute amounts of the metal may still be present to fulfill the possible requirement of a mosquito, over 95 per cent of the normally accumulated zinc is not essential for growth. As to the integrity of other physiological phenomena such as reproduction, further experimentation is required. The criterion of zinc deficiency used in these studies is based on a marked decrease in total carcass content of this metal and not on derangements of physiology which could be due to the general debility of the organism or to secondary effects.

The inhibition of larval growth by EDTA supplementation may be due to the chelation of essential mineral nutrients resulting in their unavailability to the organism. This explanation is supported by the decreased zinc content in EDTA-reared pupae. Zinc, which has a high affinity for EDTA, reversed the inhibition presumably by displacing essential metals such as calcium and magnesium from their more easily dissociable versene complexes. Although

possible, it seems unlikely that zinc is essential for the mosquito because a tenfold excess of EDTA compared to zinc on a molar basis was required for inhibition. In addition the incorporation of zinc under these conditions was nil, and yet growth and development were normal. Further evidence was provided by the fact that the addition of lead, which has an even greater affinity for EDTA, gave the same results. Obviously additional studies are required to define the essential inorganic elements for mosquito growth.

A modern criterion of micronutrient requirement is based on its demonstration as a specific cofactor in an essential enzyme system. Several zinc enzymes have been described (Vallee, 1956), and one of the best known is carbonic anhydrase, an enzyme which is absent in some insects (Sobotka and Kann, 1941). Whether or not this enzyme or similar zinc-enzyme complexes are present and essential in the mosquito is unknown, and thus the physiological role of zinc in this insect remains to be demonstrated. Teleologically, it may have an important function, but further experience is necessary to establish this view.

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