preferable strain.

Summary. Adrenalectomized mice varied in response to cortisone according to genetic constitution. Some strains were: a) highly sensitive. They responded significantly to 1 μg of cortisone, and maximally to 6 μg. b) Relatively insensitive. They responded to 3-6 μg but required 16 times the dose of a sensitive strain to approach maximum response. c) Too low in eosinophils to be practicable in calculations. Males were generally more reliable than females. Normal animals of 2 strains differed in response to mild stress. F_1 hybrid males from C57BR/cd X C57BL/6 are recommended for the assay described.

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Effects of Antibiotics on Daphnia.*† (19731)

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In his recent studies of hemoglobin formation in Daphnia, Fox has devised a method for estimation of the blood pigment in the living animal and shown that its concentration increases in response to lowering of the oxygen tension of the environment(1). It would appear that the hemoglobin system of such small aquatic animals may provide a useful tool for the study of certain problems of hemoglobin metabolism, among them aspects of nutrition. Interest in the adaptation of Daphnia to this kind of investigation has led to an attempt to cultivate the animals free of the natural microflora of the intestine, which, through synthetic or degradational enzymatic activities, could preclude certain types of experiments. At the same time, it was recognized that success in the attempt might well create a nutritional problem, inasmuch as living bacteria in the gut will doubtless be necessary for normal growth of numerous species, including Cladocera(2), until their vitamin and other requirements have been more fully defined. Accordingly, it was decided to try to eliminate bacteria and fungi by the use of various combinations of antibiotics. The results that relate especially to the nutrition of Daphnia will be reported elsewhere. Meanwhile, in the course of the work it appeared that the tolerance of Daphnia for some chemotherapeutic agents parallels that of man. This fact, together with the several convenient qualities of Daphnia as a laboratory subject suggested that the findings might be of interest from a pharmacological point of view, and could perhaps be of use to those concerned with the toxicity of antibiotics.

An obvious deficiency of the microcrustacea

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in experimentation is their size, which makes parenteral injection of materials with ordinary equipment virtually impossible. There are, on the other hand, the familiar advantages that Daphnia are readily cultivable in large numbers within a small space and that they exhibit a number of easily appreciable indices of injury, while their transparency permits direct microscopic examination in life of circulating blood cells and viscera. Moreover, metabolic rates, in terms of oxygen consumption, may be readily determined over a period of hours, or longer, by the use of Fox and Wingfield's modification(3) of the Winkler method. These points are illustrated in the data, which compare the toxicities of a number of antibiotics for Daphnia.

Methods. The strain of *Daphnia pulex* employed was cultivated from a single female. The maximum length reached is about 2.5 mm. Routine cultures of Daphnia were maintained in tanks at room temperature, about 23°C while all experiments were conducted in 250 ml bottles, each containing about 150 ml of water, which were placed in a constant temperature incubator at 20°C. Considerable difficulty was encountered in finding a water of suitable composition for certain experiments, notably those in which Daphnia were kept without food. It appears that a few workers have found it possible to keep Daphnia alive in pure distilled water for as long as 10 days (4). Such tolerance is by no means the rule for fresh water crustacea; wide variations in ability to retain chloride(4) and in requirement for cations(5) occur and may involve strain differences. The strain of Daphnia used here survived for less than 24 hours in water triple-distilled with permanganate. Nor did the animals live longer in tap-water bubbled vigorously with air and having a total content of solids of about 75 p.p.m. and a pH of 7.0. After these findings, trials were made of natural hard waters containing some 200 p.p.m. of total solids, of which there were about 20 p.p.m. each of calcium and magnesium, and having a pH of about 7.8 after equilibration with air. In such waters Daphnia usually survived regularly for 8 to 10 days without food. It was also found that an artificial water made up to contain calcium superphosphate permitted regular survival of most of the animals for 8 to 10 days when starved and it was decided to use this water routinely. The composition is as follows: To each liter of tap water are added 1) 60 mg $CaSO_4$, 2) 80 mg calcium superphosphate, 3) 80 mg magnesia alba. The mixture is bubbled vigorously with air overnight and filtered. Final pH is about 7.9; the electrolyte conductivity corresponds to about 120 p.p.m. NaCl.

Chemotherapeutic agents were added in varying concentrations to the water and each test was run in duplicate in 250 ml bottles which were then seeded with about 12 Daphnia. For each experiment controls were set up in water alone; these animals invariably survived for more than a week even though not fed. The end-point generally employed to estimate toxicity was death of all of the Daphnia. The experiments were terminated on the seventh day since the controls usually died shortly thereafter. Measurements of pH with the glass electrode showed variations only within the range 7.8 $\pm$ 0.1, *i.e.,* differences unlikely to influence the results. Where feeding was undertaken a suspension was used of *Chlorella vulgaris* grown on agar slants. The suspension was adjusted to a standard optical density in a Coleman spectrophotometer and a fixed amount added to each bottle at intervals of a few days. Oxygen content of the water was measured by the modification of Winkler's method described by Fox and Wingfield(3). The sampling syringes employed differed only in detail from theirs. The use of phosphoric acid as recommended by them was found to give somewhat lower readings than predicted values when tests were performed on distilled water equilibrated with air. The substitution of 20% HCl, employed by Krogh(6), overcame this difficulty, and it was then found possible to obtain values for oxygen content having an error of not more than 2% even when the sample was about one ml. All of the determinations were performed in duplicate. The solutes employed in these experiments did not introduce any significant error.

Results. In Table I is set forth the comparative toxicity of a number of agents in

TABLE I. Survival in Days of Starved Daphnia in Varying Concentrations of Chemotherapeutic Agents.

Group	Antibiotic	Conc. in μg/ml of water 50	25	12	6
I	Penicillin G Terramycin (HCl) Chloramphenicol Sulfonamides*	7	7	7	7
II	Streptomycin sulfate	3	4	7	7
	Dihydrostreptomycin Cl.	2	5	7	7
	Polymixin B	2	3	3	5
	Neomycin sulfate	3	3	5	6
III	Actidione	—	—	1	1
	Bacitracin†	7	7	7	7
IV	Thiolutin‡	1	1	7	7
	Rimocidin‡	1	2	5	6

* Includes sulfadiazine, sulfamerazine, sodium sulfathiazole, succinyl sulfathiazole.

† Containing 58.2 units/mg.

‡ Kindly supplied by Dr. Gladys Hobby of Chas. Pfizer & Co.

terms of length of survival of the exposed Daphnia, which were starved except for the unknown contribution to their nutrition that may have been made by the antibiotics themselves or derived from them by bacterial action. The concentrations include those that may be reached in the tissues when the substances are employed in man. The drugs are arranged arbitrarily in groups. In Group I are those compounds in common clinical use which are absorbed at least moderately well from the human intestinal tract and which exhibit comparatively little immediate toxicity for man. Aureomycin could not be included in the studies because it proved to be too unstable. Group II includes agents which are known to be rather less well tolerated by man. The substances in Groups III and IV are separated from the others merely for convenience of appraisal in what follows. It may be seen that penicillin, terramycin, chloramphenicol, and the sulfonamides exhibited no evident toxicity for Daphnia, which showed normal activity throughout the duration of the experiments. No abnormalities were noted in their appearance on microscopic examination. All of these agents are relatively stable at 20°C for several days, with the exception of penicillin. In separate experiments penicillin was added to the water every 2 days, each time in amounts sufficient in themselves to restore fully the original concentration, yet no evidence of toxicity was found. Nonetheless, it is of course possible that the apparent innocuity represents failure of absorption.

The streptomycins (Group II) were lethal in higher concentrations, but at what may be considered therapeutic levels, *i.e.*, 6 to 12 μg/ml, they did not affect survival or behavior. Polymixin and neomycin in man may cause renal damage(7,8) and neomycin may injure the 8th nerve as well(8). The same agents appeared to be highly toxic for Daphnia, which succumbed to their action in less than a week.

Of particular interest is the finding that neomycin exerted a specific pharmacological action on the gut. After about 24 hours' exposure to concentrations of 6 to 12 μg/ml, microscopic examination disclosed a very tight constriction of the intestine, always at about the junction of the first and middle thirds. No similar effect was noted with any of the other drugs tested.

Thus far there is substantial agreement for each antibiotic considered between toxicity for man and for Daphnia. In Group III is bacitracin, apparently harmless to Daphnia, yet known to be nephrotoxic in the human subject(9). But bacitracin is quite without effect clinically if given by mouth because it is destroyed in the gut, and this may well hold also for Daphnia. Actidione was by far the most poisonous material tested. In experiments not included in the data of Table I it could be shown that concentrations as low as 1 μg/ml killed Daphnia in a few days. The substance has been employed in man to a limited extent, and the acute toxicity is described as nausea (10). For rodents actidione is highly poisonous, whether instilled into the stomach or given intravenously(11); the lethal dose appears to lie between 1 and 2.5 mg/kg.

Group IV includes only 2 fungicidal agents for which data as to effects on other species are not available, although they are certainly not harmless(12).

For the more toxic antibiotics, in addition to the experiments with starved Daphnia a corresponding series was run in which the animals were fed chlorella. No significant length-

TABLE II. Oxygen Consumption of Daphnia.

Bottle No.	1*	2	3	4	5†	6
No. of Daphnia	0	42	50	57	0	0
Streptomycin, μg/ml	10	5	10	0	0	0
Chlorella	+	+	+	+	+	0
		Oxygen content of water, cc/l				
March 12, p.m.	6.05	6.04	6.06	5.99	6.08	6.03
March 14, p.m.	5.00	4.06	3.85	3.14	4.32	5.39
Oxygen loss	1.05	1.98	2.21	2.85	1.76	.64
Calculated		−1.05	−1.05	−1.76		
Total oxygen loss from Daphnia		.93	1.16	1.09		
		Oxygen consumption, μl/hr				
Per Daphnia		.11	.12	.10		
Per μg non-exoskeletal N			.05			

* Bottle 1 represents respiration of chlorella and bacteria; the value for this oxygen loss is subtracted in bottles 2 and 3.

† Bottle 5 represents respiration of chlorella and bacteria; the value for this oxygen loss is subtracted in bottle 4.

ening of life span was seen as a result of feeding.

Having examined the effect of antibiotics on the survival of Daphnia, it seemed worthwhile to find out whether the exhibition of an antibiotic might produce a measurable influence on metabolism. For this purpose it was judged best to employ an agent which in high concentration was demonstrably harmful, and therefore absorbable, but in lower concentration was seemingly innocuous. Streptomycin has been shown to have these qualities. Moreover, it was found that a concentration of streptomycin sulfate of 10 μg/ml did not, over a period of 2 weeks, affect nutrition or egg-laying in Daphnia that were fed living *Chlorella vulgaris*.

In Table II are recorded the data for oxygen consumption of Daphnia in the presence of streptomycin and in control preparations over a period of 2 days. The mean wet weight of Daphnia after drying briefly on filter paper was 0.3 mg. Eleven molted exoskeletons were found by micro-Kjeldahl analysis to contain 6 μg N. Three separate groups of 25, 28, and 28 whole Daphnia contained, respectively, 55, 73, and 78 μg N, or a mean of 2.5 μg per animal. The mean non-exoskeletal N is thus estimated to be 2 μg, and the rate of oxygen consumption may therefore be expressed in terms which permit comparison with existing data for related species(13). The oxygen loss given in bottle 5 represents the respiration of bacteria and chlorella (the preparations were kept in the dark). This value is regarded as a control and is subtracted from the total oxygen loss given in bottle 4 in order to gain an approximation to the amount of oxygen used that is attributable to respiration of Daphnia. The preparations represented in bottles 1, 2, and 3 all contained streptomycin. Otherwise, bottle 1 corresponds to control bottle 5, and likewise serves for the measurement of oxygen consumption of bacteria and chlorella. The oxygen loss is less than that in bottle 5, presumably because streptomycin has produced some bacteriostasis. Despite the obvious limitations and assumptions involved there is good agreement in the figures for oxygen consumption of Daphnia with and without added streptomycin, and therefore no evidence that streptomycin in any way has augmented or depressed respiration. In terms of non-exoskeletal N, the oxygen uptake of Daphnia under these experimental conditions appears to be in the neighborhood of 5×10^{-2} μl per μg N per hour.

Discussion. It is of interest to compare this value with those obtained by Zeuthen for marine microcrustacea of similar size, in acute experiments employing either the Cartesian diver respirometer or the Winkler method

(13). Amongst animals examined by Zeuthen were copepods about 0.85 mm long, each containing some 0.5 μg N. They respired at a rate of 7 to 8 x 10^{-2} μl O_2 per μg N per hour. Gammarus, containing about 1 mg N, consumed approximately 2 to 4 x 10^{-2} μl O_2 per μg N per hour. Daphnia is intermediate in size and the value calculated above for its oxygen consumption corresponds well to what might be anticipated from Zeuthen's data.

It would be interesting to know whether the tolerance of Daphnia for the agents listed in Group I, Table I, represents truly lack of toxicity or, rather, impaired absorption. It is probably a fair assumption for the relatively stable substances of known structure, *e.g.*, chloramphenicol and the sulfonamides, that some absorption from the gut took place. The same cannot be said for penicillin and terramycin. It might be possible to examine the question directly by placing Daphnia, carefully washed, on agar plates seeded with bacteria of known sensitivity, and so devise a modification of the inhibition-zone technic for measuring antibiotic concentrations. Such an undertaking lies beyond the scope of the present work.

The findings overall indicate a parallelism between toxicity of certain antibiotics for Daphnia, in terms of survival, and toxicity for man, in terms of untoward reactions that have limited their use. This is perhaps not surprising and in itself suggests no more than that Daphnia might profitably be employed as a test animal, bearing in mind the advantages and disadvantages already noted. The comparative fitness of Daphnia for such practical purposes could take on added significance if it were shown for any antibiotic that its potential harmful effects were reflected experimentally more clearly in Daphnia than in the rodents commonly used. The point may be illustrated by an examination of neomycin. The initial paper describing the substance reported that it exhibited little or no toxicity for animals(14). Yet within a year clinical trials indicated that of 32 patients receiving the drug in whom serial urinalyses were done, no less than 24 (75%) showed casts. Moreover, eighth nerve injury was encountered, and the conclusion was drawn that the material should probably not be released for general use(8). No doubt more exacting animal experiments would have narrowed this discrepancy. But it is noteworthy that in Daphnia neomycin demonstrated at once and unmistakably, a high degree of toxicity as judged both by its shortening of life and by its apparently specific action on the gut, which was easily seen *in vivo*. These considerations provide evidence that in particular instances a more successful prediction of potential toxicity can be made from simple experiments in Daphnia than from studies in higher animals.

Summary. 1. The survival of Daphnia following exposure to varying concentrations of chemotherapeutic agents was studied. 2. The toxicity of the antibiotics for Daphnia in terms of survival showed a correspondence to their acute toxicity for man, in terms of untoward reactions. 3. An apparently specific pharmacological action of neomycin on the gut of Daphnia is described. 4. The oxygen consumption of Daphnia was found to be about 5 x 10^{-2} μl per μg N per hour. The rate was not altered following addition of 10 μg/ml of streptomycin. 5. The use of Daphnia as a test animal for screening antibiotics is considered.

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