OSMOTIC REGULATION IN
DAPHNIA MAGNA
UNDER PHYSIOLOGICAL CONDITIONS
AND IN THE PRESENCE OF
HEAVY METALS

BY

IB HOLM-JENSEN

CONTENTS

PREFACE

The present investigations were carried out in the Zoophysio-logical Laboratory of the University of Copenhagen during 1944 and the first months of 1945.

I wish to express my sincere thanks to the leader of this laboratory, Professor, Dr. AUGUST KROGH for the never failing interest he has taken in my work and for his most valuable help and criticism.

I am greatly indebted to Dr. G. DE HEVESY, leader of the Biological Department of the Institute of Theoretical Physics of the University of Copenhagen, and to the personnel of the cyclo-tron, who put the radioactive matters applied in this study at my disposal.

The ocean water used was kindly put at my disposal by the Hydrographic Laboratory of the Danish Government Fishery Investigations, head: Professor, Dr. MARTIN KNUDSEN.

INTRODUCTION

Daphnia magna, fig. 1, is a small freshwater Crustacean belonging to the sub-order *Cladocera* of the order *Phyllopoda*, subclass *Entomostraca*. The body weights of adult individuals are about 4 to 5 mg.

Daphnia magna can easily be propagated in the laboratory all the year round (NAUMANN 1933, a), and it is specially suitable as experimental animal for a number of different purposes.

The stock of *Daphnia magna* used for the experiments to be described in this paper is the same as that used by NAUMANN for his investigations on the susceptibility of Daphniae to toxic substances (NAUMANN 1933 & 1934).—The experimental animals
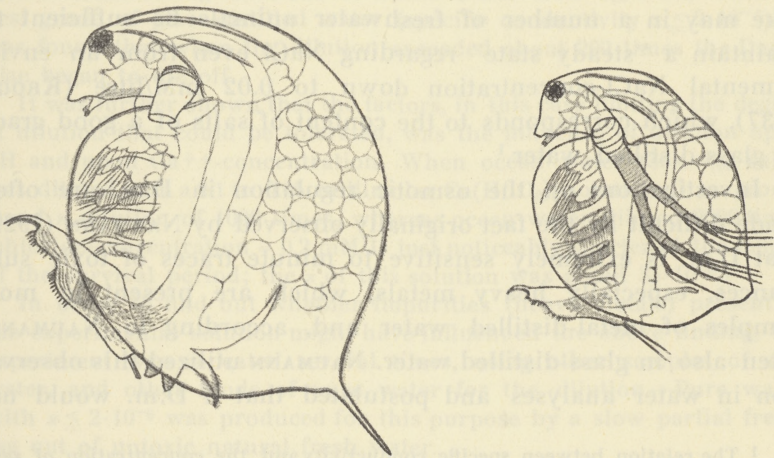

Fig. 1. *Daphnia magna* Straus from Lilljeborg (1900). Two individuals, both females, but different phaenotypes.

have been propagated by parthenogenesis since 1939. Only females have been used for the experiments.

Daphnia magna is highly permeable to water. Determinations with D_2O (Ussing, cited by Krogh 1939) demonstrated the remarkably high water exchange of 80 per cent in less than 2 minutes.

The osmotic pressure of the haemolymph of D. m. is about 3 atm. as against an osmotic pressure of the order of 0.1 atm. in the environmental fluid (Fritsche 1916). So a considerable osmotic inflow of water must take place.

In a freshwater animal permeability to water should be supposed to be connected with an unavoidable loss of salts, even if the integument be impermeable to salts, because the amount of water taken up osmotically must be eliminated by a corresponding secretion of urine, which fluid probably would contain at least traces of salts.

As D.m. will stand deprivation of food for several days, it must be supposed to be able to balance its loss of salts by uptake of ions from the surrounding fluid. This uptake, which occurs against a considerable concentration gradient, must be active, which means that it is dependent on processes in living cells supplying energy.

During recent years such adenoid uptakes of ions have been demonstrated in several aquatic animals (Krogh 1939). The uptake may in a number of freshwater animals be sufficient to maintain a "steady state" regarding NaCl even from an environmental NaCl-concentration down to 0.02 mM/litre (Krogh 1937), which corresponds to the content of salts of a good grade of glass-distilled water.[1]

Investigations on the osmotic regulation in D.m. are often made difficult by the fact originally observed by Naumann (1929) that D.m. is extremely sensitive to minute traces of toxic substances, especially heavy metals, which are present in most samples of metal-distilled water and, according to Naumann, often also in glass-distilled water. Naumann utilized this observation in water analyses and postulated that if D.m. would not

---

[1] The relation between specific conductivity and the concentration of salts may very roughly be estimated from the assumption that the specific conductivity $x = 1 \cdot 10^{-6}$ ohms^{-1}cm^{-1} per 0.01 milli equivalent of dissolved salts present in 1 litre of the solution.

survive at least 10 days in a sample of distilled or natural fresh-water, this must be due to toxic impurities.

NAUMANN does not discuss the problem whether distilled water may be made so pure that the lack of salts may be responsible for the toxic effect on the Daphniae, and he ascribes all poisoning occurring in relation to treatment with distilled water to toxic impurities.

NAUMANN carried out several determinations of the initial specific conductivity of the samples of distilled water used in his experiments; but unfortunately he never repeated his measurements during the performance of the *Daphnia magna* test, and so it is impossible to figure out from the data published how far the concentration of salts may be decreased, if the lack of ions shall not hurt the Daphniae.

In a previous communication on the D. m. test of NAUMANN the writer (HOLM-JENSEN 1944) on the basis of experiments discussed the influence of the composition of the environmental fluid upon D. m., using individuals of the same breed as used by NAUMANN. As this paper is published in Danish only, a brief survey of a few points, which are important to the present study, will be given below.

The length of the survival period of fasting D. m. was observed in a series of increasing dilutions of ocean water (36.4 $^0/_{00}$ salts) with the best grade of glass-distilled water (specific conductivity $\varkappa \leq 2 \cdot 10^{-6}$). It was found that when the dilution exceeded about 200 times the Daphniae began to die off.

It was further shown that the factors, in this case limiting the degree of dilution that could be tolerated, was the influence of dilution upon pH and upon Ca^{++}-concentration. When ocean water (36.4 $^0/_{00}$ salts) was diluted with a 0.1 to 0.05 millimolar $Ca(HCO_3)_2$ solution D. m. would stand a dilution of 1000 times, whereas ocean water diluted 2500 times (chloride-concentration = 0.2 mM/1) just noticeably shortened the length of the survival period; the $\varkappa$ of this solution was about $35 \cdot 10^{-6}$.)

In order to find out whether impurities (heavy metals) present in the experimental solution might have influenced the above finding, the experiment was repeated several times, using other samples of sea-water, and other kinds of pure water for the dilution.—Pure water with $\varkappa \leq 2 \cdot 10^{-6}$ was produced for this purpose by a slow partial freezing out of untoxic natural fresh water.

The result of these experiments confirmed that a specific conductivity of about $35 \cdot 10^{-6}$ would correspond to the lowest concentration of salts that the Daphniae would stand for days. The difference between

the behaviour of the animals in highly diluted sea water and in corresponding dilutions of pure NaCl was very small.

Planning the present study, the aim was to elucidate the physiological salt exchange between D. m. and the surrounding fluid, first and foremost because the power of absorbing ions appeared to be extraordinarily well developed in this animal.

Furthermore it was intended to try to find out whether intoxication with heavy metals to which D. m. is extremely sensitive would in some way act by interfering with the normal exchange of salts.

The reason for investigating this last named problem is partly an observation made by the writer (HOLM-JENSEN 1944) that the toxicity of Cu^{++} decreases enormously when the NaCl-concentration of the surrounding fluid approaches that of the haemolymph of the animals, and partly observations by GICKLHORN (1925) and GICKLHORN & KELLER (1925) that in Daphniae silver may be accumulated from a dilute environmental $AgNO_3$-solution in certain cells in the gills and in young individuals in the "Nackenorgan" also. These observations were interpreted by KOCH (1934) as caused by an active uptake of Ag^+ in these cells. A similar accumulation has since been demonstrated in other Arthropoda also. KOCH & KROGH (1936) demonstrated that certain cells in the larvae of Chironomus plumosus and Culex pipiens, which in a similar way are able to accumulate silver, are undoubtedly responsible for the physiological uptake of ions. SCHMIDT NIELSEN (1941) demonstrated an active uptake of silver in cells in the gills of Astacus from an $AgNO_3$-solution which was diluted sufficiently to exclude the possibility that the accumulation of silver demonstrated might be due to a simple precipitation as AgCl from Ag^+ which would enter the cells by diffusion.

Methods.

A. Experiments with radioactive elements used as tracer substances.

(1) Determination of the rate of active uptake of ions.

For the purpose of present investigations the uptake of the following ions Na^+, K^+, Br^-, Cl^- and Cu^{++} and Pb^{++} is studied by means of preparations containing radioactive isotopes of the ions in question.

Such investigations are based upon the fact that living cells cannot distinguish between the different isotopes of an element. Therefore the amount taken up actively will contain the radioactive element in the proportion present in the fluid from which it is taken up.

If for instance the exchange of Na^+ between the Daphniae and the environmental fluid is to be studied under physiological conditions, a number of the animals are transferred to an experimental solution containing radioactive sodium, $^{24}_{11}Na$, but chemically of the same composition as the fluid in which the animals had stayed before the experiment. Individuals are taken out at suitable intervals. The radioactivities of whole animals, and in a few cases of blood also, are measured, referred to the same point of time and expressed per unit of the sample.

We are now able to figure out the rate of active uptake of sodium if we assume that no uptake of sodium takes place by diffusion through the integuments, that the volume and sodium concentration of the animals are kept constant and that the ratio between the radioactivity and sodium concentration is the same in all body fluids of the animal.

For the sake of convenience we keep the relative amount of experimental solution so high that the exchange of ions does not

measurably reduce the amount of radioactivity present in the environmental fluid.

Taking the radioactivity per unit volume of the experimental fluid divided by its Na^+-concentration as unity, we use the symbol a_t for the activity per unit of the animal, or of the haemolymph of the animal, after the lapse of t hours; (a_0 when t = 0 and a_∞ when t = ∞). The concentration of Na^+ in the animal, taken as a whole or in the haemolymph, is called C and the Na^+-concentration of the surrounding fluid c.

We want to compute the rate of uptake as the uptake of Na^+ expressed in millimols per kilo (or litre) per hour.

The rate of increase in the radioactivity per unit of the animal, or of the haemolymph, is now given by the following differential equation

$$\frac{da_t}{dt} = k - k_1 a_t,$$ I

where k and k_1 are constants. k being the rate of uptake per unit volume of the animal (or haemolymph) and $k_1 a_t$ the rate of loss of the labelled sodium per unit volume.

If t = ∞, $\frac{da_t}{dt}$ will be = 0 and it is seen that $k_1 = \frac{k}{a_\infty}$.

This is inserted in equation I

$$\frac{da_t}{dt} = k - \frac{k}{a_\infty} a_t.$$ II

Integration gives

$$a_t = a_\infty - K \cdot e^{-\frac{k}{a_\infty} t}.$$ III

If we put t = 0, K is seen to be = a_∞, so we have

$$a_t = a_\infty \left(1 - e^{-\frac{k}{a_\infty} t}\right),$$ IV

or solved with regard to k,

$$k = a_\infty \cdot \frac{1}{t} \cdot \ln \frac{a_\infty}{a_\infty - a_t}.$$ V

As a_∞ = C we can write

$$k = C \frac{1}{t} \cdot \ln \frac{C}{C - a_t}.$$ VI

The facts obtained from these "steady state" experiments were supplemented by a few experiments carried out in circumstances where no steady state could be reached.

The results of these last named experiments were not obtained by means of radioactive indicators only. Also measurements of the changes in electrolytic conductivity of the environmental fluid proved able to yield valuable information.

(2) Production of radioactive isotopes for the experiments.

Radioactive isotopes of Na, K, Cl, Br and Cu were produced by bombardment with swift deuterons in the Copenhagen cyclotron. The four first named substances were treated in the form of NaCl, KCl, NaBr, or KBr, of which about 50 mg was melted on to a target $15 \times 7 \times 2$ mm made of copper.

For production of radioactive copper a piece of copper foil was placed in the beam of deuterons.

The radioactive isotopes to be considered are

$$^{24}_{11}\text{Na half-life period 14.8 hours}$$
$$^{42}_{19}\text{K} \quad - \quad - \quad 12.4 \quad -$$
$$^{38}_{17}\text{Cl} \quad - \quad - \quad 37 \text{ minutes}$$
$$^{64}_{29}\text{Cu} \quad - \quad - \quad 12.8 \text{ hours,}$$

whereas radioactive Br was obtained in form of a mixture of

$$^{82}_{35}\text{Br half-life period} \quad 34 \text{ hours}$$

and $^{80}_{35}\text{Br}$. This last named isotope apparently possesses two different half-lives of 4.4 hours and 18 minutes.

In consequence of the decay of the substances in question, figures expressing the strength of the radioactivity at the point of time t_1, t_2, t_3, and so on, must be referred to some arbitrary but fixed point of time t_0. This is carried out by the equation

$$y_0 = y_t \, e^{\lambda t},$$
$$\text{where } \lambda = \frac{\ln 2}{T},$$

T being the length of the half-life period.

When experiments had to be carried out on Na or K, they

were treated in the cyclotron in the form of ordinary NaCl and KCl, respectively. 6 to 8 hours after the end of the activation process the radioactivity of $^{38}_{17}Cl$ will, owing to its short half-life, be negligible as compared with the activity of Na or K, so that the whole of the radioactivity measured after 8 hours may be ascribed to $^{24}_{11}Na$ or $^{42}_{19}K$, respectively.

If, on the other hand, experiments on the uptake of Cl^- had to be performed, the samples should be measured within 4 to 5 hours after the end of the treatment in the cyclotron. Next day the same samples are measured again. The strength of the residual radioactivity is extrapolated backwards to the point of time of the first measurement and subtracted from the activity first measured to get the activity of $^{38}_{17}Cl$ as a difference.

Carrying out experiments with radioactive Br which was made radioactive in the form of Na- or KBr a similar procedure is not feasible. In such cases bromine was isolated as AgBr, whereas ^{24}Na or ^{42}K could be estimated in the supernatant fluid.

The first experiments carried out with radioactive NaCl were lost because the experimental solution turned out to be toxic to the Daphniae.

The toxicity in question could be removed by shaking the solution with carbon powder and subsequent filtering through filter paper. As the same effect could also be obtained by treatment with magnesium powder followed by readjustment of pH, there can be no doubt that the toxicity of the fluid before the treatment should be ascribed to contamination with heavy metals, probably copper from the target.

In all experiments on irradiated halides of the alkali metals the following procedure for preparation of the radioactive solution was consequently adopted.

The irradiated target was weighed and plunged into about 20 ml of pure distilled water. After the salt had dissolved, the target was removed, dried, and weighed again. From the difference between the weights the amount of distilled water to be added to make the solution contain exactly 10 mM/litre was figured out. This solution was now shaken with about 1 gram of carbon powder (carbo ossium "Merck") and filtered.

The resulting fluid was used for production of the final experimental solutions.

Radioactive copper $_{29}^{64}Cu$ for the experiments was obtained by bombarding a piece of copperfoil. A small piece was cut out, weighed and dissolved in one ml redistilled water, to which a drop of bromine was added. The surplus of bromine was removed by evaporating the solution after addition of 0.1 ml 1 normal HCl to about one third of its orginal volume. Usually the surplus of HCl was neutralized with NaOH.—It was ascertained by treating a blank in the same way that no toxic compound of the bromine was left.

Radioactive lead in form of ThB, half-life period 10.6 hours, was obtained from the active deposit on a negative sheet of platinum foil of a thorium preparation (HEVESY 1926). The ThB was brought into solution by dipping the platinum foil into 0.1 ml 1 normal HCl. A small quantity of $PbCl_2$ was added and the solution nearly neutralized with NaOH.

(3) Sampling.

The samples of the radioactivities which were to be measured were placed in small aluminium dishes, 14×2.5 mm (LEVI 1941), evaporated to dryness and exposed in a Geiger-Müller counter. The principal technical features concerning the measurements will be described in the next chapter.

The samples consisted of

(1) experimental solution,
(2) one or more whole Daphniae
and (3) haemolymph of the Daphniae.

Samples containing up to 0.1 ml of the experimental solution were placed as centrally as possible in the dishes, which for the present purpose were previously coated with a thin layer of paraffin wax, melting point 68—72°. After evaporation of the water at a temperature not exceeding 60° a central disc of salts, the diameter of which is about half the diameter of the dish, will be left.

Samples of animals were prepared for measurements in the following way. One single or a few individuals were captured, weighed, and placed in a single layer as centrally as possible in the aluminium dishes, and allowed to dry.—In order to get rid

of the amount of the experimental fluid contained between the shells of the animals, they were, before weighing, placed on a piece of moist filter paper in a small glass tube, the bottom of which consisted of a glass-filter plate. The tube was closed by a rubber stopper and centrifuged at 3000 revol./min. for 2 minutes. It was ascertained in previous experiments that this treatment, which strangely enough did not kill the Daphniae, would reduce the amount of fluid left between the shells to less than 2 per cent of the body weight.—Immediately after the centrifugation the Daphniae were transferred to small closed weighing bottles and weighed with an accuracy of $\pm 0,1$ mg. It was ascertained that repetition of the procedure described would not measurably diminish the weights determined.

Regarding $^{24}_{11}$Na, it was found permissible after drying to compare countings from whole animals directly with that of the evaporated experimental solution. Comparing countings from a single Daphnia containing radioactive sodium with countings from the same preparation after thorough mincing of the animal in a drop of water and subsequent drying, no measurable difference of the radioactivities could be noticed, probably because the influences of different factors would balance out each other. This result is supposed to be applicable to the more penetrating β-radiation from $^{42}_{19}$K and $^{38}_{17}$Cl also.

For sampling of haemolymph the Daphniae were placed in a moist chamber arranged under a binocular stereo-microscope. One of the antennae was cut near its base by means of a very sharp pair of scissors and haemolymph collected in a small capillary glass tube (compare FRITSCHE 1916). The end of the tube was wiped off by means of a piece of moist filter-paper and the length of the sample (usually about 20 mm) was measured. The tube was now washed out in a drop (about 0.05 ml) of distilled water, placed in an aluminium dish, and allowed to dry.

The counting from this sample was expressed in relation to a sample of a standard solution of equal volume delivered in like manner from the same capillary.

(4) Determination of the radioactivity of the samples.

The radioactivity of the samples was measured by means of a Geiger-Müller tube counter.

The dish containing the sample was placed beneath the window of the counter tube.

The counter-amplifier-register arrangement applied was of a similar design as that which during recent years has been developed in the Institute of Theoretical Physics in Copenhagen. (Levi 1941; Ambrosen, Madsen, Ottesen and Zerahn 1945.)

Two steps of scaling were introduced between amplifier and mechanical register so that groups of four impulses are registered as single counts. The counting loss, due to the finite recovery time of the register, in this arrangement is less than 1 per cent when the number of countings registered does not exceed one fourth of 1200 impulses/min. and need not be corrected for. At $\frac{1}{4} \times 1500$ imp./min. the loss is 2 per cent and at $\frac{1}{4} \times 2000$ it is 5 per cent. This counting rate was the highest practically obtainable, because the mechanical register was liable to break down at higher counting rates.

If the radioactivity of any sample to be measured corresponded to more than 2000 imp./min., the measurement was carried out after the lapse of a suitable time.

The counter arrangement was tested every day in use by several countings of a uranium standard; and the decay-curve of a radioactive preparation with an initial activity corresponding to about 2000 imp./min. of the isotope to be measured was determined in connection with every experiment carried out to make sure that no defect in the apparatus might have arisen, and to show that no "false radioactivity" might influence the countings.

The countings of the standard preparation, corrected when necessary for counting losses, were plotted on logarithmic paper against time.

The curve obtained should, if only a single radioactive substance is present and if the reaction is uncomplicated by the formation of new radioactive substances, make a straight line with a slope corresponding to the known half life period of the substance in question.

As the statistical fluctuation in the number of disintegrations from a radioactive preparation is about $\sqrt{N}$, N being the total number of impulses counted, the corresponding relative error will be $\dfrac{10^2}{\sqrt{N}}$ per cent. To obtain an accuracy of p per cent we consequently have to count $N = \dfrac{10^4}{p^2}$ impulses.

As the fluctuations from other causes can hardly be kept below 1 per cent it is generally of no use to count more than a total of 10,000 impulses from the same sample.

From the number of impulses registered in the time interval t_1 to t_2 minutes is subtracted $(t_2 - t_1)\,n$, where n is the background count per minute of the individual counter used. n is due to cosmic radiation and to contaminations of the counter with radioactive matter. The difference $N_{t_2-t_1}$ corresponds to a certain constant fraction of the sum of β-particles (and γ rays) emitted from the preparation in question in the time interval t_1 to t_2.

$N_{t_2-t_1}$ is divided by $t_2 - t_1$. If the resulting figure exceeds 1200 it should be corrected for the counting loss mentioned. The correction may conveniently be read from a curve plotting the correction to be added against the number of impulses registered per minute. However, this correction was applied on very few occasions because it was generally possible to count the samples again after the course of another half-life period.

We have now obtained a figure, which, with the fluctuation stated, represents the strength of the radioactivity at a point of time t_x which may generally be taken as equal to $\dfrac{t_1 + t_2}{2}$.

As the radioactivity in question decays at a certain rate, we must refer the strength of our preparations expressed by $\dfrac{N_{t_2-t_1}}{t_2 - t_1}$ at the time $\dfrac{t_1 + t_2}{2}$ to some fixed point of time, which may be chosen arbitrarily.

This is conveniently done by application of the well known equation

$$y_0 = y_t \cdot e^{\lambda t},$$

where y_0 and y_t are the strengths of radioactivity at t_0 and t, respectively. Instead of λ we may put $\dfrac{\ln 2}{T}$, where T is the

length of the half-life period of the isotope considered. So we have

$$y_0 = y_t \cdot e^{\frac{\ln 2}{T} \cdot t},$$

or, for the present purpose more conveniently,

$$y_0 = y_t \cdot 10^{\frac{\log_{10} 2}{T} \cdot t}.$$

The error committed above by putting

$$y_{t_x} = \frac{N_{t_2 - t_1}}{t_2 - t_1} \text{ equal to } y_{\frac{t_1 + t_2}{2}} \text{ is in most cases negligible. The approx-}$$

imation consists in replacing the decay curve

$$y_t = y_0 \cdot e^{-\frac{\ln 2}{T} \cdot t}$$

by a straight line possessing the same integral in the interval t_1 to t_2. It is obvious that this approximation will cause an error increasing with $\frac{t}{T}$.

To get a figure for the degree of approximation we shall compute the error committed when $t_2 - t_1 = T$. For the sake of convenience we put $t_1 = 0$, and we get the error

$$\Delta = y_{t_x} - y_{\frac{t_2}{2}}$$

where

$$y_{t_x} = \frac{N_{t_2}}{t_2},$$

and

$$y_{\frac{t_2}{2}} = y_0 \cdot e^{-\frac{\ln 2 \cdot t_2}{t_2 \cdot 2}} = \frac{y_0}{1.414}.$$

y_0 is found from the decay curve by integration.

$$N_{t_2} = \int_0^{t_2} y_0 \cdot e^{-\frac{\ln 2}{t_2} \cdot t} \cdot dt$$

$$N_{t_2} = \frac{y_0 \cdot t_2}{\ln 2} (e^{-\ln 2} - 1)$$

$$y_0 = \frac{N_{t_2}}{t_2} \cdot 1.3862$$

$$y_{\frac{t_2}{2}} = \frac{N_{t_2}}{t_2} \cdot \frac{1.386}{1.414} = \frac{N_{t_2}}{t_2} \cdot 0.98,$$

and we have

$$\varDelta = \frac{N_{t_2}}{t_2}(1 - 0.98) = \frac{N_{t_2}}{t_2} \cdot 0.02.$$

This means that if we extend a single counting to a duration equal to the length of the half life period and put the resulting figure in relation to some counting undertaken in a very short period, the error committed in putting y_{t_x} equal to $y_{\frac{t_1 + t_2}{2}}$ may approach 2.0 per cent. If, however, we only compare countings from periods of equal length we shall of course commit no error.

In the experiments to be described in the following it was always permissible to take $y_{t_x} = \frac{N_{t_2 - t_1}}{t_2 - t_1}$ equal to $y_{\frac{t_2 - t_1}{2}}$.

B. Measurements of electrical conductivity.

The electrical conductivity was measured on all samples of glass-distilled water used for experimental purposes in order to make sure that the concentration of salts was below a certain value. Furthermore a number of experiments were carried out in which the conductivity of an experimental solution containing Daphniae was estimated.

For measurements on distilled water or samples of experimental solutions exceeding 20 ml a commercial set of dipping electrodes, "Philips" G.M. 4140, was generally used. For smaller volumes down to 1 ml a small electrode vessel made of "Jenaer Geräte" glass was employed. (Holm-Jensen 1947). The electrodes were made of uncoated platinum foil, each about 6 × 8 mm, sealed to the inner surface of the vessel.

The measurements of the electrical resistance were carried out by means of a commercial Wheatstone-bridge arrangement, "Philips Philoscope".

Differences in the resistance of 0.5 to 1 per cent could generally be detected, whereas the error of single estimations might amount to about 2 per cent, which figure is about twice the maximum reading error.

For the measurements alternating current of 1.000 Hz and 2 volts was used.

The conductivities estimated are given as the specific conductivity $\varkappa$ = the conductivity expressed in $ohms^{-1}$ cm^{-1}.

In experiments in which the problem is to measure changes in the conductivity of the experimental fluid temperature changes may interfere. In such cases the conductivity is referred to some arbitrary mean temperature on the assumption that the conductivity increases $2.25\,^0/_0\,/1^\circ$ C.

Experiments.

The experiments to be described in the following are indended to elucidate the physiological exchange of ions in *Daphnia magna* and to reveal the existence of any influence of heavy metals upon the natural loss and uptake of ions.

A. Experiments on the physiological exchange of ions in *Daphnia magna*.

(1) Exchange of sodium.

Previous to the experiments to be described below the experimental animals were pretreated for about 24 hours with a solution chemically as nearly as possible equal to the radioactive experimental solution.

A number of Daphniae, freshly captured from the culture basins, were washed in the pretreatment solution (3 changes in the course of about 1 hour) and finally allowed to stay in this fluid for 24 hours previous to the experiments. During the period of pretreatment no food was given.

The experiments were carried out at ordinary room temperature, about 20° C.

Experiment a.

For the first experiment on the exchange of radioactive Na^+ the following two experimental solutions A and B were applied.

Solution A consisting of labelled NaCl 1.00 mM/litre and $Ca(HCO_3)_2$ 0.10 mM/litre.

Solution B consisting of equal parts of solution A and a dilution of ocean water adjusted by dilution (nearly 500 times)

to a Na^+-concentration of 1.00 mE/litre and by addition of $Ca(HCO_3)_2$ to a HCO_3^--concentration of 0.2 mE/litre.

A $Ca(HCO_3)_2$-solution containing 0.10 mM/litre (equal to 0.20 mE Ca^{++}/litre) for production of the above experimental solution was prepared by bubbling CO_2 through a solution of $Ca(OH)_2$, the concentration of which does not exceed 1 mE Ca^{++}/litre, until the electrolytic conductivity reached a minimum. This solution was finally by further dilution adjusted to a specific conductivity of $22 \cdot 10^{-6}$ corresponding to a Ca^{++}-concentration of 0.20 mE/litre.

For the conductivity measurements a "Philips" dipping conductivity cell, G.M. 4241, was used.

After pretreatment for 24 hours in ocean water diluted 500 times (i. e. to a Na^+-concentration of 0.96 mE/litre) with the above $Ca(HCO_3)_2$-solution containing 0.1 mM $Ca(HCO_3)_2$/litre about 200 Daphniae of an average weight of about 3 mg were transferred to each of the experimental solutions A and B. The concentration of Daphniae in these experimental solutions did not exceed one individual per 10 ml of the fluid.

Daphniae were sampled for measurement of their radioactivity at suitable intervals. The results of the measurements are given in fig. 2, where the ratio between the radioactivity of equal weights of the animals and the surrounding fluid (which is taken to contain 1.00 mE Na^+/litre) is plotted against time. Measurements on single Daphniae from solution A and B are marked ● and ○, respectively, and measurements on samples consisting of 5 individuals are marked ⊙ and ◎, respectively. The weights of the samples in milligrammes are entered beside the points indicating the strength of the radioactivities.

The radioactivities per weight unit of the animals showed large individual differences during the first hours of the uptake of the labelled Na^+. These differences decreased with time. When a steady state regarding labelled Na^+ was reached, the maximum difference from the mean value was in each of the experimental fluids below ± 5 per cent.

The large differences in the individual rates of exchange of Na^+ should undoubtedly be related to the moulting cycles.

When complete replacement, after the lapse of about 20 hours, has taken place, a minor difference between the animals in
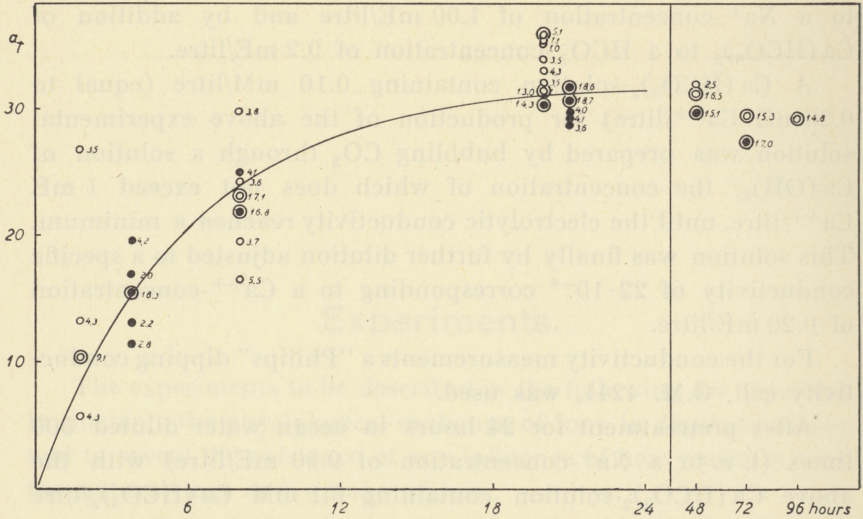
Fig. 2. The concentration of labelled Na$^+$ of Daphniae, mE/kg, plotted against time. The environmental solution contains labelled NaCl 1.0 mM/litre and Ca(HCO$_3$)$_2$ 0.1 mM/litre.

solution A and B is noticed. This difference which persists during the following days should probably be ascribed to some insufficiency of solution A (lack of K$^+$ and Mg^{++} ?). Compare Holm-Jensen (1944).

In both experimental solutions a slight decrease in the content of (labelled) sodium is noticed during the following days.

Another minor difference is noticed between the activities per weight unit of small and adult Daphniae, indicating that the sodium concentration of the smaller animals is about 10 per cent higher than that of the adults.

A few determinations of the radioactivity of the haemolymph of Daphniae from solution B were carried out. Only large specimens (weights exceeding about 4 mg) were used for this purpose. The results of these measurements are given in table 1.

After equilibrium is reached in about 20 hours, the radioactivity per (volume) unit of the haemolymph is nearly twice the radioactivity per (weight) unit of whole animals, corresponding to a concentration of sodium of about 65 mE/litre in the haemolymph.

Considering the large differences in the individual rate of uptake of Na$^+$ which was found in the preceding experiment

Table 1.

The concentration, a_{20Na}, of labelled Na^+, mE/litre, established in the haemolymph of Daphniae which have stayed for 20 hours in an environmental fluid containing labelled NaCl 1.0 mM/litre and $Ca(HCO_3)_2$ 0.1 mM/litre.

Daphnia weight	Haemolymph $a_{20\,Na}+$
4.1	62
4.3	58
3.8	68
4.0	67
5.1	68

Average: 65

it must be realized that *Daphnia magna* is unfit for use as experimental animal in experiments intending to demonstrate influences causing only minor changes in the rate of uptake of ions.

Neglecting a single observation we may estimate from fig. 1 that $a_t = \frac{1}{2} \cdot a_\infty$ corresponds to t = 4 hours $\pm \frac{4}{2}$. We are now able to figure out the rate of exchange per unit weight of the animal or per unit volume of the haemolymph of Na^+ making use of equation V page 10. Using this formula we have to put a_t, expressed as stated on page 10, equal to a_t of the haemolymph which we will take equal to twice the activity of the whole animal, and we get

$$k = 64 \cdot \frac{1}{4^{+4}_{-2}} \cdot \ln 2 .$$

$$k = 11^{-5.5}_{+11} \text{ mE/litre haemolymph and hour,}$$

or referring to whole animals,

$$k = 5.5^{-2.25}_{+5.5} \text{ mE/kg and hour.}$$
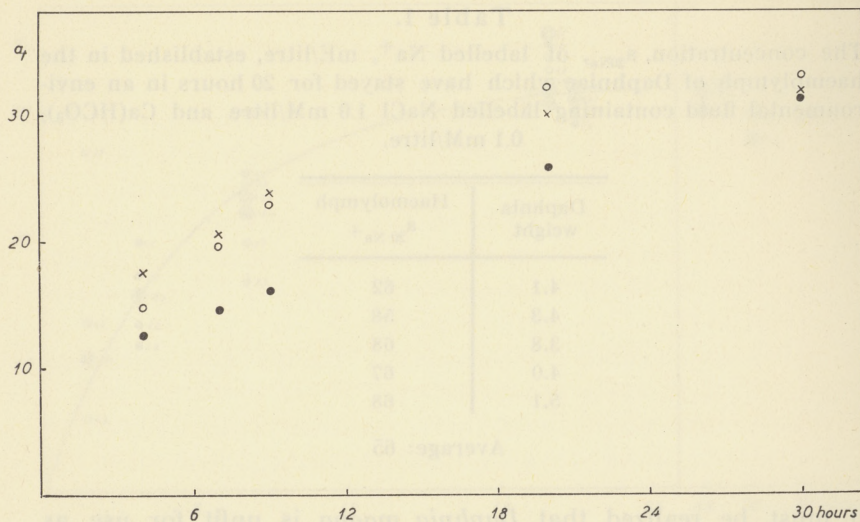
Fig. 3. The concentration of labelled Na$^+$ of Daphniae, mE/kg, plotted against time. The concentration of labelled Na$^+$ of samples from three different environmental solutions containing 49, 4.9, and 0.49 mE Na$^+$/litre are marked ●, ○ and ×, respectively.

Experiment b.

In the next experiment on labelled Na$^+$ the rate of uptake from solutions of different concentrations of Na$^+$ was studied.

The compositions of the labelled solutions applied were:

C. Ocean water diluted 10 times mixed with an equal volume of a pure NaCl-solution containing 50 mE Na$^+$/ litre. This mixture contains 49 mE Na$^+$/litre, 1.1 mE Ca^{++}/litre and 0.13 mE HCO$_3^-$/litre.

D. Ocean water diluted 100 times mixed with an equal volume of a NaCl-solution containing 5.0 mE Na$^+$/litre and 0.1 mM Ca (HCO$_3$)$_2$. This mixture contains 4.9 mE Na$^+$/litre 0.21 mE Ca^{++} and 0.11 mE HCO$_3^-$/litre.

E. Ocean water diluted 1000 times with an equal volume of a solution containing 0.5 mE Na$^+$/litre and 0.1 mM Ca (HCO$_3$)$_2$ /litre. This mixture contains 0.49 mE Na$^+$/litre and about 0.10 mE Ca^{++} and HCO$_3^-$/litre.

The pH of these solutions was nearly the same (about 7.4). Animals were sampled at different intervals, weighed, and their radioactivities measured. The results are presented in fig. 3. In
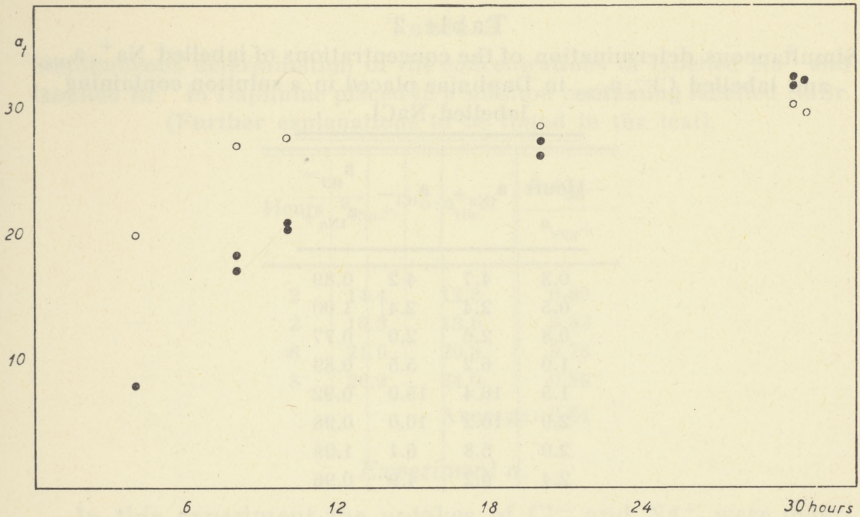
Fig. 4. The concentration of labelled Na^+ of Daphniae, mE/kg, plotted against time. The concentration of labelled Na^+ of samples from two different environmental solutions containing 1.0 and 0.05 mE Ca^{++}/litre are marked ● and ○, respectively. The Na^+ concentration and the pH of both solutions are the same.

this experiment each sample consisted of 10 to 15 Daphniae, average weight about 3.5 mg/individual.

Samples from solution C, D and E are marked ●, ○ and ×, respectively.

No significant difference was found between the rates of Na^+-exchange of animals in solutions D and E. The rate of exhange was considerably slower in animals placed in solution C.

Experiment c.

In the next experiment the influence of Ca^{++} on the rate of exchange was studied.

The following two experimental solutions were applied.

Solution F, which contained NaCl 0.8 mM/litre, $CaCl_2$ 0.5 mM/litre and $NaHCO_3$ 0.2 mM/litre.

Solution G, which contained NaCl 0.8 mM/litre, $CaCl_2$ 0.025 mM/litre and $NaHCO_3$ 0.2 mM/litre.

The results are given in fig. 4. Samples from solution F and G are marked ● and ○, respectively. Each sample consists of about 15 individuals of medium size.

Table 2

Simultaneous determination of the concentrations of labelled Na^+, a_{tNa}, and labelled Cl^-, a_{tCl}, in Daphniae placed in a solution containing labelled NaCl.

Hours	a_{tNa^+}	a_{tCl^-}	$\dfrac{a_{tCl^-}}{a_{tNa^+}}$
0.3	4.7	4 2	0.89
0.5	2.4	2.4]	1.00
0.8	2.6	2.0	0.77
1.0	6.2	5.5	0.89
1.5	16.4	15.0	0.92
2.0	10.2	10.0	0.98
2.0	5.8	6.1	1.05
2.4	5.2	4.9	0.96

Average: 0.93

There is no doubt that the exchange of Na^+ is more rapid in the solution containing less calcium.

(2) Exchange of potassium.

Only two experiments were carried out, both indicating an uptake of K^+ against the concentration gradient.

However, the author has later been informed by Mr. K. ZERAHN, M. Sc., of the Institute of Theoretical Physics of the University of Copenhagen, that the samples of radioactive KCl applied were probably contaminated with ^{24}Na.

As the experiments unfortunately were not extended beyond 30 hours, it could not be indisputably established whether the uptake determined would proceed beyond the point of time where the sodium-exchange should be complete.

Consequently the experiments on K^+ had to be discarded.

(3) Exchange of chloride and bromide.

The experiments carried out on the uptake of Cl^- and Br^- were intended to make sure that the uptake of these ions can take place against considerable concentration gradients, and furthermore to compare the rates of uptake of these two ions.

Table 3

Simultaneous determination of the concentrations of labelled Na^+ and labelled Br^- in Daphniae placed in a solution containing labelled NaBr. (Further explanations to be found in the text).

Hours	a_{tNa^+}	$5 \cdot a_{tBr^-}$	$\dfrac{5 \cdot a_{tBr^-}}{a_{tNa^+}}$
2	14.4	12.8	0.89
2	16.5	13.6	0.82
8	21.6	20.3	0.78
8	26.9	24.0	0.86

Average: 0.84

Experiment a.

In this experiment the uptakes of Cl^- and Na^+ were determined simultaneously on single individuals.—The labelled experimental solution applied was composed as solution A page 20 containing 1.00 mM NaCl/litre.

The results are presented in table 2.

The exchange of Cl^- is seen to be slightly smaller than the exchange of Na^+.

Experiment b.

In this experiment the uptake of labelled Br^- was studied.

The experimental solution, containing labelled NaBr, consisted of NaCl 4.00 mM/litre, NaBr 1.00 mM/litre and $Ca(HCO_3)_2$ 0.1 mM/litre.

The results are given in table 3. Each sample consists of 5 to 10 individuals.

If we want to compare the rate of uptake of Br^- with the rate of uptake of Cl^-, we must realize that the Br^--concentration in the experimental fluid in the present experiment makes up but one fifth of the halide-concentration. So we have to compare $5 \cdot a_{tBr}$ with a_{tCl} from the preceding experiment.

To make up for the individual difference in the rate of uptake of salts of the experimental animals we shall compare the quotients $\dfrac{5 \cdot a_{tBr}}{a_{tNa}}$ from experiment (b) with $\dfrac{a_{tCl}}{a_{tNa}}$ from experiment (a).

$\mu \cdot 10^6$
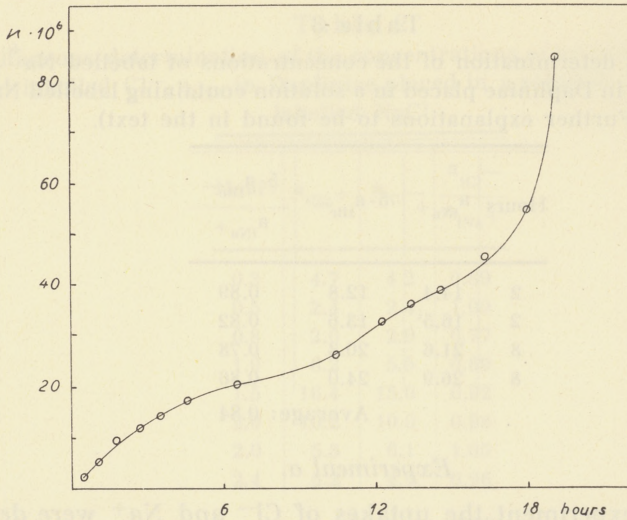
Fig. 5. The electrolytic conductivity, ohms^{-1}cm^{-1}10^6, of 1 ml of distilled water in which was placed one single Daphnia, weight about 4 mg.

$\dfrac{a_{tBr} \cdot 5}{a_{tNa}}$ is seen to be smaller than $\dfrac{a_{tCl}}{a_{tNa}}$ found in experiment a, but the difference is hardly significant, so it may be concluded that only minor differences can exist regarding the ability to take up Cl^- and Br^- in Daphniae which are placed in solutions containing equivalent amounts of Na^+ and $Cl^- + Br^-$. This result is in accordance with the prevailing assumption that cells performing active transport of ions can as a rule scarcely distinguish between Cl^- and Br^-.

(4) Determination of the lowest concentrations of salts allowing the uptake of ions to balance the loss of salts.

The problem to determine the lowest concentration of salts of the environmental fluid allowing the rate of active uptake to balance or even to exceed the loss of ions was investigated by frequent determinations of the electrolytic conductivity of different experimental fluids in which Daphniae were placed.

Owing to the large individual differences regarding the rate of the loss of salts of the Daphniae, these experiments must be carried out on single individuals. The Daphniae were placed
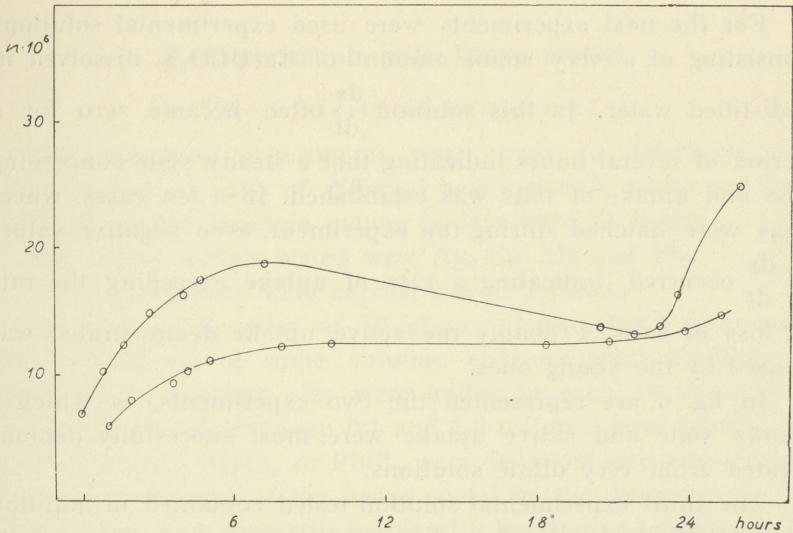
Fig. 6. Two experiments on the electrolytic conductivity, $ohms^{-1} cm^{-1} 10^6$, of 1 ml of a pure $Ca(HCO_3)_2$-solution in which was placed one single Daphnia, weight about 4 mg. In the experiment represented by the upper curve, eggs were hatched after 6 hours and moulting occurred after 21 hours.

in 1 ml of different experimental solutions in the small conductivity vessel mentioned on page 18. The experiments were carried out at about 20°.

The first experimental fluid tested in this way was distilled water, possessing an initial specific conductivity of less than $2 \cdot 10^{-6} ohms^{-1} cm^{-1}$. A typical experiment is represented in fig. 5, where x of the surrounding fluid is plotted against time. x is seen to increase until death.

$\frac{dx}{dt}$ decreases with time to begin with, later on, before death $\frac{dx}{dt}$ begins to increase again. $\frac{dx}{dt}$ was never negative in this series of experiments.

As x reaches a value corresponding to the presence of more salt in the surrounding fluid than is initially present in dissolved form in the animals, it is probable that the rise of x during the experiment partly may be due to liberation of ammonia and carbon dioxide from the animals, and to a loss of $CaCO_3$ from the shells.

The Daphniae generally did not survive moulting in these experiments.

For the next experiments were used experimental solutions consisting of a very small amount of $Ca(HCO_3)_2$ dissolved in redistilled water. In this solution $\frac{dx}{dt}$ often became zero for a period of several hours indicating that a steady state concerning loss and uptake of salts was established. In a few cases, where eggs were hatched during the experiment, even negative values of $\frac{dx}{dt}$ occurred, indicating a rate of uptake exceeding the rate of loss of salts. Probably the active uptake demonstrated was caused by the young ones.

In fig. 6 are represented the two experiments, in which a steady state and active uptake were most succesfully demonstrated from very dilute solutions.

The third experimental solution tested contained in addition to $Ca(HCO_3)_2$ also NaCl in different concentrations. The results were very much like those obtained when Daphniae were placed in pure $Ca(HCO_3)_2$-solutions.

In order to demonstrate active uptake of ions at a rate surmounting the rate of loss, a fourth series of experiments were next carried out on Daphniae pretreated with a large volume (about 10 ml per individual) of a pure $Ca(HCO_3)_2$-solution, 0.05 mM/litre, for 3 to 4 hours.

In these experiments negative values of $\frac{dx}{dt}$ indicating a rate of uptake of salts exceeding the rate of loss, were obtained also in experiments where no eggs were hatched.

The experimental solutions were produced in the following way. 1 ml of a $Ca(HCO_3)_2$-solution containing 0.05 mM/litre of $Ca(HCO_3)_2$ (x = about $11 \cdot 10^{-6}$) were placed in the electrode vessel and NaCl or ocean water was added until the desired initial x was obtained.

Negative values of $\frac{dx}{dt}$ were found in about 20 out of 50 of these experiments.

The lowest initial NaCl-concentration from which an active uptake exceeding the loss could be demonstrated in the adult Daphniae, was 0.06 mE Na^+/litre. Very few of the animals survived moulting in solutions containing less than 0.15 mM/litre.

B. Experiments with heavy metals.

(1) The toxicity of heavy metals.

The following experiments were made to determine the concentration of salts of different heavy metals which are just sufficient to kill *Daphnia magna* in less than 24 hours.

The heavy metals tested were Ag, Cu, Hg and Pb.

The experiments were carried out as follows.

In each of a series of 10 glass vessels $(10 \times 3$ cm) were delivered 20 ml of some suitable environmental solution, 4 Daphniae of medium size were added to each vessel, and a measured quantity between 0.1 and 1.0 ml, of a pure solution of $AgNO_3$, $CuSO_4$, $HgCl_2$ or $PbCl_2$ were delivered into each vessel. The concentrations of the heavy metal in the vessels were arranged to make a geometric series with the quotient $\sqrt{10}$ $(= 3.2)$.

The environmental solutions used for these experiments were:

A: Ocean water diluted 10 times with distilled water. This solution contains 48 mE Na^+/litre 2.2 mE Ca^{++}/litre and 0.26 mE HCO_3^-/litre.

B: Ocean water diluted 100 times with a solution in distilled water of 0.2 mM $NaHCO_3$/litre. Solution B contains Na^+ 5.0 mE/litre, Ca^{++} 0.22 mE/litre and HCO_3^- 0.23 mE/litre.

C: Ocean water diluted 1000 times with a solution of 0.1 mM Ca $(HCO_3)_2$/litre. Solution C contains Na^+ 0.5 mE/litre Ca^{++} 0.23 mE/litre and HCO_3^- 0.20 mE/litre.

The pH of the above solutions is about 7.6.

The Daphniae were observed at suitable intervals. It was noticed whether the animals were alive or not, and signs of intoxication in the living individuals were looked for.

The results of a few representative experiments are given in tables 4, 5 and 6. In these tables the degree of intoxication is indicated by means of the figures 1 to 6 incl.

0 means no visible signs of intoxication.

1　—　movements slightly unsteady.

2　—　　　　—　　　　unsteady.

3　—　　　　—　　　　very unsteady.

4　—　very faint and unsteady, the animals are mainly collected near the bottom.

Table 4.

The toxic effect of Cu on Daphniae placed in different experimental solutions. The degree of intoxication is indicated by the figures 1 to 6.

Experimental solution	Cu		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
A. 1/10 ocean-water	$1 \cdot 10^{-1}$	3.2	2	2	4	4 or 5	6	6	6
	$3.2 \cdot 10^{-2}$	1.0	1	1	3	4	6	6	6
	$1.0 \cdot 10^{-2}$	0.32	0	0	0	1	a 4, b c d } 1	a 4, b c d } 2	a 6, b c d } 4
	$3.2 \cdot 10^{-3}$	0.10	0	0	0	0	0	a 6, b c d } 1	a 6, b c d } 3
	$1 \cdot 10^{-3}$	0.032	0	0	0	0	0	0	3 or 4
	$3.2 \cdot 10^{-4}$	0.010	0	0	0	0	0	0	pale and faint, but no typical signs of Cu-intoxication
	0		0	0	0	0	0	0	0

(to be continued)

5 means the animals are permanently resting on the bottom, unable to swim up.
6 — dead.

When the experimental animals in the same vessel are intoxicated to different degrees, the individuals are marked a, b, c, and d.

By repetition of these experiments the findings could be reproduced with only minor variations apart from the experiments on $PbCl_2$, which turned out to give most inconstant

Table 4 (continued).

Experi-mental solution	Cu		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
B. 1/100 ocean-water	1·10⁻¹	3.2	2	a 6 / b c d } 4 or 5	6	6	6	6	6
	3.2·10⁻²	1.0	2	a b } 6 / c d } 4 or 5	6	6	6	6	6
	1·10⁻²	0.32	3	4 or 5	a 6 / b c } 5 / d 4	6	6	6	6
	3.2·10⁻³	0.10	1	3	a 6 / b c } 5 / d 4	a 6 / b c } 5 / d 4	6	6	6
	1·10⁻³	0.032	0	0	0	0	3	a 6 / b c d } 5	6
	3.2·10⁻⁴	0.010	0	0	0	0	0	0	a 6 / b c d } pale and faint, but no typical signs of Cu-intoxication
	0	0	0	0	0	0	0	0	0

(to be continued)

and contradictory results. These findings will be considered in a following chapter.

The main results of the above experiments are:

(1) The intoxication and death caused by Ag, Cu, and Hg will develop more slowly in solution A than in solution B, and more rapidly in solution C than in solution B.

(2) Concerning Hg the concentration sufficient to kill the Daphniae in 48 hours is independent of the environmental solution applied, whereas in the case of Ag and Cu the Daphniae seem to be able to stand a somewhat higher concentration in solution A than in B and C.

Table 4 (continued).

Experimental solution	Cu		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
C. 1/1000 ocean-water	1·10⁻¹	3.2	4	6	6	6	6	6	6
	3.2·10⁻²	1.0	4	a,b 6 / c,d 5	6	6	6	6	6
	1·10⁻²	0.32	4	a,b,c 6 / d 5	6	6	6	6	6
	3.2·10⁻³	0.10	4	a,b,c 6 / d 5	6	6	6	6	6
	1·10⁻³	0.032	1	a 6 / b,c,d 3 or 4	a,b 6 / c 5 / d 3	a,b 6 / c,d 3	6	6	6
	3.2·10⁻⁴	0.010	0	0	0	0	0	3	6
	0	0	0	0	0	0	0	0	0

The concentrations just sufficient to kill the Daphniae in less than 24 hours in the experimental conditions in solution B are:

$$\text{Ag about } 1 \cdot 10^{-4} \text{ mE/litre} = 0.01 \text{ mg/litre}$$
$$\text{Hg} \quad - \quad 3 \cdot 10^{-4} \quad - \quad = 0.03 \quad -$$
$$\text{Cu} \quad - \quad 1 \cdot 10^{-3} \quad - \quad = 0.03 \quad -$$

Minor variations in the concentration of Daphniae would not significantly influence these results.

Pb^{++}-concentrations of $3 \cdot 10^{-2}$ mE/litre = 3.2 mg Pb^{++}/litre were tolerated for more than 5 days by several animals in

Table 5.

The toxic effect of Hg on Daphniae placed in different experimental solutions. The degree of intoxication is indicated by the figures 1 to 6.

Experi-mental fluid	Hg		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
	$3.2 \cdot 10^{-2}$	3.2	a b c} 6 d 5	6	6	6	6	6	6
	$1 \cdot 10^{-2}$	1.0	1	a 6 b c} 5 d 3	6	6	6	6	6
	$3.2 \cdot 10^{-3}$	0.32	0	0	a 5 b c} 3 d	6	6	6	6
A. 1/10 ocean-water	$1 \cdot 10^{-3}$	0.10	0	0	0	a 6 b c} 1 d	6	6	6
	$3.2 \cdot 10^{-4}$	0.032	0	0	0	0	a b} 5 c d} 4	a b} 6 c d} 5	6
	$1 \cdot 10^{-4}$	0.010	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0

(to be continued)

one experimental series; whereas 0.10 mg Pb^{++}/litre in other experiments proved to be fatal to at least some of the animals in 24 to 48 hours.

When Daphniae, which had been subjected to concentrations of Ag, Cu, and Hg which by permanent influence had turned out to be just sufficient to kill all the animals in less than 24 hours, after the lapse of 2 to 3 hours were transferred to non-toxic solutions, most of the animals would completely recover.

3*

Table 5 (continued).

Experi-mental fluid	Hg		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
B. 1/100 ocean-water	$3.2 \cdot 10^{-2}$	3.2	a, b, c } 6 d 5	6	6	6	6	6	6
	$1 \cdot 10^{-2}$	1.0	1	3 or 4	6	6	6	6	6
	$3.2 \cdot 10^{-3}$	0.32	0	0	a, b, c } 5 d 4	6	6	6	6
	$1 \cdot 10^{-3}$	0.10	0	0	1	a, b, c } 6 d 4	6	6	6
	$3.2 \cdot 10^{-4}$	0.032	0	0	0	a 5 b, c, d } 1 or 2	a, b, c } 6 d 5	6	6
	$1 \cdot 10^{-4}$	0.010	0	0	0	1	0	0	0
	0	0	0	0	0	0	0	0	0

(2) Accumulation of copper.

The accumulation of copper was demonstrated by means of radioactive copper. The amount of copper taken up from solutions of different compositions was determined in order to reveal the influence of the concentrations of Cu^{++} and natural salts upon the Cu-accumulation.

Experiments on different solutions containing 0.05 mg Cu^{++}/litre ($= 0.05 \cdot 10^{-3}$ $\mu g/\mu l$) showed the following uptakes.

From solution A (page 31):

Table 5 (continued).

Experimental fluid	Hg		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
C. 1/1000 ocean-water	3.2·10⁻²	3.2	a⎫ b⎬ 6 c⎫ d⎭ 5	6	6	6	6	6	6
	1·10⁻²	1.0	1	3 or 4	a⎫ b⎬ 6 c⎭ d 5	6	6	6	6
	3.2·10⁻³	0.32	0	1	6	6	6	6	6
	1·10⁻³	0.10	0	0	0	a⎫ b⎬ 6 c⎫ d⎭ 5	6	6	6
	3.2·10⁻⁴	0.032	0	0	0	1	6	6	6
	1·10⁻⁴	0.010	0	0	0	0	a 6 b⎫ c⎬ 0 d⎭	a 6 b⎫ c⎬ 0 d⎭	a 6 b⎫ c⎬ 0 d⎭
	0	0	0	0	0	0	0	0	0

after 2 hours 2.4·10⁻³ µg Cu/mg Daphniae

    —   -   —   2.8·10⁻³   —

    —   4   —   2.5·10⁻³   —

    —   -   —   2.9·10⁻³   —

    —   20   —   3.9·10⁻³   —

    —   -   —   3.1·10⁻³   —

from solution B:

after 2 hours 3.0·10⁻³ µg Cu/mg Daphniae

    —   -   —   2.8·10⁻³

Table 6.

The toxic effect of Ag on Daphniae placed in different experimental solutions. The degree of intoxication is indicated by the figures 1 to 6.

Experi-mental fluid	Ag		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
A. 1/10 ocean-water	$3.2 \cdot 10^{-3}$	0.34	1	1	2	3	4 or 5	6	6
	$1 \cdot 10^{-3}$	0.11	0	0	1	2	4	4	a 6 b c } 5 d
	$3.2 \cdot 10^{-4}$	0.034	0	0	0	0	1	2	1
	$1 \cdot 10^{-4}$	0.011	0	0	0	0	0	1	0
	$3.2 \cdot 10^{-5}$	0.003	0	0	0	0	0	0	0
	$1 \cdot 10^{-5}$	0.001	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0

(to be continued)

and from solution C:

after 1.5 hours $3.9 \cdot 10^{-3} \mu$g Cu/mg Daphniae

— — — $3.5 \cdot 10^{-3}$ —

Each sample consisted of about 5 individuals of medium size.

The above experiments demonstrate an accumulation of Cu in the Daphniae which, independent of the concentration of the natural salts, will reach a concentration of copper of the order of 50 times the concentration of the experimental fluids. The maximum concentration is nearly obtained in less than 2 hours.

Table 6 (continued).

Experimental fluid	Ag		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mE/1							
B. 1/100 ocean-water	$3.2 \cdot 10^{-3}$	0.34	2	3	a,b} 5 c,d} 4	6	6	6	6
	$1 \cdot 10^{-3}$	0.11	1	2	a,b} 5 c,d} 4	6	6	6	6
	$3.2 \cdot 10^{-4}$	0.034	0	2	a,b} 5 c,d} 4	a,b} 6 c,d} 5	6	6	6
	$1 \cdot 10^{-4}$	0.011	0	0	1	3	a,b} 6 c,d} 4	a,b} 6 c,d} 4 or 5	a,b,c} 6 d} 5
	$3.2 \cdot 10^{-5}$	0.003	0	0	0	1	2	3	a} 6 b,c,d} 3
	$1 \cdot 10^{-5}$	0.001	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0

(to be continued)

Next the accumulation of Cu was investigated from solutions containing 0.5 and 0.005 mg Cu/litre (= (0.5 or 0.005)·10^{-3} μg Cu/μl fluid). In these experiments solution B (page 31) was used as environmental solution.

From the solution containing 0.5 mg Cu^{++}/litre the following uptakes were found:

after 2 hours $12.2 \cdot 10^{-3}$ μg/mg Daphniae
— 2.5 — $13.6 \cdot 10^{-3}$ —

and from the solution containing 0.005 mg Cu/litre:

after 2 hours $1.1 \cdot 10^{-3}$ μg/mg Daphniae
— 4 — $1.2 \cdot 10^{-3}$ —

Table 6 (continued).

Experimental fluid	Ag		1 h	2 h	4 h	8 h	16 h	24 h	48 h
	mE/1	mg/1							
C. 1/1000 ocean-water	$3.2 \cdot 10^{-3}$	0.34	a 6 b 5 c 4 d 4	a 6 b 6 c 6 d 6	6	6	6	6	6
	$1 \cdot 10^{-3}$	0.11	a 6 b 6 c 4 d 4	a 6 b 6 c 5 d 5	6	6	6	6	6
	$3.2 \cdot 10^{-4}$	0.034	4	a b 6 c d 5	6	6	6	6	6
	$1 \cdot 10^{-4}$	0.011	a 6 b 4 c 4 d 4	a 6 b 5 c 5 d 5	6	6	6	6	6
	$3.2 \cdot 10^{-5}$	0.003	0	0	1	1	1	2	3
	$1 \cdot 10^{-5}$	0.001	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0

In all the above experiments, samples were taken for measurement of the radioactivity of the experimental solutions both before and after the experiments in order to make sure that the tiny amounts of copper present had not disappeared from the fluids, e. g. by absorption to the vessel during the experimental period.

These experiments indicate that the amount of Cu taken up during the first hours, will increase with increasing concentration of Cu^{++} in the environmental fluid.

(3) Accumulation of lead.

The experiments on the accumulation of lead were made first of all in order to reveal the cause of the extraodinary individual differences (mentioned on page 34) in the faculty of Daphniae to tolerate Pb^{++}.

A few preliminary experiments clearly demonstrated that it is extremely difficult to maintain Pb^{++} in the experimental solutions.

By means of radioactive lead it was shown that a solution containing 1 mg Pb^{++}/litre as $PbCl_2$ in pure redistilled water will often deposit a considerable, but very inconstant, fraction of its Pb-content on the walls of the glass vessels. Furthermore each transfer of the fluid by means of glass pipettes may remove from the solution several per cent of the amount of Pb^{++} present. The major part of the amount of Pb retained by the vessels and pipettes could easily be brought into solution again by a little diluted hydrochloric acid.

Repeating this experiment with Copenhagen tap water as solvent instead of distilled water, the following results were obtained.

In freshly drawn tap water, pH = about 7.5, a concentration of 1 mg Pb^{++}/litre would be fairly stable for several hours in an open glass vessel and for days in a closed bottle.

Tap water aged for a fortnight in an open vessel would be unfit for use as a solvent for 1 mg Pb^{++}/litre (introduced as $PbCl_2$), leaving after 24 hours only a few per cent of the amount of Pb added.

The loss of Pb^{++} found in this experiment would be greatly reduced, even if it still made up a large fraction of the amount initially present, if the fluid before addition of $PbCl_2$ was filtered and transferred to a clean vessel without deposits of $CaCO_3$ on the walls.

The above findings are probably caused by the very small solubility product of lead carbonate $(3 \cdot 10^{-14})$, which will cause the solutions containing lead at concentrations of the order of 1 mg Pb/litre (= 0.01 mE Pb/litre) to be highly supersaturated. Consequently Pb^{++} will be very liable to exchange for Ca in $CaCO_3$, present in the glass or as sediment, and to precipitate as $PbCO_3$.

These considerations will probably explain the inconstancy of the toxic effect of Pb^{++} on *Daphnia magna*.

However, a few experiments were carried out in order to elucidate the accumulation of Pb in *Daphnia magna*.

The experimental solution used was solution B (page 31.), to which was added $PbCl_2$ containing radioactive Pb, to a concentration of 1 mg Pb/litre.

Daphniae were added and specimens sampled at different intervals.

After 1 hour the radioactivities of the Daphniae per weight unit turned out to be of the order of 1000 times the initial activity per unit of the experimental fluid corresponding to an accumulation of the order of $1 \mu g$ Pb/mg Daphniae.

A number of Daphniae, which had stayed for 1 hour in the above radioactive solution, were transferred to a solution not containing lead (only one individual in each vessel) and left there until moulting. The dead shells and the animals were now sampled for measurement of their radioactivities. In these experiments the isolated shells proved to contain about 90 per cent of the sum of the amount of lead present in the dead shell and in the animal.

Finally the accumulation of lead in isolated shells introduced into the above experimental solution was investigated. The uptake of lead expressed per shell (dry weight about 0.1 mg) was found to be of the same order of magnitude as the accumulation met with in living animals, which means an uptake per weight unit of the shells about 50 times the uptake per weight unit in whole animals.

Consequently we must assume that the accumulation in Daphniae of Pb from solutions containing Pb^{++} mainly depends on a precipitation of Pb in the shells.

(4) Interference of heavy metals with the physiological exhange of ions.

Owing to the considerable individual differences in the physiological rate of the exchange of ions between Daphniae and the surrounding fluid, it should beforehand be considered questionable whether any interference between heavy metals and the rate
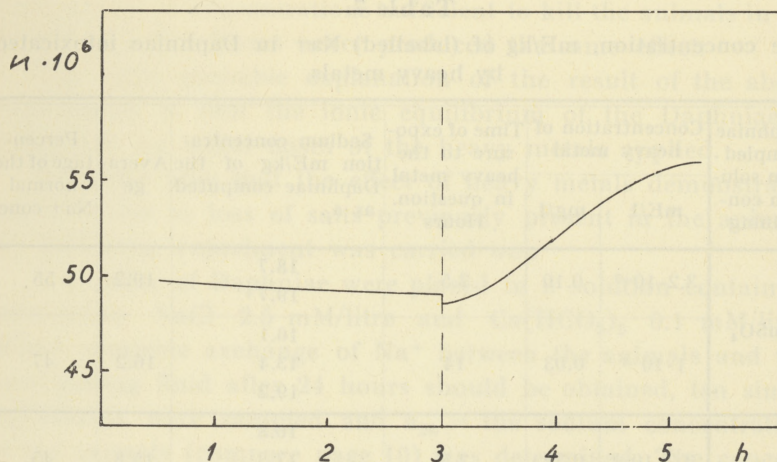
Fig. 7. The electrolytic conductivity, ohms^{-1}cm^{-1}10^6, of 1 ml of a dilute so-lution containing NaCl and Ca(HCO$_3$)$_2$ in which was placed one single Daphnia, weight about 4 mg. At 3 o'clock a Cu^{++}-concentration of 0.003 mE/litre was established by addition of a small amount of a CuSO$_4$-solution.

of uptake of the natural ions might readily be demonstrated from determination of the rate of uptake of the natural ions.

Therefore the following procedure for elucidating the influ-ence of heavy metals on the salt exchange of *Daphnia magna* was adopted.

Single Daphniae, which were pretreated with a pure Ca(HCO$_3$)$_2$-solution, 0.1 m M/litre, for a few hours, were placed in the one ml conductivity vessel in a solution containing a little Ca(HCO$_3$)$_2$ and NaCl, each corresponding to a specific conduc-tivity equal to about 20·10^{-6}. $\varkappa$ was measured every quarter of an hour. When $\dfrac{d\varkappa}{dt}$ had become zero, or even attained negative values, for a couple of hours a quantity sufficient to kill the animals of the poisonous substance to be investigated was added to the fluid. Determinations of $\varkappa$ were continued at short intervals.

In all experiments on Ag, Cu and Hg, $\varkappa$ began to rise im-mediately after the addition of poisonous quantities of salts of the heavy metals and the rise continued until the death of the animals.

A typical experiment of this kind is represented in fig. 7: The broken vertical line indicates the point of time when about 10 μl

Table 7.

The concentration, mE/kg of (labelled) Na^+ in Daphniae intoxicated by heavy metals.

Daphniae sampled from solution containing	Concentration of heavy metal		Time of exposure to the heavy metal in question. Hours	Sodium concentration mE/kg of the Daphniae computed as a_1.	Average	Percentage of the normal Na^+ conc.
	mE/1	mg/1				
CuSO$_4$	$3.2 \cdot 10^{-3}$	0.10	2.5	18.7 19.7	19.2	55
	$1 \cdot 10^{-3}$	0.03	14	16.1 13.4 19.2	16.2	47
HgCl$_2$	$3.2 \cdot 10^{-3}$	0.32	2.5	16.8 17.3 13.3	15.8	45
	$1 \cdot 10^{-3}$	0.10	4	15.9 18.3	17.1	49
	$3.2 \cdot 10^{-4}$	0.032	19	17.4 26.9	22.2	64
AgNO$_3$	$1 \cdot 10^{-3}$	0.11	3	10.3 13.1	11.7	35
	$3.2 \cdot 10^{-4}$	0.034	3	13.9 16.7	15.3	44
	$1 \cdot 10^{-4}$	0.011	8	12.4 18.6	15.5	45
No heavy metal				34.0 32.8 35.5 35.5 36.4	34.8	

of a solution containing 0.3 mE $Cu^{++}/litre$ was added. The resulting Cu^{++}-concentration in the experimental fluid is about 0.003 mE $Cu^{++}/litre$ (equal to 0.1 mg Cu/litre).

Experiments on Pb (up to 5 mg/litre) failed to reveal any similar effect.

A number of inorganic poisons were tested in similar experiments. Phenole, tannic acid, urethane, ethyl alcohol, ethyl ether

and benzene at concentrations sufficient to kill the animals in the course of a few hours never produced the same effect.

The most probable explanation of the result of the above experiments is that the ionic equilibrium of the Daphniae is influenced by lethal doses of the heavy metals applied.

To make sure that the effect of heavy metals demonstrated above is due to loss of salts previously present in the animals the following experiment was carried out.

A number of Daphniae were placed in a solution containing radioactive NaCl 2.5 mM/litre and $Ca(HCO_3)_2$ 0.1 mM/litre. When complete exchange of Na^+ between the animals and the surrounding fluid after 24 hours should be obtained, ten single individuals were sampled and a_{24} = the sodium concentration of the animals (compare page 10) was determined. The concentration of Na^+ in the animals was found to be 34 ± 2 mE/litre.

Now a number of the Daphniae together with about 10 ml of the radioactive environmental fluid per individual were transferred to a number of vessels to each of which was added a quantity of the different poisonous substances just sufficient to kill the animals in 8 to 24 hours. After at least 25 hours Daphniae were sampled again and determinations of their radioactivity carried out.

Daphniae were only sampled when the movements of the animals unmistakably indicated that they were severely poisoned. Only living individuals which were able to swim freely through the fluid were investigated.

The results of this experiment are given in table 7.

The concentrations of sodium in the animals poisoned by heavy metals are seen to be reduced down to about one third of the normal concentration. Whereas Daphniae seriously poisoned by organic compounds did not suffer any loss of sodium.

It is probable that death in Daphniae poisoned by heavy metals present in the surrounding fluid in concentrations just sufficient to kill the animals is due mainly to deprivation of the natural ions.

(5) Investigations into the mechanism of the effect of heavy metals upon the sodium equilibrium of *Daphnia magna*.

The experiments to be described in this chapter were made in order to determine whether heavy metals will influence the ionic equilibrium of the Daphniae by establishing an increased permeability or by retarding the physiological uptake of ions.

For this purpose the rate of uptake of radioactive sodium was determined in Daphniae intoxicated by heavy metals and other toxic substances and compared with the normal rate of uptake.

The experimental solution used was a solution of 1.0 mM radioactive NaCl/litre and 0.1 mM $Ca(HCO_3)_2$/litre in distilled water.

100 ml of this solution were delivered into each of a number of glass vessels and a small volume of a solution containing salts of Ag, Cu or Hg was added to each vessel. Samples, each consisting of about 5 individuals were taken out after 1 to 3 hours. The rates of uptake of ^{24}Na of the intoxicated and the unintoxicated Daphniae were compared. No animal was sampled which was poisoned to such a degree that it was unable to swim freely through the fluid.

In this experiment we have to take $\frac{at}{t}$ to represent the rate of uptake of Na^+ per weight unit of the animals.

The results are given in table 8.

It is seen that when Daphniae are placed in solutions containing heavy metals in concentrations sufficient to kill the animals in a few hours, the rate of uptake of sodium will be reduced (to about one third of the normal rate). But on the other hand, concentrations of heavy metals which are known to require 12 to 24 hours to kill the animals will not significantly reduce the rate of uptake of sodium during the first couple of hours.

This experiment clearly demonstrates that the presence of heavy metals in the environmental fluid may reduce the rate of uptake of sodium.

It would be reasonable to assume that the addition of ions of a heavy metal will not instantaneously influence the active uptake

Table 8.

The rate of uptake of labelled Na^+ per weight unit, $\frac{at}{t}$, expressed in mE/kg and of hour of Daphniae placed in environmental solutions containing heavy metals.

	m E/l	mg/l of the heavy metal	$\frac{a_t}{t}$ after:					average	$\dfrac{a_t \text{ of intox. D.}}{a_t \text{ of unintox. D.}}$
			1.0 h	1.5 h	2.0 h	2.5 h	3.0 h		
$AgNO_3$	$3 \cdot 10^{-4}$	0.03	2.6 2.1					2.4	0.36
	$3 \cdot 10^{-5}$	0.003		6.4	7.5			7.0	1.1
$HgCl_2$	$1 \cdot 10^{-2}$	1.0				2.0		2.0	0.30
	$5 \cdot 10^{-4}$	0.05				6.6		6.6	1.0
$CuSO_4$	$1 \cdot 10^{-2}$	3.2		1.3		1.4		1.4	0.21
	$1 \cdot 10^{-4}$	0.03					4.7	4.7	0.71
$PbCl_2$	$5 \cdot 10^{-1}$	50.0					3.2	3.2	0.48
	$1 \cdot 10^{-2}$	1.0					4.3	4.3	0.65
no heavy metal			7.6 6.4	6.2	6.6	7.9 5.9	5.6	6.6	

of ions. So the possibility exists that we should be able to demonstrate still greater reductions in the rate of uptake of sodium if the Daphniae were pretreated with heavy metal before addition of labelled sodium to the environmental fluid. For this reason the following experiment was carried out.

20 Daphniae were transferred to a series of vessels each containing 50 ml of ocean water diluted 500 times with the usual $Ca(HCO_3)_2$-solution. $CuSO_4$ was added until concentrations of 1×10^{-1} and 1×10^{-2} mE Cu^{++}/litre, equal to 3.2 and 0.32 mg Cu/litre. After the lapse of 0.5 hour 50 ml of a solution of 1.0 mM labelled NaCl/litre, containing $Ca(HCO_3)_2$ as usual, was added to each vessel. After the further lapse of 1.0 hour about 15 individuals were sampled and a_t determined. — The results are given in table 9.

The ratio $\dfrac{a_t \text{ of intoxicated Daphniae}}{a_t \text{ of unintoxicated Daphniae}}$ is seen to be lower than that in the preceding experiment. So there can hardly be any doubt that the retarding effect upon the uptake of ions caused by Cu^{++} and probably by ions of other heavy metals also, will increase with time.

Table 9.

The rate of uptake of labelled Na^+ per weight unit of Daphniae
pretreated with a solution containing Cu^{++} before being placed
in an experimental solution containing labelled Na^+ and the
same concentration of Cu^{++} as the petreatment fluid.

CuSo$_4$		a_1	$\dfrac{a_1 \text{ of intoxicated Daphniae}}{a_1 \text{ of unintoxicated Daphniae}}$
mE/1	mg/1		
$1 \cdot 10^{-2}$	3.2	0.74	0.18
$1 \cdot 10^{-3}$	0.32	0.82	0.20
0	0	4.13	..

The above experiments are not sufficient to exclude completely
the possibility that heavy metals may also influence (increase)
the permeability of *Daphnia magna*. However, we have to take
into consideration:

(1) that the physiological rate of exchange of Na^+ is about
$5.5^{+5.5}_{-2.3}$ mE/kg and hour (compare page 23).

(2) that the rate of uptake of Na^+ in Daphniae intoxicated
by heavy metals is often decreased and never increased.

(3) that the Daphniae will die when the concentration of
Na^+ expressed per weight unit is reduced to about one third
of the normal value, and

(4) that the lengths of the survival period of Daphniae placed
in solutions containing enough heavy metal finally to kill the
Daphniae may extend to 24 hours or even longer.

So we may conclude that the permeability in these circum-
stances can hardly be significantly greater than under physio-
logical conditions.

(6) Elimination of the toxic effect of ions of heavy
metals by formation of complex compounds.

In consequence of the effects of heavy metals demonstrated
in the preceding chapters it would be of interest to consider
the influence upon the toxicity of these metals which could be
obtained by adding to the experimental solutions substances
which will form complex compounds with heavy metals.

Table 10.

The Influence of glutathione and cysteine on the toxicity of copper and mercury.

a

Experimental solution		18 hours	24 hours	42 hours	96 hours
Cu	Glutathione				
0.02 mE/1	0.125 mM/1	{ alive { natural	alive natural	alive natural	alive natural
—	0.050 —	—	—	—	—
—	0.025 —	—	—	—	{ 2 dead { 3 natural
—	0.0125 —	all dead	dead	dead	dead
—	0 —	all dead	dead	dead	dead
0	0 —	natural	natural	natural	{ 1 dead { 3 natural
0	0.125 —	natural	natural	natural	natural

b

Cu	Cysteine	18 hours	24 hours	42 hours	96 hours
0.02 mE/1	0.125 mM/1	{ alive } { natural}	natural	natural	natural
—	0.050 —	—	—	—	—
—	0.025 —	dead	dead	dead	dead
—	0.0125 —	dead	dead	dead	dead
—	0 —	dead	dead	dead	dead
0	0 —	natural	natural	natural	{ 1 dead { 3 natural
0	0.125 —	—	—	—	natural

(to be continued)

For this purpose the influence of glutathione and cysteine was investigated.

The experimental solution was ocean water diluted 100 times with a solution in a pure distilled water of 0.1 mM $Ca(HCO_3)_2$ /litre. 20 ml of this solution was delivered into each of the usual experimental vessels (10×3 cm), and small quantities of a more concentrated solution of either $CuSO_4$ or $HgCl_2$ were introduced to establish concentrations of 0.64 mg Cu^{++} /litre and 20. mg Hg/litre respectively, these concentrations are equivalent

Table 10 (continued).

c

Experimental solution.		18 hours	24 hours	42 hours
Hg	Glutathione			
0.02 mE/1	0.125 mM/1	{ all alive / natural }	all alive	all dead
—	0.050 —	{ 1 dead / 3 natural }	{ 3 dead / 1 dying }	all dead
—	0.025 —	{ all alive / motions / slow }	{ 3 dead / 1 alive / very weak }	all dead
—	0.0125 —	all dead	dead	all dead
—	0 —	all dead	dead	all dead
0	0 —	natural	natural	natural

d

Hg	Cysteine	18 hours
0.02 mE/1	0.125 mM/1	all dead
—	0.050 —	all dead
—	0.025 —	all dead
—	0.0125 —	all dead
—	0 —	all dead
0	0 —	natural

to 0.02mE/litre of heavy metal.—Different amounts of glutathione or cysteine were added from freshly prepared solutions of these substances containing 1.0 mM/litre. 4 Daphniae were added to each vessel.

The results of this experiment are given in table 10. Considering the results of this experiment it should be borne in mind that the concentration of the heavy metals applied is about 20 resp. 70 times the concentrations which without addition of glutathione or cysteine would have been fatal to the animals in less than 24 hours.

A lasting protective effect of glutathione is found in the Cu-experiment where an equivalent concentration of glutathione only slightly in excess of the concentration of Cu is sufficient.

The protective effect of glutathione on Hg appears as a retardation of the process of intoxication. The protective effect extends in time with the concentration of glutathione.

Cysteine causes nearly the same protection as glutathione against Cu, but it is without any protective effect against Hg-intoxication.

In a similar experiment glutathione proved to be able to protect against Ag-intoxication in the same way as against Cu.

In a third experiment on $HgCl_2$ an amount of glutathione of 10 times the amount equivalent to the amount of Hg present was added twice a day. In this way it proved possible to extend the protective action of glutathione on Hg-intoxication for several days.

A similar detoxicating effect of glutathione on intoxications caused by a number of organic substances could not be demonstrated.

Probably the protective effect of glutathione against Ag, Cu and Hg would apply also to other heavy metals.

Making use of the above observations it should probably be possible by means of glutathione to decide whether the toxicity of some unknown solution is caused by the presence of heavy metals.

Furthermore it should be considered that there might be a possibility of utilizing the stoechiometric relation between glutathione and Cu or Ag in analytic work with *Daphnia magna* as an indicator of surplus of the heavy metal in question.

Another possibility of practical application of the detoxicating effect of glutathione and cysteine is in the therapy of intoxications caused by heavy metals in man.[1]

(7) Comparison of the toxic effect of heavy metals on *Daphnia magna* with the effect on another freshwater animal which is less permeable than *Daphnia magna*.

In consequence of the results of the experiments described in the preceding chapters it is reasonable to assume some con-

---

[1] Note added in print: When this paper was written the author had not yet heard about BAL $(CH_2SHCHSHCH_2OH)$ which is able most effectively to neutralize the toxic effect of heavy metals in the organism.
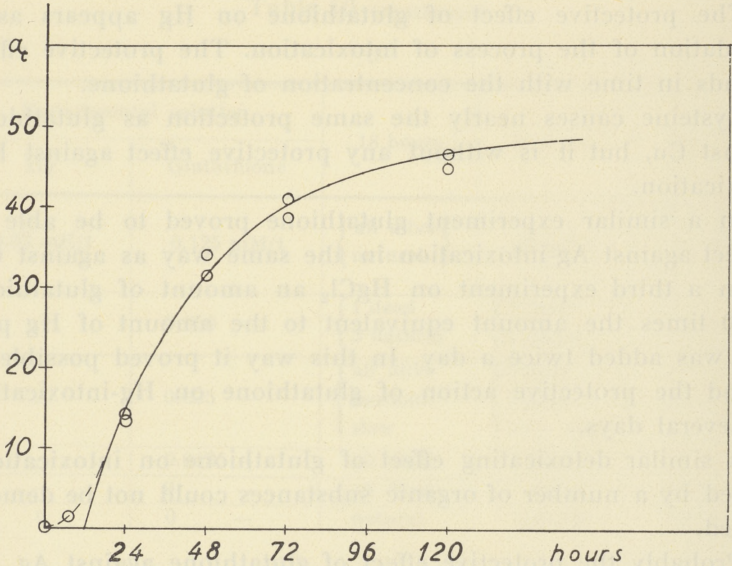
4*

Fig. 8. The concentration of labelled Na+ of *Lebistes reticulatus* placed in a
solution containing labelled NaCl 1.0 mM/litre and $Ca(HCO_3)_2$ 0.1 mM/litre.

nection between the great sensitivity of *Daphnia magna* and
its dependence upon the process of active uptake of ions.

On this background it would be of interest to compare the
toxic effects of a heavy metal upon Daphniae with the toxic
effects of the same substance on a freshwater animal which,
under physiological conditions, will exchange its natural ions
at a slower rate.

As experimental animal for this comparison was chosen
Lebistes reticulatus, a small West-Indian fish belonging to the
Cyprinodonts, which is easily accessible being a very popular
fancy fish among aquarium keepers.

The experiments were carried out principally like experiment
(a) page 20 on Daphniae. The experimental solution was solution
B page 20.

7 days old individuals weighing 6.7 mg $\pm$ 0.7 were used.

Before the experiment the animals were pretreated for 20
hours with a solution of the same chemical composition as the
experimental fluid.

The results of this experiment are given in fig. 8.

The samples consist of single animals.

The individual difference between the concentration of the radioactivities per weight unit of animals samples at the same time during the uptake is very small as compared with the large individual differences met with in the corresponding experiments on *Daphnia magna*.

The solid curve in fig. 8, represents the equation

$$a_t = 49\left(1 - e^{\frac{\ln 2}{24} \cdot (t - 12)}\right).$$

This equation, compare equation IV on page 10, expresses a_∞ to be equal to 49 and the half period to be equal to 24 hours.

Neglecting the initial value of $a_t = 0$, we find that the above equation is in close accordance with the experimental results. Making use of equation V on page 10 we get the rate of uptake of Na^+ from

$$\frac{k}{49} = \frac{\ln 2}{24}$$

$$k = 1.4 \text{ mE/kg and hour.}$$

The deviation shown by the experimental curve from the curve drawn in accordance with the above equation is expressed by the s-shape of the first ascending part. This deviation is probably significant. The course of the s-shape is hardly of importance to the present discussion.

The explanation of the s-shape may be:

(1) The temperature during the pretreatment period may have differed from the temperature during the experiment (unfortunately the temperature was not registered during the pretreatment period).

(2) The manipulations in connection with the transference of the animals to the experimental vessels may cause the active uptake of Na^+ to stop for some hours (compare KROGH 1937).

(3) The animals are given no food during the pretreatment and experimental periods, for this reason the ionic balance may eventually be influenced. Or

(4) The solution may have become contaminated with traces of heavy metal, which during the experimental period are given off from the vessel used.

Now the abilities of *Daphnia magna* and *Lebistes reticulatus* to stand difference concentrations of heavy metals were compared.

Table 11.

Comparison between the toxicity of Cu and Hg to Daphniae and Lebistes.

4 Daphniae and 1 Lebistes in each vessel.

a

Cu mg/1	2 hours Lebistes	Daph-niae	16 hours Lebistes	Daph-niae	40 hours Lebistes	Daph-niae	64 hours Lebistes	Daph-niae
0.32	natural	irritated. motions rapid	a little dull	dead	dead	dead	dead	dead
0.1	natural	natural	natural	dead	dead	dead	dead	dead
0.032	natural	natural	natural	2 dead 2 dull	natural	2 dead 2 nearly natural	natural	3 dead 1 natural
0.01	natural	natural	natural	natural	natural	natural	natural	natural
0.00	natural	natural	natural	natural	natural	natural	natural	natural

b

Hg mg/1	2 hours Lebistes	Daphniae	16 hours Lebistes	Daphniae	40 hours Lebistes	Daphniae
1.00	natural	slightly irritated?	dead	dead	dead	dead
0.32	natural	natural	dull	dead	dead	dead
0.10	natural	natural	natural	dead	natural	dead
0.032	natural	natural	natural	dead	natural	dead
0.01	natural	natural	natural	natural	natural	natural

This experiment was carried out in the same way as the experiments on Daphniae described on page 31. The heavy metals tested were Cu and Hg added as $CuSO_4$ and $HgCl_2$. The experimental solution applied was solution B page 31. The results are given in table 11.

The intoxication in Lebistes is seen to develop at a slower rate in solutions containing Cu or Hg, than in *Daphnia magna*.

Concerning Cu the concentration which could be permanently tolerated in Lebistes did not differ significantly from the concentration that the Daphnia would stand. Whereas, in the case of Hg, Lebistes would apparently stand for several days concentrations of Hg which are 3 times as high as those tolerated by the Daphniae.

The experiments on Hg were repeated with the only change that the experimental animals three times in the course of the first 24 hours were transferred to freshly prepared experimental solutions. Under these circumstances also the difference between the final toxic effect of Hg upon Daphniae and Lebistes disappeared. So it is probable that the difference noticed in this experiment should be ascribed to the disappearance of Hg from the environmental solutions during the experimental period.

In consequence of the above findings the problem arises whether there is any real difference between the ability of fasting freshwater animals permeable to water to stand permanently the presence of heavy metals in the surrounding water.

Comments.

It is impossible to conclude this paper without mentioning the principal differences between the point of view of the late Professor E. Naumann (loc. cit.) and that of the present writer concerning the cause of death of *Daphnia magna* when placed in distilled water.

The discrepancy between Naumann and the writer may be expressed as follows:

Naumann ascribes all damage to *Daphnia magna* caused by distilled water to be due to the presence of traces of toxic substances, whereas, in consequence of the present study, we have to consider both damages caused by toxic substances and by lack of natural ions, principally Na^+, Ca^{++}, Cl^- and HCO_3^-.

However, it should not be forgotten that when Naumann during the years 1928 to 1933 performed his investigations on *Daphnia magna* very little was known about the permeability and need for active uptake of ions in freshwater animals.—In this connection it should be remembered that deuterium (heavy hydrogen), which applied as deuterium oxide (heavy water) made it possible to study the physiological exchange of water, and the artificial radioactive elements which made it possible to undertake corresponding investigations into the ionic exchange of aquatic animals, were not accessible when Naumann performed his studies.

It is a remarkable fact that the influence of heavy metals upon the ionic exchange in *Daphnia magna*, as demonstrated in this paper, apparently does not affect the permeability of the animals. So the influence of heavy metals upon the ionic exchange of *Daphnia magna* cannot directly be compared with for instance the influence of lead upon the permeability of red

blood corpuscles which was demonstrated by ØRSKOV (1935). In spite of this discrepancy it would be highly interesting to study the influence of heavy metals upon the active uptake of ions in single cells also.

The observation that Daphniae which are poisoned by salts of heavy metals present in the environmental fluid in concentrations which will kill them in the course of 4 to 24 hours, are always extremely deprived of Na^+, naturally gives rise to a suspicion that under such circumstances deprivation of the natural ions might be the direct cause of death.

As far as the present writer knows it has not yet been attempted to include considerations on the influence of heavy metals on the active transport of ions in discussions on the mechanism of the toxic action of very dilute solutions of heavy metals on organisms and single cells as well as on bacteria.

Suggestions.

On the basis of the experimental results of this study and the conclusions and interpretations discussed in relation to the experiments described, the present writer would like to put forward the following considerations, which may serve as a starting point for further investigations into the mechanism of the toxic effect of heavy metals in aquatic animals.

The following facts have to be considered.

An initial concentration of the order of 0.03 mg/litre of a number of heavy metals present as salts in the environmental fluid will kill *Daphnia magna* and certain other freshwater animals.

The amount of heavy metal present in one ml of a solution containing 0.03 mg/litre, it equal to 0.03 μg. This amount would in a compact monolayer cover an area of about 0.1cm^2. As the ratio between the inner surface and the volume of the experimental vessels considerably exceeds 0.1 cm^2/ml we may expect the experimental solutions in question to be very unstable, owing to processes of adsorption and sorption.

Consequently it is highly probable that concerning the experimental solutions in question, we have always to deal with concentrations decreasing with time.

We take it for granted that the only noxious effect of these very dilute solutions of heavy metals seems to be due to a blockade of the ion-absorbing mechanism, and we take into consideration that the blockade has been shown to be reversible, or in other words that the blockade may be abolished when the concentration of the heavy metal in the environmental fluid decreases below a certain level.

In consequence of the above considerations it would be reason-

able to imagine that the differences concerning the initial concentration of a heavy metal that could be tolerated by different aquatic animals should be correlated first and foremost to the individual differences in the length of the periods for which the animals will do without their ion-absorbing mechanism.

This means that experiments ought to be carried out for the purpose of correlating differences in the ability of freshwater animals to stand different initial concentrations of heavy metals with the rate of the physiological exchange of ions in these animals.

Furthermore it would be of the greatest interest to investigate more conclusively than has been possible in the present study, whether heavy metals will also influence the permeability of freshwater animals as well as the renal excretion of ions.—As experimental animal for this purpose should be chosen an animal of larger size than *Daphnia magna*, offering the possibility of collecting urine. Probably *Eriocheir sinensis* (the Chinese wool-handed crab) would be suitable for this study, as being very permeable (KROGH 1939) and very sensitive to heavy metals (unpublished observations of the writer).

As a supplement to the studies suggested above it would be very interesting to undertake a comparison between the effect of heavy metals upon aquatic animals living in salt- and in freshwater. Also for this purpose *Eriocheir sinensis*, which is adapted to both salt and freshwater, would probably be especially suitable.

————

Summary.

The present paper is an account of an experimental study of the osmotic regulation in *Daphnia magna* under natural conditions and in the presence of different heavy metals.

I.

The reasons for expecting a very rapid ionic exchange between *Daphnia magna* and the surrounding fluid are given and the possibility is pointed out that ions of heavy metals when present in the environmental fluid may in some way interfere with the normal exchange of ions in *Daphnia magna*.

II.

The technical features concerning the application of radioactive isotopes in the study of the physiological exchange of the natural ions and in investigations on the accumulation of heavy metals in *Daphnia magna* are discussed.

The applicability of measurements of the electrolytic conductivity in studies on the ionic balance of freshwater animals is pointed out.

III A.

Active uptakes of ions, i. e. uptakes against the concentration gradient, are demonstrated, and the rate of uptake of Na^+, Cl^- and Br^- is determined to be of the order of 6 mE per kg and hour. As for Br^- and Cl^-, it is shown that the mechanism responsible for the active uptake can hardly distinguish between these ions.

The ionic exchange in *Daphnia magna* placed in very dilute solutions is studied. The minimum requirements enabling the

animals to obtain ionic equilibrium are found to be concentrations of 0.1 to 0.2 mE/litre of each of the following ions: Na^+, Ca^{++}, Cl^-, and HCO_3^-.

III B.

The toxicities of Ag^+, Cu^{++}, Hg^{++}, and Pb^{++} are studied in different experimental solutions and a very rough estimate of the initial concentrations of these heavy metals which are just sufficient to kill *Daphnia magna* is obtained.

It is demonstrated that *Daphnia magna* will accumulate copper from a solution containing Cu^{++}. The very small amount taken up, about $3 \cdot 10^{-3}$ μgCu/mg of the animals, is nearly independent of the concentration of natural salts, but varies with the concentration of Cu^{++}.

Lead is accumulated mainly passively in the shells probably by exchange of Ca for Pb^{++}.

Ag, Cu, and Hg are shown to influence the ionic equilibrium in *Daphnia magna*, causing a loss of ions. Just before death the animals which are killed by heavy metals will show a considerably reduced sodium content. Sodium concentrations down to one third of the normal values are measured.

It is demonstrated that this loss of salts is probably due to a blockade of the mechanism responsible for the active uptake of ions.

A similar effect could not be demonstrated when the Daphniae were intoxicated by organic poisons.

The toxic effects of Ag, Cu and Hg could be eliminated by addition to the experimental fluids of glutathione in quantities which in the case of Ag and Cu need hardly exceed the amount equivalent to the concentration of the heavy metal in question. For Hg a surplus of glutathione is required.

The above-mentioned detoxications with Ag and Cu are permanent, whereas a lasting detoxication of Hg requires additional amounts of a surplus of glutathione added at suitable intervals.

Cysteine shows a similar effect to that of glutathione on Ag- and Cu- but not on Hg-intoxications.

Finally the toxic effect of salts of Cu and Hg upon *Daphnia magna* is compared with the toxic effect on another fresh water animal, *Lebistes reticulatus* which is less permeable than *Daphnia magna*.

The Cu-intoxication will develop more rapidly in *Daphnia magna* than in *Lebistes reticulatus* but no significant difference was found between the concentrations which could permanently be tolerated by the two experimental animals.

For Hg the same difference regarding the time required for development of the intoxication was noticed. It was further shown that an apparently greater resistance of *Lebistes reticulatus* to Hg would disappear if the Hg-concentration of the environmental solution was kept constant by renewal of the experimental solutions during the experiment. The significance of these findings is discussed.

From the Zoophysiological Laboratory
of the University of Copenhagen.

REFERENCES

AMBROSEN, J., MADSEN, B., OTTESEN, J., & ZERAHN, K., A Geiger-Müller Counter Arrangement for Biological Research. Acta Physiol. Scand. **10,** 195, (1945).

FRITSCHE. H., Studien über die Schwankungen des osmotischen Druckes der Körperflüssigkeiten bei Daphnia magna. Int. Rev. Hydrobiol. **8,** 22 & 125, (1916)

GICKLHORN, J., Über specifische und lokale Reduktion von Silber- und anderen Metallsalzen in den Kiemensäckchen von Daphnia M. Lotos. **73, 83,** (1925).

GICKLHORN, J. & KELLER, R., Zschr. Zellf. und Mikr. Anat. II (1925).

HEVESY, G., and PANETH, F., A Manual of Radioactivity. Oxford Univ. Press, London, 1926.

HOLM-JENSEN, I., *Daphnia magna* som Giftindikator. Lunds Universitets Aarsskr. N. F. Afd. 2. **40,** Nr. 5 (1944).

HOLM-JENSEN, I., Microestimation of Electrolytic Conductivity. Acta Physiol. Scand. **13.** 353, (1947)

KOCH, M.H., Essai d'interpretation de la soi-disant "reduction vitale" de sels d'argent par certains organes d'Arthropodes Ann. Soc. Sci. Bruxelles. **54,** 346, (1934).

KOCH, H. & KROGH, A., La fonction des papilles anales des larves de Diptères. Ann. Soc. Sci. Bruxelles. 56, 459, (1936).

KROGH, A., Osmotic Regulation in Fresh Water Fishes by Active Absorption of Chloride Ions. Z. vergl. Physiol. **24,** 656, (1937).

— Osmotic Regulation in Aquatic Animals. Cambridge Univ. Press. (1939).

LEVI, H., A Geiger Counter Arrangement Applied to Measurements of Radioactive Indicators in Biological Research, Acta Physiol. Scand. **2,** 311, (1941).

LILLJEBORG, W., Cladocera sueciæ. Uppsala. (1900).

NAUMANN, E., Grundlinien der experimentellen Planktonforschung. Sammlung Binnengewässer. 6, Stuttgart. (1920).

— *Daphnia magna* Straus als Versuchstier. Kungl. Fysiogr. Sällskabets i Lund Förhandlingar (Proceedings of the Royal Physiographic Society at Lund.) 3, Nr. 2, (1933, a).

Naumann, E., Die D. m.-Probe zur Prüfung von Materialien für Wasserbauten. Ibid. 3, Nr. 3, (1933, b).
— Die Anwendung der D. m.-Probe für Luftanalysen. Ibid. 3, Nr. 4, (1933, c.)
— Über einige Fehlerquellen der D. m.-Probe bei toxikologichen Untersuchungen. Ibid. 3, Nr. 12, (1933, d).
— Über Wachstumbeschleunigung bei *Daphnia magna* durch anorganisch-chemische Reize. Ibid. 3, Nr. 13, (1933,e).
— Über einige Ursachen der sinkenden Toxizität abgestandenen Wasserleitungswassers. Ibid. 3, Nr. 14, (1933, f).
— Das destillierte Wasser gegenüber der D. m.-Probe. Ibid. 3, Nr. 15, (1933, g).
— Über die Beeinflussung von *Daphnia magna* durch einige Salze und Salzmischungen. Ibid. 4, Nr. 2, (1934, a)
— Einige Bemerkungen über die Abhängigkeit der *Daphnia magna* von pH- Standard des Wassers unter experimentellen Bedingungen. Ibid. 4, Nr. 3 (1934, b).
— Über die Beeinflussung von Orientierung, Bewegung und Verteilung von *Daphnia magna* durch freie CO_2. Ibid. 4. Nr. 4, (1934, c).
— Über die Toxizität des Kupfersulfats für *Daphnia magna*. Ibid. 4, Nr. 5, (1934, d).
— Über die biologische Verwertung einiger Methoden zur »Entkupferung« durch Austauschverfahren von in gewöhnlicher Weise metalldestilliertem Wasser. Ibid. 4, Nr. 6, (1934, e).
— Über einige Giftwirkungen des glasdestillierten Wassers durch Apparat- und Luftverunreinigungen. Ibid. 4, Nr.11, (1934, f).
— Über die Toxizität einiger vom Laboratoriumsbetrieb abhängiger Glasverunreinigungen für *Daphnia magna*. Ibid 4, Nr. 12, (1934, g).
— Über die Toxizität des Zinksulfats für *Daphnia magna*. Ibid. 4. Nr. 13, (1934, h).
— Über die Toxizität des Bleiclorids für *Daphnia magna*. Ibid. 4, Nr. 14, (1934, i).
Schmidt-Nielsen, K., Aktiv Ionoptagelse hos Flodkrebs og Strandkrabbe. D. Kgl. Danske Vidensk. Selskab, Biol. Medd. XVI, 6, (1941).
Ørskov, S., Untersuchung über den Einfluss von Kohlensäure und Blei auf die Permeabilität der Blutkörperchen für Kalium und Rubidium. Biochem. Z. 279, 250, (1935).