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The Effect of Short-Term Exposure to Pentachlorophenol and Osmotic Stress on the Free Amino Acid Pool of the Freshwater Amphipod *Gammarus pseudolimnaeus* Bousfield

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Abstract. The effects of acute stressors on the concentration of free amino acids (FAA) in the freshwater amphipod *Gammarus pseudolimnaeus* was investigated. Forty-eight hours of exposure to acutely toxic concentrations of pentachlorophenol resulted in a significant decrease in the total FAA pool at the greater concentrations and a significant change in the FAA profile at the lesser concentration. Hyperosmotic conditions did not alter the FAA concentrations while hypoosmotic conditions caused a significant decrease in the total FAA pool. Pentachlorophenol exposure did not impair the ability of amphipods to resist hypoosmotic conditions; however, osmotic stress did influence the organisms ability to recover from toxicant exposure, as measured by changes in the concentration of FAA. The observed alterations in the concentration of total FAA in stressed amphipods is probably related to a disruption in the osmoregulatory ability of the organism. Changes in the FAA pool has potential as in *in-situ* biochemical indicator of toxicant-induced stress in freshwater invertebrates.

A variety of techniques exist for determining the response of aquatic organisms to chemical stressors. Organismal responses, such as growth and reproduction, have routinely been measured as end points; however, these techniques are expensive and time-consuming and are not easily adapted to monitoring effects under field conditions. Biochemical indicators of toxicant-induced stress have potential application in both laboratory hazard assessment and field monitoring. Because most effects originate at the biochemical level of organization, changes at this level should be the most sensitive indicators and provide the earliest possible warning of future adverse effects. In the context of field monitoring, one of the most difficult tasks is determining whether the aquatic biota are under stress. By utilizing alterations in specific biochemical parameters, one can identify organisms which are stressed by natural perturbation and those affected by exposure to toxic chemicals. This can only be accomplished after background variability associated with the parameter of interest is established, such that any changes caused by toxicant-induced stress can be identified.

A large array of specific and general biochemical indicators of stress (BIS) have been developed, including: changes in enzyme activity, lysosomal stability, concentrations of adenylates, glycogen, lipids, and free amino acids (FAA) and the RNA/DNA ratio (Bayne *et al.* 1985). In marine invertebrates, FAA pools have received a considerable amount of attention and have been successfully used as general indicators of toxicant exposure (Roesijadi *et al.* 1976; Kasschau *et al.* 1980). Interest in FAA pools arose after the discovery of the important role of these compounds in intracellular osmoregulation in euryhaline invertebrates (Gilles 1975; Duchateau-Bosson and Florkin 1961). Since then, additional research showed that the FAA pool of marine organisms responds to a variety of natural and man-made perturbations (Sansone *et al.* 1978; Augenfeld *et al.* 1980). With freshwater organisms however, the situation is quite different. Whereas information on amino acid metabolism in

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freshwater organisms is available (Gilles 1979), the effect of toxicant-induced stress on the FAA pool is unknown.

A variety of mechanisms may be responsible for toxicant induced alterations in the concentration of free amino acids. Direct and indirect changes in protein metabolism initiated by toxicant exposure can affect the intracellular FAA pool. In addition, toxicant induced changes in osmoregulatory ability can cause subsequent alterations in the concentration of FAA. Anaerobic metabolism, which can be increased under stressful conditions, can also cause changes in specific amino acids. All of the above mechanisms may be involved in FAA changes observed in toxicant stressed organisms, although little empirical data supports the predominance of any single mechanisms.

Given the paucity of information available on the effects of stress on the FAA pool of freshwater invertebrates, a comprehensive study was initiated to determine if alterations in the FAA pool could be used as a biochemical indicator of stress (BIS). This study encompassed the establishment of the natural variations in the FAA pool of specific freshwater invertebrates (Graney and Giesy 1986), determining the effects of acute and chronic exposure to specific toxicants on the FAA pool and establishing a relationship between these alterations and higher level effects such as growth.

This paper discusses the effects of acute stressors on the FAA pool of the freshwater amphipod *Gammarus pseudolimnaeus*. Acute toxicity tests were conducted with pentachlorophenol (PCP). This compound was chosen because of the large toxicity data base and its specific mode of action. Pentachlorophenol uncouples oxidative phosphorylation and thus disrupts the energy metabolism of the organism. Such an effect may alter protein metabolism and potentially cause changes in the FAA pool. In the first experiment, amphipods were exposed to PCP for 96 hr and the effects of this exposure on whole body FAA pools were determined. The second experiment investigated the influence of osmotic shock on the FAA pool of the amphipods. This experiment was based on the results of the first study, in which the reduction in the FAA pool caused by PCP exposure may have been caused by a disruption in osmoregulatory ability. In a third experiment, the combined effects of osmotic shock and PCP exposure were investigated.

Materials and Methods

General Procedures

All experiments were conducted with populations of the freshwater amphipod *Gammarus pseudolimnaeus*, Bousfield (Crustacea; Amphipoda) collected from the field. Organisms were collected by D-frame dip net from Glass Creek, a "pollution free" second-order stream located in Barry County, Michigan. They were returned to the laboratory and, prior to test initiation, acclimated for at least 96 hr and for no longer than two weeks. All tests were static renewal and conducted with unfiltered river water collected from Glass Creek, MI. During testing, *G. pseudolimnaeus* were fed Ash leaves which were preconditioned by soaking them in river water for two weeks. Amphipods were exposed to measured concentrations of pentachlorophenol (>99% pure) obtained from Fluka Chemical Company (Hauppauge, NY). Pentachlorophenol concentrations in water were measured by the method of Carr *et al.* (1982). Briefly, this method entails extracting one hundred ml of acidified water with 10 ml chloroform and back-extracting into 2.0 ml of 200 mM NaOH. The aqueous fraction is measured spectrophotometrically at 320 nm.

On designated days, five organisms were sampled from each treatment and immediately frozen on dry ice. Whole amphipods were subsequently dried at 95°C for 24 hr, weighed, and extracted by homogenizing the entire organism in methanol. After centrifugation, the supernatants were decanted and stored in a freezer (−10°C) until analysis by high pressure liquid chromatography. The extract was derivatized one min prior to injection by mixing 100 μL extract with 50 μL *o*-Fluoraldehyde® (*O*-phthaldialdehyde:OPA) (Pierce Chemical Co., Rockford, IL). Since the reaction of the amino acids with OPA requires the presence of a free amine group, this method does not allow quantification of proline or hydroxyproline. Separation of 19 amino acids was achieved with a 5 μm, ultrasphere® ODS C-18 column preceded by a pellicular ODS ocatadecyl (37–53 μm) guard column. A 40 min linear gradient (17–73%) of solvent A (100% methanol) to B (0.1 *M* sodium acetate; pH 7.0) was utilized. All solvents were filtered and degassed before use. LDS/Milton Roy constametric® pumps were interfaced with a Kratos FS-970 L.C. fluorometric detector (activation wavelength 330 nm; emission wavelength 418 nm; sensitivity 5.0; range 1.0 μAmps; time constant 0.5 sec).

A standard amino acid mixture was also dried at 95°C for 24 hr to ensure that the drying procedure did not result in the loss of more labile amino acids such as glutamine (Jacob and Barrett 1982). No significant loss of the standard amino acid mixture occurred during the drying process.

Patterns of relative FAA concentrations of stressed and unstressed amphipods were compared by univariate and multivariate statistical techniques. Total FAA concentrations of different treatment groups were compared by analysis of variance (ANOVA) followed by Duncans Multiple Range Test. The patterns of relative concentrations of individual FAA were compared by profile analysis (Morrison 1967). This technique uses multivariate analysis of variance to compare the differences between adjacent amino acids. Significance was determined by Wilk's criterion (SAS Institute 1982).

Pentachlorophenol Exposure

Gammarus pseudolimnaeus were exposed for 96 hr to 0.66, 1.13 or 1.68 mg/L pentachlorophenol (measured concentrations). Each test concentration consisted of triplicate exposure chambers, with each chamber containing 30 amphipods. Tests were conducted at 20°C (±1.0), pH 8.0, and the water was continuously aerated during the test. Amino acids were analyzed in amphipods sampled at test initiation and at 48 hr.

Osmotic Stress

Amphipods were subjected to both hypo- and hyperosmotic conditions for 48 hr. Hypoosmotic conditions were obtained by adding conditioned leaves (food) to distilled water, aerating the water for 24 hr and then adding the amphipods. The resulting water had a hardness of 1.9 mg/L as $CaCO_3$ (1.9×10^{-5} osmolal). Hyperosmotic conditions consisted of a 1.05% salt solution (NaCl) and were equivalent to approximately 30% saltwater or 10.5 0/00 (0.18 osmolal). Organisms were sampled at test initiation and after 48 hr exposure to each osmotic condition. Test water was continuously aerated and temperature and pH were 20.0(±1.0)°C and 8.0, respectively.

Pentachlorophenol-Osmotic Stress Exposure

The combined effect of PCP and osmotic conditions on the FAA pool was investigated by exposing *G. pseudolimnaeus* first to pentachlorophenol and then to osmotic stress. Because organisms collected from the field were utilized and this test was conducted during the winter months, the temperature during this exposure was 10°C, considerably lower than the other tests (20°C). Amphipods were exposed for 48 hr to 0.86, 1.16, 1.51, 1.80 or 2.24 mg/L pentachlorophenol (measured concentrations), after which they were immediately transferred and exposed for 24 hr to either clean dilution water (river water) or "hypoosmotic" water, similar in quality to that used in the osmotic shock experiment. Organisms were sampled and analyzed for FAA at test initiation and after 48 hr of toxicant exposure. Organisms which were transferred to hypoosmotic conditions after 48 hr PCP exposure were sampled and analyzed for FAA after 24 hr of osmotic stress.

Results and Discussion

Pentachlorophenol Exposure

The 96-hr LC_{50} was 1.15 mg/L (C.L. 1.04–1.36). The total amino acid concentration of *G. pseudolimnaeus* was significantly reduced by exposure to 1.68 mg L/PCP for 48 hr (Table 1). Except for the sulfo-amino acid taurine, this reduction was due to decreases in all individual amino acids. Glutamine was reduced approximately four-fold, with all the remaining amino acids being reduced by approximately one-half. A reduction of the total FAA concentration was also noted in individuals exposed to 1.13 mg/L PCP, however this decrease was not statistically significant ($\alpha = 0.05$). Exposure to 1.13 mg/L PCP caused both increases and decreases in concentrations of specific amino acids. These changes resulted in a qualitative profile which was significantly different from the control organisms (Figure 1). This difference was established by conducting multivariate analysis of variance (MANOVA) on the differences between adjacent amino acids. For example, the differences between

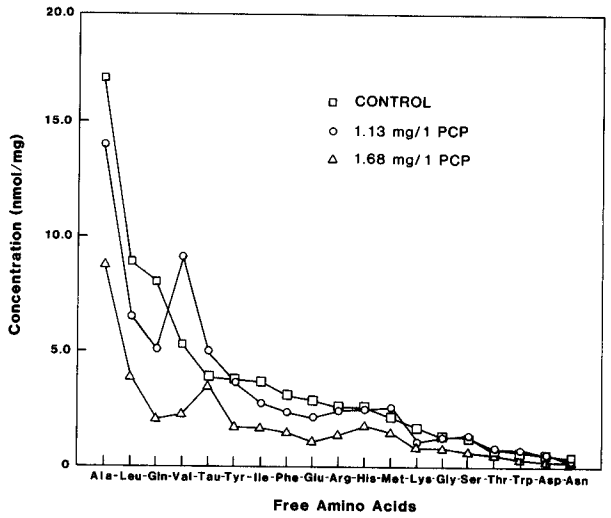

Fig. 1. Profile of free amino acid (FAA) concentrations in *G. pseudolimnaeus* exposed to 1.13 and 1.68 mg/L PCP for 48 hr. Organisms exposed to 1.13 mg/L exhibited a statistically significantly different profile ($\alpha < 0.0001$, Wilk's criterion, profile analysis) (SAS Inc 1982)

alanine and leucine, leucine and glutamine, glutamine and valine, etc., were calculated for each treatment (Figure 1). A MANOVA was then conducted on these differences, essentially testing for parallelism, and significance was established, using Wilks' criterion. In the present study, the calculated F-value was 50.28 and was highly significant ($\alpha = 0.0001$). As discussed below, qualitative changes such as these may represent a more sensitive indicator of stress than the changes in the total FAA concentrations observed when organisms were exposed to greater toxicant concentrations.

In marine invertebrates, specific amino acids have been identified as being fairly responsive to certain kinds of stress. Increases in the molar ratios of taurine to glycine were used as a quantitative index of stress in the hard clam *Mercenaria mercenaria* (Jeffries 1972). Similar results were observed for the clam *Macoma inquimator* (Roesijadi and Anderson 1979) and mussel *Mytilus edulis* (Widdows *et al.* 1981) exposed to petroleum hydrocarbons. However, a decrease in the taurine/glycine ratio was observed in oil stressed bivalves (Augenfeld *et al.* 1980). Kasschau *et al.* (1980) found that glycine concentrations in sea anemones (*Bunodosoma cavernata*) were too variable to be an effective indicator of toxicant-induced stress. Other amino acids in marine invertebrates which have been shown to be specifically affected by toxicants include glutamate (Kasschau *et al.* 1980), alanine and aspartate (Riley and Mix 1981; Powell *et al.* 1982; Roesijadi *et al.* 1976) and arginine, lysine, and threonine (Augenfeld *et al.* 1980; Roesi-

Table 1. Concentrations of individual and total amino acid (nmol/mg) of *G. pseudolimnaeus* exposed to pentachlorophenol for 48 hr

	Day 0	Day 2		
Amino acid	Control (S.D.)[a]	Control (S.D.)	1.68 mg/L (S.D.)	1.13 mg/L (S.D.)
Alanine	17.21 (2.3)	16.88 (1.15)	8.80 (4.3)	14.05 (4.5)
Arginine	4.06 (0.5)	2.64 (0.69)	1.36 (0.61)	2.48 (0.69)
Asparagine	0.66 (0.07)	0.36 (0.04)	0.18 (0.76)	0.23 (0.084)
Aspartic acid	0.79 (0.07)	0.52 (0.08)	0.22 (0.16)	0.52 (0.175)
Glutamine	8.5 (1.7)	8.05 (4.49)	2.07 (1.77)	5.14 (2.48)
Glutamic acid	3.04 (0.26)	2.93 (0.23)	1.10 (0.86)	2.16 (0.801)
Glycine	1.83 (0.46)	1.35 (0.91)	0.80 (0.34)	1.28 (1.37)
Histidine	2.04 (0.03)	2.59 (0.48)	1.75 (0.56)	2.46 (0.615)
Isoleucine	3.59 (0.71)	3.67 (0.7)	1.66 (0.63)	2.75 (0.66)
Leucine	8.07 (1.35)	8.87 (1.67)	3.90 (1.57)	6.50 (1.56)
Lysine	2.16 (0.42)	1.72 (0.41)	0.82 (0.37)	1.09 (0.21)
Methionine	1.85 (0.24)	2.21 (0.48)	1.53 (0.75)	2.62 (1.03)
Phenylalanine	3.2 (0.46)	3.11 (0.64)	1.52 (0.52)	2.45 (0.58)
Serine	2.4 (0.74)	1.35 (0.19)	0.64 (0.30)	1.40 (0.81)
Taurine	5.75 (0.99)	3.87 (1.1)	3.49 (0.88)	5.01 (1.74)
Threonine	1.17 (0.26)	0.76 (0.07)	0.47 (0.22)	0.80 (0.36)
Tryptophan	0.74 (0.19)	0.64 (0.16)	0.31 (0.15)	0.75 (0.31)
Tyrosine	4.04 (0.78)	3.75 (0.6)	1.75 (0.65)	3.72 (1.08)
Valine	5.07 (0.79)	5.31 (0.72)	2.34 (0.86)	9.06 (0.99)
Total	76.17(14.89)	70.58(16.30)	34.71[b](7.28)	64.46(18.41)

[a] S.D.—standard deviation, n = 5

[b] Significantly (α = 0.05) lower than control organisms based on Duncans Multiple Range Test

jadi and Anderson 1979). In general, the inconsistency of organismal responses under different conditions and exposed to different toxicants makes interpretation of alterations in the concentration of FAA extremely difficult.

Compared with marine organisms, relatively little work has been conducted on toxicant-induced alterations in the FAA pool of freshwater organisms. Changes in the total FAA concentration was measured in freshwater crabs exposed to Sumithion®; however, concentrations of individual amino acids were not reported (Bhagyalakshmi *et al.* 1983). These authors found that the total concentration of amino acids increased during acute exposure and declined during chronic exposure. Differences in total FAA were also reported for freshwater bivalves collected from polluted and unpolluted environments (Gardner *et al.* 1981). The mantle tissue of bivalves collected from habitats contaminated by acid and metals had a significantly greater total concentration of free amino acids. The increase in total FAA concentrations reported by Gardner *et al.* (1981), is opposite to the effect observed in our study, where a significant decrease in FAAs occurred. The reason for the differences is unknown; however, given the variety of mechanisms by which the total FAA concentration can be altered, it is not surprising that the response of the total FAA concentration varies in different organisms stressed under different conditions.

Two types of changes in the FAA pool of organisms normally occur under toxicant-induced stress. The first involves either an increase or decrease in the total FAA concentration. In the present study, acutely toxic concentrations of PCP caused a decrease in the FAA pool. The second type of response results in alterations in specific amino acids (either increases or decreases), which may or may not cause changes in the total amino acid concentration. This effect was observed in amphipods exposed to lower PCP concentrations. The different responses of the FAA pool may reflect the presence of a variety of mechanisms responsible for amino acid alterations. These mechanisms, or the rationale for expecting changes in the FAA pool of toxicant stressed organisms, can be separated into two categories. The first category covers the effect of stress on protein metabolism and subsequent changes in the FAA pool. The second encompasses the complex mechanisms controlling osmotic regulation and the role of FAA in this process.

Protein metabolism is an extremely complex, homeostatically-regulated, process which can be altered during toxicant-induced stress and result in changes in concentrations of specific amino acids as well as the total FAA concentration. Protein degradation and subsequent utilization of the released amino acids for anaplerotic reactions and/or energy production represents an important mechanism for changes in the FAA pool. For many invertebrates,

amino acids may contribute significantly to the total energy budget of the organism (Gilles 1970; Bayne 1973; Bursell 1966). Adenosine 5′-monophosphate (AMP) and/or ADP are covalent modifiers of many of the enzymes involved in nitrogen metabolism, such as glutamate dehydrogenase (Stryer 1981), so that changes in the energy status of an organism can modulate the oxidation of amino acids, thus altering the relative proportions or profile of the FAA pool. Therefore, under the increased energy demand associated with toxicant-induced stress, invertebrates may degrade proteins to augment the available energy supply thus altering the FAA pool (Gould *et al.* 1976; Bhagyalakshmi *et al.* 1983; Powell *et al.* 1982).

In addition, changes in the rate of amino acid transfer between intracellular and extracellular compartments can influence the FAA pool. Amino acid transport across membranes is an extremely complex process, controlled by a variety of interrelated processes. Toxicants can interfere directly with the energy requiring transport processes, thus affecting the intracellular pool, or they can indirectly influence FAA by altering the hormonal milieu of the organism. Hormones, such as cortisol, insulin and glucagon can directly influence amino acid uptake and distribution and are thought to be key factors controlling the metabolism of amino acids (Munro 1970; Guidotti 1978).

Toxicants may also influence the FAA pool by directly or indirectly altering the osmoregulatory processes of an organism. In all organisms, the major objective of osmoregulation is to maintain a constant intracellular milieu such that normal metabolic processes can proceed. In marine or euryhaline organisms amino acids are extremely important intracellular osmolytes and can be adjusted during salinity fluctuations. In freshwater organisms however, the role of FAA in osmoregulation is less clear. Free amino acids constitute 10–20% of the active intracellular osmolytes of freshwater invertebrates (Gilles 1979) and can play an important role in intracellular osmoregulation (Hanson and Dietz 1976). However, freshwater crustaceans are generally considered to be efficient osmoregulators. Hyperosmotic conditions are maintained by decreased water permeability, active salt uptake and the production of hypotonic urine. Stressful conditions can interfere with osmoregulatory ability, resulting in a decrease in blood osmolality; however, it is unknown whether the intracellular FAA pool concomitantly decreases to maintain isotonic conditions. Acute stress, such as handling, often results in a transitory loss of osmoregulatory ability and may cause a decrease in the entire FAA pool.

The complexity of nitrogen metabolism in aquatic invertebrates, coupled with the lack of information on the regulatory processes that control metabolism, makes it extremely difficult to discern mechanisms by which individual amino acids may be altered during toxicant-induced stress. However, when developing a general biochemical indicator of stress, understanding the mechanism is not always essential as long as the changes observed are consistent, can be quantitatively or qualitatively related to exposure, can be separated from the "background noise" or variability normally associated with field conditions and are related to ecologically relevant end points.

The primary effects of acute toxicant-induced stress in the present study were a decrease in the entire FAA pool at the greater exposure concentrations and an alteration in the FAA profile at the lower concentration. Presently, it is difficult to speculate on which mechanisms may be responsible for the observed changes. Pentachlorophenol is a strong metabolic poison which uncouples electron transport from ADP phosphorylation (Weinbach 1956) and can result in increased metabolic rates (Peer *et al.* 1983; Weinbach and Nolan 1956). This can accelerate the utilization of tissue energy reserves (Holmberg *et al.* 1972), and for invertebrates which can utilize protein as an energy source, possibly alter the concentration of FAA. With respect to the decline observed in the total pool, because all amino acids except taurine decreased, it was hypothesized that the mechanism responsible was hypoosmotic stress caused by impaired osmoregulatory ability. The breakdown in osmoregulation could be caused by changes in the hormonal status of the organism induced by the stressor or could be the result of direct damage to the gills and/or excretory organs. In either situation, dilution of the hemolymph would require adjustment of the intracellular osmolality, possibly *via* reduction in the FAA pool. If this was the mechanism responsible in the present study, then one would also expect non-toxicant induced osmotic stress to cause a similar change in amino acid concentrations. To test this hypothesis, a study was conducted to determine the influence of extraorganismal hypoosmotic and hyperosmotic conditions on the FAA pool of *G. pseudolimnaeus*.

Osmotic Stress

Hyperosmotic (1.05% NaCl; 0.18 osmolal) and hypoosmotic (hardness = 1.9 mg/L $CaCO_3$; 1.9×10^{-5} osmolal) conditions caused changes in the

Table 2. Concentrations of individual and total amino acids (nmol/mg) of *G. pseudolimnaeus* exposed to hyper- and hypoosmotic shock for 48 hr

Amino acid	Control (S.D.)[a]	Hyperosmotic (S.D.)[b]	Hypoosmotic (S.D.)[c]
Alanine	18.80 (4.4)	22.76 (1.08)	11.3 (0.92)
Arginine	7.59 (1.36)	5.14 (3.9)	2.59 (0.74)
Asparagine	0.14 (0.02)	0.31 (0.08)	N.D.[d]
Aspartic acid	1.02 (0.13)	1.30 (0.73)	0.53 (0.22)
Glutamine	13.16 (5.84)	9.71 (3.2)	5.20 (0.89)
Glutamic acid	3.60 (0.10)	2.87 (0.68)	1.23 (0.22)
Glycine	1.38 (0.32)	3.63 (3.48)	0.12 (0.02)
Histidine	3.81 (0.43)	4.2 (1.1)	2.94 (1.1)
Isoleucine	3.70 (0.63)	4.34 (0.94)	2.45 (0.73)
Leucine	9.12 (1.59)	10.26 (2.5)	6.05 (2.0)
Lysine	3.06 (0.73)	2.32 (1.7)	1.10 (0.42)
Methionine	3.00 (0.37)	2.48 (0.63)	1.72 (0.54)
Phenylalanine	3.30 (0.73)	3.80 (0.98)	2.67 (1.19)
Serine	2.49 (0.81)	5.31 (6.4)	0.94 (0.21)
Taurine	4.07 (1.3)	5.95 (0.27)	7.85 (1.59)
Threonine	1.52 (0.38)	1.57 (0.88)	0.66 (0.13)
Tryptophan	0.72 (0.10)	0.96 (0.14)	0.69 (0.22)
Tyrosine	4.54 (1.09)	5.07 (0.88)	5.11 (2.8)
Valine	5.55 (0.99)	6.11 (1.27)	3.67 (1.09)
Total	90.57(21.34)	98.09(24.61)	56.89[e](14.27)

[a] S.D.—standard deviation, n = 5
[b] 1.05% NaCl; 0.18 osmolal
[c] Distilled water (1.9 mg/L as $CaCO_3$; 1.9×10^{-5} osmolal)
[d] N.D.—Amino acid not detectable
[e] Significantly ($\alpha = 0.05$) lower than control organisms based on Duncans Multiple Range Test

concentrations of FAA of *G. pseudolimnaeus* exposed for 48 hr (Table 2). Under hyperosmotic conditions, there was a slight increase in the total FAA pool; this increase was not statistically significant ($p > 0.05$). The concentration of most free amino acids were elevated, although the majority of the increase in the total pool could be attributed to alanine, glycine, serine, and taurine. The concentrations of arginine, glutamine, and glutamate decreased in hyperosmotically stressed organisms. The inability of *G. pseudolimnaeus* to significantly increase their FAA pool under conditions of hyperosmotic shock does not mean that under less severe osmotic conditions, intracellular osmoregulation via increased FAAs does not occur. Hyperosmotic regulation is generally a much slower process than hypoosmotic regulation and may require a more gradual acclimation to saline conditions than were utilized in this study.

Hypoosmotic conditions resulted in a significant decrease in the total concentration of FAA (Table 2). No mortality occurred after 48 hr osmotic stress; however, changes in amphipod swimming behavior was observed, indicating that the organisms' were stressed by the hypoosmotic conditions. Concentrations of arginine, glutamate, glutamine, and glycine decreased by the largest percentage. Exceptions to this were tryptophan, which did not change at all, and taurine and tyrosine, which increased relative to the control organisms. The changes in the pattern of FAA concentrations observed during hypoosmotic conditions were very similar to those measured for acute PCP exposure (Tables 1 and 2). In both cases, a significant decrease was observed in the total FAA concentration.

The similarity of changes in concentrations of FAA observed in organisms exposed to PCP and hypoosmotic conditions indicate that similar mechanisms may be responsible for the changes in both cases. Although theoretically different mechanisms and/or stressors can cause the same alterations in the FAA pool, the wide range or variety of FAA responses reported in the literature indicates that when consistent changes in the FAA profile occur, they are likely due to interferences with the same or similar processes. In the present study, both of the stressors, although completely different in nature, may have had the same effect on the organism; that is, a disruption in osmoregulatory ability with subsequent dilution of the hemolymph. This type of response is indicative of acutely or severely stressed individuals and does not represent a sublethal response. Therefore, decreases in the concentration

of total FAA are indicative of severe stress and do not represent a very sensitive biochemical indicator. Changes in the pattern of concentrations of individual FAA, such as those observed at the lower PCP concentrations, are more likely the type of response which will be observed in sublethally stressed organisms.

Combined PCP Exposure and Osmotic Stress

Prior to presenting the results of this experiment, a discussion of the rationale behind the study design is warranted. Many of the concepts presented below have been reviewed in an article by Stebbing (1981) that discusses the relationship between stress and homeostatic regulation in aquatic organisms. All organisms contain specific control systems, such as thermoregulation or osmoregulation, which maintain preferred conditions for a large variety of processes. Stress associated with exposure to toxicants can exert a "workload" on these homeostatic control processes. The ability of an organism to effectively resist changes in its preferred state over an extended period of time can be referred to as its "counteractive capacity." Because homeostatic control mechanisms require energy, an organism's counteractive capacity is dependent upon its initial health status. In addition, these control processes have a finite capacity, in that excessive or long-term stress can eventually force the organism to deviate from its preferred state. An organism may be able to adjust or cope with a single stressor (*i.e.*, toxicant); however, if a second stressor is applied (*i.e.*, osmotic), the organism may not maintain homeostasis, even though under normal unstressed conditions, the organism could have resisted the second stressor. Therefore, the ability of an organism to maintain homeostasis upon exposure to a particular stressor is influenced by how much of its "counteractive capacity" has been utilized to cope with prior stressors.

In the present study, we hypothesized that the stress of toxicant exposure would make the organism more susceptible to osmoregulatory breakdown due to a lesser counteractive capacity, such that osmotic stress would cause changes in the FAA pool which otherwise would have not occurred. This concept could provide an approach to use biochemical measurements to assess the health of organisms collected from the field. Secondary stressors, such as osmotic stress, could be applied to organisms collected from potentially polluted sites, and their ability to resist that stressor would be indicative of prior stress and the effect of that stress on their "counteractive capacity."

The 96-hr-LC_{50} for the pentachlorophenol exposure was 1.37 mg/L (Confidence limits 1.18–1.57), considerably greater than the previous exposure. The difference is most likely due to the lower water temperature at which this test was conducted.

A significant ($p < 0.05$) decrease was observed in the total FAA concentrations (nmol/mg) of *G. pseudolimnaeus* exposed to PCP for 48 hr followed by osmotic shock for 24 hr (Tables 3 and 4). As with the PCP exposure without subsequent hypoosmotic stress (Table 1), exposure to concentrations slightly greater than the 96-hr LC_{50} caused a significant reduction in the total FAA concentration at 48 hr. Exposure to sublethal concentrations of PCP (1.16 mg/L) had no effect on the total FAA concentration. However unlike the previous exposure, the FAA profiles of organisms exposed at the lower PCP concentration were not significantly different from control organisms. Hypoosmotic conditions for 24 hr did not significantly influence the total FAA pool of control organisms, although concentrations of all amino acids, except glycine, did decrease slightly. The concentrations of FAA in *G. pseudolimnaeus* which had been exposed to 1.51 mg/L PCP for 48 hr returned to pre-exposure values after the organisms were transferred to clean "normal" water. Organisms which were transferred to clean hypoosmotic conditions instead of clean "normal" water did not recover and had total FAA concentrations very similar to those measured after 48 hr PCP exposure. The FAA concentrations of *G. pseudolimnaeus* exposed to sublethal PCP concentrations were not significantly affected after 24 hr of hypoosmotic conditions. Therefore, the stress associated with 48 hr sublethal exposure to PCP did not permanently influence the ability of *G. pseudolimnaeus* to osmoregulate, as measured by changes in the FAA pool.

We can conclude that short-term toxicant-induced stress did not deplete the "counteractive capacity" of the organism to further resist osmotic stress. The conclusion assumes that a reduction in this capacity would be realized as a change in the FAA pool which, although theoretically possible, has not been supported by experimentation. In addition, the exposure was extremely short in this study. A longer-term toxicant-induced stress, for which the concept of counteractive capacity would be more applicable, may be more effective in reducing the organism's ability to cope with subsequent osmotic stress such that the FAA pool may be altered. The effect of osmotic stress on the organism's ability to reestablish its normal FAA pool

Table 3. Concentrations of individual and total amino acids (nmol/mg) of *G. pseudolimnaeus* exposed to pentachlorophenol for 48 hr, prior to 24 hr of hypoosmotic stress

	Day 0	Day 2		
Amino acid	Control (S.D.)	Control (S.D.)	1.16 mg/L (S.D.)[a]	1.51 mg/L (S.D.)
Alanine	11.21 (2.49)	8.54 (2.25)	8.73 (2.36)	5.72 (1.87)
Arginine	6.86 (1.90)	6.24 (1.38)	8.41 (1.93)	3.18 (0.74)
Asparagine	0.64 (0.24)	0.52 (0.23)	0.42 (0.22)	0.13 (0.06)
Aspartic acid	0.54 (0.26)	0.66 (0.25)	0.75 (0.50)	0.57 (0.15)
Glutamine	4.25 (0.86)	3.96 (0.72)	4.34 (1.47)	2.55 (0.38)
Glutamic acid	2.19 (0.74)	2.33 (0.63)	2.50 (1.21)	1.97 (0.26)
Glycine	2.62 (0.58)	1.44 (0.32)	1.60 (0.62)	1.05 (0.19)
Histidine	1.53 (0.50)	1.64 (0.52)	1.43 (0.51)	0.92 (0.32)
Isoleucine	3.23 (1.01)	2.59 (0.64)	2.57 (0.95)	1.77 (0.30)
Leucine	7.45 (2.36)	6.17 (1.32)	5.75 (2.11)	3.88 (0.65)
Lysine	5.09 (1.84)	4.02 (0.90)	4.33 (1.87)	2.46 (0.52)
Methionine	1.90 (0.64)	1.77 (0.51)	1.76 (0.56)	1.33 (0.36)
Phenylalanine	2.56 (0.75)	2.24 (0.47)	2.10 (0.81)	1.31 (0.29)
Serine	1.48 (0.67)	1.48 (0.41)	1.36 (0.63)	0.89 (0.19)
Taurine	2.20 (0.64)	2.11 (0.74)	2.09 (0.93)	1.52 (0.28)
Threonine	1.12 (0.37)	1.06 (0.30)	1.15 (0.54)	0.59 (0.13)
Tryptophan	0.70 (0.24)	0.62 (0.15)	0.56 (0.36)	0.39 (0.12)
Tyrosine	4.18 (0.78)	3.43 (1.20)	3.65 (0.90)	1.98 (0.49)
Valine	3.81 (1.11)	3.36 (0.82)	3.23 (1.12)	2.27 (0.39)
Total	62.4 (16.7)	56.6 (11.45)	54.6 (17.14)	34.9 (7.05)[b]

[a] S.D.—standard deviation, n = 5

[b] Significantly (α = 0.05) lower than control organisms

indicates that in these organisms, toxicant stress had exhausted its capacity to maintain normal hemolymph osmotic conditions.

Before concluding this discussion, an additional point needs to be discussed concerning the initial total FAA pool of the control organisms from all three experiments. The initial mean total FAA concentrations for organisms used in experiments 1, 2 and 3 were 76.17, 90.57 and 62.4 nmol/mg, respectively. These values are considerably different, and, when developing a BIS, differences such as these need to be explained; especially, when changes in the FAA, which are considered to be stress-related in one experiment, overlap with the "normal range" for control organisms in a subsequent experiment. This was the case in the present experiments, where FAA concentrations found to be significantly reduced due to osmotic stress (56.89 nmol/mg) were very similar to control levels in the study of the combined effects of PCP-osmotic stress (62.4 nmol/mg). These discrepancies can be explained by comparing the time of year the organisms were collected with the seasonal variation in total FAA pools (Graney and Giesy 1986). Organisms used in the combined PCP-osmotic stress experiment were collected in March when total FAA concentrations of *G. pseudolimnaeus* are the smallest (66.9 nmol/mg). For the other experiments, the amphipods were collected in June and July, during which the total FAA concentration varied from 83–120 nmol/mg. Therefore, when conducting experiments on the effects of stress on biochemical parameters of field collected organisms, accessory factors such as seasonal variation must be considered.

Summary and Conclusions

The FAA pool was altered during acute exposure to PCP and osmotic shock. In both cases, the mechanism by which the amino acids were effected was most likely a breakdown in osmoregulatory ability. At the lower PCP concentrations, changes in concentrations of individual FAA were observed; however, these changes were not consistent. Free amino acids are not a very sensitive indicator for short-term sublethal toxicant-induced stress, although changes in the FAA pool in chronically (60 day) exposed organisms were observed prior to significant effects on the organism's growth (Graney 1986).

Table 4. Concentrations of individual and total amino acids (mmol/mg) of *G. pseudolimnaeus* after 48 hr exposure to pentachlorophenol followed by 24 hr hypoosmotic stress. (See Table 3 for values after PCP exposure but before hypoosmotic shock)

	Control		1.51 mg/L		1.16 mg/L	
Amino acid	Control[a]	Osmotic[b]	Control[c]	Osmotic[d]	Control[c]	Osmotic[d]
Alanine	8.20[e](0.66)[f]	7.05 (0.99)	8.16 (2.36)	4.95 (0.89)	8.31 (1.69)	7.66 (0.94)
Arginine	5.39 (0.26)	5.36 (2.02)	5.97 (1.22)	4.41 (1.21)	5.72 (1.63)	5.82 (1.37)
Asparagine	0.24 (0.05)	0.28 (0.15)	0.42 (0.14)	0.24 (0.07)	0.36 (0.11)	0.26 (0.07)
Aspartic acid	0.76 (0.17)	0.86 (0.26)	0.71 (0.42)	0.51 (0.27)	0.77 (0.31)	0.85 (0.19)
Glutamine	3.49 (0.27)	4.13 (1.08)	3.62 (1.21)	2.48 (0.78)	3.91 (1.30)	3.31 (0.76)
Glutamic acid	2.00 (0.33)	1.99 (0.43)	2.19 (1.10)	1.56 (0.36)	2.05 (0.69)	2.55 (1.01)
Glycine	2.10 (0.19)	1.50 (0.25)	1.43 (0.41)	0.88 (0.18)	1.48 (0.39)	1.79 (0.29)
Histidine	0.67 (0.33)	0.78 (0.30)	1.61 (0.48)	0.79 (0.18)	1.46 (0.61)	1.09 (0.41)
Isoleucine	2.40 (0.31)	2.68 (0.83)	2.73 (1.24)	1.72 (0.45)	2.71 (1.82)	2.33 (0.86)
Leucine	5.54 (0.56)	5.89 (1.18)	5.95 (2.77)	3.98 (0.80)	6.04 (2.11)	5.44 (1.02)
Lysine	3.83 (0.66)	3.73 (1.13)	4.48 (1.88)	2.93 (0.83)	3.95 (1.62)	3.28 (1.11)
Methionine	1.57 (0.18)	1.54 (0.30)	2.14 (0.92)	1.34 (0.29)	1.76 (0.49)	2.06 (0.22)
Phenylalanine	1.76 (0.22)	1.91 (0.32)	2.34 (1.05)	1.39 (0.28)	2.04 (0.62)	1.99 (0.27)
Serine	1.38 (0.23)	1.38 (0.36)	1.27 (0.78)	0.84 (0.31)	1.31 (0.69)	1.49 (0.52)
Taurine	1.65 (0.12)	1.83 (0.45)	1.62 (0.38)	1.34 (0.75)	1.71 (0.42)	1.88 (0.36)
Threonine	0.68 (0.22)	0.77 (0.21)	1.00 (0.42)	0.57 (0.18)	0.88 (0.39)	0.66 (0.09)
Tryptophan	0.52 (0.03)	0.56 (0.09)	0.67 (0.30)	0.39 (0.09)	0.71 (0.19)	0.92 (0.14)
Tyrosine	2.93 (0.48)	3.30 (1.10)	3.67 (0.86)	2.39 (0.63)	3.45 (0.93)	3.03 (0.92)
Valine	2.75 (0.40)	3.03 (0.50)	3.37 (1.39)	2.18 (0.46)	3.09 (0.65)	2.61 (0.74)
Total	47.8 (2.97)	48.57(11.00)	53.3 (18.2)	34.9[g] (6.44)	51.71 (12.92)	49.02 (9.18)

[a] Control organisms subjected to no osmotic shock
[b] Control organisms hypoosmotically shocked for 24 hr
[c] Exposed organisms transferred to clean water for 24 hr
[d] Exposed organisms hypoosmotically shocked for 24 hr
[e] Mean, n = 5
[f] Standard deviation in parentheses
[g] Significantly (α = 0.05) lower than all other organisms

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