INTERNAL REPORT

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CHARACTERISATION OF ENZYME SYSTEMS FOR DEVELOPMENT AS A RAPID SCREENING METHOD FOR ENVIRONMENTAL POLLUTANTS: FIRST REPORT FEBRUARY 1990

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RESEARCH REPORT FOR SEPTEMBER 1989 - mid FEBRUARY 1990

Characterisation of enzyme systems for development as a rapid screening method for environmental pollutants

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1. General Introduction

This report presents data collected from September 1989 to mid February 1990 which arose from research into the use of enzymes as markers for toxicants (see Research Proposal, File JR/05/38). We were therefore looking at inducible enzymes, particularly Glutathione S-Transferase (GST), that are used by a wide range of animal and plant species to detoxify anthropogenic and naturally occurring substances. The research involved characterising the optimum *in vitro* temperature, pH and protein titre for the GST assay (Section 3) and determining whether feeding affected GST rates (Section 4). Further, we looked at the extent of induction of GST by some model compounds (Section 5). Preliminary studies on GST activity in a mussel (Section 6) and cytochrome P-450 activity in a fish (Section 7) are included. Also, whether RP4 water causes induction is of interest because of the suspected organic nature of the toxicant. Therefore, GST rates obtained from animals used in our laboratories routine RP4 water bioassays are presented in Section 8. The possibility of temporal changes in GST activity from control animals has been begun to be looked at (Section 9).

Our aim is to eventually develop an early warning system and a possible environmental monitoring system for organic toxicants, particularly for the organic toxicant in RP4 water, using enzymes involved in detoxification. We have endeavoured to look at both cytochrome P-450 and GST but most of our work has been with the GST assay because of its simplicity. The GST assay also allows us to easily obtain results from our main laboratory test animals (water fleas and green hydra). This is not to dismiss the cytochrome P-450 assay since intial results appear promising (Section 7). It is hoped that with the recently purchased ultracentrifuge, the cytochrome P-450 method may be refined and its sensitivity improved.

Therefore, results will be presented under discrete subject headings with a focus on the GST assay and results from water flea and hydra. Where possible, figures will be used in the presentation of results. Data tables for these figures will be given in Appendix 6 so they can be used for future reference.

2. General methods for GST assay

At the start of the project, animals were collected from stock tanks when needed. But for work with toxicants, there was an obvious need for better controlled conditions. These conditions are described in Section 2.1. Section 2.2 describes what is now felt to be the optimal conditions (*i.e.* volumes, pH *etc.*) for the GST assay in relation to the number of animals given in Section 2.1.

2.1 Animal Preparation

Water fleas

50 - 60 juvenile water fleas (*Moinodaphnia macleayii)* up to 6 hours old are collected from gravid female water fleas that have already had two broods (our standard laboratory method). They are placed in 500ml polymethylpentene wide mouth bottles (Nalgene Plastics) with 450ml of control water (either Buffalo Billabong or Magela Creek water - our standard laboratory control water) and 450 μl of food. Because of the volume of the containers, it was thought necessary to aerate the water to circulate the food. This initial set-up day is called Day 0. The water and food are changed after about 24h (normally early afternoon of Day 1) with the water fleas transferred by pipette. On Day 2, feeding or toxicant regimes are imposed from the change of water. This is the start of the test (0h). Some time later, depending on whether it is a feeding trial or a 24h acute

toxicity test, the animals are retrieved and homogenised (see below). For the specific animal treatments and times refer to the appropriate result section.

The animals are kept at 30°C in a 12h/12h light/dark cycle (photophase starting 0600h) for the duration of the experiment and counted at each water change and when retrieved for homogenising.

Hydra

30[1] pink hydra (*Hydra vulgaris)* or green hydra (*H. viridissima*) are placed in a 9cm plastic petri dish with 50ml of control water (either Buffalo Billabong or Magela Creek water as per standard laboratory method). The hydra are fed individually with brine shrimp naupilii. After 30min the petri dishes are cleaned (brine shrimp removed) and the water changed. This initial set-up day is called Day 0. The hydra are fed and cleaned and water changed about 24h later (normally early morning of Day 1). The treatments are started at the time of the water change (*i.e.* 30min after feeding on Day 1 and is called 0h). Some time later, depending on whether it is a feeding trial, 24h or 48h acute toxicity test, the animals are retrieved and homogenised (see below). For the specific animal treatments and times refer to the appropriate result section.

The animals are kept at 30°C in a 12h/12h light/dark cycle (photophase starting 0600h) for the duration of the experiment and counted at each water change and when retrieved for homogenising.

2.2GST assay

Protocols covering some of the methodology have been written for technical staff to follow and are included in the Appendices (Appendix 1 - 4). These include specific instructions on how to retrieve the animals, the volumes to be used *etc.* in step by step instructions. The sections below will therefore only give an overview of the methods where enough specific detail is given in the Appendices.

Enzyme preparation

All procedures are carried out at 0-4°C. After the animals are retrieved, they are placed in a 3ml, all glass homogenising vessel. Excess water is removed and cold potassium phosphate buffer added. The animals are homogenised with 10-15 grinding strokes of the pestle. The homogenate is centrifuged at 10000g for 10min and the supernatant retained for protein determination and as the source of GST (see Appendix 2 and 3).

Protein determination

Protein is determined using a modified method of Bradford (1976)[2,3]. A stock solution of Coomassie Brilliant Blue G (Sigma) is made by dissolving 10mg of dye in 5ml of 100% AR ethanol. 10ml of orthophosphoric acid (BDH, 85% w/v) is added to this. For use on the day of the assay the stock solution is diluted with water 1:5 to give the protein reagent.

Protein titre is determined by adding 50µl of the sample and 2.5ml of protein reagent to a plastic disposable 3ml cuvette. The sample and protein are mixed

1. This number of hydra can sometimes lead to fungal contamination in the water due to uneaten or egested food. Therefore, we are now currently trying 10 - 15 animals.
2. At the start, we used a commercial kit (Sigma total protein determination kit No. 610A) based on the same method but because of problems of supply now make up our own stock solutions.
3. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analyt. Biochem. 72, 248 - 254.

and then left standing for two minutes for colour development. The absorbance is determined at 595nm.

A standard curve is obtained by using three concentrations of BSA (Sigma) in the range of 30 - 5μg (normally 30, 10 and 6μg). The blank and dilution solution used is normal saline (0.85% NaCl).

Rate determination

GST rates are determined spectrophotometrically on a Perkin-Elmer UV-Vis Lambda 2 spectrophotometer according to the method of Habig *et al.* (1974)[4]. This involves measuring the increase in absorbance at 344nm as 1 chloro 2,4 dinitrobenzene (CDNB) is converted to its more polar product. Because of the difficulty in rearing, handling and processing large number of animals, we have developed the assay to a minimum final volume of 750μl to minimise the number of animals needed.

The assay reaction mixture is made up in an Eppendorf vial which is kept on ice. All solutions have been pre-chilled on ice. An aliquot of enzyme is added to the vial and and the volume adjusted to 150μl with potassium phosphate buffer of pH 7.3. A further 420μl of potassium phosphate buffer of the appropriate buffer is added to give the required pH (see Section 3.3, 4.2 and 4.3 and Appendix 4). 30μl of 25mM CDNB is then added and the reaction is started with 150μl of 5mM reduced glutathione (GSH). Immediately on addition of GSH, the reaction mixture is vigorously mixed and aspirated into the spectrophotometer. The absorbance is determined at 9s intervals for a period of 2min with the temperature at 25°C ± 1°C.

The above reaction mixture with pH 7.3 buffer substituted for enzyme is used as a control. Three controls are run for each pH with the average used in calculation of rates.

Calculations

Rates are calculated from the fastest and most linear absorbance changes in the 2min period (normally cycle 5 to cycle 12 over 1.05min). The rate for the control for this same period is used to correct for any chemical reaction. Therefore, the rate is calculated from:

$$\text{GST rate} = (\Delta A_{enz} - \Delta A_{con}) \times 1 / \Delta\epsilon / \Delta t$$

where ΔA_{enz} = Change in Absorbance at 344nm due to enzyme over the period relating to Δt;

ΔA_{con} = Change in Absorbance at 344nm due to chemical over the period relating to Δt;

l = Light path length (cm);

$\Delta\epsilon$ = Extinction coefficient for CDNB/product difference spectrum ($mM^{-1}cm^{-1}$);

Δt = The period of the fastest and most linear Absorbance change (min);

and is expressed in terms of nmol/min. The specific rate is the GST rate divided by the protein titre and is expressed as nmol/min/μg protein. Another estimator for GST activity which was found useful for water flea was the rate per animal

4. Habig, W.H., M.J. Pabst and W.B. Jakoby (1974). Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 7130 - 7139.

which was simply the rate divided by the number of animals homogenised. It is expressed as pmol/min/animal.

3. Preliminary work on water flea GST

Results presented in this section give the initial characterisation of GST in water flea with regard to basic operating parameters, *i.e.* temperature, pH and protein titre. All experiments were carried out on animals taken from stock tanks kept outside under shelter.

3.1 Effect of temperature on rates

Two equal aliquots of an enzyme preparation were assayed as described in Section 2.2 but one enzyme preparation was held at 30°C. Similarly, one control was run at 30°C. Figure 1 shows that the enzyme preparation had a faster rate and reached a maximum Absorbance when it was held at 25°C. Not only did the enzyme preparation at 30°C have a slower rate but the chemical reaction (the control curve) was nearly as fast. It was therefore decided that 25°C would be used for the GST assay.

3.2 Correlation of protein with rate

Different volumes of two enzyme preparations were assayed as described in Section 2.2. The first enzyme preparation covered a range of 15 - 60 μg of protein (Figure 2a) and for that part of the curve which shows linearity, has a correlation coefficient of 1. The second enzyme preparation covered a range of 5 - 30 μg of protein (Figure 2b) and had a correlation coefficient of 0.67. It would appear that the second enzyme preparation gave a stronger rate per unit protein (*i.e.* a higher specific activity) and that there were two points that lie well off the regression line. Overall, the data suggests that consistent results in rates may be expected when the protein titre is in the range of 5 - 40μg and there are corresponding rates of 2 - 10nmol/min. But when the specific rate is considered, results may need to be interpreted with caution. For instance, the specific rate for the maximum rate in Figure 2a is 132% that of minimum while for Figure 2b, the difference between the two endpoints is 152%. To obtain the best results then, the protein added to the assay needs to be very similar between treatments within any one experiment.

3.3 Determination of optimum pH

Enzyme preparations were made from animals collected at three different times through the study period. The effect of an *in vitro* pH of 7.0, 7.3 and 7.6 was tested by adding to 150μl of these enzyme preparations 420μl of potassium phosphate buffer at a pH of either 6.9, 7.3 or 7.7, respectively (see Appendix 4). The buffered enzyme preparations were then assayed as described in Section 2.2.

Although the results are somewhat variable for pH 7.0 and 7.6, an *in vitro* pH of 7.3 always gave the highest specific rate (Table 1). Therefore, pH

FIGURE 1

Effect of Temperature on Rates.

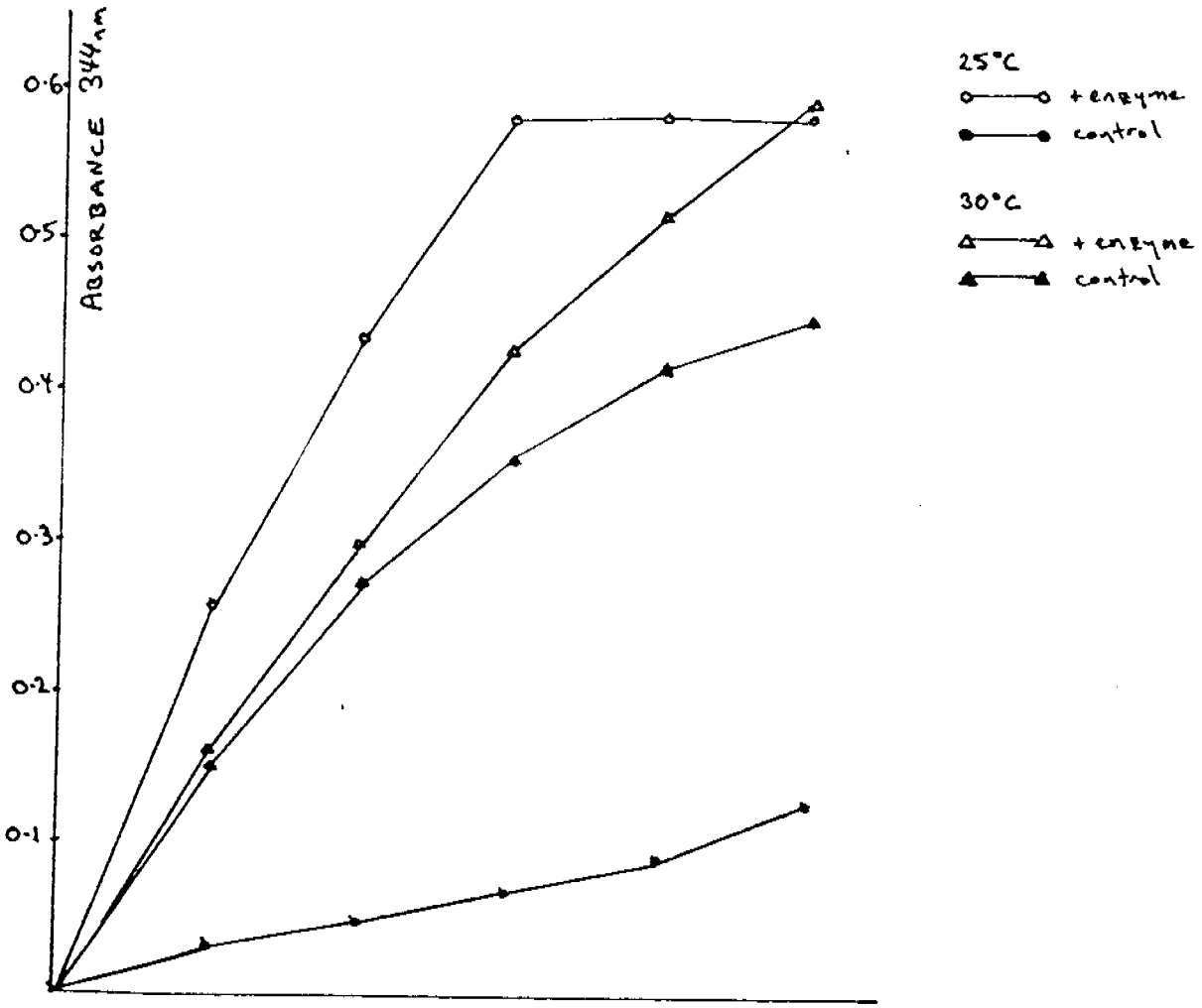


FIGURE 2A

GST Rates over the first protein concentration range

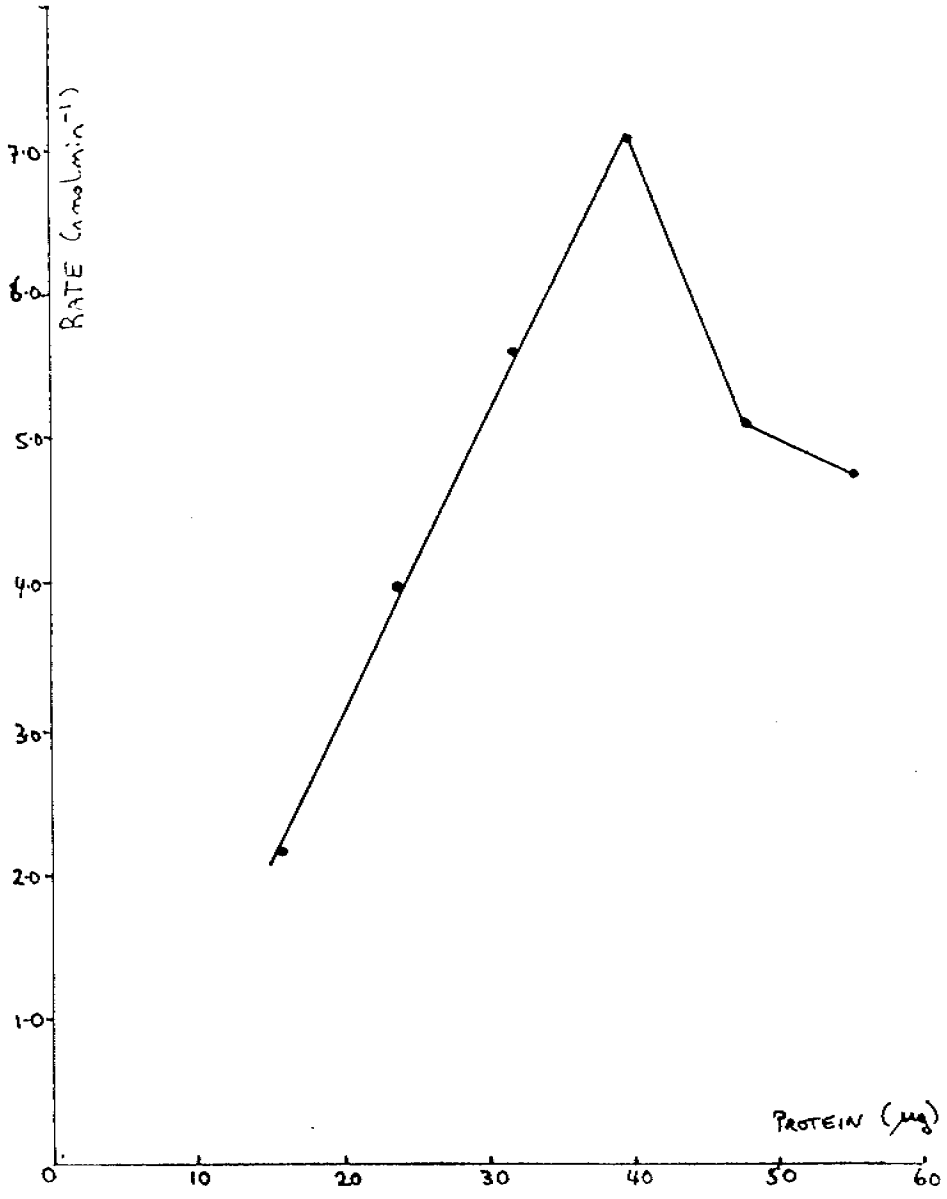
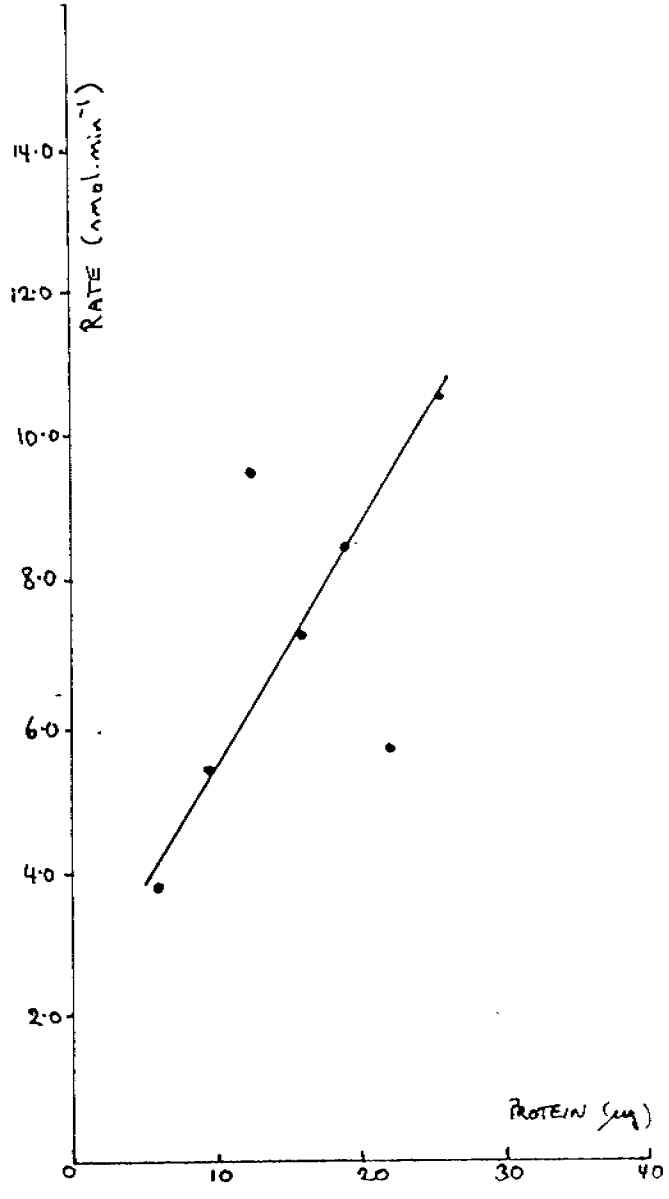


FIGURE 2B

GST Rates over the second protein concentration ra



7.3 was accepted as the optimum *in vitro* pH for water flea GST.

TABLE 1 : Effect of pH on GST activity in water flea

pH	7.0	7.3	7.6
Specific Rate	133	277	240
(nmol/min/μg pr)	268	326	36
	92	186	132
Average	164	263	136
(S.D.)	75	58	83

4. Effect of feeding on water flea and hydra GST activity

Before experiments could be carried out on the nature of the response of GST activity in animals exposed to toxicants, we had to determine whether feeding had any effect. Further, preliminary results indicated that the pH optimum may change for hydra depending on time after their last feed. Therefore, GST rates for hydra were determined for a range of pHs *in vitro* at different times after a meal. Desired *in vitro* pHs were achieved as in section 3.3 for water flea. The additional pH of 8.0 was obtained by adding 420μl of potassium phosphate buffer at pH 8.1 to 150μl of enzyme at pH 7.3.

4.1 Effect of feeding on water flea GST activity

Two experiments have so far been carried out to determine the effect of feeding on GST activity in water fleas. Groups of 50 animals per time treatment were transferred into water containing food or water not containing food. One group from each water treatment was retrieved and homogenised at 0, 4, 12, and 24h after the start of the test (0h, see Section 2.1).

The results are summarised in Table 2 and Figures 3 and 4. There appears to be no difference betweer water treatments until 24h after the start. At this stage, the specific rate (Table 2), rate per animal (Figure 3), and protein per animal (Figure 4) are distinctly different. The rate per animal is greater for those animals that were fed as is also the protein per animal value. The lower specific activity in the fed group may be explained by the higher protein per animal value. If the apparent extra protein in the fed group does not contribute to an increase in rate, then this would lower the specific rate.

TABLE 2 : Effect of feeding on water flea GST activity

Time[a] (h)	0	4	12	24
		Food Added		
Mean Specific Rate[b]	224	199	269	171
(S.D.)[c]	(159)	(150)	(112)	(19)
		Food Not Added		
Mean Specific Rate[b]	256	349	232	241
(S.D.)[c]	(208)	(241)	(215)	(10)

a Time after placement into water with or without food
b (nmol/min/μg pr)
c n = 2

To achieve a more sensitive specific rate value in toxicity tests, we decided that all animals would not be fed in 24h toxicity tests, or for the last 24h in 48h toxicity tests. The specific rates would not then lose resolution due to non-contributing protein. Differences between treatments may therefore be more apparent.

4.2 Effect of feeding on green hydra GST activity

Earlier attempts to try and find an optimum pH appeared impossible to reproduce and it was thought that the time after feeding may be critical. Feeding *per se*, or growth (*i.e.* leading to budding) due to feeding, may cause the induction of different forms of GST with differing pH optima. Also, feeding may introduce an exogenous source of the enzyme via the food (brine shrimp naupilii) which would itself have GST. This had obvious importance in standardising future assays with toxicants. Two experiments have so far been carried out to determine the effect of feeding on GST activity in green hydra. In Experiment 1, the first initial experiment on hydra feeding, four groups of 30 animals were set up as described in Section 2.1 while Experiment 2 had five groups of 30 animals. The start of the experiment (0h) was also as described (*i.e.* 30min after feeding on Day 1). One group was retrieved and homogenised for

FIGURE 3. Effect of food on Rate per Animal

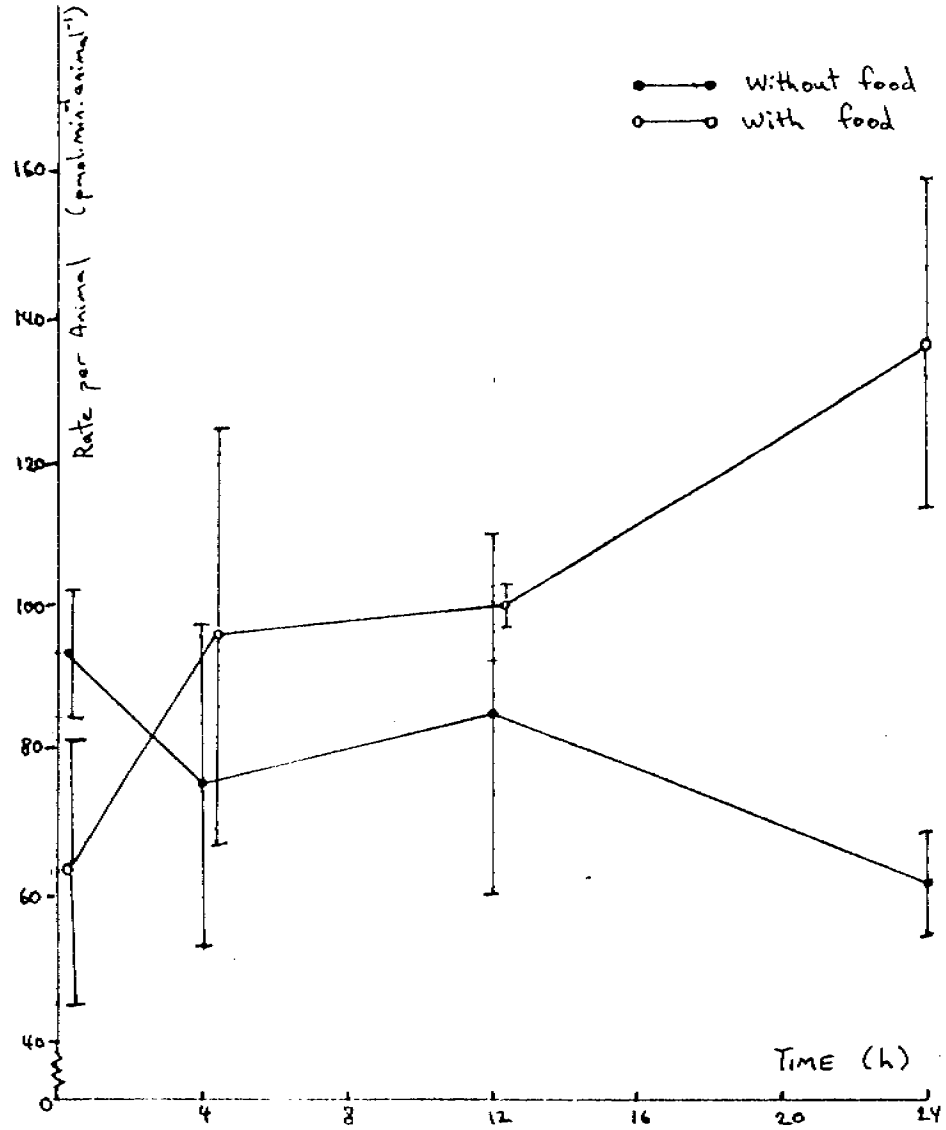
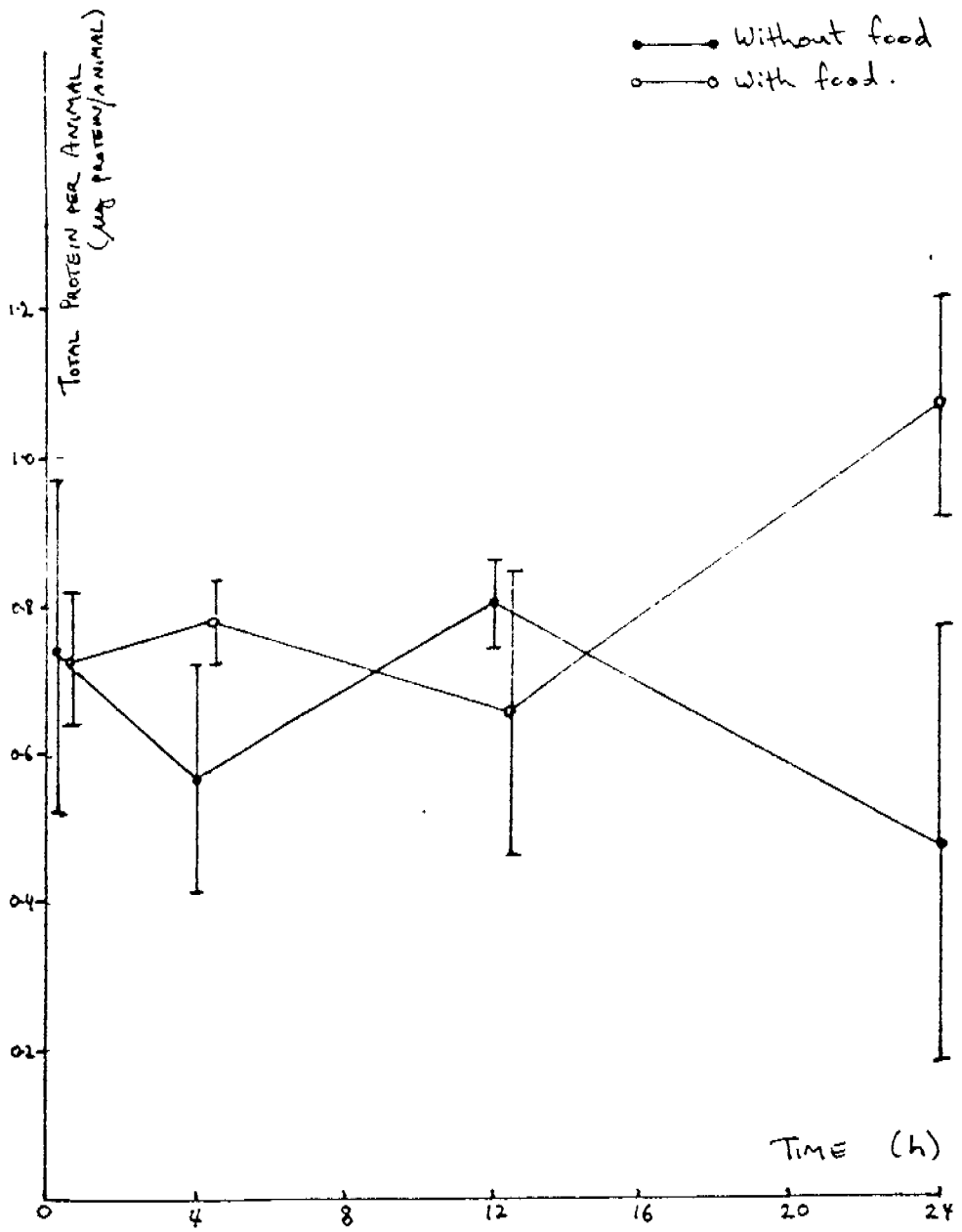


FIGURE 4. Effect of food on Total protein per Animal



each time treatment (*i.e.* for Experiment 1 at 2, 6, 24 and 84h after the start of the test; for Experiment 2 at 0, 2, 6, 24 and 30h after the start of the test).

The results are summarised in Table 3 and show specific rates at different *in vitro* pH. Despite its missing data, it is interesting that at 84h there was no GST activity observed for an *in vitro* pH of 7.6 but there was still a good specific rate for pH 7.3. At this stage we wondered to what extent the food (brine shrimp naupilii) contributed to specific activity at the earlier time points of the study. Therefore, brine shrimp naupilii were assessed for their optimum *in vitro* pH for GST (Appendix 5). It appears to lie in the range of pH 7.6 - 8.0 although more data is needed for confirmation.

TABLE 3 : Variation in green hydra GST activity with pH and time after feeding.

Time[a] (h)	Expt No.[b]	pH	7.0	7.3	7.6	8.0
			Specific Rates[c]			
0	1		-	-	-	-
	2		2591	1705	1705	1675
2	1		1352	1501	1203	-
	2		1744	1704	1179	677
6	1		-	1132	2170	-
	2		3133	2568	2316	846
24	1		1364	1418	1759	1041
	2		1811	2208	1914	2263
30	1		-	-	-	-
	2		2724	2131	2368	1954
84	1		1347	1852	0	-
	2		-	-	-	-

a Time after placement into water with or without food
b Experiment Number
c (nmol/min/μg pr)

We then undertook to repeat the experiment, with some time modifications (Experiment 2), to see if we could discern any trend in optimum pH and whether there was any activity at high pHs at 0h. The results do not clearly indicate any trend which relates to the immediate feeding history. Rather, they seem to indicate that GST in green hydra can tolerate a broad pH range. It was decided therefore, to always assess GST rates 24h after a water change and at two *in vitro* pHs, that of 7.3 and 7.6.

4.3 Effect of feeding on pink hydra GST activity

As for green hydra, it was necessary to determine the optimum *in vitro* pH for GST in pink hydra at different times after feeding. Two experiments have so far been carried out in relation to this aim. Five groups of 30 animals were set up as described in Section 2.1. The start of the experiment (0h) was also as described (*i.e.* 30min after feeding on Day 1). One group was retrieved and homogenised for each time treatmen: (*i.e* at 0, 2, 6, 24, and 30h after the start of the test).

The results are summarised in Table 4 and show specific rates at different *in vitro* pH. There is no apparent trend although in Experiment 1 there was a very high specific activity obtained at 0h for pH 8.0. Whether this activity was due to the brine shrimp used as food is hard to determine, especially since there were elevated levels (although not to the same extent) at other times in both experiments. As for green hydra, the results seem to indicate that GST in pink hydra can tolerate a broad pH range and does not relate to the immediate feeding history. It was decided therefore, to always assess GST rates 24h after a water change and at two *in vitro* pHs, that of 7.3 and 7.6.

TABLE 4 : Variation in pink hydra GST activity with pH and time after feeding.

Time[a] (h)	Expt No.[b]	pH	7.0	7.3	7.6	8.0
			Specific Rates[c]			
0	1		516	362	488	1227
	2		1413	1492	1572	1811
2	1		769	769	750	637
	2		1366	1295	1110	1865
6	1		335	359	586	389
	2		2285	2018	2232	2592
24	1		762	788	512	420
	2		2418	2393	1985	2775
30	1		332	455	430	479
	2		3513	3713	4365	3733

a Time after placement into water with or without food
b Experiment Number
c (nmol/min/μg pr)

5. Effect of toxicants on animal survival and GST activity

Animals were prepared for treatment with pentachlorophenol (PCP), butylhydroxy toluene (BHT), or malathion as described in Section 2.1. Results from only one test per animal per treatment are reported here. Therefore, any interpretation drawn from the data (*i.e.* whether the toxicant had an effect) is tentative. The toxicant is introduced with the water (at the water change (0h) on Day 2 for water flea, day 1 for hydra). The carrier used for PCP and BHT was ethanol with the ethanol having an end
dilution with water of 1/1000. No carrier was used for malathion at the concentrations used but adequate mixing had to be ensured. Food is not added for water fleas whereas the hydra are fed (see Section 4). The water at this water change has the toxicant. In 48h acute toxicity tests, the water is again changed on Day 3 at t = 24h.

5.1 Effect of toxicants on animal growth and survival

Results are presented here for the effect of PCP, BHT and malathion on water flea survival. Survival, when discussed here in relation to water fleas, specifically refers to the number of water fleas surviving over the experimental period. It is expressed as a percentage relative to the initial starting number (%survival).

Due to an error in data recording, results can only be given for the effect of PCP on hydra growth. Animal growth here, refers specifically to the whole number increment in individual hydra over the experimental period. Survival, when discussed in relation to hydra, here specifically refers to the number decrement in individual hydra below the starting number of individuals. Obviously, these definitions do not address the problems of static numbers but is outside the scope of this study.

Effect of PCP, BHT and malathion on water flea survival

At 5600μg/l PCP appeared to have an effect on %survival relative to the rest of the body of the data (Figure 5). There appeared to be no effect of BHT at the concentrations used on %survival (Figure 6) even though the top concentration was 1042μg/l. Malathion in contrast, had a marked effect on %survival at a concentration as low as 0.5ng/l.

Effect of PCP green hydra growth and survival

Figures 8 and 9 show that in a 24h acute toxicity test green hydra do not survive concentrations of $\geq$112μg/l. Further, there is an effect on growth, and presumably survival, at 11.2μg/l and clearly an effect on survival at 56μg/l in a 48h acute toxicity test (Figure 10).

Effect of PCP pink hydra growth and survival

Results are similar for pink hydra compared with green hydra in 24h acute toxicity tests (Figures 11 and 12). There did appear to be some effect of PCP at 56μg/l though in one experiment (Figure 11).

5.2 Effect of toxicants on water flea and hydra GST activity

Table 5a and 5b shows the results for ethanol controls which gives some indication of the variation that may be expected for the parameters assayed. As a general guide, we would not consider that the toxicant had an effect unless there was greater than 130% change in rates and even then at least a 20% difference between the treatment and the control. For protein per animal used in water fleas, greater than 20% difference would seem to be an acceptable indication of

FIGURE 5. Effect of PCP on Water Flea Survival

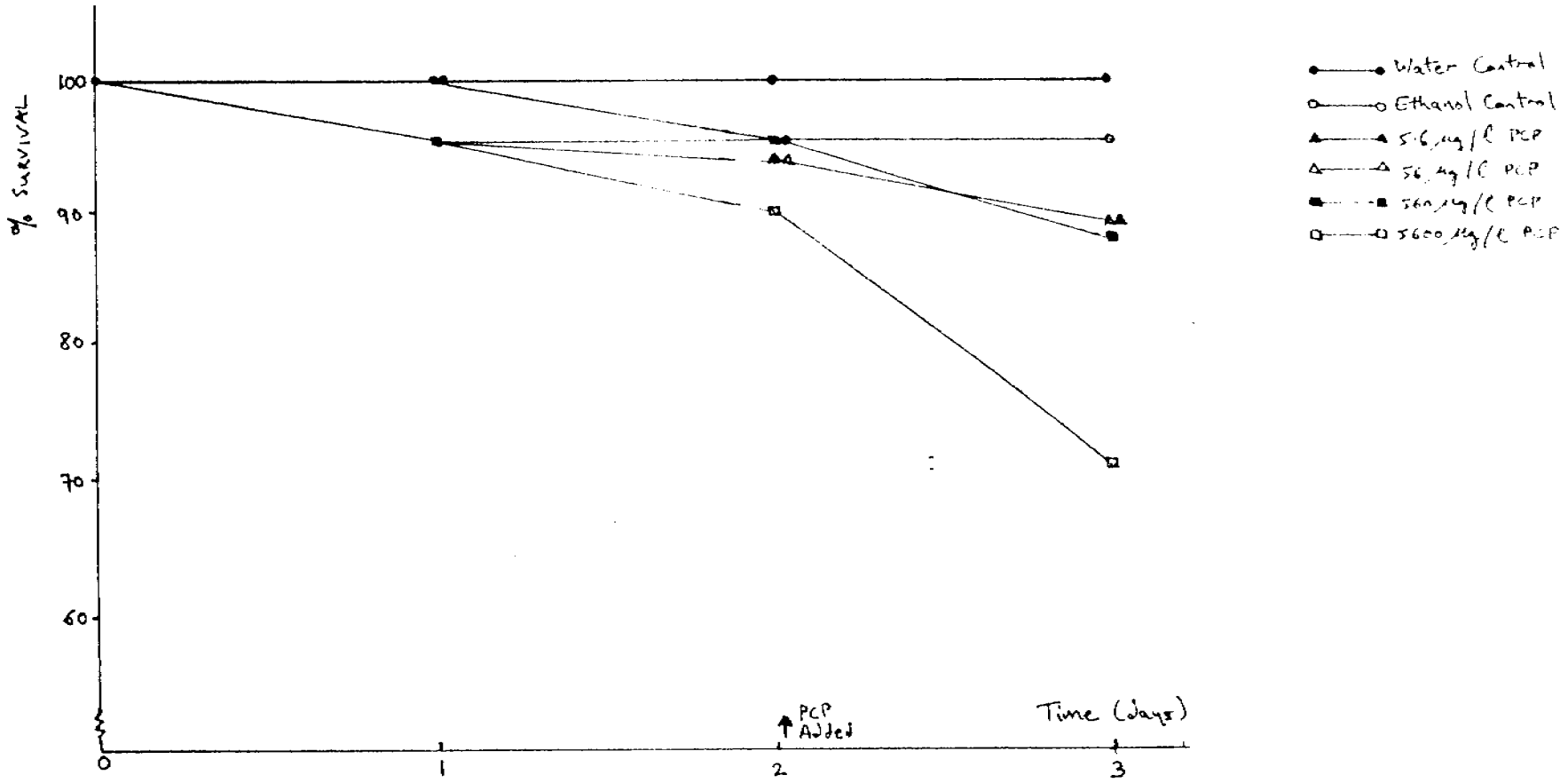


FIGURE 6 Effect of BHT on Water flea Survival

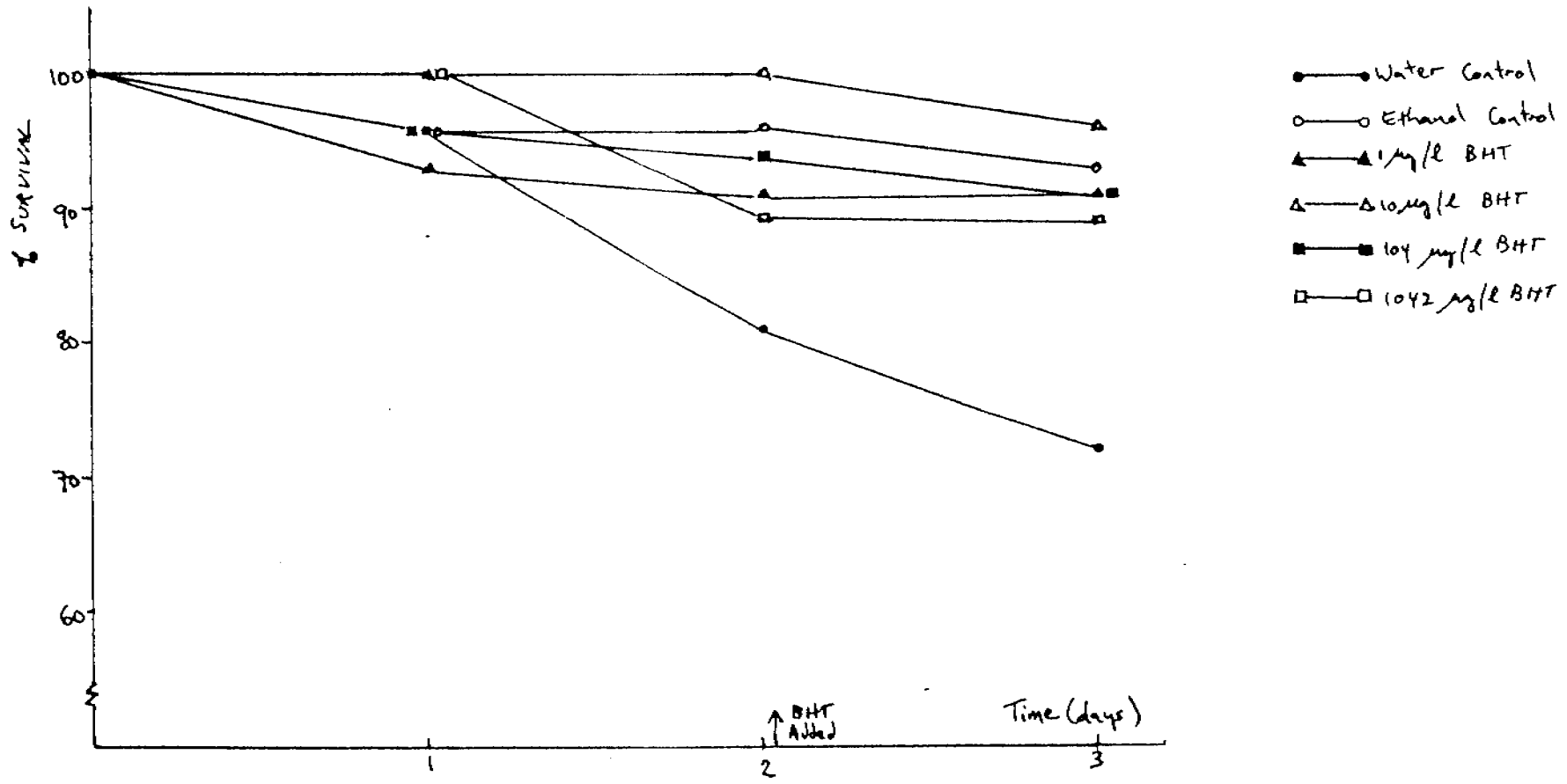


Figure 7. Effect of Malathion on Water flea Survival.

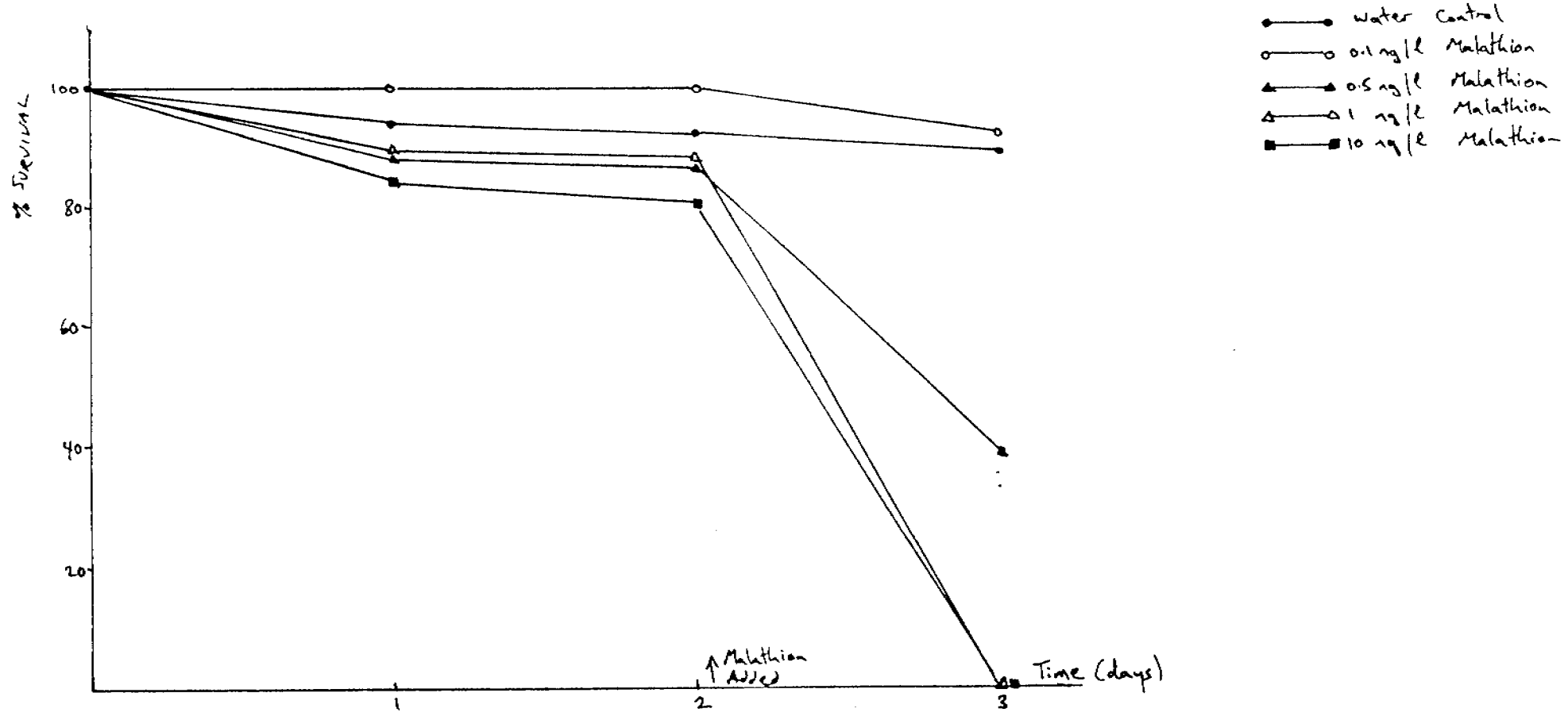


Figure 8. Effect of higher concentrations of PCP (>56 µg/l) on Green hydra growth

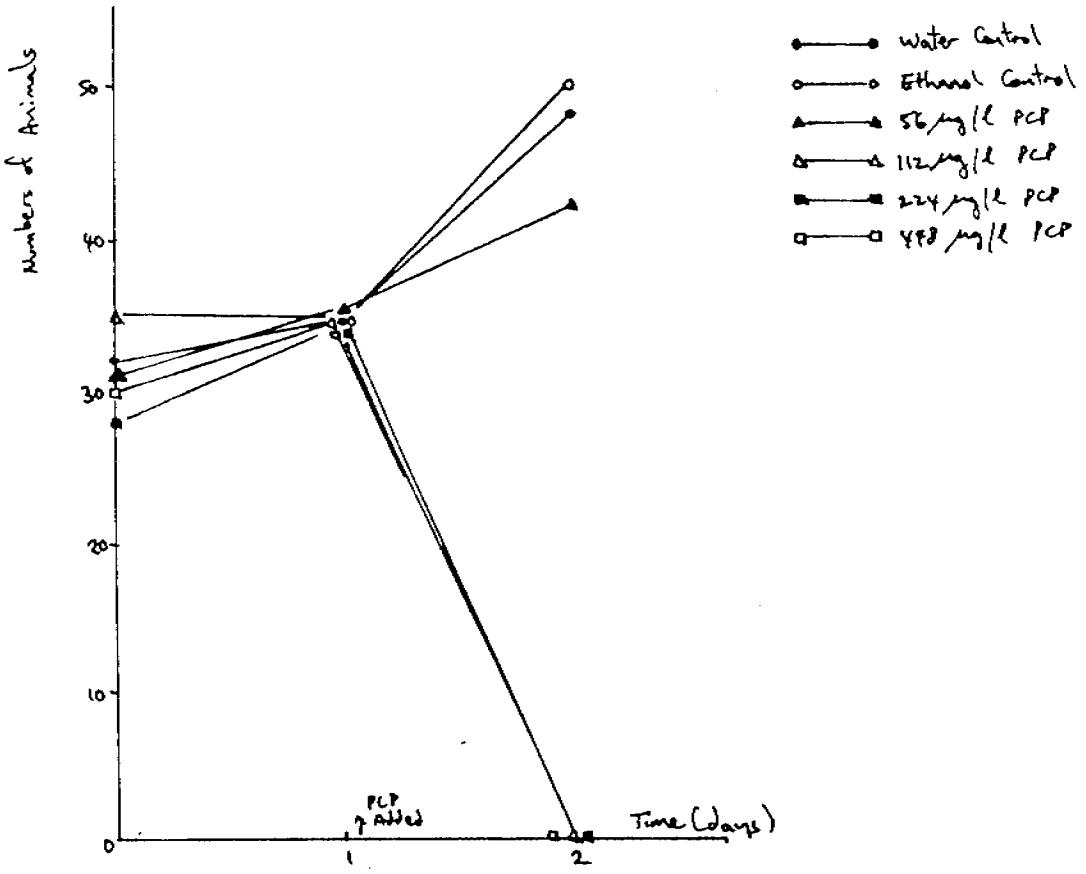


Figure 9. Effect of lower concentrations of PCP (< 56 µg/l) on Green hydra growth

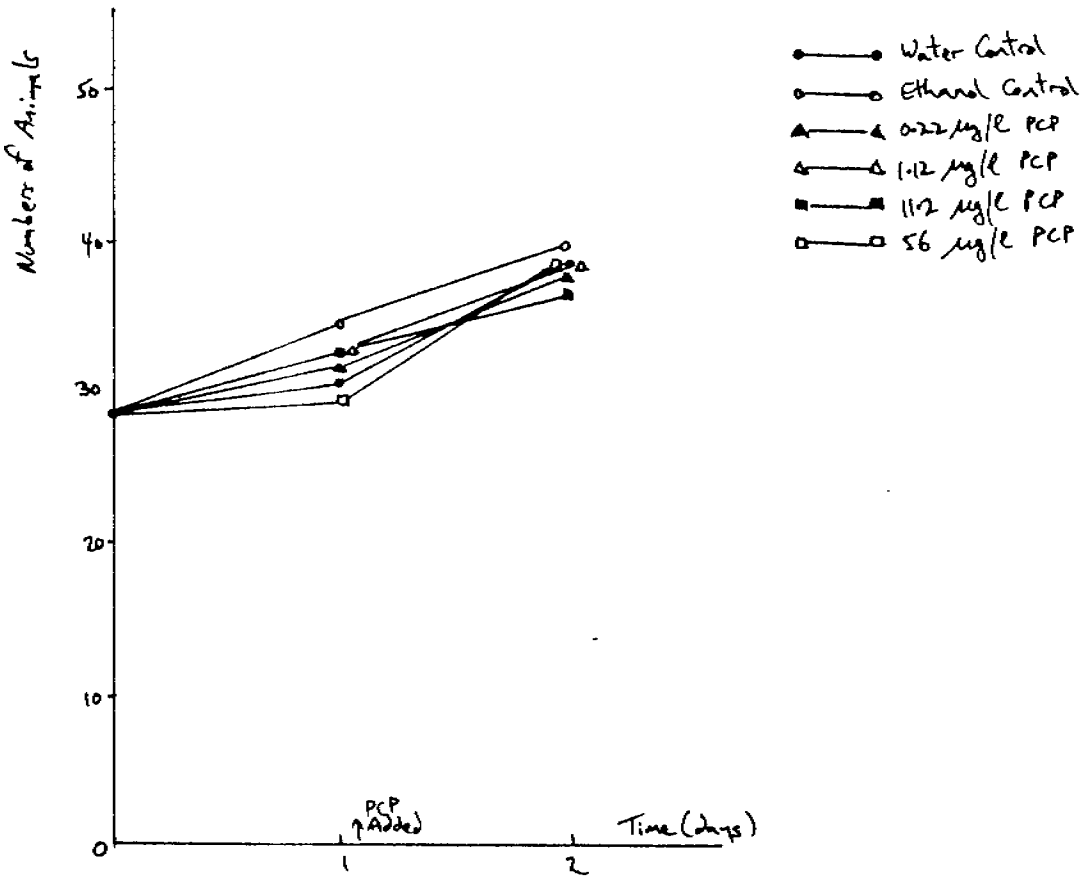


Figure 10. Effect of 48h exposure to lower concentrations of PCP (≤56 µg/l) on Green hydra growth

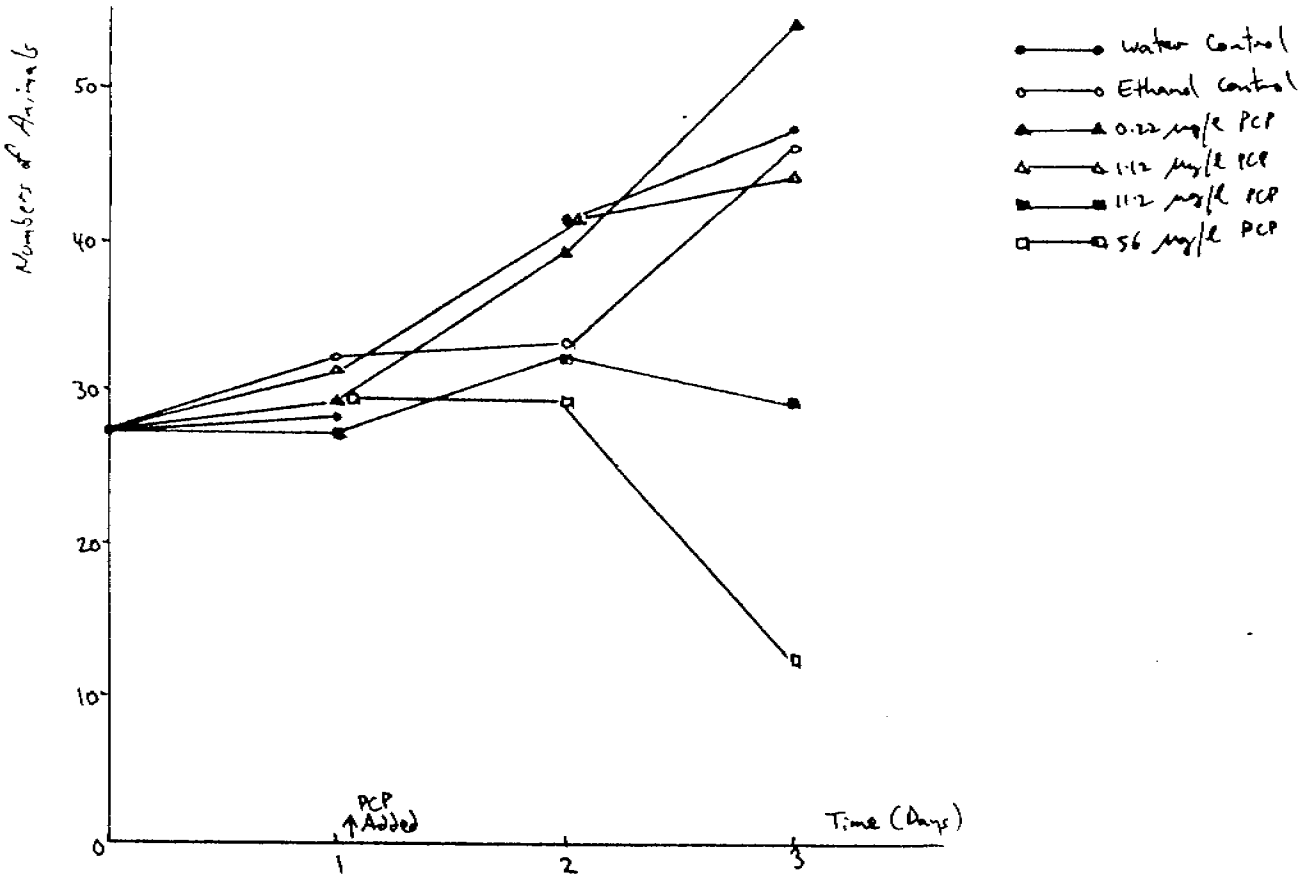


Figure 11. Effect of higher concentrations of PCP (≥ 56 µg/l) on Pink hydra growth

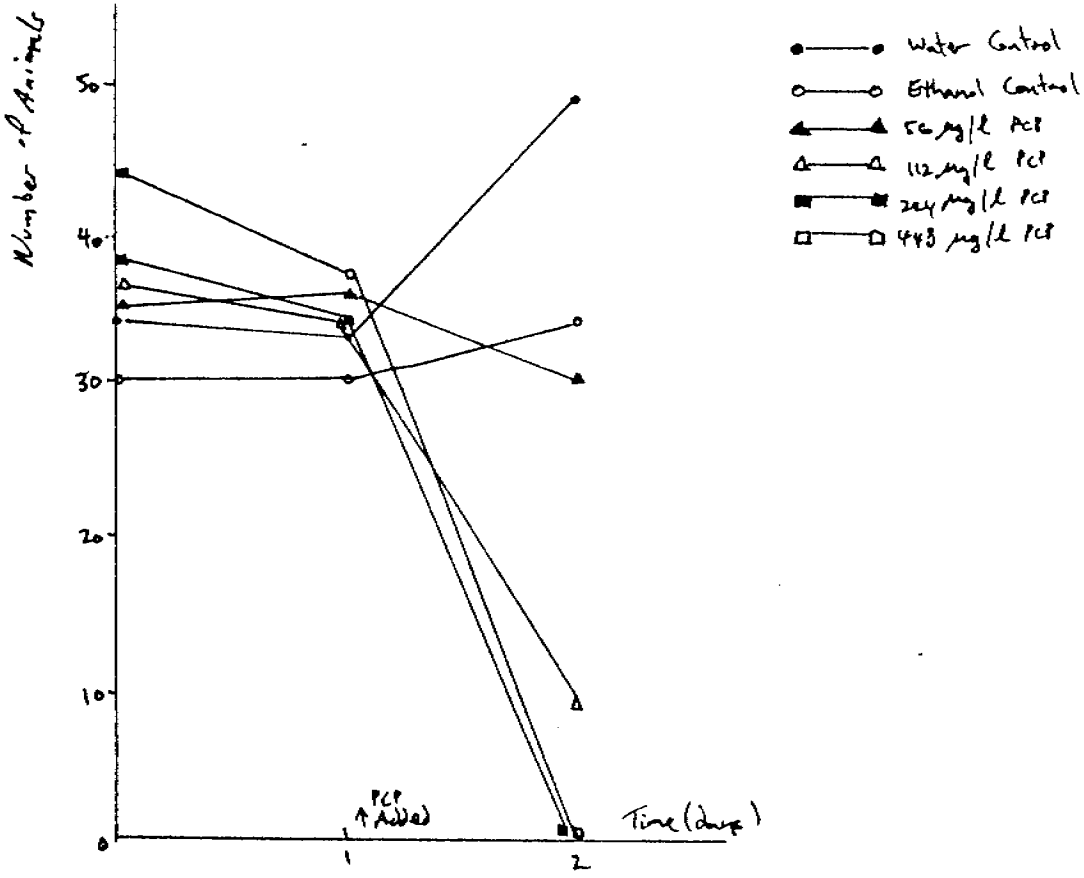


Figure 12. Effect of lower concentrations of PCP (≤56 µg/l) on Pink hydra growth

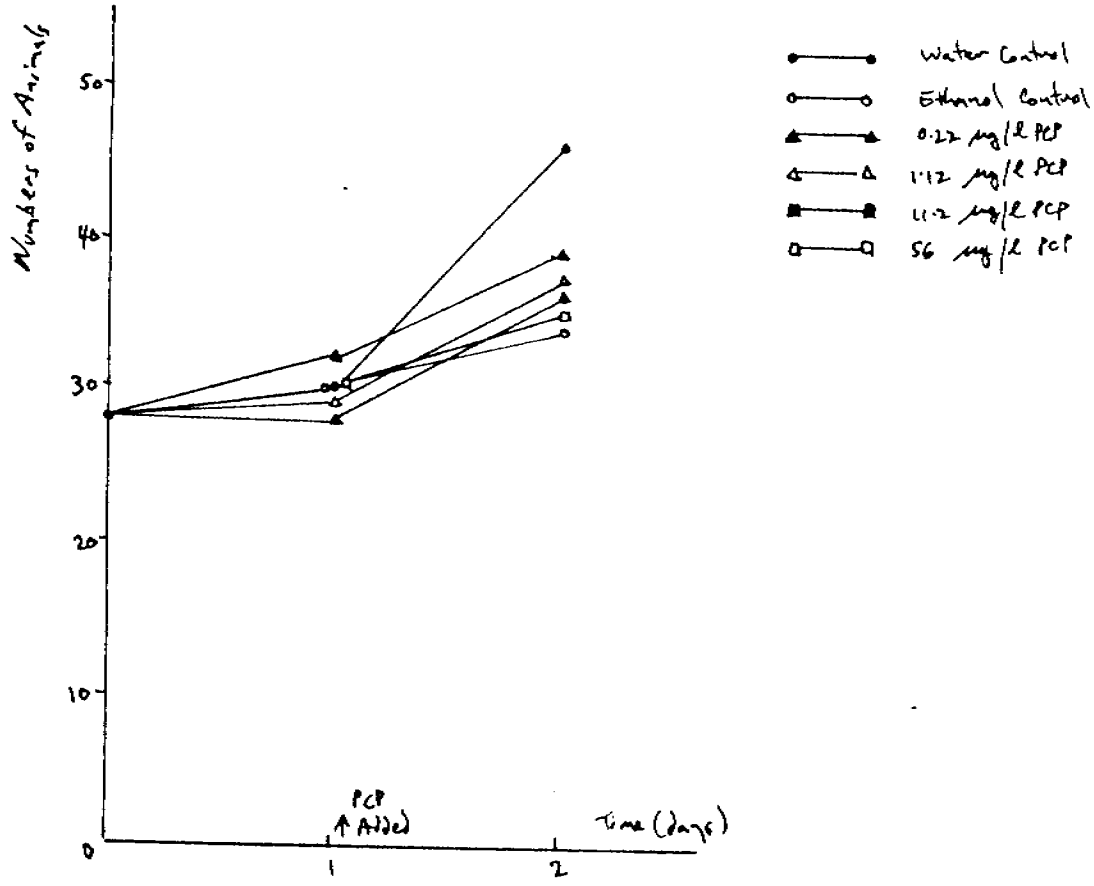


Figure 13. Effect of PCP on GST activity & Total protein per animal in water flea

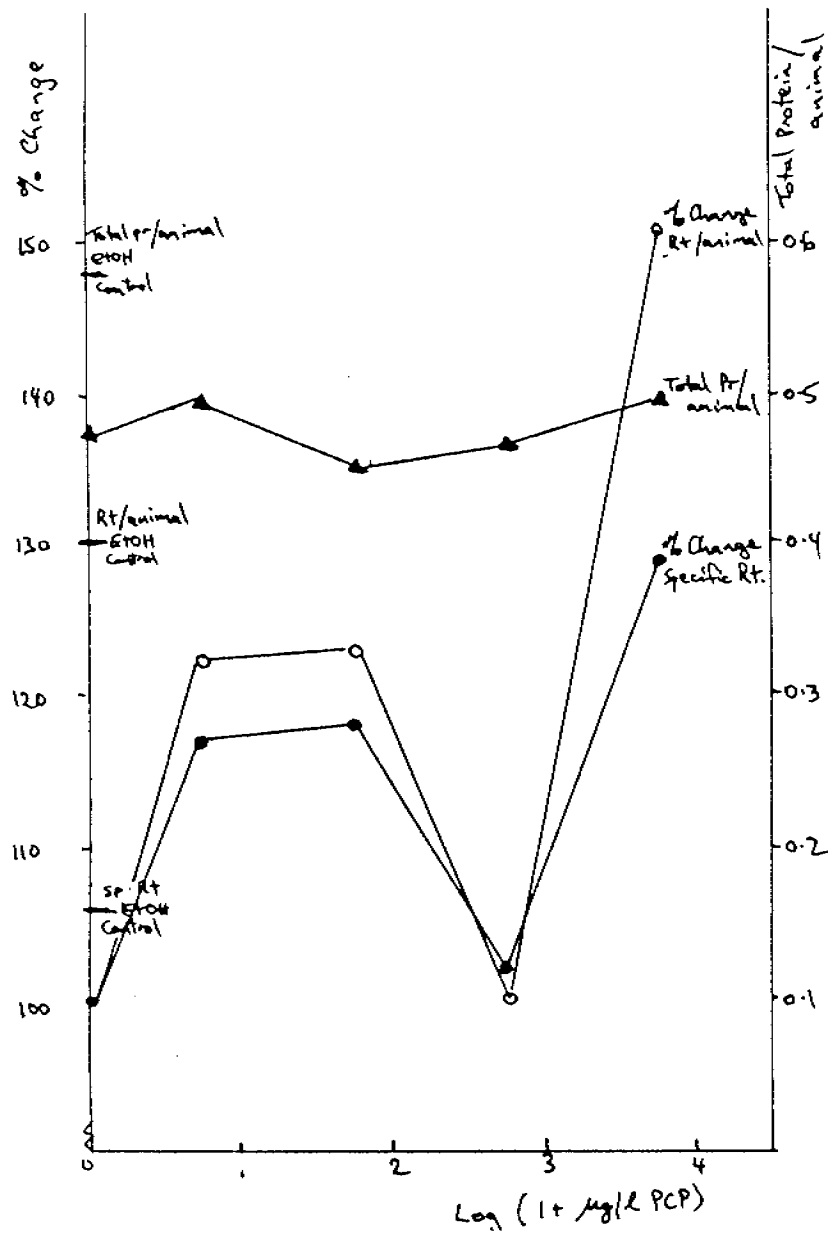
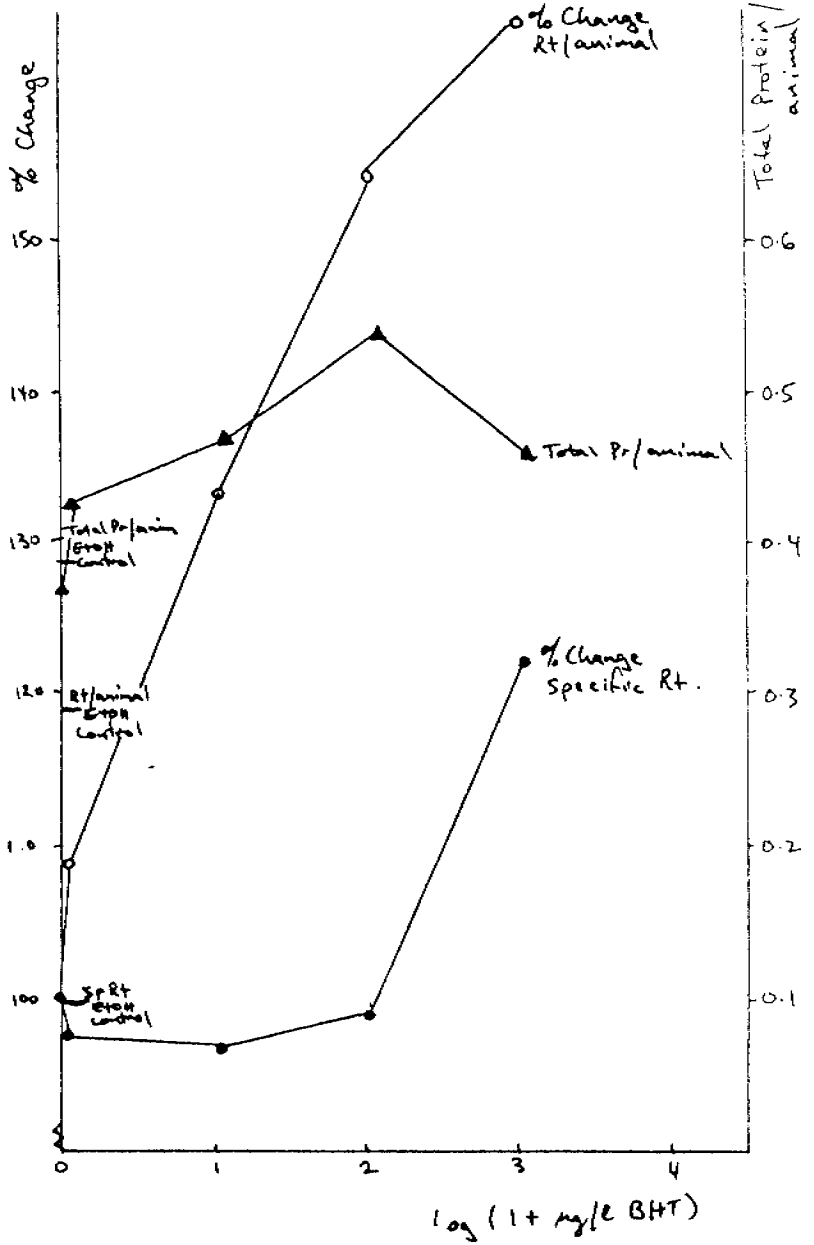


Figure 14. Effect of BHT on GST activity & Total protein per animal in water flea



change. We will have better idea of confidence limits when more data is gathered.

TABLE 5a : Ethanol control values for GST activity in water flea

Toxicant	%Δ Sp Rt[a]	Pr/animal[b]	%Δ Sp Rf
PCP	106	.581	130
BHT	100	.390	119

a The change in specific rate (nmol/min/μg protein) compared with water control values expressed as a percentage.
b μg protein/animal
c The change in rate per animal (pmol/min/animal) compared with water control values expressed as a percentage.

TABLE 5b : Ethanol control values for GST activity in hydra

Species	Toxicant	pH 7.3	7.7
		%Δ Sp Rt[a]	
Green hydra	PCP[b]	101	125
	PCP[c]	105	87
	BHT[b]	87	93
Pink hydra	PCP[b]	100	129
	BHT[b]	119	131

a The change in specific rate compared with water control values expressed as a percentage.
b 24h acute toxicity test.
c 48h acute toxicity test.

The specific rate of hydra GST was determined at ph 7.3 and 7.6 and results reflect the need to continue to screen hydra GST activity at these two pHs.

Effect of PCP, BHT and malathion on water flea GST activity

PCP appeared to have little effect on any of the parameters assayed although there may have been an effect on protein per animal at the highest PCP concentration of 5600μg/l (Figure 13).

There was a marked elevation on specific activity at the highest BHT concentration of 1042μg/l in contrast to the specific activities at the lower concentrations. It would appear to correspond to a lower protein per animal value. Therefore, the (assumed) effect of BHT was more clearly seen with rate per animal which showed a linear response with dose. If this relationship holds in further studies then it shows that there can be elevated GST rates without any obvious change in %survival.

Malathion, if anything, suppressed GST activity overall but with a peculiar increase in protein per animal at 0.5ng/l (Figure 15). This elevated value is presumably reflects whole animal changes which precede death (*e.g.* tissue breakdown, cell lysis, *etc.*)

Effect of PCP, BHT and malathion on green hydra GST activity

PCP appeared to have an effect on rates (Table 6, Figures 16 and 17). This effect was most clearly seen in the 48h acute toxicity test (Figure 17). The elevation of specific rate at an *in vitro* pH of 7.7 at the PCP concentration of 56μg/l (Figure 16) appears to precede any obvious effect of PCP on growth (Figures 8 and 9) and an even bigger elevation in specific rate after 48h (Figure 17). It is surprising therefore that after 48h there are not larger increases in specific rate at lower concentrations of PCP.

TABLE 6 : Effect of PCP on green hydra GST activity

PCP (μg/l)	0[a]	0[b]	56
pH 7.3			
Specific Rate[c]	659	557	1695
%Δ Sp Rt[d]	100	85	257
pH 7.7			
Specific Rate[c]	516	548	1095
%Δ Sp Rt[d]	100	106	200

a Water control
b Ethanol control
c nmol/min/μg protein
d The change in specific rate compared with water control values expressed as a percentage.

Figure 18 is hard to interpret because of a missing value for 11μg/l BHT. It would appear though that specific rates may peak (and level off?) at 10μg/l. Therefore, this would allow only a small window for GST induction unless, like the pink hydra, there was a plateau in specific rate in the 10 - 100μg/l concentration range. A high concentration of BHT (1042μg/l) appears to suppress the GST specific rate.

The data for malathion is extremely difficult to interpret (Figure 19) but like the data for the other animals, GST activity appears to be suppressed. More data is needed to assess whether the high specific activity value for pH 7.3 at 1ng/l is just random fluctuation.

Figure 15. Effect of Malathion on GST activity & Total protein per animal in Water flea.

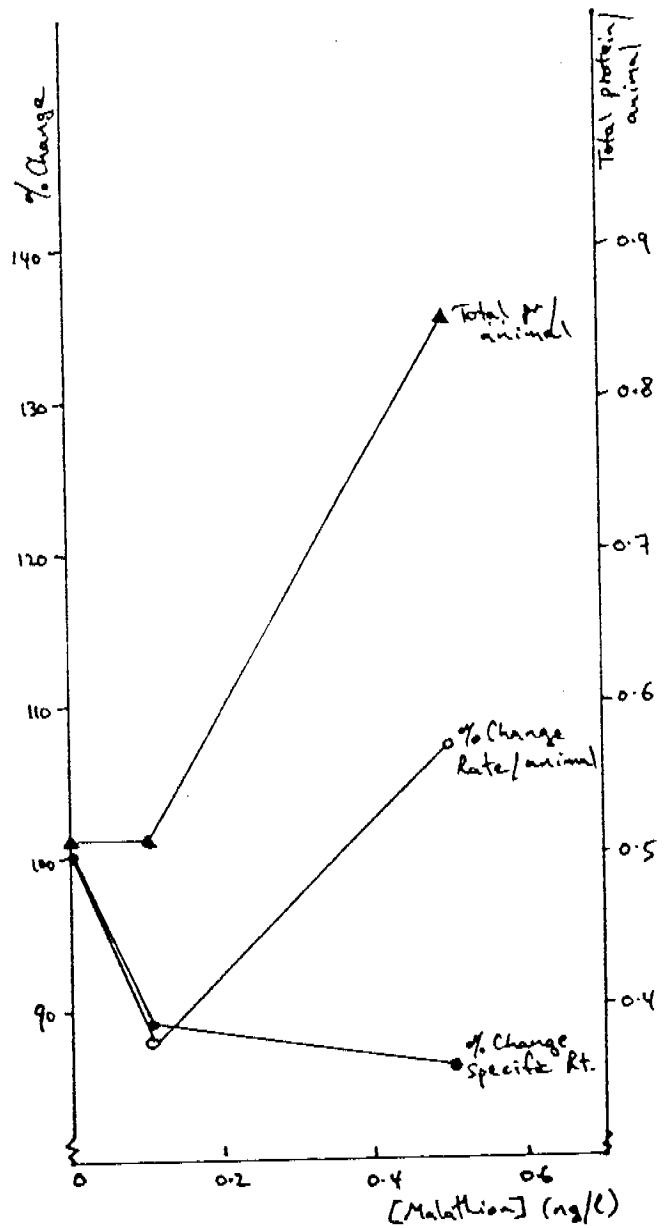


Figure 16. Effect of PCP on GST activity in Green hydra (24 h Test)

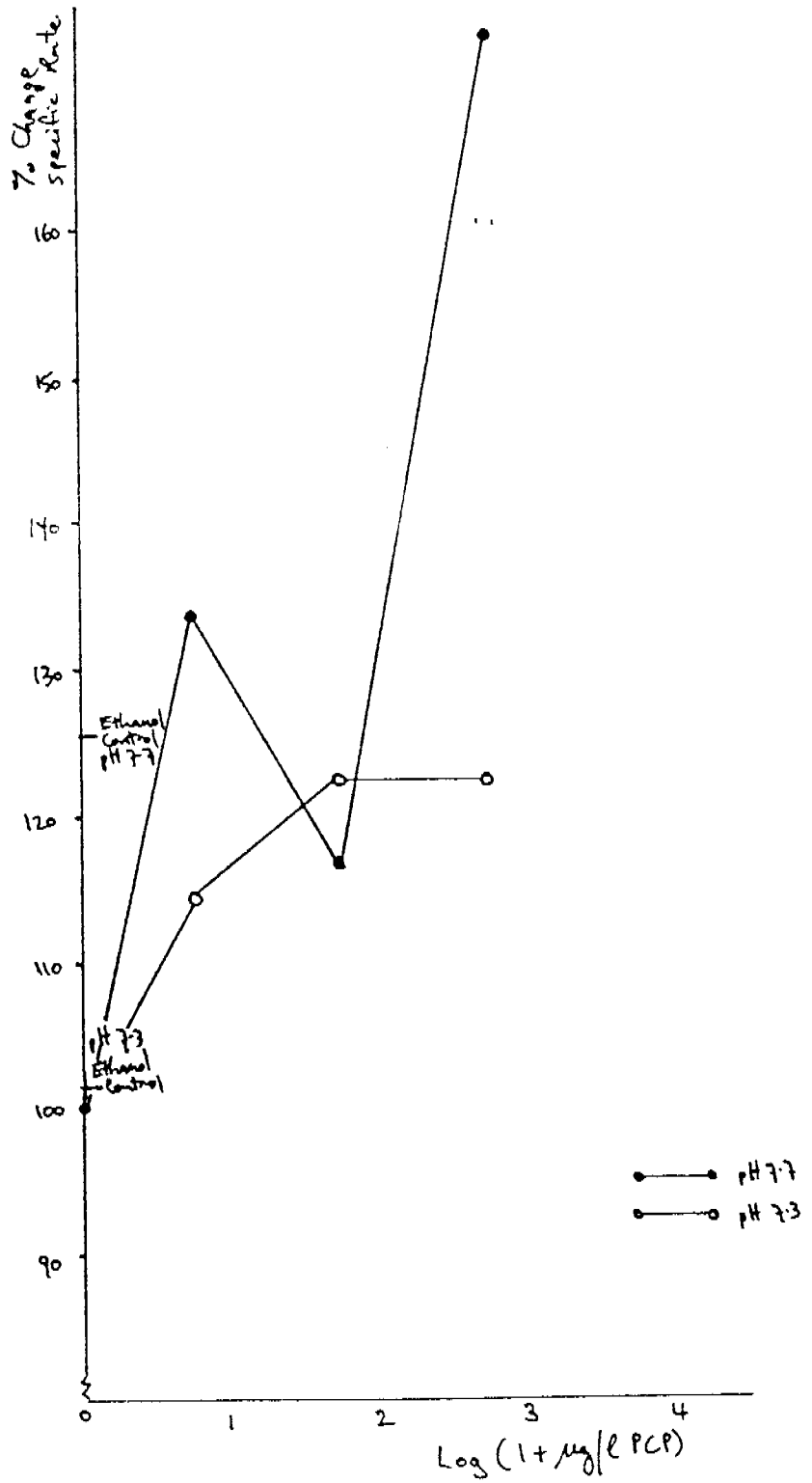


Figure 17. Effect of PCP on GST activity in Green hydra (48h Test)

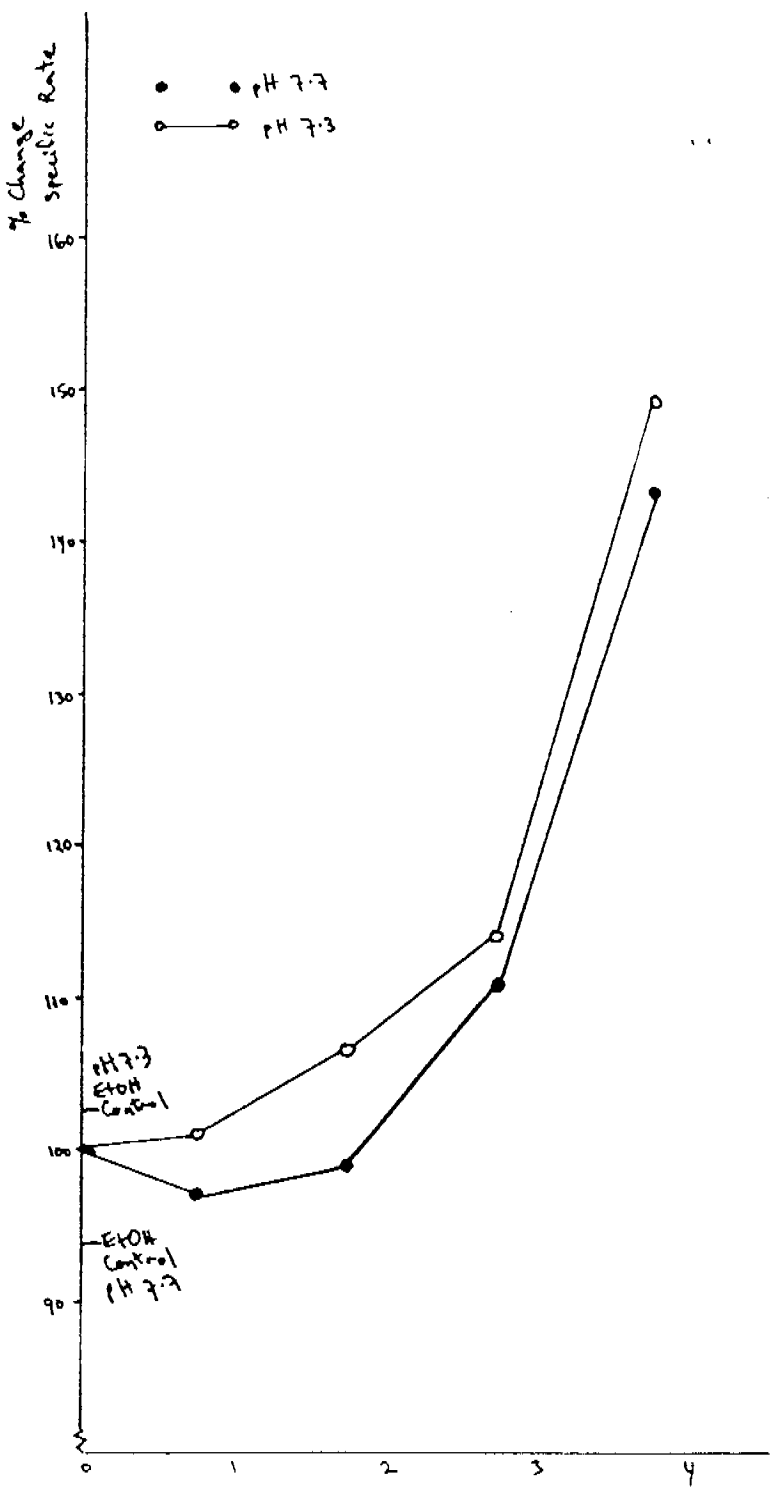


Figure 18. Effect of BHT on GST activity in Green hydra (24h Test)

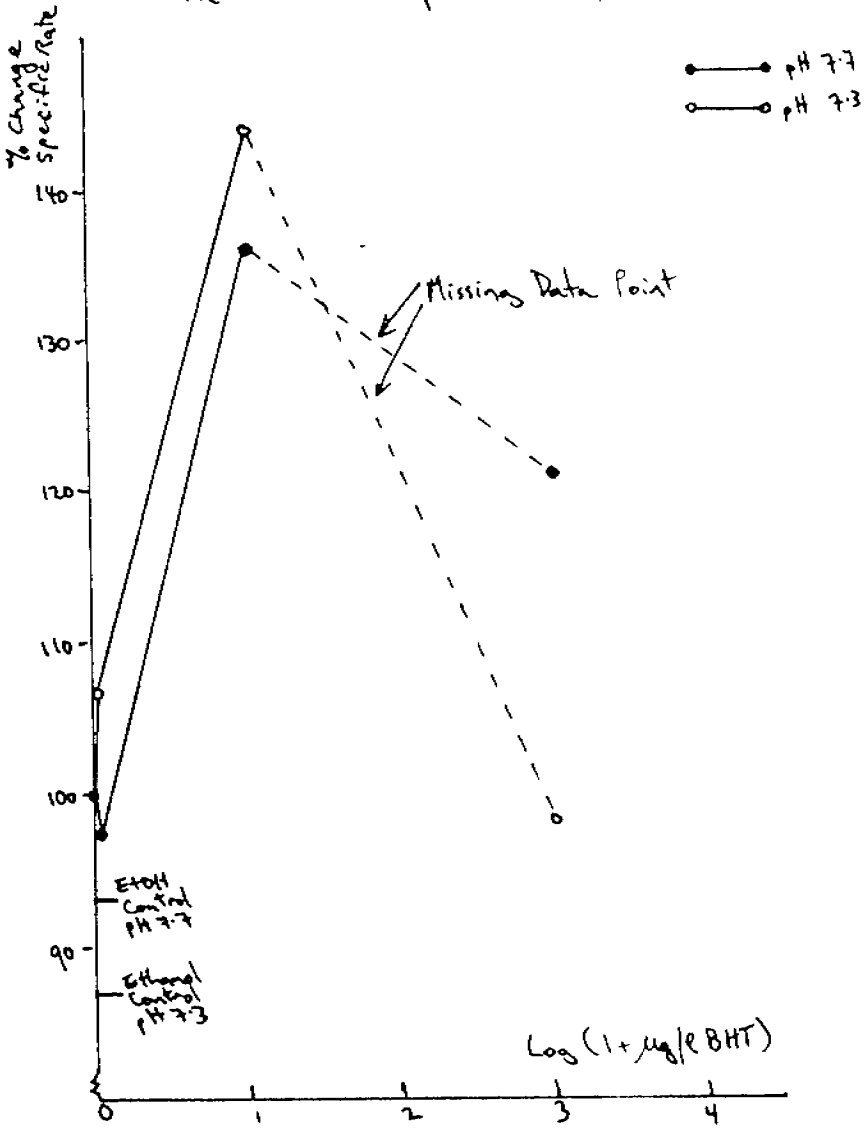


Figure 19. Effect of Malathion on GST activity in Green hydra. (24h Test)

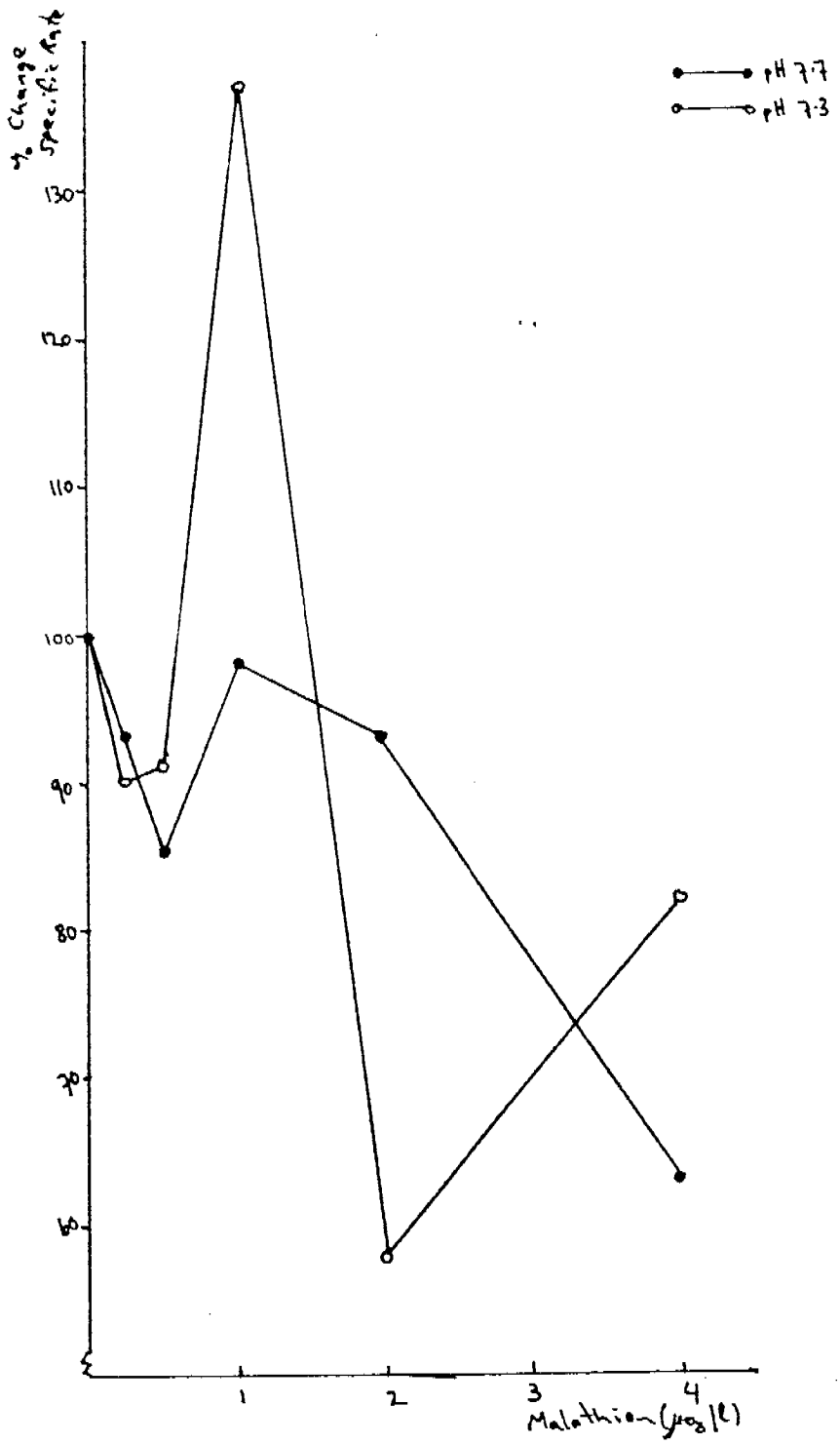


Figure 20. Effect of PCP on GST activity in Pink hydra (24h Test)

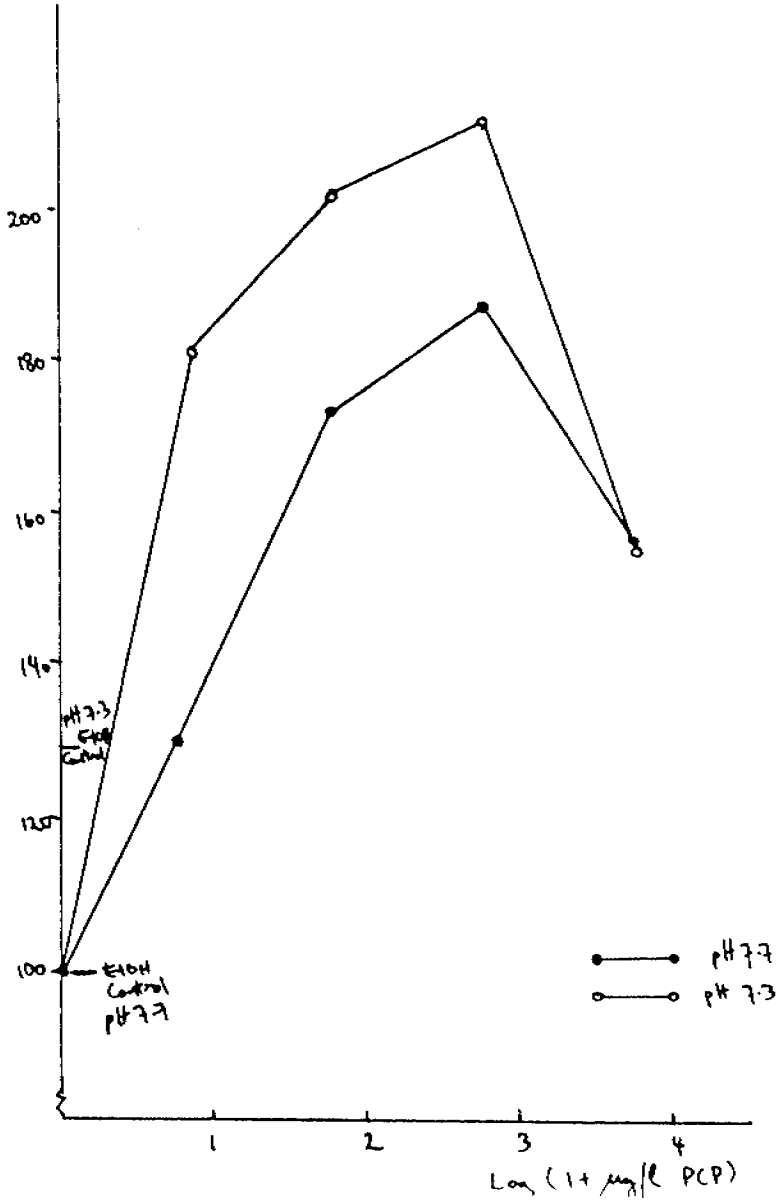


Figure 21. Effect of BHT on GST activity in Pink hydra (24h Test)

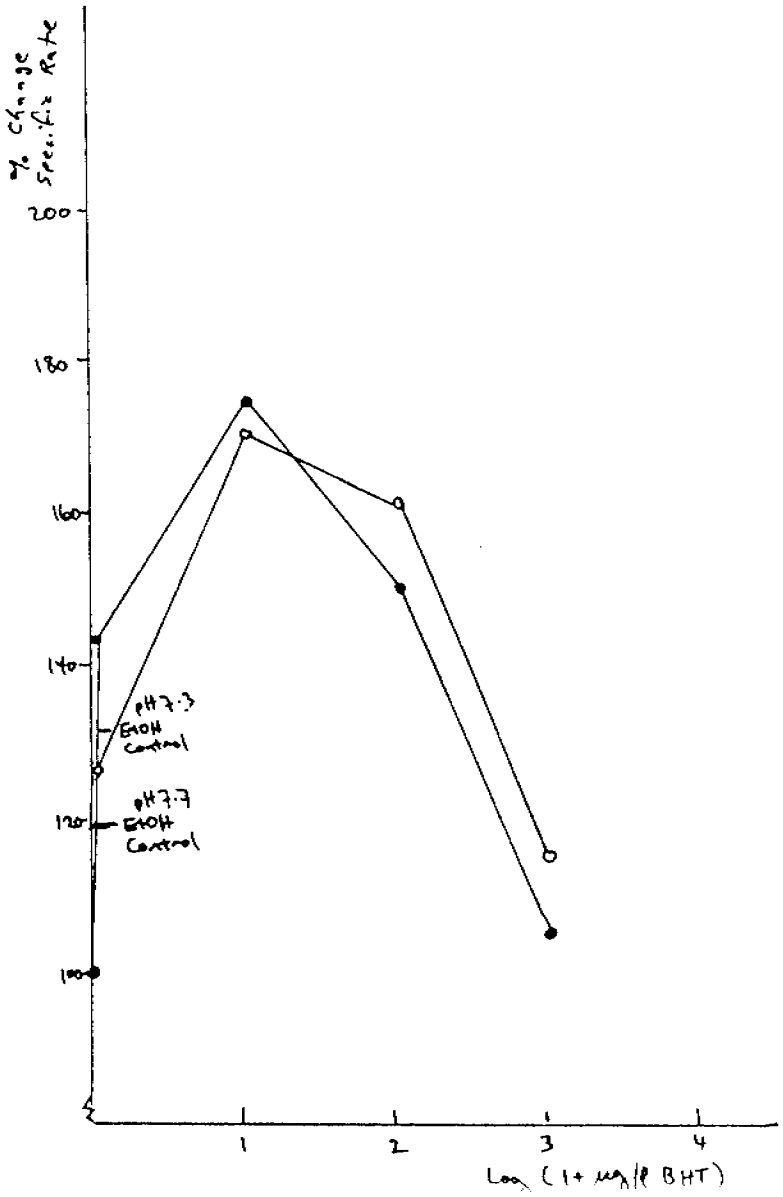
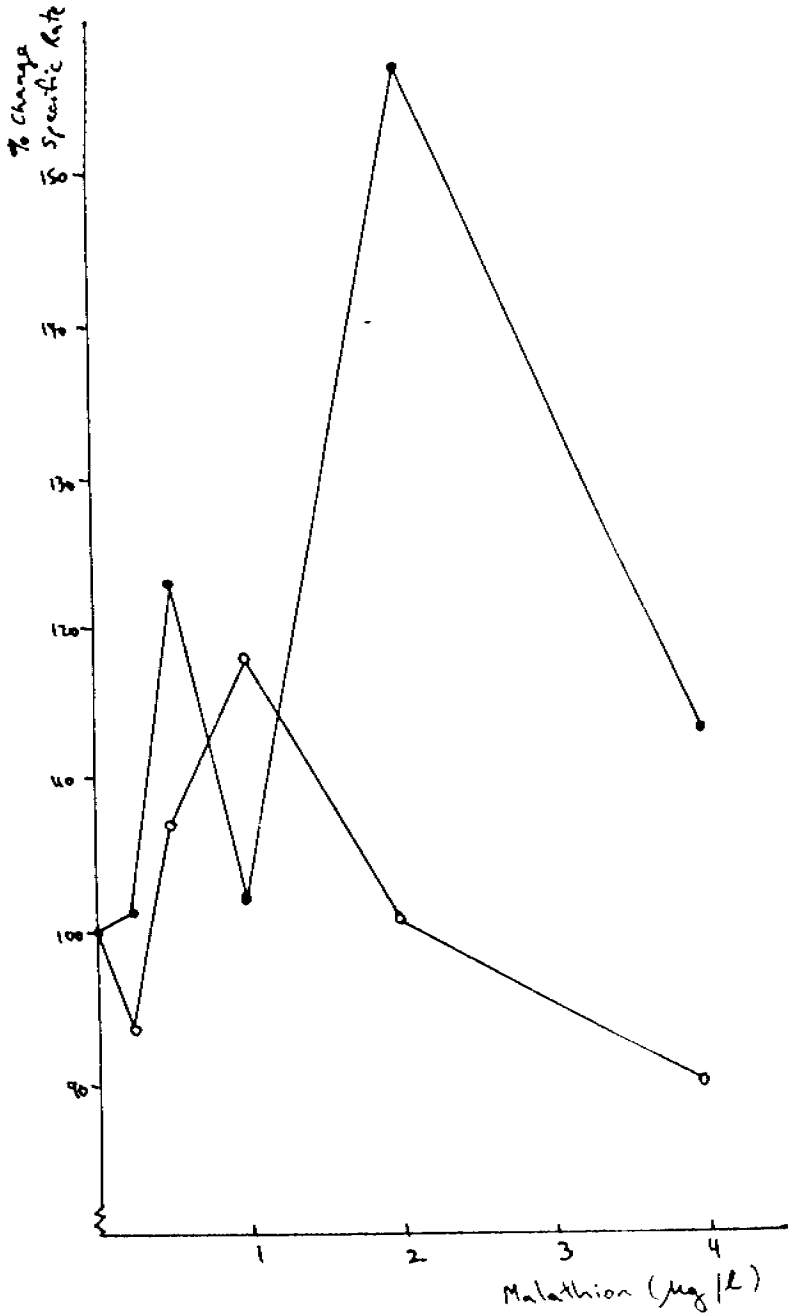


Figure 22. Effect of Malathion on GST activity in Pink hydra (24h Test)



Effect of PCP, BHT and malathion on pink hydra GST activity

PCP appeared to have a marked effect on specific rates irrespective of *in vitro* pH (Figures 20). Specific rate appeared to reach a peak at 11μg/l and then begin to fall.

Figure 21 shows that there may be an effect of BHT on specific rate at 1μg/l when the *in vitro* pH is 7.7. There is a clear effect irrespective of pH at 10μg/l at which concentration the specific rates peak. The rates appear to plateau and then fall at the higher concentrations.

The data for malathion is extremely difficult to interpret (Figure 21) different responses for each of the *in vitro* pHs. The data, like that for the other animals, appears to be suppressed except for a curious elevated value for pH 7.7 at 2ng/l. More data is needed to assess whether this high specific activity value is just random fluctuation.

6. Preliminary studies on GST activity in mussels

Twelve mussels were collected from Buffalo Billabong on 15/11/89. One was dissected on the 16/11/89 (Mussel 1), five on the 17/11/89 (Mussels 2 - 6) and the rest on the 23/11/89 (Mussels 7 - 12). Tissues dissected from Mussel 1 were kept on ice and then homogenised with cold potassium phosphate buffer (pH 7.3) in a 15ml all glass homogeniser. About 4ml of buffer was needed for each gram of tissue. Some tougher tissues had to be diced before homogenising (*e.g.* foot, mantle, visceral mass). Enzyme was prepared as described in Section 2.2 and stored at -80°C. The tissues dissected out were the digestive gland, gill, palps, visceral mass, anterior and posterior abductor muscles, foot, mantle, and the 'rest' which consisted of the heart, kidney, and aorta. The rectum was not taken with the 'rest' but discarded. Also, some of the granular substance found around the digestive gland and between the foot and the visceral mass was isolated and collected from Mussels 2 - 12. All tissues from Mussels 2 - 12 were placed in Eppendorf vials and immediately frozen in liquid nitrogen. These tissues have been stored at -80°C for subsequent GST and cytochrome P-450 assay.

Mr M. Mannion routinely samples caged mussels in RP4 and I have started to dissect and store the tissues as above for subsequent assay. So far, only one has been collected (7/2/90) and dissected (RP4 Mussel 1 on 8/2/90).

GST rates were determined for Mussel 1 tissues on two different occasions (20/11/89 and 1/12/89). On the first occasion, the enzyme was thawed, used and then refrozen (having been kept on ice after thawing) since a considerable amount remained for most tissues. Rates were determined over a range of protein titres at an *in vitro* pH of 7.3. After a pH check on digestive gland, pH 7.3 appeared to be suboptimal and 7.6 better (Figure 23a). Therefore, on the second occasion, rates were determined for pH 7.6 over a range of protein titres. A comparison was also made of rates at an *in vitro* pH of 7.3 for 50μl of enzyme. This last comparison provided a check on whether the repeated freezing/thawing caused a deterioration in rates.

On the first occasion, all tissues assayed appeared to have GST activity but with only half the tissues showing a correlation of GST rate with protein (Figure 23 b - i). The highest specific activity was in digestive gland with most other tissues also having a moderate specific activity. Palps and gill had low specific activity.

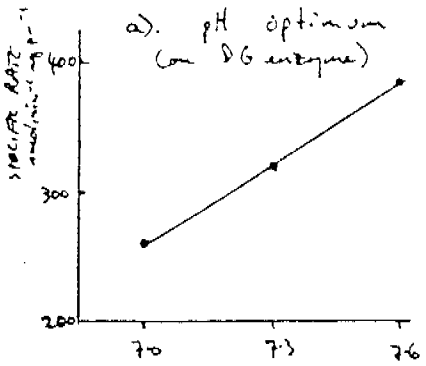
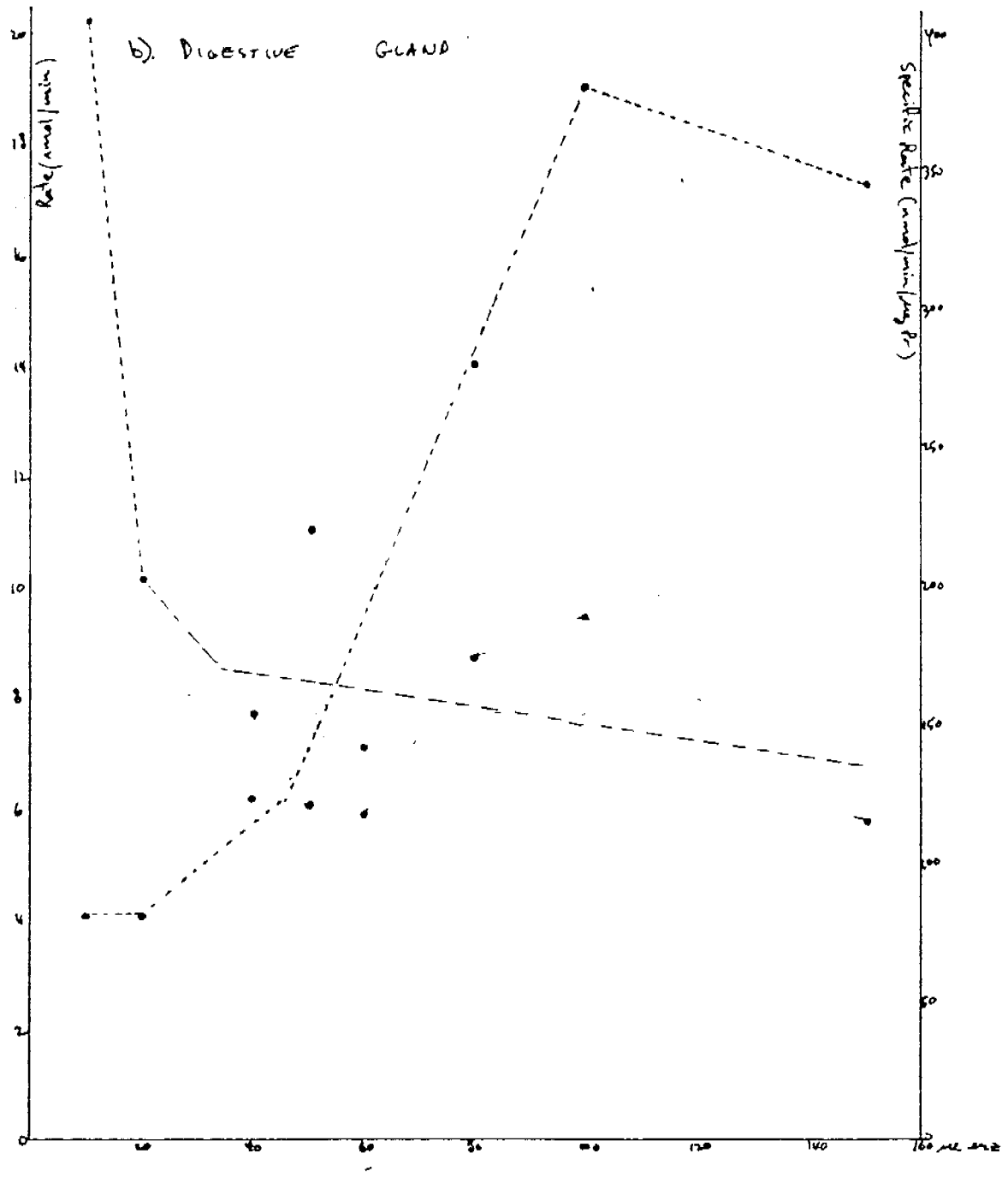
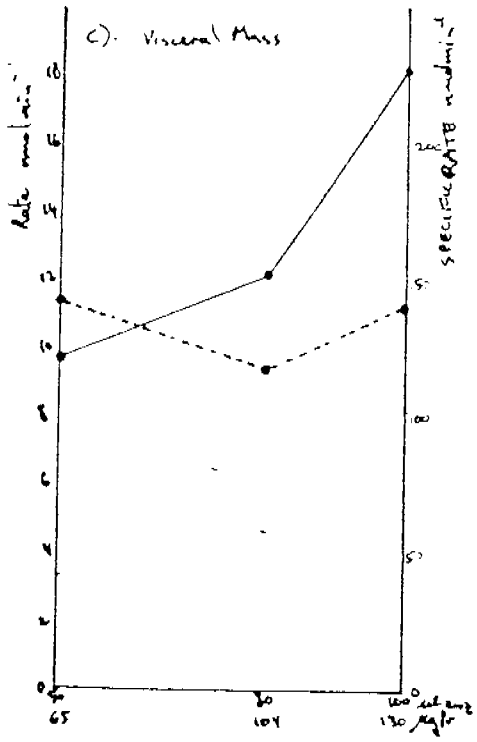
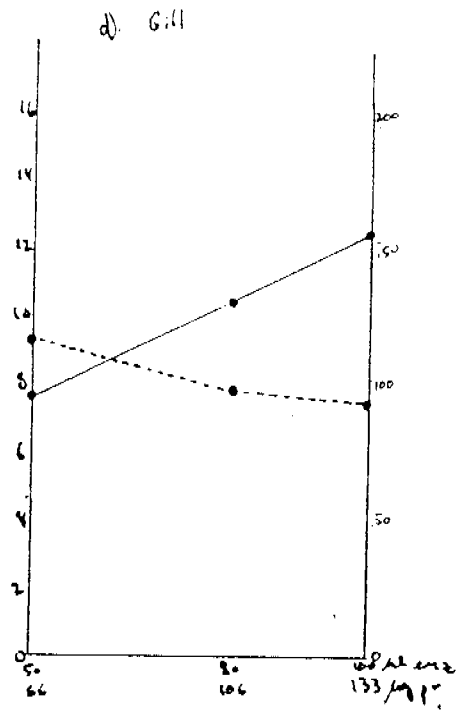


Figure 23. GST activity in Tissues of a freshwater Mussel.

(Fig 23 c) - i) over page)







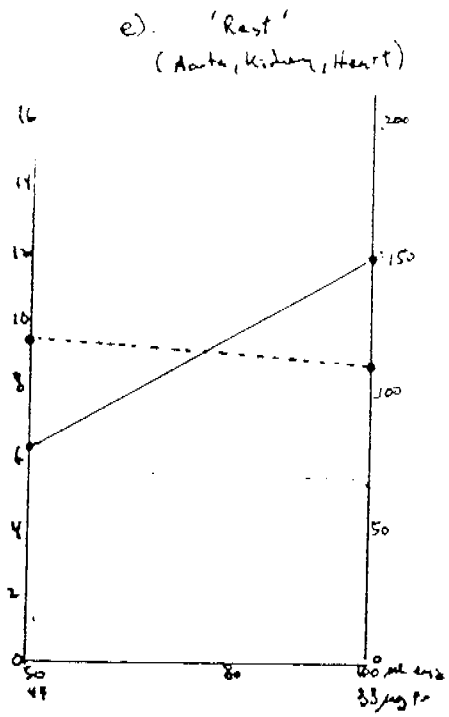
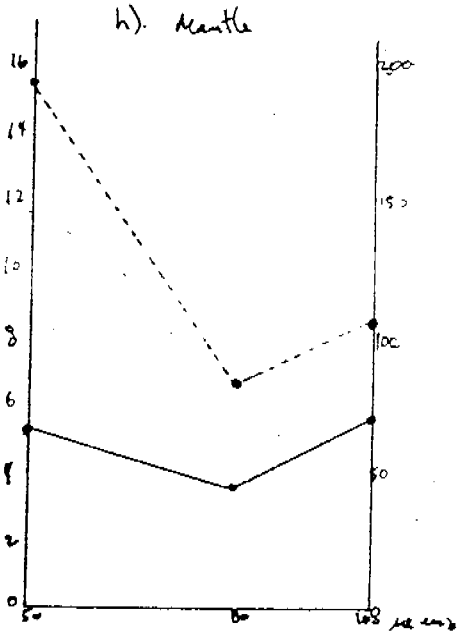
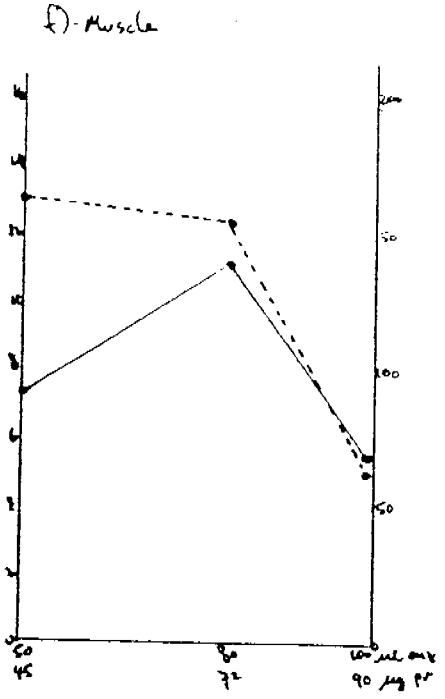
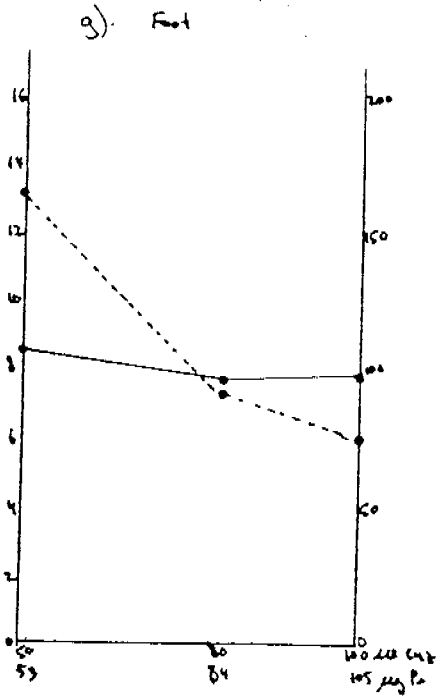
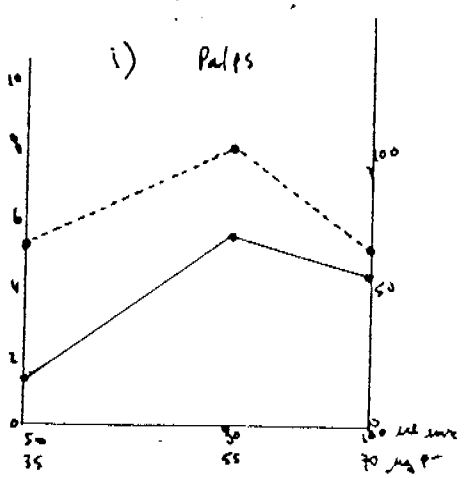


Figure 23 continued.









GST activity in a freshwater mussel tissues after repeated freezing thawing.

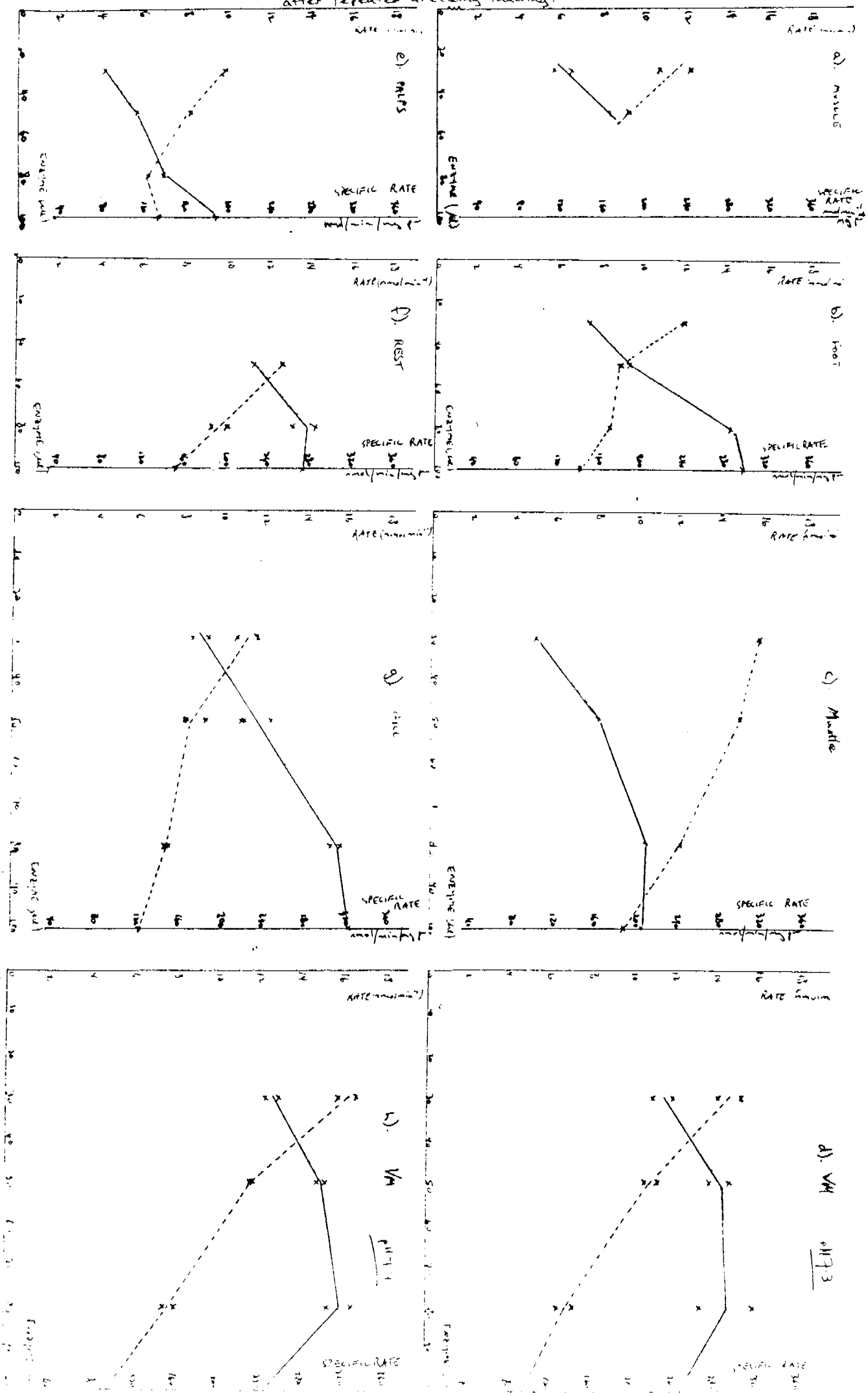


TABLE 7 : Comparison of GST specific rates at different *in vitro* pHs for various tissues of the freshwater mussel[a]

	7.3[b]	7.3[c]	7.6[c]
	Specific Rate (nmol/min/μg pr)		
Mantle	192	230	299
'Rest'[d]	142	193	255
Visceral Mass	142	220	232
Muscle	162	203	185
Foot	163	185	181
Gill	115	150	172
Palps	40	138	164

a Comparison made on 50μl of enzyme
b Assayed on 20/11/89 after freezing and the thawing
c Assayed on 1/12/89 after 2 cycles of freezing and the thawing
d The 'rest' consists of the heart, kidney and aorta

There was an unexpected increase in GST specific activity on the second occasion despite the repeated freezing/thawing (Table 7). Further, it confirms that an *in vitro* pH of 7.6 is optimal for most tissues. The exceptions may be muscle and foot. The correlation of GST rate with protein titre seemed to improve in most tissues, especially foot and palps (Figure 24 a - f). Visceral mass GST rates appeared to reach a plateau irrespective of *in vitro* pH (Figure 24 g and h).

7. Preliminary studies on liver cytochrome P-450 activity in black bream

Cytochrome P-450 activity was determined for black bream liver using the ethoxyresorufin O-deethylation assay (EROD) modified from Burke and Mayer (1974)[5].

Black bream liver was obtained fresh on dissection (courtesy of Biological Monitoring) on the 20/9/89. All subsequent steps until fluometric assay were carried out on ice. 1g of the liver was homogenised in 1 ml of homogenising buffer. Homogenising buffer was made with 38.02mg EDTA, 15.42mg dithiothritol, 1.982mg phenanthroline and 100mg trypsin inhibitor made up in 100ml of 100mM potassium phosphate buffer, pH 7.4. The homogenate was centrifuged for 5min at 1000g to remove cellular debris. The supernatant was removed to a clean vial and spun at 12000g for 20min at 4°C. The supernatant from the 12000g spin was used as the enzyme source. Because of the soluble contaminants of the 12000g and cytochrome P-450 requirement of NADPH, a NADPH-generating system was included in the assay. This is obtained by making

5. Burke, M. D. and R. T. Mayer (1974). Ethoxyresorufin: Direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metabolism and Disposition 2, 583 - 588.

up fresh 3.5mg NADPH, 7mg glucose-6-phosphate, 50μg of 10I.U. glucose-6-phosphate dehydrogenase and 10mg $MgCl_2$ in 1ml of 100mM potassium phosphate buffer, pH 7.4. The assay reaction mixture consisted of 100μl of enzyme, 445μl of 100mM potassium phosphate buffer, pH 7.4, 5μl of 50μM ethoxyresorufin (with 1.25% w/v Tween 80) and 100μl of NADPH-generating system.

The blank was 100μl of enzyme plus 650μl of potassium phosphate buffer, pH 7.4.

On addition of the NADPH-generating system, the assay reaction mixture was vigorously mixed and aspirated into a Perkin-Elmer LS-30 Luminescence Spectrometer. The excitation wavelength was set at 530nm and the spectrometer recorded emission scans over 530 - 610nm at 5min intervals through a 30min period. Temperature was uncontrolled but probably between 25 - 30°C due to machine heat.

Figure 25 shows the development of the product (resorufin) peak at 580nm and the diminishing substrate (ethoxyresorufin) peak at 554nm. Peak height of the substrate peak and the difference height between the maximum and minimum for the product (both as a percentage of the 10min substrate peak height) were plotted versus time (Figure 26). Although only a guide of the rate of *in vitro* product clearance and substrate formation, it illustrates a linear rate of removal of the product and a partly linear rate of formation for the product. Product formation appears to reach an upper limit and then gradually fall. Since the product alone appears stable over a similar period (Figure 27), product formation perhaps reflects enzyme saturation and then some further binding of the product by other cellular compounds (possibly soluble compounds like endogenous GSH). The continual disappearance of substrate, which also appears to be stable for this amount of time (Figure 28) seems to argue that there are other pathways for ethoxyresorufin catabolism.

Calculation of the rate of formation of product using the 5 and 10min values gives a rate of 12pmol/min. But it should be noted that this is arrived from only one time interval and a change of only 5mm in peak height. Attention should be paid to improving the sensitivity of the assay by obtaining a better enzyme preparation with the ultracentrifuge.

8. GST activity in green hydra and water flea from routine bioassays of RP4 toxicity testing

Regular assaying of animals used in our laboratory's routine bioassay toxicity testing has been started. This will provide baseline data which can be used for future reference. Therefore, some future experiments will investigate the response in GST rates in animals exposed to toxicants, including RP4 water, in the short- and long-term toxicity tests. If a relationship exists, then the enzyme assay could be used to give an early warning of the nature and magnitude of toxicity. Tables 8 - 10 are therefore included here for such future reference.

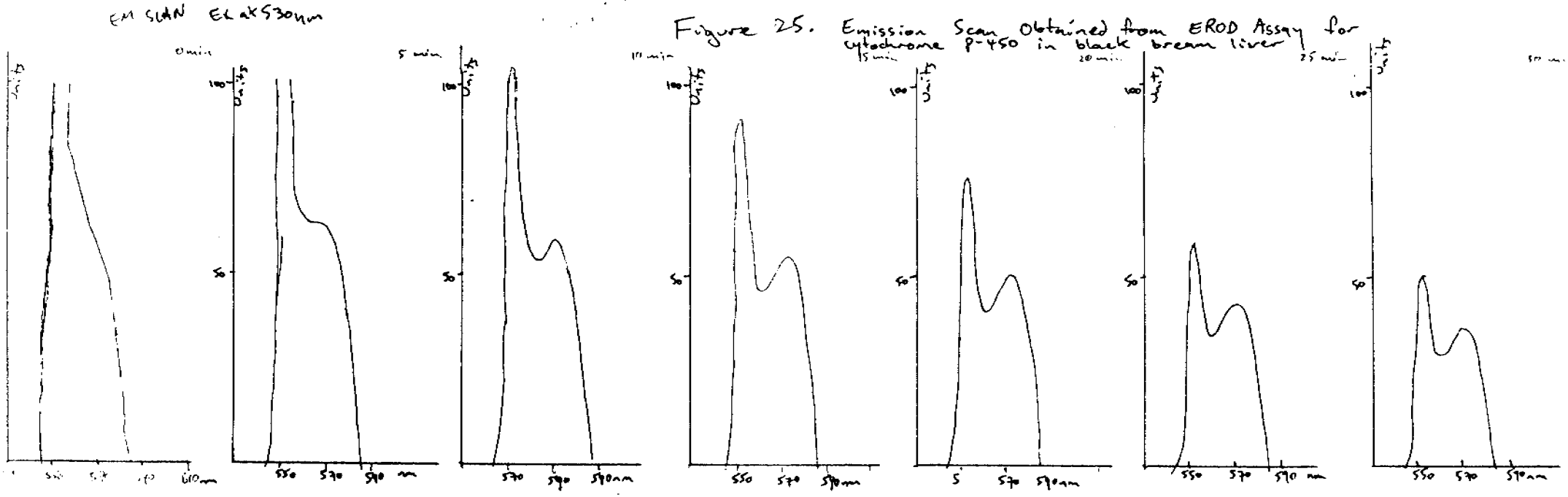


Figure 25. Emission Scan Obtained from EROD Assay for cytochrome p-450 in black bream liver

Figure 26. Product clearance/formation & substrate clearance in the EROD Assay

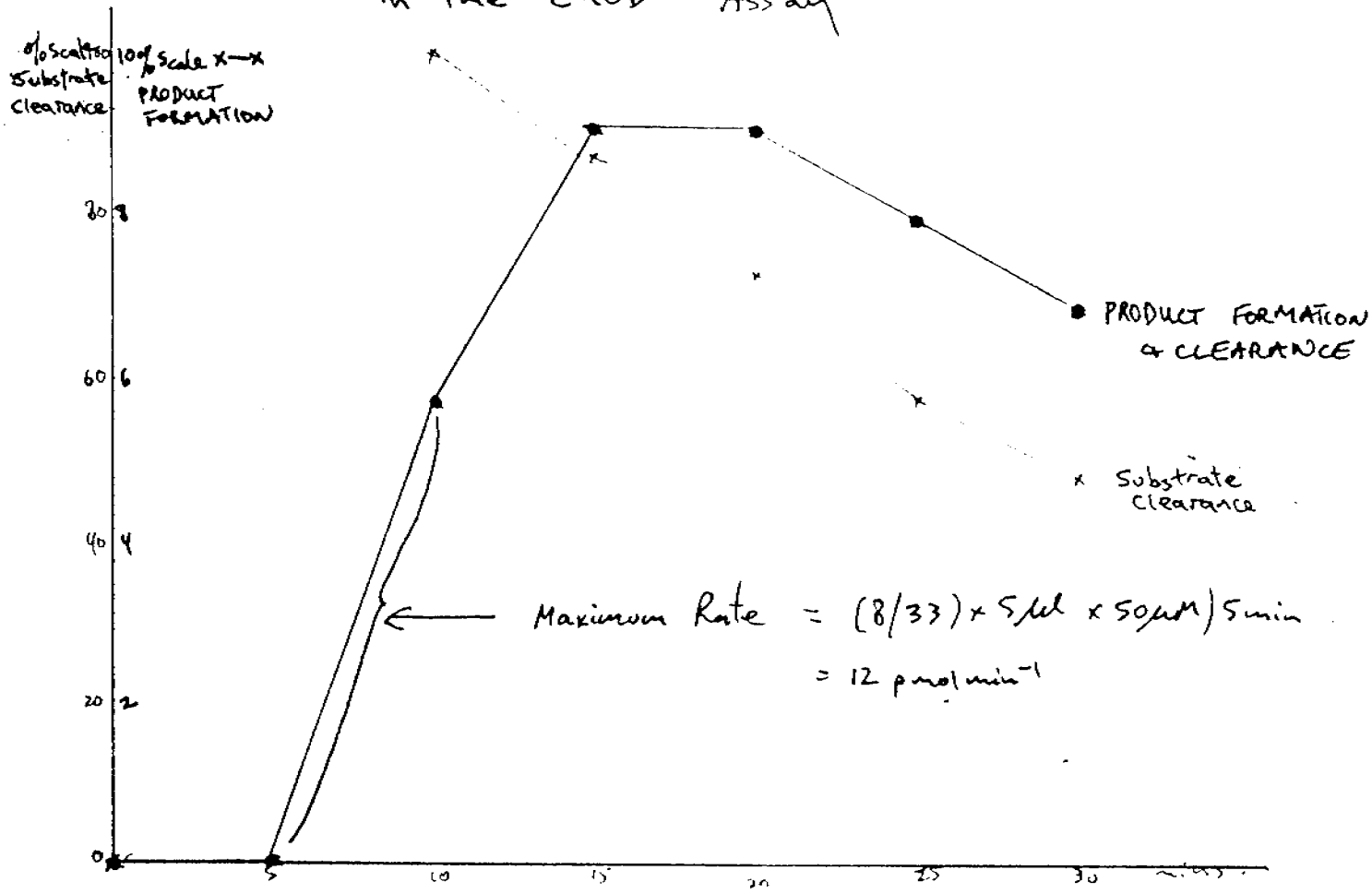
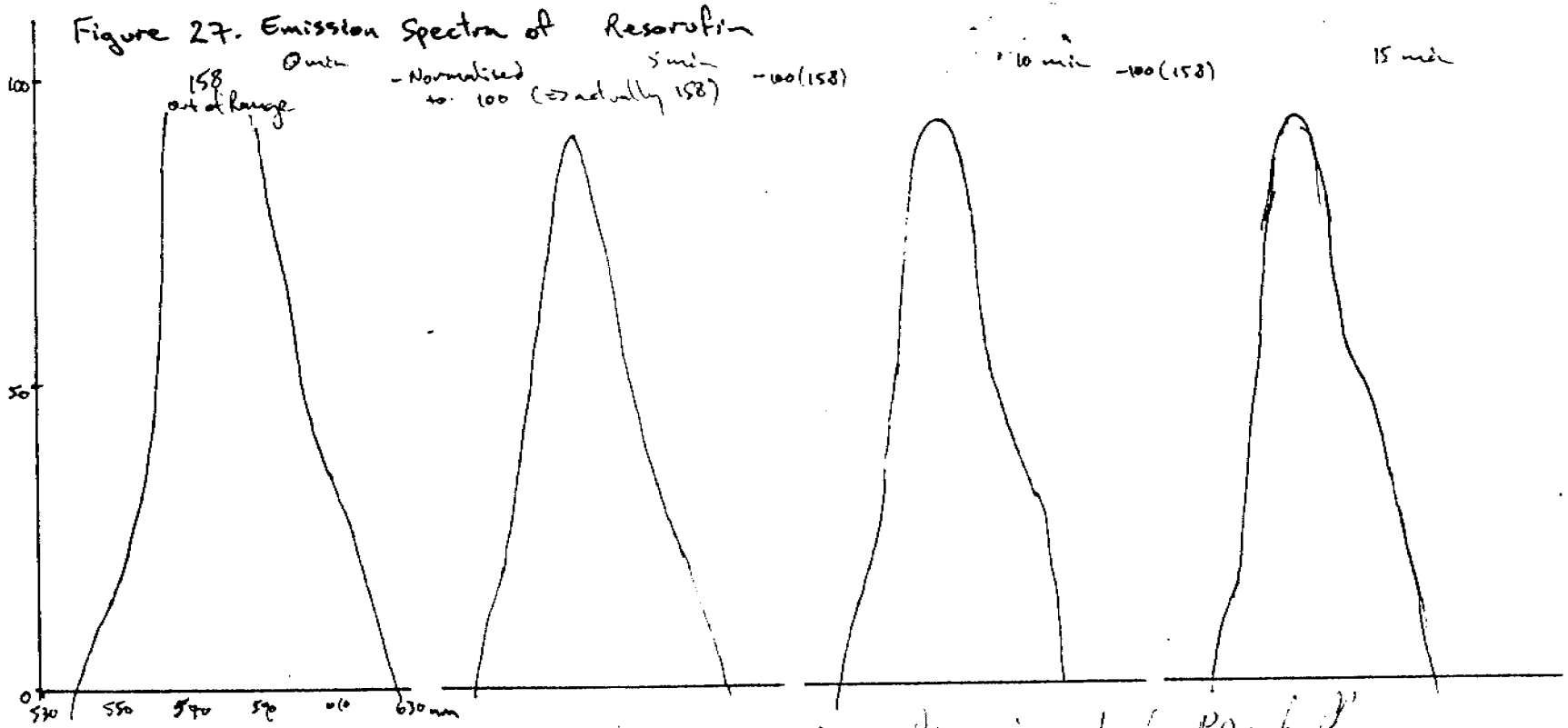
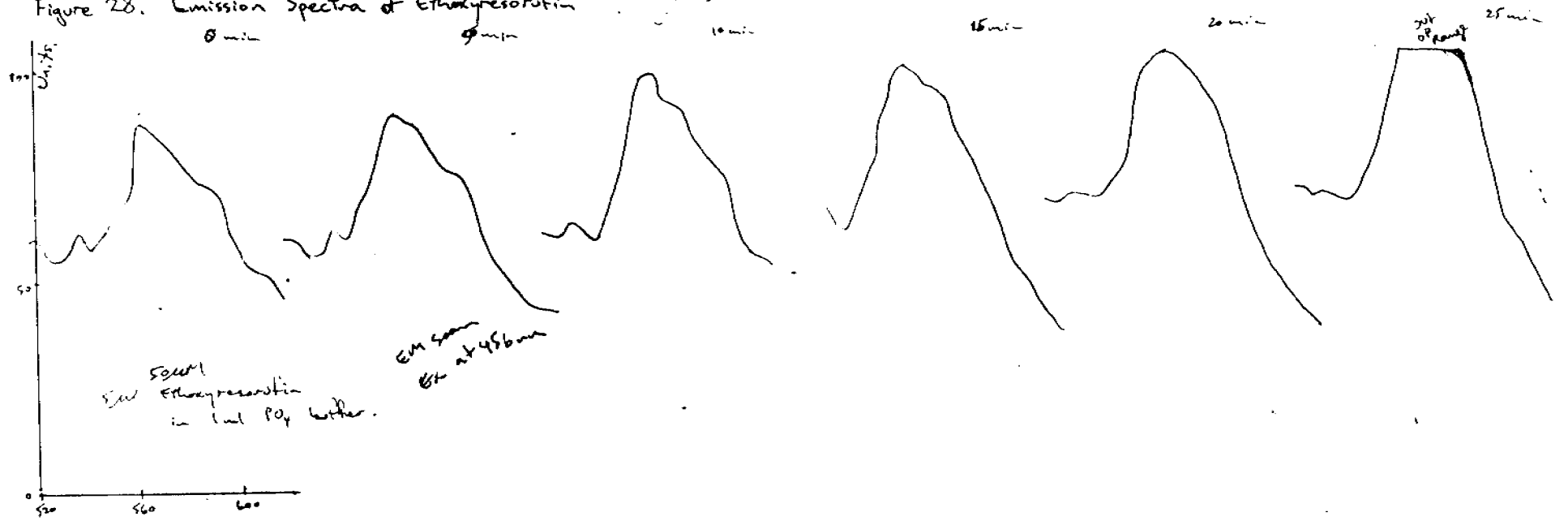


Figure 27. Emission Spectra of Resorufin



5μL of 53μM Resorufin in 1ml PO4 buffer showing stability of peak.

Figure 28. Emission Spectra of Ethoxyresorufin



5 ml 5 μM Ethoxyresorufin in 1 ml PO_4 buffer.

TABLE 8 : Data for Experiment 29 (14/12/89) on green hydra using RP4 water[a]

Treatment ID	A	B	C	D	E	F	G
Descriptor	Control	T1 3.2%	T2 3.2%	T3 3.2%	T1 10%	T2 10%	T3 10%
pH 7.3							
Specific Rate[b]	1977	541	455	495	585	519	449
(%Δ)[c]		(27)	(23)	(25)	(30)	(26)	(23)
pH 7.6							
Specific Rate[b]	2157	521	542	465	540	430	508
(%Δ)[c]		(24)	(25)	(22)	(25)	(20)	(24)

a computer code : RP4-5
b nmol/min/mg protein
c The change in rate of treatment compared with control expressed as a percentage

TABLE 9 : Data for Experiment 44 (22/1/90) on water flea using RP4 water[a]

Treatment ID	A	B	C	D	E	F
Descriptor	Control	1% RP4	C18[b]	DIOL[b]	CM[b]	NH2[b]
Specific Rate[c]	209	137	211	162	183	154
(%Δ)[d]		(66)	(101)	(78)	(88)	(74)
Protein/animal[e]	1.550	1.427	0.953	1.650	1.704	1.624
Rate/animal[f]	204	144	149	198	231	185
(%Δ)[d]		(71)	(73)	(97)	(113)	(91)

a computer code : SEP-P1
b 1% RP4 with different treatments
c nmol/min/mg protein
d The change in rate of treatment compared with control expressed as a percentage
e μg protein/animal
f pmol/min/animal

TABLE 10 : Data for Experiment 47 (29/1/90) on water flea using RP4 water[a]

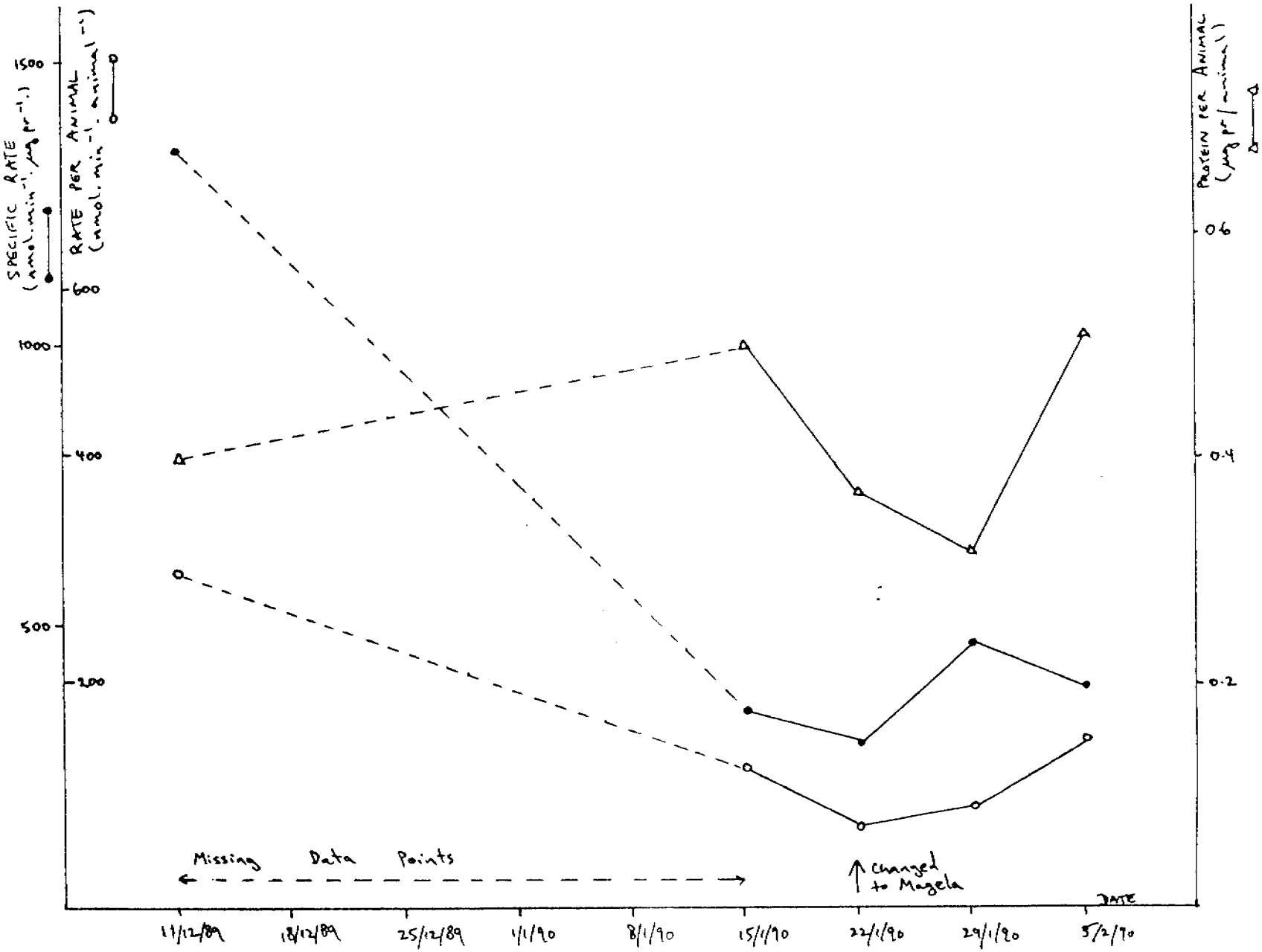
Treatment ID Descriptor	A Control	B NH2	C NaCl	D NaHCO3	E 1%RP4	F 10%RP4
Specific Rate[b]	224	175	201	202	181	183
(%Δ)[c]		(78)	(90)	(90)	(81)	(82)
Protein/animal[d]	2.679	2.139	2.039	2.159	2.052	2.076
Rate/animal[e]	401	278	303	323	275	281
(%Δ)[d]		(69)	(76)	(81)	(69)	(70)

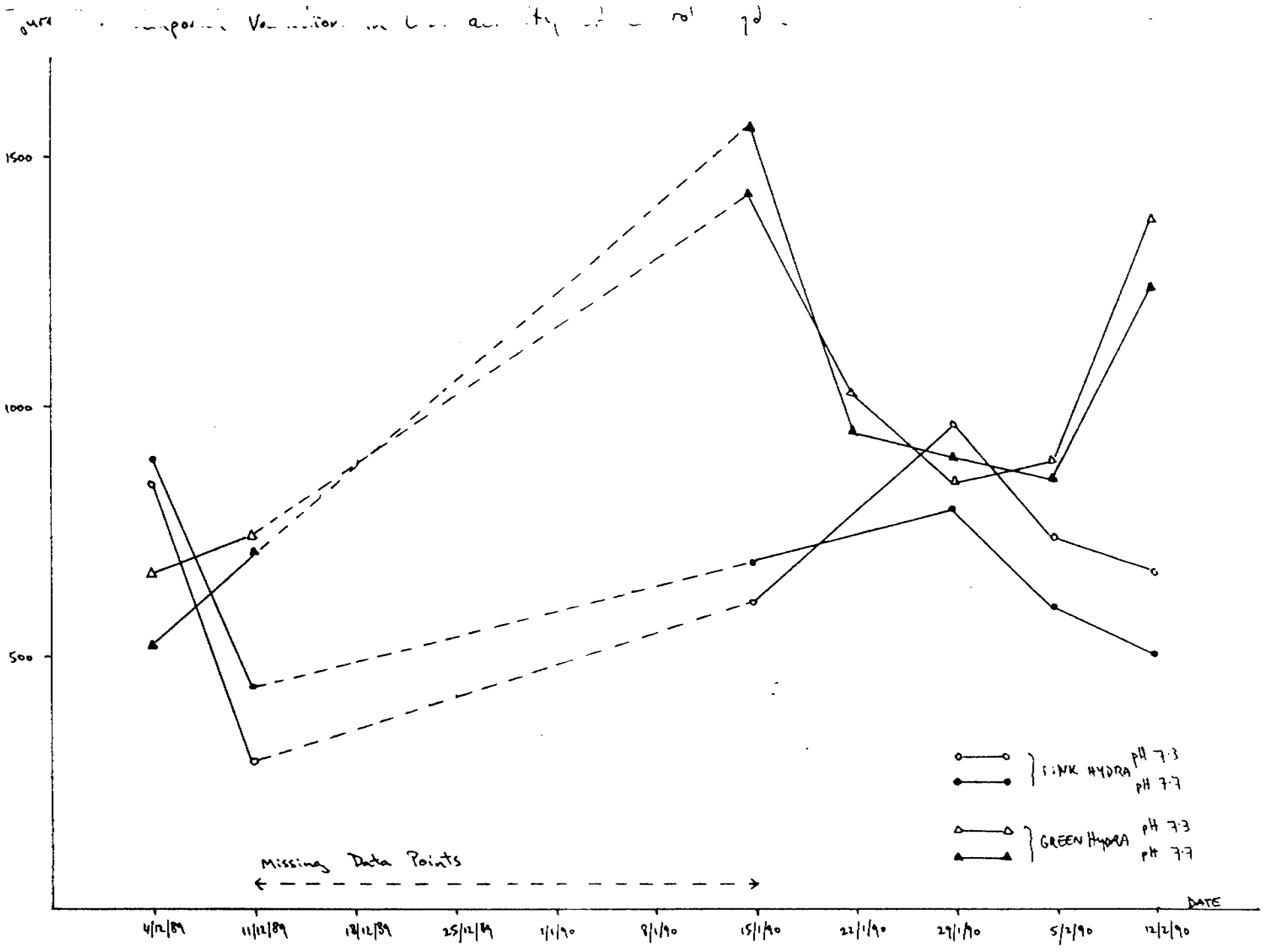
a computer code : NH2E1
b nmol/min/mg protein
c The change in rate of treatment compared with control expressed as a percentage
d μg protein/animal
e pmol/min/animal

9. Temporal GST activity of control animals

Quality of control water varies through the year because of seasonal changes in precipitation and evaporation rates. This may effect GST activity because of associated organic material breakdown with a release of organic compounds that might induce GST. Therefore, GST activity from control animals were plotted against the date of the collection of water (Figures 29 and 30). There is not enough data as yet to determine whether changes in GST activity reflect any temporal cycle.

Figure 29. Temporal Variation in GST activity of Control water fleas.




1500
1000
500
Missing Data Points
PINK HYDRA pH 7·3
pH 7·7
GREEN HYDRA pH 7·3
pH 7·7
DATE
4/12/89
11/12/89
18/12/89
25/12/89
1/1/90
8/1/90
15/1/90
22/1/90
29/1/90
5/2/90
12/2/90

10. Appendices

Appendix 1: Initial preparation protocol

Preparation in Biochemistry Lab

1. Turn on centrifuge.

2. Prepare ice bucket with crushed ice and vial block.

3. Put buffer bottle (pH = 7.3) on ice.

4. Label appropriate number of vials with ID for centrifuging.
   e.g. *A, B, C, ... F.*

5. Label appropriate number of vials with ID for storage of enzyme at -80^{o}C.

   i.e.Species ID, date, toxicant, Sample ID
   where GH = green hydra, PH = pink hydra, and
   WF = water flea (*Moinodaphnia macleayi*), and
   Sample ID is usually A, B, ... F (control to highest concentration of toxicant).

6. Label appropriate number of plastic disposable cuvettes with ID for storage at 4 ^{o}C (for future protein determination - see "Enzyme preparation protocol").

   i.e.Species ID, date, volume, Sample ID
   where GH = green hydra, PH = pink hydra, and
   WF = water flea (*Moinodaphnia macleayi*), and
   Sample ID is usually A, B, ... F (control to highest concentration of toxicant), and
   volume is the amount of supernatant (in μl) taken for protein determination (see "Enzyme preparation protocol").

7. Put all vials in vial block to cool down.

8. Take in 200 - 1000μl pipette (+ tip) and ice bucket into microscope room.

NB: Items 5 and 6 above can be done while the centrifuge is running (see "Enzyme preparation protocol").

Appendix 2: Enzyme preparation protocol for water flea

1. Count and separate animals:

   Adult water fleas are decanted into a petri dish and counted on a white background. They are pipetted from the petri dish onto filter paper (Whatman Grade 1, 9cm diam.) in a buchner funnel/flask water vacuum set-up and a gentle suction applied. One sample of 400ml needs to be broken up into a series of about 5 subsamples with the fleas being pooled on the filter paper again..

2. Rinse fleas with control water leaving them slightly wet.

3. Transfer fleas to a 3ml glass homogenising vessel with 270μl of cold buffer (pH 7.3):

   Pick the water fleas up as a group with fine forceps. This works better when the fleas are still slightly wet as the surface tension of the water "glues" them together but a balance between too dry and too wet needs to be reached. Transfer the water flea mass to the tip of the homogeniser pestle and then place pestle in the homogeniser vessel.

4. Homogenise on ice with 10 grinding strokes of the pestle.

5. Pipette homogenate into prepared vial (Item 4, "Preparation in Biochemistry Lab" sheet).

6. Repeat 1 to 5 for each sample.

7. Spin samples (as one group in one run) at 10000g for 10 min (centrifuge method No. 3)

8. Pipette off as much supernatant (enzyme) as possible into prepared vials (Item 5, "Preparation in Biochemistry Lab" sheet). STORE at -80^{o}C.

9. Pipette a 50μl aliquot of supernatant into prepared cuvettes (Item 6, "Preparation in Biochemistry Lab" sheet). Cover cuvettes with parafilm and STORE at 4^{o}C.

Appendix 3: Enzyme preparation protocol for hydra

1. Count animals and place in 3ml glass homogenising vessel: Animals are counted using the microscope on low power.

2. Pipette hydra into the homogenising vessel.

3. Pipette excess water off.

4. Rinse with cold buffer (pH 7.3) and then remove excess:

   Pipetting will remove most of the buffer but a combination of decanting/pipetting/draining will remove the rest. It might also be necessary to roll up a filter paper and blot the inside of the top of the homogenising vessel to remove some buffer.

5. Add 360μl of cold buffer (pH 7.3).

6. Homogenise on ice with 10 grinding strokes of the pestle.

7. Pipette homogenate into prepared vial (Item 4, "Preparation in Biochemistry Lab" sheet).

8. Repeat 1 to 7 for each sample.

9. Spin samples (as one group in one run) at 10000g for 10 min (centrifuge method No. 3)

10. Pipette off as much supernatant (enzyme) as possible into prepared vials (Item 5, "Preparation in Biochemistry Lab" sheet). STORE at -80^{O}C.

11. Pipette a 50μl aliquot of supernatant into prepared cuvettes (Item 6, "Preparation in Biochemistry Lab" sheet). Cover cuvettes with parafilm and STORE at 4^{O}C.

Appendix 4: Determination of pH protocol

AIM :

To determine the optimum in vitro pH for glutathione transferase isolated from Moinodaphnia macleayii.

METHOD :

Homogenising

Isolate 250 adult females from general culture and homogenise in 1.1ml of potassium phosphate buffer, pH 7.3, with this and all subsequent procedures at 4°C. Centrifuge the homogenate at 10000g for 10min. Take the supernatant to use as the enzyme preparation.

Separate the enzyme into six 150µl aliquots (for enzyme assays)in eppendorf tubes and three 50µl aliquots (for protein determinations) in 3ml plastic disposable spectrophotometer cuvettes. Close or cover all enzyme preparations and store as appropriate, either on ice (enzyme assays) or in the fridge (protein determinations).

Enzyme assay preparation

For enzyme assays at different pH's, add 420µl of test potassium phosphate buffer (either pH 6.9, 7.3 or 7.7), 30µl of 1-chloro-2,5-dinitrobenzene, 150µl of glutathione to the enzyme preparation. Agitate the assay mixture and assay immediately.

Controls are made up the same except that the enzyme is replaced with 150µl of potassium phosphate buffer, pH 7.3.

Rate determinations

Rates are measured spectrophotometrically on a Perkin-Elmer UV-vis Lambda 2 spectrophotometer with kinetic capability. Ensure that the water bath is held at 25°C ± 1°C. Background correction is made with water and a rinse with water between runs aids in washing. At the beginning and end of the experiment, wash the line with ethanol and then water. Measure absorbance at 344nm with a cycle time of 0.15min for 15 cycles with the pump off for over the cycling period.

Protein determination

Protein determinations are made with Sigma total protein determination kit (No. 610A) as per kit instructions.

Appendix 5: Determination of pH for brine shrimp naupilii

Brine shrimp naupilii are used as food for hydra and could conceivably contribute to GST activity assayed in hydra close to the time of feeding. The optimum pH for brine shrimp naupilii GST was therefore determined. The results were to be used in the interpretation of data collected on the effect of feeding by hydra on GST activity (Section 4.2 and 4.3).

3ml of brine shrimp naupilii were separated from water by filtration and then homogenised in 1ml of cold potassium phosphate buffer, pH 7.3. The procedure described in Appendix 4 was then followed.

Results are shown in Table 11.

TABLE 11 : Effect of pH on GST activity in brine shrimp naupilii

pH	7.0	7.3	7.6	8.0
Specific Rate (nmol/min/μg pr)	1401	1042	1823	1731
	1598	923	1671	1929
	1580	1153	1846	
	1433	1382		
Average	1503	1125	1782	1830
(S.D.)	(100)	(195)	(96)	(140)

Appendix 6: Data tables for figures shown in report

The data used in figures throughout the report are presented in Tables 12 - 22. It is provided for future reference.

TABLE 12[a]: Data for effect of temperature on GST activity

	Time (min)	0	5	10	15	20	25
		Absorbance[b] at 344 nm					
Control[c]	25°C	0.000	0.033	0.049	0.068	0.091	0.127
Treatment[d]	25°C	0.000	0.258	0.434	0.582	0.581	0.582
Control[c]	30°C	0.000	0.153	0.271	0.355	0.415	0.447
Treatment[d]	30°C	0.000	0.158	0.297	0.426	0.517	0.590

a Data for Figure 1
b Absorbance adjusted so all curves start at origin
c Reaction mixture without enzyme
d Reaction mixture with enzyme

TABLE 13[a]: Data for correlation of protein concentration with GST rate

Protein Range 1							
Protein[b,c]	16.02	24.04	32.06	40.07	48.08	56.10	
Rate[d]	2.15	3.99	5.66	7.08	5.07	4.72	
Protein Range 2							
Protein[b,c]	6.38	9.58	12.77	15.96	19.15	22.34	25.54
Rate[d]	3.91	5.21	8.75	7.14	8.23	5.63	10.26

a Data for Figure 2a,b
b μg protein (bovine serum albumin reference)
c Protein extracted at different times
d $nmolmin^{-1}$

TABLE 14[a]: Data for effect of feeding on water flea GST activity

Time[b] (h)	0	4	12	24
		Food Added		
Protein/animal	0.736	0.781	0.651	1.072
(S.D.)[c]	0.088	0.057	0.192	0.148
Rate/animal[d]	63	96	100	137
(S.D.)[c]	18	29	3	23
		Food Not Added		
Protein/animal	0.742	0.569	0.799	0.477
(S.D.)[c]	0.227	0.154	0.057	0.294
Rate/animal[d]	93	75	85	62
(S.D.)[c]	9	22	25	7

a Data for Figures 3 and 4
b Time after placement into water with or without food
c n = 2
d (nmol/min/animal)

TABLE 15[a]: Data for effect of PCP, BHT and malathion on water flea survival in 24h acute toxicity tests

PCP							
Concentration[b]		0	0[c]	6	56	560	5600
%Survival[d,e]	Day 2	100	100	100	100	96	96
	Day 3	100	96	94	94	96	90
	Day 4	100*	96*	89*	89*	88*	72*
BHT							
Concentration[f]		0	0[b]	1	10	104	1042
%Survival[d,e]	Day 2	96	96	93	100	96	100
	Day 3	81	96	91	100	94	89
	Day 4	77*	93*	91*	96*	93	89
Malathion							
Concentration[b]		0	0.1	0.5	1	10	
%Survival[d,e]	Day 2	94	100	89	88	84	
	Day 3	92	100	87	88	80	
	Day 4	89*	92*	39	0	0	

a Data for Figures 5 - 7
b ng/l
c Ethanol control
d (number on day *x*/number on day 1)x100; Day 1 always 100%
e Toxicant added with no added food at water change on day 3
f µg/l
* Brood observed

TABLE 16[a]: Data for effect of PCP on hydra growth in 24h or 48h acute toxicity tests

GREEN HYDRA							
High PCP range	24h Test						
Concentration[b]		0	0[c]	56	112	224	448
No. of animals	Day 1	32	31	31	35	28	30
	Day 2	34	34	35	35	34	34
	Day 3[d]	48	50	42*	0	0	0
Low PCP range	24h Test						
Concentration[b]		0	0[c]	0.2	1.1	11	56
No. of animals	Day 1	28	28	28	28	28	28
	Day 2	30	34	31	32	32	29
	Day 3[d]	38	39	37	37	36	38
Low PCP range	48h Test						
Concentration[b]		0	0[c]	0.2	1.1	11	56
No. of animals	Day 1	27	27	27	27	27	27
	Day 2	28	32	29	31	27	29
	Day 3[d]	41	33	39	41	32	29
	Day 4	47	46	54	44	29	12
PINK HYDRA							
High PCP range	24h Test						
Concentration[b]		0	0[c]	56	112	224	448
No. of animals	Day 1	34	30	35	36	38	44
	Day 2	33	30	36	34	34	37
	Day 3[d]	49	34	30	9	0	0
Low PCP range	24h Test						
Concentration[b]		0	0[c]	0.2	1.1	11	56
No. of animals	Day 1	28	28	28	28	28	28
	Day 2	30	30	32	29	28	30
	Day 3[d]	46	34	39	37	36	35

a Data for Figures 8 - 12
b µg/l
c Ethanol control
d Toxicant added on day 2 after counting feeding and cleaning hydra
* Tentacles retracted

TABLE 17[a]: Data for effect of PCP, BHT and malathion on water flea GST activity in a 24h acute toxicity test

PCP						
Concentration[b]	0	0[c]	6	56	560	5600
Specific Rate[d]	267	282	313	316	273	345
(%Δ)[e]		(106)	(117)	(118)	(102)	(129)
Protein/animal[f]	0.473	0.581	0.495	0.450	0.463	0.497
Rate/animal[g]	84	109	103	106	84	127
(%Δ)[e]		(130)	(123)	(126)	(100)	(151)
BHT						
Concentration[h]	0	0[c]	1	10	104	1042
Specific Rate[d]	286	287	272	269	280	348
(%Δ)[e]		(100)	(95)	(94)	(98)	(122)
Protein/animal[f]	0.367	0.390	0.422	0.468	0.537	0.458
Rate/animal[g]	70	83	76	93	111	118
(%Δ)[e]		(119)	(109)	(133)	(159)	(169)
Malathion						
Concentration[b]	0	0.1	0.5			
Specific Rate[d]	392	347	336			
(%Δ)[e]		(89)	(86)			
Protein/animal[f]	0.509	0.510	0.849			
Rate/animal[g]	149	131	159			
(%Δ)[e]		(88)	(107)			

a Data for Figures 13 - 15
b ng/l
c Ethanol control
d nmol/min/mg protein
e The change in rate of treatment compared with control expressed as a percentage
f μg protein/animal
g pmol/min/animal
h μg/l

TABLE 18[a]: Data for effect of PCP, BHT and malathion on green hydra GST activity in acute toxicity tests

PCP - 24h test						
Concentration[b]	0	0[c]	0.2	1.1	11	56
pH 7.3						
Specific Rate[d]	731	738	836	893	888	-
(%Δ)[e]		(101)	(114)	(122)	(122)	-
pH 7.6						
Specific Rate[d]	702	878	934	817	1229	-
(%Δ)[e]		(125)	(133)	(116)	(175)	-
PCP - 48h test						
Concentration[b]	0	0[c]	0.2	1.1	11	56
pH 7.3						
Specific Rate[d]	841	882	857	951	1075	1662
(%Δ)[e]		(105)	(102)	(113)	(128)	(198)
pH 7.6						
Specific Rate[d]	888	771	833	869	1075	1649
(%Δ)[e]		(87)	(94)	(98)	(121)	(186)
BHT - 24h test						
Concentration[b]	0	0[c]	1	10	104	1042
pH 7.3						
Specific Rate[d]	1421	1240	1517	2039	-	1388
(%Δ)[e]		(87)	(107)	(144)	-	(98)
pH 7.6						
Specific Rate[d]	1551	1445	1505	2105	-	1881
(%Δ)[e]		(93)	(97)	(136)	-	(121)

continued next page

TABLE 20a[a]: Effect of pH on mussel GST activity

pH	7.0	7.3	7.6
Specific Rate (nmol/min/μg pr)	261	323	385

a Data for Figure 23a

TABLE 20b[a]: Correlation of protein and GST rate in mussel digestive gland

Protein[b]	10	20	40	50	60	80	100	150
Rate[c]	4.05	4.05	6.19	6.02	7.06	14.01	18.98	17.25
Specific Rate[d]	404	202	155	222	117	174	189	115

a Data for Figure 23b
b μg protein (BSA protein reference)
c nmol/min
d nmol/min/μg pr

TABLE 18 *continued*

Malathion - 24h test

Concentration[b]	0	0.1	0.5	1.0	2.0	4.0
pH 7.3	1021	965	915	1038	926	-
(%Δ)[e]		(95)	(90)	(102)	(91)	-
pH 7.6						
Specific Rate[d]	942	965	939	951	1135	-
(%Δ)[e]		(102)	(100)	(101)	(121)	-

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a Data for Figures 16 - 19
b μg/l
c Ethanol control
d nmol/min/mg protein
e The change in rate of treatment compared with control expressed as a percentage

TABLE 20c[a]: Correlation of protein and GST rate in other tissues of mussel

	Visceral mass			**Gill**		
Protein[b]	65	104	130	67	106	133
Rate[c]	9.61	12.15	18.17	7.64	10.30	12.27
Specific Rate[d]	142	117	140	115	97	92
	'Rest'[e]			**Muscle**		
Protein[b]	44	-	88	45	72	90
Rate[c]	6.25	-	11.92	7.29	11.11	5.56
Specific Rate[d]	142	-	134	162	154	62
	Foot			**Mantle**		
Protein[b]	53	84	105	27	43	54
Rate[c]	8.57	7.76	7.87	5.21	3.59	5.67
Specific Rate[d]	163	92	75	192	83	104
	Palps					
Protein[b]	35	55	69			
Rate[c]	1.39	5.56	4.40			
Specific Rate[d]	40	101	64			

a Data for Figure 23c-i
b μg protein (BSA protein reference)
b nmol/min
c nmol/min/μg pr
d The 'rest' consists of heart, kidney and aorta

TABLE 20d[a]: Correlation of protein and GST rate[b] in other tissues of mussel after two freezing/thawing cycles

	Visceral mass[c]				Visceral mass			
Protein[d]	39	65	104	130	39	65	104	130
Rate[e]	11.46	14.24	14.58	12.65	12.60	15.03	15.92	12.45
Specific Rate[f]	295	220	141	98	324	232	154	96
	Gill				**'Rest'[g]**			
Protein[d]	40	67	106	133	44	71	88	
Rate[e]	8.93	11.44	15.53	16.17	11.31	13.74	13.79	
Specific Rate[f]	224	172	146	122	255	194	155	
	Foot				**Muscle**			
Protein[d]	32	53	84	105	27	45		
Rate[e]	7.54	9.52	14.39	14.98	6.15	8.33		
Specific Rate[f]	239	181	170	142	228	185		
	Mantle				**Palps**			
Protein[d]	16	27	43	54	21	35	56	70
Rate[e]	5.16	8.14	10.52	10.32	4.067	5.65	6.94	9.43
Specific Rate[f]	316	299	242	190	196	164	124	135

a Data for Figure 24a-h
b at an *in vitro* pH of 7.6 except for visceral mass where GST activity was determined for pH 7.3 and 7.7
c *in vitro* pH of 7.3
d µg protein (BSA protein reference)
e nmol/min
f nmol/min/µg pr
g The 'rest' consists of heart, kidney and aorta

TABLE 21[a]: Data from EROD assay on black bream liver

Time (min)	0	5	10	15	20	25	30
Substrate clearance[b]	-	-	88	77	64	50	42
% Scale[c]	-	-	100	88	73	57	48
Product formation[d]	0	0	5	8	8	7	6
% Scale[c]	0	0	6	9	9	8	7

a Data for Figure 26
b peak height on an arbitrary scale of 0 - 100mm at 552nm
c Conversion of mm scale to % using 10 min product formation value (*i.e.* 88mm) as the 100% value
d Difference on an arbitrary scale of 0 - 100mm between the maximum at 574nm and minimum at 560nm

TABLE 22[a]: Data for control GST rates plotted against date of control water collection

Date	11/2	18/12	15/1	22/1	29/1	5/2	12/2
Water Flea							
Sp. Rate[b]	1346		345	286	466	392	
Protein per animal [c]	0.399		0.497	0.367	0.315	0.509	
Rate per animal[d]	298		127	70	87	149	
Green Hydra							
Sp. Rate[b] pH 7.3	659	731	1421	1021	841	880	1367
Sp. Rate[b] pH 7.7	516	702	1551	942	888	849	1235
Pink Hydra							
Sp. Rate[b] pH 7.3	843	286	601		956	631	564
Sp. Rate[b] pH 7.7	887	436	684		789	631	564

a Data for Figures 29 and 30
b Specific Rate (nmol/min/μg protein)
b μg protein/animal
c pmol/min/animal