

THE MODES OF ACTION OF TOXIC AGENTS

I. OBSERVATIONS ON THE POISONING OF CERTAIN CRUSTACEANS BY COPPER AND MERCURY

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(Text-figs. 1-5)

Reports of recent attempts to discover how copper and mercury act as poisons to crustaceans are to be found in papers by Clarke (1947), Pyefinch & Mott (1948), Barnes & Stanbury (1948), Hoffmann (1950) and Russell Hunter (1950). Most of the evidence obtained has been interpreted in the light of two general theories (cf. Pyefinch & Mott, 1948). One is that these poisons exert their toxic effects by inactivating vital processes which occur at the animal's surface: the other is that they are absorbed by the animal and act internally by inhibiting metabolic changes. However, conclusive evidence supporting or excluding either possibility has yet to be obtained.

The present account describes some preliminary experiments which have formed part of a series on the effects of toxic agents on crustaceans, and is concerned with testing the importance of penetration in the toxic action of a heavy metal. In these experiments the relative toxicities of copper (as copper sodium citrate) and mercury (as mercuric chloride, ethylmercuric chloride and mercuric iodide) have been tested using (1) larvae of the brine shrimp *Artemia salina* (L.), a species which has been found to be very resistant to heavy metal poisons, and (2) adults of the marine copepod *Acartia clausi* (Giesbrecht) and larvae of the barnacle *Elminius modestus* (Darwin), two crustacean species which, compared with *Artemia*, show little resistance to these poisons. Copper sodium citrate ($\text{NaCuC}_6\text{H}_5\text{O}_7 \cdot n\text{H}_2\text{O}$; Cu = 17.5%) was used in this investigation because, of the various copper salts tested in experiments with *Artemia*, this was the only one which gave lethal concentrations of copper which were soluble in sea water at pH 8.1. Mercuric chloride was tested because of its widespread use by other workers in this field of research. Ethylmercuric chloride was used because studies of its action as a bactericide and fungicide have led to the view that it possesses a rapid rate of penetration (cf. Sexton, 1953). Finally, mercuric iodide was tested because of the results of further studies carried out in this laboratory (to be published later) concerning the influence of iodine compounds (e.g. sodium iodide and sodium iodoacetate) on the susceptibilities of certain crustaceans to mercury poisoning.

Experiments have also been carried out in which the toxic effects of bipartite mixtures of copper and mercury have been tested, and the results of these studies have provided further information on the modes of action of these poisons which, in some respects, appear to be different.

METHODS

Animals

Artemia salina was reared in sea water. Two days after the larvae had hatched they were removed by filtration on a fine gauze, transferred to a small volume (5–10 ml.) of filtered sea water, and samples (0.01–0.02 ml.) of the thick suspension of animals so formed were then added to the various solutions under test.

Acartia clausi was collected on the day of each experiment with a medium tow-net in Wembury Bay. Separation of these animals from unwanted material was facilitated by their swarming in an area of high light intensity at the surface of the sample, from which they were pipetted first into filtered sea water and then into the toxic solutions.

Elminius modestus larvae were obtained from adults collected from rocks at Tinside in front of the Plymouth Laboratory. The animals were placed in fresh sea water and the larvae which they liberated were collected after 1 h and added to the toxic solutions.

Toxic agents

Solutions of the poisons in sea water were prepared immediately before each experiment and adjusted to pH 8.1. The amounts of mercuric iodide and ethylmercuric chloride which dissolved in sea water were small and limited the range of toxic concentrations which could be studied. However, by shaking suspensions of these compounds in sea water overnight, it was possible to dissolve a concentration of each equivalent to 25 mg Hg^{++}/l .

The concentrations of the mercury and copper compounds used in the present work are expressed throughout in terms of mg Hg^{++} and Cu^{++}/l ., a terminology used purely for the sake of convenience in comparing toxicities and not intended to imply that these are the ions to which the compounds give rise in sea water.

Toxicity measurement

Glass tubes (10 by 2 cm), each containing the solution to be tested (5 ml.), and 50–100 animals, were placed on a glass plate marked in squares (0.25 cm²) and examined under a binocular microscope. Sufficient replicates were used to give a total of 200–300 animals in each of several concentrations of the same poison and in the control solutions. With the three species used it was found that when the animals lost their activity they settled on the bottom of the tube so that the number which lay above each square section of the glass

plate could readily be counted. In this way the total number of animals which had lost all signs of movement, including that of their appendages, was estimated at suitable time intervals. These experiments were usually conducted until at least 90% of the test animals had become sufficiently quiescent to be presumed dead. The remaining 10% were then killed (by adding one or two drops of a solution of sodium azide in dil. HCl to the sea-water medium), the total number of animals was estimated, and the percentage of the total which were dead at each time interval was calculated. These percentage values plotted against time gave sigmoid curves, from which the time required for 50% of the test animals to die was calculated. Such '50% death' values were found for each of several concentrations of a given poison. Usually duplicate determinations of the time of 50% death in a particular concentration of any of the poisons used gave values not differing by more than 5% of the mean.

No experiment on *Acartia* and *Elminius* lasted for more than 24 h. Some on *Artemia*, however, were continued for 4 days, during which time animals immersed in toxic solutions and in sea water moulted from the first to the second instar (Heath, 1924). As yet, however, no quantitative examination has been made of the effects of the poisons used on the mechanism of moulting or on the toxicities of these poisons to animals at different stages of development. No food was given to the animals during the toxicity experiments, but both *Acartia* and *Elminius* remained normally active for one day and *Artemia* for at least 5 days in filtered sea water.

EXPERIMENTS AND RESULTS

Comparison of Toxicities

When the time of 50% death was plotted against the concentration the toxicity curves shown in Fig. 1 (*Artemia*), Fig. 2 (*Elminius*) and Fig. 3 (*Acartia*) were obtained. It seemed that the most satisfactory way of estimating the relative toxicities of the poisons to each species was to determine the ratios of the concentrations which caused 50% death in the same time. Because, however, of the marked differences between the toxicities of the poisons used, it was not possible to find a time of 50% death which was common to all four toxicity curves. Accordingly, times of 50% death which were common to the toxicity curves of mercuric chloride, ethylmercuric chloride and mercuric iodide were used to estimate the equitoxic concentrations and hence the relative toxicities of these three mercury compounds; and times of 50% death which were common to the toxicity curves of copper sodium citrate and mercuric chloride were used in the same way in order to calculate the relative toxicities of these two poisons. The toxicities of the four poisons tested with each species, expressed in terms of that of mercuric chloride as a standard, are shown in Table I.

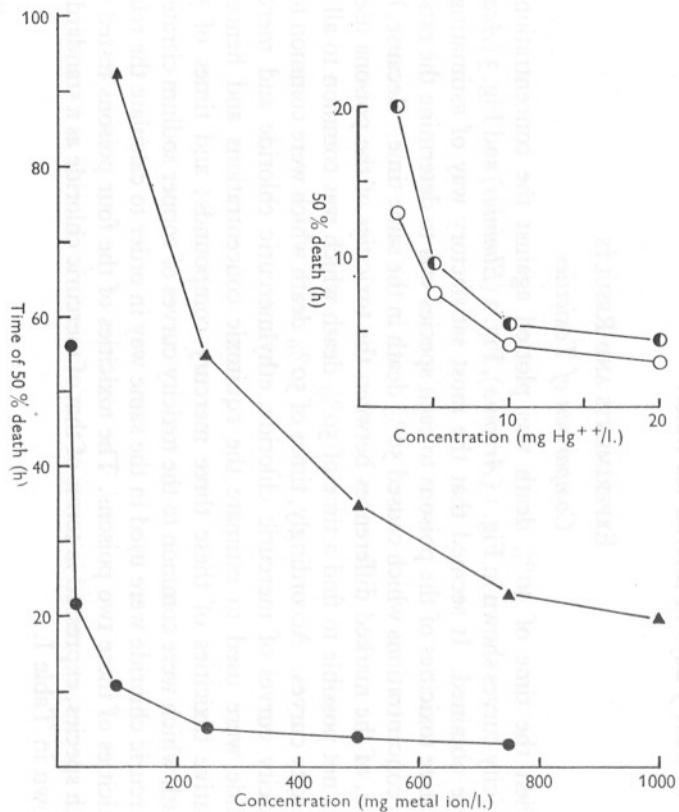


Fig. 1. Survival of larvae of *Artemia salina* in sea water containing copper sodium citrate (▲—▲), mercuric chloride (●—●), ethylmercuric chloride (◐—◐) and mercuric iodide (○—○).

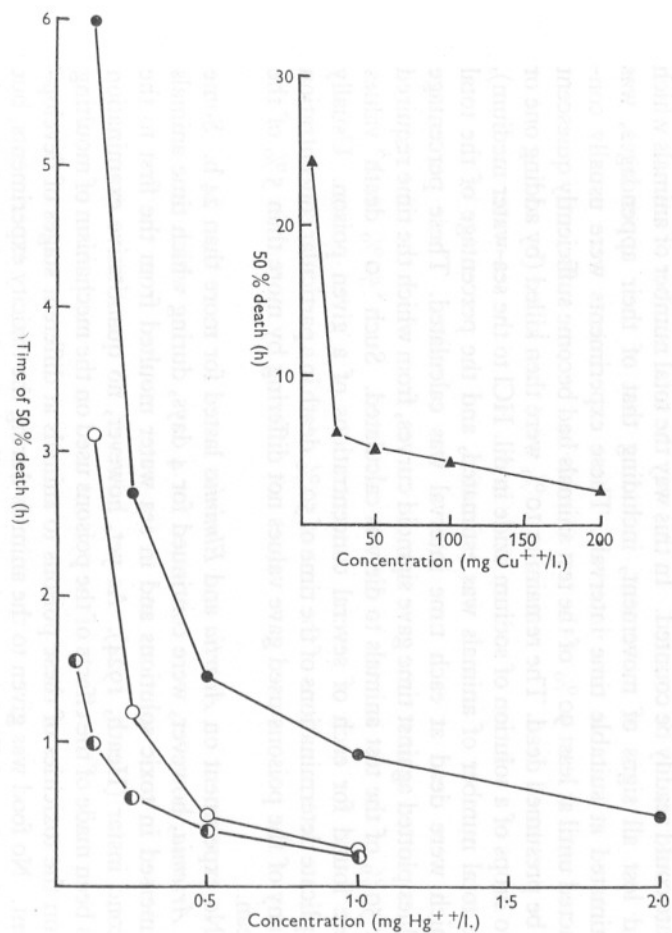


Fig. 2. Survival of larvae of *Elminius modestus* in sea water containing copper sodium citrate (▲—▲), mercuric chloride (●—●), ethylmercuric chloride (◐—◐), and mercuric iodide (○—○).

The results showed that differences between the toxicities of mercuric iodide and ethylmercuric chloride on the one hand and mercuric chloride on the other varied with the resistance to mercury poisoning of the species used as test animal, being minimal with the least resistant *Acartia* (50% death in 2.5 h in 0.05 mg Hg⁺⁺/l.), slightly greater with *Elminius* (50% death in 2.5 h in 0.30 mg Hg⁺⁺/l.) and maximal with the very resistant *Artemia* (50% death in 2.5 h in 800 mg Hg⁺⁺/l.). No such trend was observed, however, in the differences between the toxicities of copper sodium citrate and mercuric chloride: the results simply indicated that copper sodium citrate was much less toxic than mercuric chloride to the three species examined.

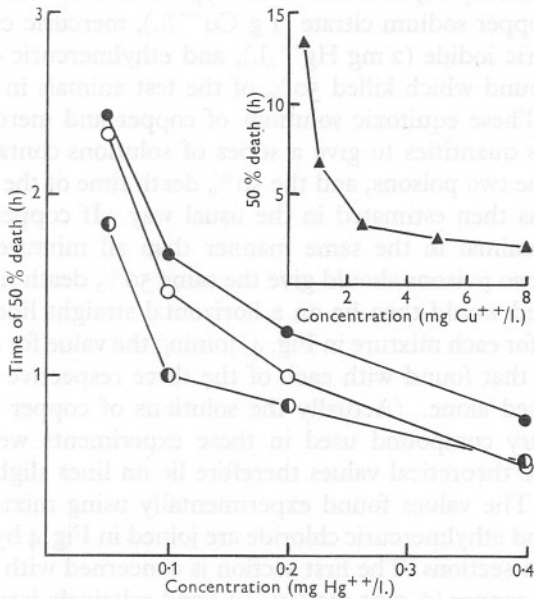


Fig. 3. Survival of *Acartia clausi* in sea water containing copper sodium citrate (▲—▲), mercuric chloride (●—●), ethylmercuric chloride (◐—◐) and mercuric iodide (○—○).

TABLE I. RELATIVE TOXICITIES OF COPPER AND MERCURY POISONS

Compounds in sea water, pH 8.1. Experiments conducted at room temperatures between 21 and 25° C. Toxicity of each poison relative to that of mercury as mercuric chloride, expressed as unity.

Poison	Relative toxicity		
	<i>Artemia</i>	<i>Elminius</i>	<i>Acartia</i>
Mercuric chloride	1.0	1.0	1.0
Ethylmercuric chloride	24	4.4	2.0
Mercuric iodide	31	2.6	1.7
Copper sodium citrate	0.005	0.002	0.01

Studies with Bipartite Mixtures of Poisons

Previous workers have reported that toxic mixtures of copper and mercury show marked supplemental synergisms (more-than-additive toxic effects) when used against crustaceans (Barnes & Stanbury, 1948; Pyefinch & Mott, 1948; Hoffmann, 1950; Russell Hunter, 1950). These observations have led to the view that copper and mercury act on the animals in different ways and that the changes induced by one poison reinforce those caused by the other. To examine this possibility with *Artemia* as the test animal the following procedure was used.

By means of toxicity experiments of the type described earlier certain concentrations of copper sodium citrate (1 g $\text{Cu}^{++}/\text{l.}$), mercuric chloride (50 mg $\text{Hg}^{++}/\text{l.}$), mercuric iodide (2 mg $\text{Hg}^{++}/\text{l.}$), and ethylmercuric chloride (2 mg $\text{Hg}^{++}/\text{l.}$) were found which killed 50% of the test animals in approximately the same time. These equitoxic solutions of copper and mercury were then mixed in various quantities to give a series of solutions containing different proportions of the two poisons, and the 50% death time of the test animals in each solution was then estimated in the usual way. If copper and mercury act on the test animal in the same manner then all mixtures of equitoxic solutions of the two poisons should give the same 50% death time. The series of values obtained would then lie on a horizontal straight line (shown as an interrupted line for each mixture in Fig. 4) joining the value for copper sodium citrate only and that found with each of the three respective mercury compounds when used alone. (Actually the solutions of copper sodium citrate and each mercury compound used in these experiments were not exactly equitoxic and the theoretical values therefore lie on lines slightly inclined to the horizontal.) The values found experimentally using mixtures of copper sodium citrate and ethylmercuric chloride are joined in Fig. 4 by a curve which is shown in three sections. The first section is concerned with mixtures containing traces of copper (0-0.05 mg $\text{Cu}^{++}/\text{l.}$) and relatively large quantities of mercury (*ca.* 2 mg $\text{Hg}^{++}/\text{l.}$) and in this region of the curve it will be seen that the differences between the theoretical and experimental values are insignificant. The second section of the curve includes a value obtained using a mixture of approximately equal quantities of the two poisons (2.0 mg $\text{Cu}^{++}/\text{l.}$; 1.9960 mg $\text{Hg}^{++}/\text{l.}$), and this again is not significantly different from the theoretical value. The third and largest section of the curve deals with mixtures containing small quantities of mercury and large amounts of copper, and in this region of the curve values found experimentally are consistently lower than the theoretical values. Similar results were obtained when mixtures containing mercuric iodide and copper sodium citrate were studied (central curve of Fig. 4).

The lowest curve in Fig. 4 describes the results of experiments using toxic mixtures of copper sodium citrate and mercuric chloride. The first section of

this curve is concerned with mixtures which contained traces of copper (1.0-2.5 mg Cu⁺⁺/l.) and a large excess of mercury (49.75-49.95 mg Hg⁺⁺/l.), and it will be seen that, unlike the corresponding sections of the curves described earlier, more-than-additive effects are already manifest. Results depicted in the other section of the curve show that as the quantity of copper in the mixture exceeded that of mercury the differences between theoretical and experimental values became greater until a ratio of 20:1 in favour of copper was reached; thereafter the differences became less marked except for the mixture which contained 975 mg Cu⁺⁺/l. and 1.25 mg Hg⁺⁺/l. which again showed a large more-than-additive effect. This latter mixture has been used many times in the course of these studies and has always been found to show a marked supplemental synergism.

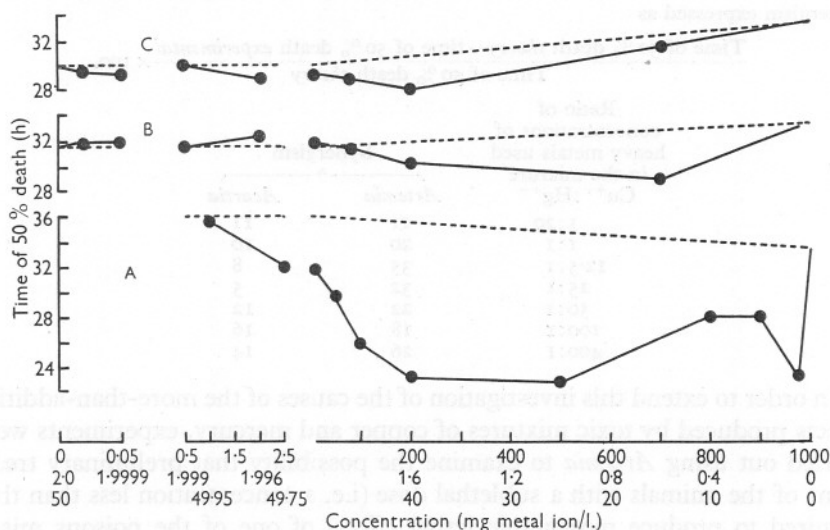


Fig. 4. Effects of bipartite mixtures of equitoxic concentrations of copper sodium citrate and mercuric chloride (A), mercuric iodide (B), and ethylmercuric chloride (C) on the survival of *Artemia* larvae in sea water (●—●, curves obtained experimentally; ---, theoretical curves expected if toxic effects of the mixed poisons were exactly additive). Concentrations of the metal ion measured as follows: Cu⁺⁺ as citrate (upper row, 0...1000), Hg⁺⁺ as C₂H₅HgCl or as HgI₂ (middle row, 2.0...0), and Hg⁺⁺ as HgCl₂ (lower row, 50...0).

Further experiments of a similar nature were carried out with *Artemia* as the test animal in order to examine the toxic effects of bipartite mixtures of mercuric chloride, mercuric iodide and ethylmercuric chloride. In these experiments, however, theoretical and experimentally determined values were always found to coincide and more-than-additive effects were not observed.

For the purposes of comparison, attempts were made to carry out studies similar to those just described for *Artemia* with the much less resistant species *Acartia*. Because of a shortage of test material, however, experiments with

this species were restricted to an examination of the toxic effects shown by mixtures of equitoxic concentrations of copper sodium citrate and mercuric chloride. The results showed that although the mixtures studied gave more-than-additive effects, in nearly every instance the sizes of the effects produced were considerably smaller than those observed when mixtures containing the same proportions of copper and mercury were examined using *Artemia* (see Table II).

TABLE II. COMPARISON OF THE SIZES OF THE SUPPLEMENTAL SYNERGISMS SHOWN BY TOXIC MIXTURES OF COPPER SODIUM CITRATE AND MERCURIC CHLORIDE WHEN TESTED WITH *ARTEMIA* AND *ACARTIA* IN SEA WATER

Synergism expressed as

$$\frac{\text{Time of 50\% death theory} - \text{time of 50\% death experimental}}{\text{Time of 50\% death theory}} \times 100.$$

Ratio of concentrations of heavy metals used in the mixture Cu ⁺⁺ :Hg ⁺⁺	Synergism	
	<i>Artemia</i>	<i>Acartia</i>
1:20	11	11
1:1	20	10
12.5:1	35	8
25:1	32	5
50:1	22	12
100:1	18	16
400:1	26	14

In order to extend this investigation of the causes of the more-than-additive effects produced by toxic mixtures of copper and mercury, experiments were carried out using *Artemia* to examine the possibility that preliminary treatment of the animals with a sublethal dose (i.e. a concentration less than that required to produce measurable lethal effect) of one of the poisons might influence the rate of death of the animals in toxic solutions of the other.

The effect of mercury on Artemia previously treated with copper. A large number of animals were immersed in sea water containing 1 g Cu⁺⁺/l. as copper sodium citrate. After 1 h in this solution the animals were removed by filtration on a fine gauze, washed several times with plain sea water and then transferred to a series of solutions containing different concentrations of mercuric chloride (250, 125 and 62.5 mg Hg⁺⁺/l.) mercuric iodide (10, 7.5 and 2.5 mg Hg⁺⁺/l.) and ethylmercuric chloride (10, 7.5 and 2.5 mg Hg⁺⁺/l.). For comparison with this copper-treated series, animals were immersed for 1 h in sea water to which no copper had been added and were then transferred to each of a second and similar series of the toxic solutions just described, while samples of these untreated as well as of the copper-treated animals were also placed in plain sea water. The 50% death time of animals present in each solution under test was then estimated in the usual way.

It was found in this experiment that animals which had been immersed in sea water containing 1 g Cu^{++} /l. as copper sodium citrate did not die at a rate significantly faster than that of the untreated animals in either sea water itself or in sea water to which various concentrations of copper had been added. They did, however, die at a much faster rate in each of the sea-water solutions which contained mercury as mercuric chloride and at a slightly faster rate in sea-water solutions of mercuric as mercuric iodide and ethylmercuric chloride (see Table III). Consequently, it seemed that although the amount of poison

TABLE III. THE EFFECT OF A PRELIMINARY TREATMENT OF *ARTEMIA* WITH COPPER ON THE RATES OF DEATH OF ANIMALS SUBSEQUENTLY IMMERSSED IN TOXIC SOLUTIONS OF VARIOUS MERCURY COMPOUNDS AND OF COPPER IN SEA WATER

Time of preliminary treatment 1 h. Effect expressed as:

$$\frac{\text{Time of 50 \% death untreated} - \text{time of 50 \% death treated}}{\text{Time of 50 \% death untreated}} \times 100.$$

Poison used to test copper-treated animals	Concn. (mg/l.)	Effect
Hg^{++} as chloride	250	33
	125	48
	62.5	47
Hg^{++} as ethylmercuric chloride	10	21
	7.5	15
	2.5	18
Hg^{++} as iodide	10	18
	7.5	24
	2.5	23
Cu^{++} as citrate	1000	4
	500	3
	250	11

which was taken up by the animals which were given a preliminary treatment with copper was insufficient to render them less viable in plain sea water or more susceptible to subsequent copper poisoning, it did markedly lower their resistance to the toxic effects of mercury.

A period of 1 h was used for the preliminary treatment with copper, but

TABLE IV. THE INFLUENCE OF TIME OF PRELIMINARY TREATMENT OF *ARTEMIA* WITH COPPER ON THE RATES OF DEATH OF ANIMALS SUBSEQUENTLY IMMERSSED IN TOXIC SOLUTIONS OF MERCURY IN SEA WATER

Concentration of copper = 1 g Cu^{++} /l. as citrate: concentration of mercury = 125 mg Hg^{++} /l. as chloride. Effect expressed as in Table III.

Time of preliminary treatment in copper solution (min)	Effect
5	12
20	34
60	41

later experiments showed that it was possible to induce a considerable influence on the susceptibility of the animals to mercury poisoning by initially exposing them to a copper treatment lasting only 5 min. (see Table IV). On the other hand, prolonging the copper treatment to more than 1 h produced only a slight further increase in the death rate of the animals in the mercury solutions, and in some experiments it was found that after this prolonged treatment the animals died at a rate faster than that of untreated animals in plain sea water.

Effect of copper on Artemia previously treated with mercury. These experiments followed a pattern similar to that described previously. The test solutions contained copper sodium citrate (250, 500, 750 and 1000 mg $\text{Cu}^{++}/\text{l.}$) and before they were immersed in these solutions, the animals were given a preliminary treatment with either mercuric chloride (250 mg $\text{Hg}^{++}/\text{l.}$), ethylmercuric chloride (10 mg $\text{Hg}^{++}/\text{l.}$) or mercuric iodide (10 mg $\text{Hg}^{++}/\text{l.}$) for 1 h. It was found that animals treated with each mercury compound died at the same rate as that of untreated *Artemia* in sea water and in a series of solutions containing different concentrations of the mercury compound. They died, however, at a rate significantly faster than that of the untreated animals in sea

TABLE V. THE EFFECTS OF PRELIMINARY TREATMENTS OF *ARTEMIA* WITH DIFFERENT MERCURY COMPOUNDS ON THE RATES OF DEATH OF THE ANIMALS IN TOXIC SOLUTIONS OF COPPER IN SEA WATER

Time of preliminary treatment 1 h. Effects expressed as in Table III.

Concn. of Cu^{++} as citrate in solution used to test mercury-treated animals (mg/l.)	Mercury compound used		
	HgCl_2	HgI_2	$\text{C}_2\text{H}_5\text{HgCl}$
	(250 mg $\text{Hg}^{++}/\text{l.}$)	(10 mg $\text{Hg}^{++}/\text{l.}$)	(10 mg $\text{Hg}^{++}/\text{l.}$)
1000	51	23	26
500	30	25	23
250	42	33	28

water to which various amounts of copper sodium citrate had been added (see Table V). Apparently, therefore, the quantity of mercury taken up by the animals during their preliminary treatment with either mercuric iodide, mercuric chloride or ethylmercuric chloride was insufficient to render them less viable in sea water or to increase their susceptibility to subsequent mercury poisoning. It did, however, make them far less resistant to copper poisoning.

In the experiments just described the concentrations of the mercury compounds used to 'sensitize' *Artemia* to copper poisoning had approximately 5 times the potential toxicity of the concentration of copper used in earlier experiments to 'sensitize' the animals to mercury poisoning. Preliminary experiments, however, showed that although concentrations

of the mercury compounds of the same potential toxicity as 1 g Cu^{++}/l . 'sensitized' the animals to copper poisoning, the extent to which they so acted varied greatly from experiment to experiment and in some instances the effect was not detected.

Experiments with cysteine and reduced glutathione. It seemed possible that the effect of non-toxic amounts of copper on the susceptibility of *Artemia* to mercury poisoning was sufficiently large to be used as a means of examining the way in which copper acted on this animal. Thus, the possibility was borne in mind that if copper exerted this influence when adsorbed on the surface of the animal, the effect might be abolished if the animal were treated with cysteine, or reduced glutathione, before it was placed in solutions containing mercury. Accordingly, the following experiments were carried out.

TABLE VI. THE INFLUENCE OF CYSTEINE AND REDUCED GLUTATHIONE ON *ARTEMIA* 'SENSITIZED' BY COPPER TO MERCURY POISONING

Concn. of mercury as mercuric chloride in test solution (mg/l.)	Effect expressed as in Table III.		
	Effect after copper-treated animals washed with		
	Sea water	Glutathione	Cysteine
250	40	27	20
125	49	45	27
62.5	33	25	20

Artemia were immersed for 1 h in sea water containing copper sodium citrate (1 g Cu^{++}/l). After the animals had been removed from the solution by filtration, approximately equal numbers were added to sea-water solutions of 0.01 M cysteine, and 0.01 M glutathione. Others were added to plain sea water. The animals were suspended in these solutions for 5 min, filtered off, washed several times with sea water and then transferred to solutions of mercuric chloride in sea water equivalent to 250, 125 and 62.5 mg Hg^{++}/l . The rates at which the four sets of animals died in the mercury solutions were compared with those shown by animals which had not been immersed in the copper solution, but simply treated with either cysteine, glutathione or sea water before they were added to sea water containing mercuric chloride. The results of this experiment (see Table VI) showed that animals treated with copper and then washed with sea water died at the same enhanced rate in the mercury solutions. However, copper-treated animals which had subsequently been washed with cysteine or glutathione, died at a rate which, though still enhanced, was usually considerably closer to that shown by animals which had not been given an initial immersion in the copper solution. These findings, therefore, were consistent with the view that some at least of the copper which was instrumental in lowering the resistance of *Artemia* to mercury poisoning might be localized on the surfaces of these animals.

Further, this attached copper was removable with cysteine and glutathione but not with sea water.

Similar experiments were carried out in which animals were immersed for 1 h in sea water containing mercuric chloride (250 mg $Hg^{++}/l.$), and then washed with either sea water, cysteine or glutathione and transferred to solutions of copper sodium citrate (1000, 750 and 500 mg $Cu^{++}/l.$) in sea water. The results of this experiment (see Table VII) showed that the mercury-treated animals which had been subsequently washed with cysteine or glutathione died at a rate considerably slower than that of the animals which had been washed in sea water. This finding, therefore, indicated that some, at least, of the mercury responsible for raising the sensitivity of *Artemia* to copper poisoning was attached to the surfaces of these animals, and could be removed with cysteine or glutathione.

TABLE VII. THE INFLUENCE OF CYSTEINE AND REDUCED GLUTATHIONE ON *ARTEMIA* SENSITIZED BY MERCURY TO COPPER POISONING

Concn. of copper as citrate in test solution (mg/l.)	Effect expressed as in Table III.		
	Effect after mercury-treated animals washed with		
	Sea water	Glutathione	Cysteine
1000	64	42	27
750	61	24	26
500	50	15	25

RESPIRATION EXPERIMENTS

The experiments carried out to investigate the toxic effects of bipartite mixtures of copper sodium citrate and different mercury compounds provided evidence in support of the view that the toxic action of copper reinforces that of mercury, and that when mixtures of copper sodium citrate and mercuric chloride are tested, using *Artemia*, large supplemental synergisms are produced. In order to extend these studies it was considered worth while to compare the effects, if any, of copper (as copper sodium citrate) and mercury (as mercuric chloride) on a fundamental biological activity of the animals, namely their respiration.

Manometric Procedure for Measuring the Respiration Rate of Artemia Treated with Copper and Mercury

Oxygen consumption was measured in the conventional Warburg apparatus at a temperature of 25° C and the gas phase used was air. Flasks of approximately 15 ml. capacity equipped with single sidearms were used. Filtered sea water (2 ml.) containing 100–150 mg wet weight of larvae was placed in the main compartment of each flask, samples of filtered sea water (1 ml.) to which no additions had been made or to which an appropriate quantity of copper

sodium citrate, sodium citrate, or mercuric chloride had been added were pipetted into the sidearm, and 0.2 ml. 20% KOH together with 3 cm² of starch-free filter-paper were placed in the centre well. Equilibration was for 30 min., after which time the rate of oxygen consumption was measured at 30 min. intervals for 2 h. The contents of the sidearms were then tipped into the main compartments of the flasks and measurements of respiration rate were continued for a further 5 h. It was found that the rate of oxygen consumption was independent of the shaking rate over the range 70–100 oscillations/min. (5 cm traverse) and determinations of qO_2 ($\mu\text{l. O}_2/\text{mg wet wt./h}$) were unaffected by the quantities of animals used between 100 and 300 mg wet wt. The pH of the contents of the flasks was measured at the beginning and the end of each experiment and was usually found to have changed from 8.1 to 7.5 during the 7 h experimental period.

The Effects of Copper and Mercury on Respiration and Motility

During the time of these manometric experiments (7 h) the respiration rate of the animals in ordinary sea water, or sea water to which an appropriate quantity of sodium citrate had been added (as a control in the experiments in which copper sodium citrate was used), remained linear, and when the animals were inspected at the end of the experiment they appeared to have suffered no loss of motility and showed no signs of damage. In the course of these studies some twenty determinations of the qO_2 of *Artemia* larvae were made and the values obtained were between 1.01 and 1.15 $\mu\text{l. O}_2/\text{mg wet wt./h}$. The respiration rates of the animals which had been subjected to poisoning by copper and mercury, however, decreased considerably during the experiment and the percentage reduction in oxygen consumption was estimated over each hour subsequent to tipping. To compare these changes in respiration rate with changes in motility the following method was used. Exactly 1 h after tipping took place one of the flasks to which a solution of the poison had been added was detached from its manometer, representative samples (0.2 ml.) of its contents were withdrawn and the numbers of animals which had lost all signs of motility in these samples were determined as percentages of the total numbers present. Other flasks were removed exactly 2, 3, 4 and 5 h after tipping took place and their contents were examined in the same way for percentage loss of motility. By this means percentage reductions in respiration and motility were compared during the same time intervals.

Results of typical experiments are shown in Fig. 5. In these experiments the quantity of copper used was 1 g $\text{Cu}^{++}/\text{l.}$ and the quantity of mercury was 250 mg $\text{Hg}^{++}/\text{l.}$, concentrations identical with those employed in the 'sensitizing' studies described earlier. The results showed that whereas copper caused a marked and immediate decrease in respiration, this poison had only a small effect on motility. By contrast, mercury reduced motility faster than it inhibited respiration. A reduction in motility might be expected to precede

a fall in respiration on the grounds that animals in a completely motionless state might still possess a small but measurable oxygen consumption. Consequently the action of mercury was considered to be typical of many poisons. The effect of copper on the animals, however, appeared to be one of specific inhibition of respiratory mechanisms, and this finding indicated a difference between the modes of toxic action of the two heavy metals. Similar conclusions have been reached by Russell Hunter (1950) from studies using *Marinogammarus marinus*.

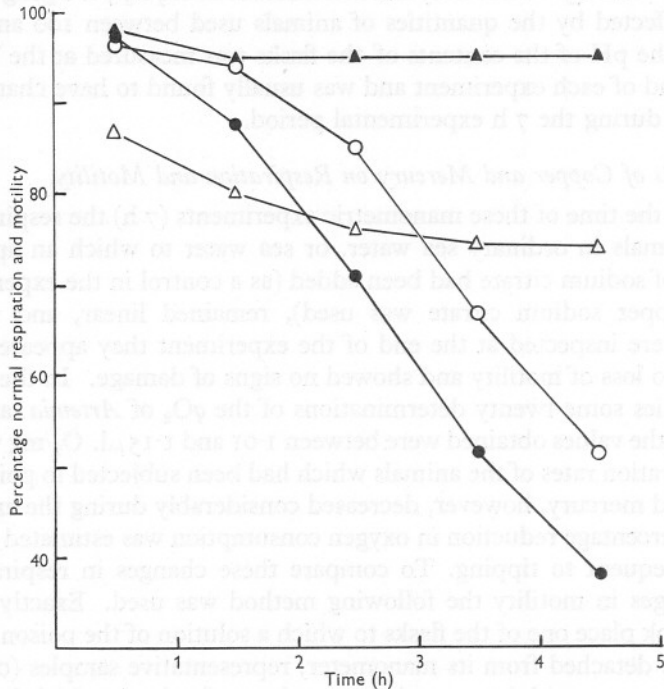


Fig. 5. Effects of copper sodium citrate and mercuric chloride on the motility and respiration rate of *Artemia* larvae in sea water (▲—▲, % decrease of motility in copper sodium citrate; △—△, % decrease of respiration in copper sodium citrate; ●—●, % decrease of motility in mercuric chloride; ○—○, % decrease of respiration in mercuric chloride).

DISCUSSION

The experiments described above were mainly of an exploratory nature and therefore only tentative conclusions may be drawn from their results. Even so the findings reported are worth examining in the light of current theories concerning the mechanisms of action of heavy metal poisons.

At the outset of the present work it was found that *Artemia* is far more resistant than either *Elminius* or *Acartia* to poisoning by mercuric chloride,

mercuric iodide, ethylmercuric chloride, and copper sodium citrate. In addition it was found that, whereas mercuric iodide and ethylmercuric chloride are far more toxic than mercuric chloride to *Artemia*, all three mercury compounds are of the same order of toxicity to *Elminius* and to *Acartia*. It appears, therefore, that when the test animal used is one which possesses a considerable resistance to mercury poisoning the differences between the toxicities of the mercury compounds are far greater than those observed when these compounds are tested on an animal which is much more readily poisoned by mercury. These results would be expected if differences between toxicities were closely allied with differences between rates of penetration, for the latter probably play an important part in determining relative toxicities to a highly impermeable animal, but exert a much smaller influence when the test animal used is one which is rapidly penetrated. The results of these toxicity studies therefore are consistent with the view that rates of penetration are, in fact, important factors influencing the toxicities of mercury compounds, and a corollary of this is that these poisons act inside the test animal.

This view assists interpretation of the results of studies made in the present work concerning the toxic effects shown by bipartite mixtures of mercury compounds and copper. Thus it has been found, using *Artemia* as the test animal, that whereas large more-than-additive toxic effects are shown by mixtures of copper sodium citrate and mercuric chloride these effects are much less marked when the mercuric chloride used in the mixtures is replaced by the more toxic iodide or ethylmercuric chloride. A possible explanation of these findings is that the copper used in the mixtures enables the mercury compounds to penetrate the test animal more readily, for it might be expected that this effect of the copper would enhance considerably the toxicity of mercuric chloride but influence to a much smaller extent that of the faster penetrating mercuric iodide or ethylmercuric chloride.

Furthermore, if copper increases the permeability of the test animal to mercury poisons it might be expected that this would have more pronounced effects when mixtures of copper and mercury are tested on a species highly impermeable to mercury than when the test animal used is one which this poison penetrates rapidly. Concerning this aspect of copper poisoning, therefore, interest attaches to further studies made in the present work which have demonstrated that the more-than-additive toxic effects shown by mixtures of copper (as citrate) and mercury (as chloride) when tested on *Artemia* are usually far greater than those observed when similarly proportioned mixtures of the two poisons have been applied to *Acartia*.

Although the results so far discussed support the view that mercury compounds are poisons which act internally, other findings during the present work have suggested that additional toxic effects of mercury as mercuric chloride may be induced at the surfaces of the test animals. Thus it has been found that *Artemia* which have been treated with a sublethal dose of mercuric

chloride become more sensitive to poisoning by copper, and that this 'sensitizing' effect can be appreciably reduced if the mercury-treated animals are washed with cysteine or with reduced glutathione. These experiments were similar in principle to those carried out by various workers in the course of studies of enzyme inhibition in which it was observed that the inactivation of enzymes such as urease (Hellerman, Chinard & Deitz, 1943), papain (Hellerman & Perkins, 1934) and yeast carboxylase (Stoppani, Actis, Deferrari & Gonzalez, 1953) by small amounts of copper, mercury and various 'mercaptide-forming' substances derived from the latter (e.g. phenylmercuric chloride) could be partially reversed by thiol compounds such as cysteine. These findings have led to the view that heavy metal poisons inhibit these enzymes by attaching to the surfaces at sulphhydryl groups responsible for catalytic activity. The results of analogous experiments carried out with *Artemia* in the present investigation, therefore, lend support to the view that mercury (as chloride) becomes attached to the surfaces of these test animals, possibly by interaction with sulphhydryl groups, and that when it is adsorbed at these surface sites it effectively lowers the resistance of *Artemia* to copper poisoning. Evidence consistent with the view that at least part of the toxic action of copper may be explained in a similar way has been obtained from complementary studies in which this poison has been found to 'sensitize' *Artemia* to the toxic effects of mercuric chloride: and in which this 'sensitizing' effect has been observed to diminish after the copper-treated animals have been washed with cysteine and reduced glutathione.

Two further significant observations have been made in the course of these 'sensitizing' experiments using *Artemia*. One is that sublethal doses of mercuric chloride, mercuric iodide, and ethylmercury chloride which have been found to render *Artemia* more susceptible to copper poisoning have been observed to show no marked influence on the susceptibility of the animals to poisoning by the respective mercury compound: and the other is that sublethal doses of copper which have been found to lower markedly the resistance of *Artemia* to mercuric chloride and, to a lesser extent, mercuric iodide and ethylmercuric chloride have no significant effect on the susceptibility of these animals to copper poisoning.

These findings, therefore, together with the results of other experiments discussed earlier in which bipartite mixtures of copper and mercury have been used, provide considerable evidence in support of the view that these two poisons act on *Artemia* in different ways, and evidence of one such difference has been obtained from an investigation of the effects of copper (as citrate) and mercury (as chloride) on the respiration rate of *Artemia* larvae. In these studies it has been found that copper depresses the respiration of the animals by approximately 25% without significantly affecting their motility, whereas mercury reduces their motility at a rate much faster than that at which it inhibits their respiration. As the concentrations of copper and of

mercury used in these respiration experiments were equivalent to those used in the 'sensitizing' experiments, there can be little doubt that the animals which were treated with copper in these studies had a reduced respiration when they were later placed in the mercury solutions. It seems possible, therefore, that one of the toxic effects ascribed to copper earlier, namely an ability to increase the permeability of *Artemia* to mercury compounds, may be correlated with its effect as a respiratory depressant.

It is clear that further experimental work is needed before a proper conspectus of heavy-metal poisoning in crustaceans can be formed. In experiments already begun attempts are being made to apply histochemical methods to the problem of determining the sites, either on or in these animals, at which heavy metal poisons exert their toxic effects. Other studies are contemplated in which it is hoped that further information concerning the causes of more-than-additive toxic effects will be gained by testing mixtures of poisons as inhibitors of isolated enzymes and multi-enzyme systems.

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SUMMARY

Studies have been made of the toxicities of copper (as copper sodium citrate) and mercury (as mercuric chloride, mercuric iodide and ethylmercuric chloride) to the three crustaceans *Artemia salina*, *Elminius modestus* and *Acartia clausi*.

Compared with *Artemia* the other two species examined have been found to be much more easily poisoned by each of the four toxic agents tested. Moreover, the differences between the toxicities of mercuric iodide and ethylmercuric chloride on the one hand and mercuric chloride on the other have been found to be far greater with *Artemia* than when *Elminius* and *Acartia* were used as the test animals.

Bipartite mixtures of copper (as citrate) and mercury (as chloride, iodide and ethylmercuric chloride) have been found to show more-than-additive toxic effects (supplemental synergisms) when tested with *Artemia*. Whereas the effects shown by mixtures of copper and mercuric chloride were found to be considerable, those displayed by mixtures of copper and either mercuric iodide or ethylmercuric chloride were, by comparison, very small. In

addition, it has been observed that the more-than-additive effects shown by mixtures of copper and mercury (as chloride) to *Acartia* were significantly smaller than those observed when corresponding mixtures were tested using the more resistant species *Artemia*.

Artemia have been found to be markedly 'sensitized' by pretreatment with a sublethal dose of copper to poisoning by mercuric chloride and, to a lesser extent, to poisoning by mercuric iodide and ethylmercuric chloride. Animals so treated, however, were not rendered more susceptible to copper poisoning. Similarly, it has been found that *Artemia* can be 'sensitized' to copper poisoning by pretreatment with mercuric chloride, mercuric iodide or ethylmercuric chloride in sublethal doses which do not render the animals less resistant to the respective mercury compound. These 'sensitizing' effects induced by copper and mercury (as mercuric chloride) have been found to be partially eliminated by washing the treated animals with solutions of either cysteine or reduced glutathione.

Further experiments with *Artemia* larvae have shown that copper depresses their respiration without significantly affecting their motility. Mercury, however, does not appear to have this effect.

The findings made in this investigation have been discussed in relation to current theories concerning the mechanisms of action of heavy metal poisons, and future studies have been indicated.

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