0.35 M mannitol) to sedimented protoplasts[9]. Freshly isolated protoplasts did not have any remnants of cell walls as indicated by their failure to stain with 0.1% Calcofluor White M2R.

Our main interest being focused on grape acid metabolism and photosynthetic carbon flow, we first compared the fraction of non-volatile organic acids in protoplasts with that of intact leaves. The 2 extracts were found to be indistinguishable (fig. 1), which implies that the protoplasts in this respect precisely represent the situation within the intact mesophyll. The viability of the isolated protoplasts was controlled by determining their ability to assimilate CO_2 from $NaH^{14}CO_3$. Total $^{14}CO_2$ incorporation was found to be 18.25 µmoles/h and mg Chl in protoplast suspensions, which compares favourably with values of 4–30 µmoles/h and mg Chl in intact leaves[7] and leaf discs[10], depending on the experimental conditions. Similar fixation rates were observed in experiments with mesophyll protoplasts from tobacco and *Antirrhinum*[11]. Qualitative analyses of the $^{14}CO_2$ fixation products in intact tissues[7,10] and grape leaf protoplasts (fig. 2) show that the distribution of radioactive label is virtually the same, with the bulk of the radioactivity appearing in sucrose, glucose, fructose, malic acid and glycine/serine. The low amount of label in the phosphorylated intermediates must be attributed to the relatively long incubation time. This also seems to account for the high labelling of citrate and glutamate, both of which were also found to accumulate radiocarbon after several hours of metabolism[12,13] in experiments with intact leaves.

Consequently, we believe our protoplast preparations reflect the metabolic state of the source tissue and thus provide a new and useful method for physiological and biochemical research on grape metabolism.

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Effect of ascorbic acid on biotransformation and modification of the toxicity of mercurials in goldfish *(Carassius auratus)*[1]

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Summary. Ascorbic acid mediated a small but significant degradation of methylmercury to inorganic mercury in goldfish *(Carassius auratus)* and reduced the toxicity of mercuric chloride despite its substantial conversion into organic form.

Because ascorbic acid is a strong reducing agent it is supposed to have potent detoxifying properties and has been used in cases of intoxication by heavy metals, including mercury[3]. Recently, Gage[4] demonstrated biodegradation of organic mercury compounds by ascorbate in vitro. This study examines the effect of ascorbic acid on biotransformation and modification of the toxicity of mercuric and methylmercuric chlorides in vivo in goldfish.

Material and methods. Details about the goldfish *(Carassius auratus)*, aquaria, and radioactive mercuric chloride and methylmercuric chloride used for this work have been described previously[5]. The modification of acute toxicity of these mercury compounds by L-ascorbic acid (BDH, Analar) was determined in a series of experiments in various dosage and application regimes as before[6,7] and the results were assessed both on the basis of acute toxicity, as determined by 24-h survival rates, and on the effect of treatment on the uptake of mercury, which was measured by counting the fish for gamma activity due to ^{203}Hg in a Packard Autogamma Scintillation Spectrometer Model 5130 as was done earlier[6].

In each experiment 10 goldfish were kept in an all-glass aquarium containing 10 l of water. Different batches of goldfish were used in different experiments. The average weight of the goldfish was 1.726 g. The lid was placed on each tank in all the experiments, except experiment III, in order to minimize the oxidation of ascorbic acid by air. Methylmercuric chloride (80 ng Hg/ml) or mercuric chloride (400 ng Hg/ml) was added together with ascorbic acid at 10, 100, or 1000 times the Hg level on a molar basis. The simultaneous uptake of Hg and vitamin C was allowed to take place for 24 h. In another mode of application regimen, the fish were first exposed to ascorbic acid for 24 h and then to mercurial for the next 24 h using the same concentration. At the end of 24 h of Hg uptake the fish were taken out of the aquarium, washed, divided into 2 groups of survivors and non-survivors, and counted for ^{203}Hg activity, then kept frozen.

The frozen fish were thawed and a homogenate of the whole fish was prepared in 5.0 ml of 0.5 M NaOH and 0.05 M L-cysteine in an Ultra Turrax homogenizer using 2 drops of antifoam tri-n-butyl phosphate (BDH). 1.0 ml of this crude homogenate was used for the study of biotransformation of mercury compounds by specific determination of inorganic mercury[8] using a Conway microdiffusion unit at 20 °C for 24 h. Our batch of methylmercuric chloride was found to contain $1.40 \pm 0.20\%$ of inorganic Hg on 5 different determinations on different days.

Results and discussion. The results of the effect of ascorbic acid treatment on the survival of goldfish, the uptake of mercury and the percentage of it found in the inorganic fraction in the fish exposed to mercuric and methylmercuric chlorides is shown in the table. The effect of pretreatment with ascorbic acid in reducing methylmercury toxicity was not consistent, nor was it always statistically significant. However, the percentage of the inorganic fraction of Hg in fish was always more than in the original methylmercuric chloride preparation which indicated that the conversion of

Whole-body uptake of methylmercury/inorganig mercury, its biotransformation and survival of goldfish under ascorbate treatment

Treatment	Methylmercuric chloride group (80 ng Hg/ml)						Mercuric chloride group (400 ng Hg/ml)					
	Total Hg uptake (ng Hg/g fish)		Inorganic Hg (ng Hg/g fish)		%		Total Hg uptake (ng Hg/g fish)		Inorganic Hg (ng Hg/g fish)		%	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
Experiment I												
Control	7565 ± 1925 (10)	--	--	--	--	--	7270 ± 1468 (10)	--	--	--	--	--
L-Ascorbic acid (× 1000) 24 h before	9627 ± 1976 (7)	12128 ± 1552 (3)	--	--	--	--	14435 ± 8496 (4)	14394 ± 3549 (6)	--	--	--	--
Experiment II												
Control	8949 ± 2217 (6)	12920 ± 1672 (4)	208 ± 74 (6)	236 ± 107 (4)	2.40 ± 0.98 (6)	1.84 ± 0.79 (4)	9478 ± 1366 (9)	17811 (1)	3792 ± 402 (9)	8591 (1)	39.80 ± 13.57 (9)	48.23 (1)
L-Ascorbic acid (× 10) 24 h before	14249 ± 2405 (4)	17751 ± 3850 (6)	396 ± 78 (4)	400 ± 132 (6)	2.83 ± 0.69 (4)	2.23 ± 0.46 (6)	11589 ± 1970 (3)	10968 ± 1802 (7)	--	--	--	--
L-Ascorbic acid (× 100) 24 h before	16213 ± 3152 (9)	19003 (1)	1213 ± 373 (9)	432 (1)	7.59 ± 2.48 (9)	2.27 (1)	13688 ± 4393 (2)	12720 ± 1978 (8)	4408 ± 1210 (2)	4098 ± 1108 (8)	32.45 ± 1.56 (2)	32.01 ± 5.48 (8)
L-Ascorbic acid (× 1000) simultaneously	8395 ± 1365 (10)	--	420 ± 296 (10)	--	5.52 ± 4.80 (10)	--	5496 ± 1552 (9)	13613 (1)	1616 ± 210 (4)*	NA	26.35 ± 2.77 (4)*	NA
Experiment III												
Control	5396 ± 555 (10)	--	152 ± 34 (10)	--	2.82 ± 0.50 (10)	--	7055 ± 1838 (8)	12648 ± 880 (2)	--	--	--	--
L-Ascorbic acid (× 1000) 24 h before, without lid	6083 ± 599 (10)	--	162 ± 35 (10)		2.65 ± 0.41 (10)	--	10342 ± 5012 (4)	12978 ± 7359 (6)	--	--	--	--

All values represent mean ± SD. Figures in parentheses represent number of fish. *Only 4 fish analyzed. NA, Not analyzed.

methylmercury took place either in the fish body or in the aquarium water. The difference in the percentage of inorganic Hg in surviving and non-surviving fish indicated that the transformation took place in the fish, rather than in the aquarium. The higher percentage of inorganic Hg in non-surviving fish than in surviving fish pointed to a definite correlation between biodegradation and manifestation of the toxicity of mercury, which is supported by a similar finding recently reported for methylmercury neurotoxicity in rats[9]. The increasing percentage of inorganic Hg in fish with increasing concentration of ascorbic acid in aquarium water revealed a role of vitamin C in biodegradation of methylmercury to inorganic Hg. This contention was supported by identical values for inorganic Hg in the control and ascorbic acid-treated fish in experiment III in which the lid was not placed on the aquarium and hence ascorbic acid could be oxidized by the air.

The biodegradation of methylmercury salts in rats[8] was found to be mediated by ascorbic acid, possibly by forming a free radical of ascorbate[4]. In our view the reductive cleavage of R-Hg-X compounds could occur in the presence of proton donors[10]:

$$R\text{-}Hg\text{-}X + e^- \rightarrow RHg^{\cdot} + X^-$$

$$R\text{-}Hg^{\cdot} + e^- + H^+ \rightarrow R\text{-}H + Hg$$

The table also shows that in control fish exposed to mercuric chloride, there was a surprisingly large biotransformation of $HgCl_2$ into some organic form, presumably dimethylmercury or methylmercury[11,12]. It was already known that methylation of inorganic mercury takes place in fish liver in vitro[11,12], but that it occurs in vivo and at such a high level is a new finding. The exposure of goldfish to ascorbic acid resulted in reduction of $HgCl_2$ toxicity, as evidenced by the significantly greater number of surviving fish in the majority of sub-groups (χ^2 was 8.57, 7.53 and 10.0, giving $p < 0.01$). A similar effect of ascorbic acid in decreasing inorganic mercury toxicity in chicks has recently been reported[13]. In our study, the protective effect was not due to conversion of Hg^{2+} into less toxic Hg^{1+} or Hg^0 because an even greater proportion of $HgCl_2$ was found to be converted into some organic form. Therefore the suggestion that the ascorbic acid forms complexes with many biologically important elements including mercury[13], thus modifying its toxicity, is worthy of further investigation.

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Diurnal rhythm of hemocyte population in an insect, *Schizodactylus monstrosus* Drury

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Summary. In *Schizodactylus monstrosus* the total hemocyte count appeared to be much lower during the day than at night. At night, the percentage of prohemocytes and spherule cells increased substantially over that of granular hemocytes, plasmatocytes and adipohemocytes. The percentage of sessile hemocytes was much higher during the day. Hemogram rhythmicity was much affected in decapitated insects; altered photoperiod had little effect on it.

Studies on insect hemocytes under various experimental conditions have received much attention due to their importance as an index of various physiological conditions[1-3]. Most animals show circadian rhythms in their behavior; circadian patterns are also reflected in the physiology of various systems in many insects[4-6]. Biochemical changes of the hemolymph are reported to occur following a definite diurnal periodicity[7,8]; similar rhythms in the mitotic activities of hemocytes have also been reported in some insects[9-11]. From various studies it is evident that all hemocytes do not circulate at the same time, some remain adhering to tissue surfaces; their appearance in and disappearance from the circulation also seems to follow a definite rhythm[12,13]. In view of these facts, the present investigation attempts to report the changes in the hemogram of a nocturnal, sand burrowing insect, *Schizodactylus monstrosus* (Orthoptera, Schizodactylidae) during different hours of day and night under normal and experimental conditions.

To avoid the effects of varying reproductive cycles on the hemocyte population in females, only adult males were used in this investigation. After collection, insects were kept separately in moist sand jars under a) complete darkness (maintaining natural conditions) and b) altered photoperiodic conditions subjecting them to 12 h of light and 12 h of dark conditions. Observations on the total hemocyte count (THC), differential hemocyte count (DHC) and hemolymph volume were made every 4 h during the day and every 2 h during the night (because of their nocturnal behavior). Hemolymph samples were collected by amputating one of the 3rd legs. For THC, hemolymph was allowed to fill the Neubauer hemocytometer chambers by capillary action about 5 sec after leg amputation. No anticoagulant was used, to avoid errors in counting; gelification of plasma in *Schizodactylus monstrosus* occurs at room temperature at about 200–210 sec after blood is shed. DHC was made by staining air-dried blood films with Leishman's stain. Cell size, position of the nucleus, nature of cytoplasmic inclusions and staining reactions were used as the main criteria for identification of hemocyte types.

To evaluate the proportions of free and sessile hemocytes