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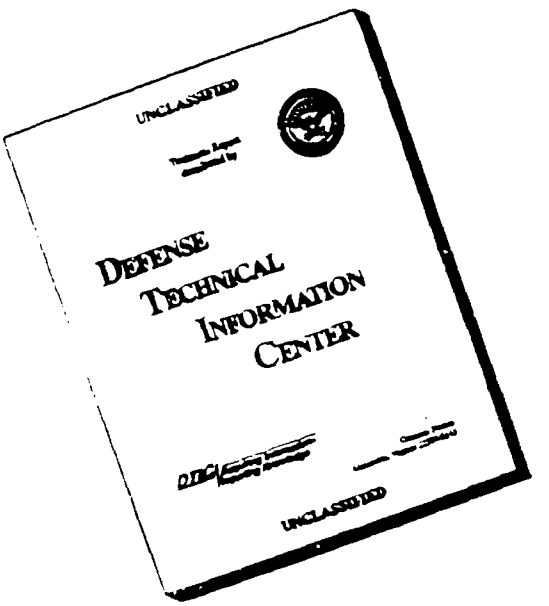

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MYCOTOXINS TOXINS AND DETOXICATION OF [redacted] AND OTHER COMPOUNDS OF MILITARY INTEREST

ANNUAL SUMMARY REPORT

ALTON MEISTER, PROFESSOR

January 14, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-86-C-6047

Cornell University Medical College

Department of Biochemistry

1300 York Avenue, New York, N.Y. 10021

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The inclusive dates of this Annual Report are November 15, 1985 to January 14, 1987 and the date of this report is January 14, 1987.

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This research, was initially concerned with detoxication of certain trichothecene toxins, and has recently changed its emphasis and is now directed toward the cyanoginosin toxins. Studies on the tricothecenes have been brought to a conclusion. A newly recognized naturally occurring glutathione derivative (γ-glutamyl glutathiome) has been prepared and methods for large scale preparation of this compound are being developed. Other glutathione derivatives that may be useful in detoxication are also under study. Cyanoginosin toxin, supplied to us by the Army, has been identified as of the LR type. Structural features of this toxin have been investigated and its toxicity has been studied after chemical modification. Preliminary studies indicate that the dehydropeptide moiety of the cyanoginosin LR toxin is essential for toxicity. This is an interesting point because dehydropeptides undergo facile reaction with certain SH-containing molecules. The possible role of glutathione in the modification of toxicity due to cyanoginosin peptides is currently being explored.

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SUMMARY

This research was initially concerned with the detoxication of mycotoxins, especially tricothecene T2 toxin. Our studies on tricothecenes have been brought to a conclusion. Studies on the formation of glutathione conjugates with tricothecene T2 toxin were carried out.

Studies have also been carried out on the synthesis of glutathione monoesters; for example a satisfactory method for the preparation of glutathione monoisopropyl ester hydrochloride and of glutathione monoethyl ester sulfate were developed. Administration of these compounds to mice leads to increased levels of glutathione in the liver and kidney. Chemical studies on the aminoethanol analog of glutathione have been continued. Methods for the large scale preparation of γ-glutamyl glutathione are being perfected.

Initial studies on cyanoginosin toxin (supplied by U.S.Army Medical Research and Development Command (USAMRIID)) have been carried out and the results obtained indicate that this sample of the toxin is of the LR type. Structural studies are consistent with earlier work and thus indicate the absence of a free amino group and presence of a dehydropeptide moiety. The dehydropeptide moiety appears to be essential for toxicity. This may constitute a significant finding because dehydropeptides are known to react effectively with SH-compounds. Studies have been initiated on the role of the glutathione system as a protectant against the toxic effects of cyanoginosin.

Foreward

a. Not applicable; copyrighted material is not quoted.

b. Not applicable; material from documents designated for limited distribution is not quoted.

c. Commercial organizations and trade names are used in this report. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

d. Animal experimentation was approved and is described in the report. In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the committee on care and use of laboratory animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication no. (NIH) 78-23, revised 1978).

e. Not applicable; human subjects were not involved in this work.

f. Not applicable; studies with recombinant DNA were not carried out as a part of this report.

TABLE OF CONTENTS

LIST OF APPENDICES

(i) Body of the Report

1. Statement of Problem

This research is concerned with the detoxication of toxins of potential military interest. Approaches based on augmentation of the glutathione system as well as other approaches are under investigation. Initially toxins of the trichothecene group such as T-2 toxin have been examined. In accordance with a change of scope of the research (carried out after mutual agreement between the Department of the Army and the principal investigator), the trichothecene-related research has been brought to a close and examination of toxins of the cyanoginosin group have been undertaken. The studies on trichothecenes and our initial work on the cyanoginosin toxins are considered in paragraphs (a) and (b), respectively, below.

(a) The trichothecene toxins are extremely poisonous and produce severe symptomatology including skin blistering, dizziness, nausea, hematemesis and other symptoms. There is evidence that such toxins have been used as weapons. The effects of such toxins are known to develop rapidly and might incapacitate members of a military force if used effectively by an enemy. Pretreatment of military personnel with a protective agent might have significant potential value. Clearly the development of an effective treatment for this type of toxicity is important.

(b) Toxins of the cyanoginosin group are produced by blue-green algae. These toxins, which have been found throughout the world, have been implicated in animal mortality and human morbidity. They have been found as toxic contaminants of municipal water reservoirs and in ponds and lakes in many locations. After the toxin is administered to experimental animals, a variety of serious symptomatology develops. Symptoms include thrombocytopenia, pulmonary thrombi, and hepatic congestion. Toxicity seems to begin in the liver and liver damage is apparently the primary cause of death.

A few reports have appeared on the chemical structure of cyanoginosins. One such toxin appears to be a cyclic hepta-peptide, which contains only two amino acid residues that are also found in proteins. The other five amino acid residues include D-alanine, β-methyl-D-aspartate, D-glutamate, N-methyldehydroalanine, and an unusual β-amino acid (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; adda). It is possible that some of these toxins may be penta or hexa peptides. On the basis of current information, these toxins contain three invariant amino acid residues; the variant amino acid residues include the L-isomers of alanine, arginine, leucine, and methionine. Thus far, these toxins have not been found to contain free amino groups.

Our current research relates to elucidating the chemical structures of these toxins. Another objective is to develop procedures for quantitative determination of the toxins. We wish to investigate enzymatic and chemical methods for the modification of the toxins with the goal of identifying the structural features associated with toxicity. This information will be used in efforts to develop procedures for detoxication.

2. Background of Studies on the Glutathione System

Animal cells use several mechanisms for protection against toxic compounds of endogenous and exogenous origin. The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine), which is present in virtually all animal cells, plays a major role in cellular protection. Glutathione functions in the detoxication of many types of compounds including reactive oxygen species, compounds possessing electrophilic centers, and other compounds into which electrophilic moieties may be introduced by metabolic reactions. There is an extensive literature on this subject; see for example (1-7). The pathway by which many foreign compounds undergo detoxication involves reaction with glutathione to form the corresponding glutathione conjugates; such reactions are catalyzed by glutathione S-transferases, but may occur nonenzymatically with certain compounds. The conjugates are converted by the action of γ-glutamyl transpeptidase to the corresponding S-substituted cysteinylglycine derivatives. The latter are converted to the corresponding S-substituted cysteine derivatives by the action of dipeptidase. S-Substituted cysteine derivatives of this type may undergo N-acetylation to form the corresponding N-acetyl derivatives. An additional pathway of metabolism of S-substituted cysteines involves transpeptidation between glutathione and S-substituted cysteines to form the corresponding γ-glutamyl-S-substituted cysteines (5,6).

Many of the compounds that are of interest from the military standpoint may be expected, by virtue of their chemical structures and properties, and in some instances on the basis of experimental work, to undergo detoxication reactions involving glutathione. Although the available data do not support the belief that glutathione is a panacea for all toxic compounds, there is sufficient evidence to believe that this tripeptide can substantially ameliorate many types of toxicity. For example, research in our laboratory and independent work at USAMRIID has shown that the toxicity of the trichothecene toxin T-2 is significantly decreased in mice treated with L-2-oxothiazolidine-4-carboxylate, a compound that increases tissue levels of glutathione, especially the liver. Acetaminophen has been used as a model compound for the study of toxicity and detoxication. In animals treated with large doses of acetaminophen, a substantial amount of the highly reactive toxic intermediate produced by oxidation of acetaminophen is conjugated with glutathione. It is significant that procedures that increase liver glutathione levels are dramatically effective in protecting experimental animals from acetaminophen toxicity. Thus, administration of L-2-oxothiazolidine-4-carboxylate to mice was completely effective in protecting mice from an LD:90 dose of acetaminiophen (12).

The glutathione levels of tissues can be increased by several procedures, as described below. Tissue levels of glutathione may also be decreased by administration of selective inhibitors of γ-glutamyl cysteine synthetase, the enzyme that catalyzes the first step in the biosynthesis of glutathione. A very useful compound in this series is L-buthionine-SR-sulfoximine (9), but other sulfoximines and still other compounds may be of value in the selective depletion of tissue glutathione levels.

Glutathione levels may be increased by administering compounds that increase the tissue levels of cysteine or γ-glutamylcysteine; these compounds are used, respectively, by γ-glutamylcysteine synthetase and by glutathione

synthetase for the synthesis of glutathione. Another approach to increasing the glutathione levels of tissues is to administer a pro-drug consisting of a derivative of glutathione, which, in contrast to glutathione, is readily transported into cells. After transport, such compounds are effectively converted to glutathione intracellularly. A compound of this class which has been examined in some detail is glutathione monoethyl ester (γ-glutamylcysteinylglycyl ethyl ester) (8,10,11). In this research, pro-drugs of cysteine, other thiols, and glutathione are being examined. These and the other compounds of interest are being synthesized by chemical and by enzymatic methods and tested for their ability to protect against toxicity. Another approach to the detoxication of various toxins will involve efforts to isolate enzymes that act on toxins. In this work, enrichment culture techniques will be employed.

The studies carried out under this contract are being conducted by prior arrangement and agreement with scientists concurrently studying this problem at U.S. Army Medical Research Institute of Infectious Diseases. The research carried out at USAMRIID is submitted under work unit S-10-AQ-197; research unit number S-10-AQ-O-12.

3.Results

3a. Experiments to probe the formation of glutathione conjugates with trichothecene T-2 toxin.

Introduction. Experiments were performed to probe for GSH conjugate formation with T-2 toxin and the excretion of this conjugate and/or its metabolites in the bile and urine. These experiments were carried out on rats (300-400 g). The animals were treated with [^{35}S]cysteine and subsequently prepared for bile collection. Prior to bile collection, the animals were treated with T-2 toxin, and bile and urine were obtained at intervals. We developed procedures for the analysis of bile and urine for glutathione metabolites, T-2 toxin metabolites, and glutathione-toxin conjugates. The samples are treated with dithiothreitol and subsequently with 2-vinylpyridine, to derivatize sulfhydryl containing compounds. Analyses were carried out by chromatography on a model 500 Durrum amino acid analyzer equipped for fraction collection.

Methods. [^{35}S]Cysteine (1.2 μCi, carrier-free) was injected intraperitoneally into 300-400 g rats, in 0.5 ml of phosphate-buffered saline, in order to label the hepatic GSH pool. Four hours later the rats were anaesthetized (60 mg/kg; pentobarbital), tracheotomized (PE 205), and cannulated in the jugular vein (PE 10), and in the bile duct (PE 10) above the entry of pancreatic ductules. Bile was collected for 0.5 hour to obtain a background profile of ^{35}S excretion, and then [^{3}H]-labeled-T-2 toxin (5 μCi; 0.36 μmole) was injected via the jugular vein cannula. Bile was collected for two additional one hour periods. Urine was then withdrawn from the bladder by needle puncture. Portions of each sample (0.2 ml) were treated with 10 μl each of 1 M dithiothreitol and 2-vinylpyridine to reduce and derivatize the sulfhydryl compounds; 1 hour later, 50 μl of 1.5 M sulfosalicylic acid was added and the samples were centrifuged to remove the precipitated protein. The pH was adjusted to 2.2 by adding NaOH, and portions of each sample were applied to (1) a Durrum 500 amino acid analyzer using the "physiological" elution procedure (Na citrate buffers); and (2) a reverse phase HPLC column

(Biosil ODS-5S), and eluted isocratically with 30% isopropanol in Sorensen's citrate buffer, pH 2.2 (flow rate; 0.45 ml/min). Eluate fractions were collected, (40 x 5 min from the Durrum, and 30 x 1.5 min from HPLC). Dimiscint (National Diagnostic) scintillation fluid was added, and the samples were counted on an LKB Beta Rack liquid scintillation counter programmed for dual label analysis. The HPLC eluate was also monitored by UV absorbance at 220 nm. The elution times of the radioactive peaks were compared with standards prepared as the vinylpyridine derivatives of known sulfur-containing compounds (cysteine, GSH, γ-glutamyl-GSH, γ-glutamylcysteine, and cysteinylglycine) determined on the Durrum, and with trichothecene standards on the HPLC, (28.5 min for T-2 and 21.5 min for HT-2).

Results. Amino acid analysis of bile samples obtained prior to T-2 dosing contained ^{35}S-labeled GSH, γ-glutamyl-GSH, cysteine and cysteinylglycine. A large fraction of the label also eluted earlier from the amino acid analyzer at times corresponding to taurine, inorganic sulfate and other oxidized metabolites of cysteine sulfur. (The conjugated bile acid taurocholate, which is secreted in bile at high concentrations, could also contain this radiolabel, and would be expected to elute in these early fractions). Reverse phase chromatography of the same samples gave a similar number of radiolabelled peaks. Following the administration of T-2, a single large peak of ^{3}H activity eluted in the early fractions of the amino acid analyses of both urine and bile. The ^{35}S activity peaks appearing in bile were unchanged by this treatment, and no new peaks appeared. In the urine samples, almost all of both radiolabels eluted in the acidic or uncharged fractions. The reverse phase analyses yielded two ^{3}H containing peaks in bile, both of which eluted earlier than T-2 or HT-2; urine contained peaks with similar elution times, but with different amounts in each, and also contained a third peak with the same elution time as HT-2. The level of ^{35}S appearing in the bile was about 70,000 dpm/ml for all collections, and did not appear to be altered by the administration of T-2 or the concomitant biliary appearance of its metabolites. About 3.4×10^{6} DPM of ^{3}H were excreted in the bile during the first two hours after giving T-2, while about 2.06×10^{6} DPM appeared in the urine over the same time. Excretion of ^{35}S over the same period was 175,000 DPM in bile and 685,000 DPM in urine.

Interpretation. The experimental approach used here was designed to reveal a small detoxification pathway involving the direct conjugation of T-2 or its metabolites with GSH. Both the toxin and the GSH pool were labeled in order to optimize detection of trace amounts of any conjugate. Samples were analyzed by two chromatographic methods to detect the coelution of the two radiolabels in such a way as to be consistent with the chemical properties of such a conjugate. The amino acid analyzer is primarily a cation exchange system, but exhibits additional separation based on hydrophobicity. Underivatized GSH elutes at about 20 minutes in this system, whereas its vinylpyridine derivative, which is more hydrophobic and contains an additional cationic site, elutes at 86 min. Conjugation of GSH with a trichothecene molecule would increase its hydrophobicity, and the corresponding elution time would be expected to occur somewhere between these two standards. No detectable ^{3}H label was delayed in this manner however; all this label had eluted in fractions earlier than those corresponding to underivatized GSH, and no shifts in the ^{35}S label of the latter compound were noted with T-2 treatment. Although the ultimate product of the mercapturic pathway in urine would be expected to be an N-acetylcysteine conjugate, the more immediate GSH,

cysteinylglycine, cysteine, and γ-glutamylcysteine conjugates would be expected to appear in bile; however, these were not detected. Similar results were obtained with the HPLC analyses. Separations with this system are based on hydrophobicity. HT-2 and T-2 are both strongly hydrophobic, with three (HT-2), or all four (T-2) of the trichothecene hydroxyl groups converted to acetyl or valeryl esters. Both toxins are known to undergo de-esterification to a tetrol nucleus followed by glucuronidation or sulfoesterification on one or more of these hydroxyls. The ^3H containing peaks appearing in HPLC fractions earlier than those corresponding to the T-2 and HT-2 standards would be consistent with the biliary and urinary appearance of such metabolites. The absence of radioactivity eluting at times corresponding to the parent compounds reflects their extensive hepatic metabolism prior to their biliary excretion. The small amount of HT-2 in the urine may have resulted from direct filtration of this initial metabolite. The early appearance of portions of both labels in the chromatographic procedures is assumed to be coincidental, reflecting a failure in resolution of the compounds responsible, and is not consistent with the chemical identity of a GSH conjugate In particular, the presumptive conjugate would be expected to retain a free amino group on the glutathione moiety and on all subsequent metabolites prior to acetylation of the cysteinyl derivative, (the final step in the mercapturic acid pathway). All such species would bind to the cation exchange column and elute at considerably later times than those observed.

Thus, considering the sensitivity of the radiochemical and chromatographic methods employed, we estimate that less than 1% of T-2 metabolism involves the direct conjugation with GSH. However, we note that reactions involving participation of GSH as a free radical scavenger and in the maintenance of the cellular thiol redox status may be of considerable importance in mitigating the biochemical lesions produced by T-2 intoxication. These reactions would be expected to be of particular importance at later stages of poisoning as the cellular mechanisms of regeneration and recovery emerge. Indeed, such reactions may account for the previous findings that administration of buthionine sulfoximine, an inhibitor of glutathione synthesis, increases sensitivity to trichothecene toxin, and that administration of L-2-oxothiazolidine-4-carboxylate increases resistance to the toxin.

3b. Studies on Glutathione Esters

Synthesis of Glutathione Monoisopropyl Ester Hydrochloride

This compound was prepared as part of an effort to study the effects of various glutathione derivatives on tissue glutathione levels. The procedure for preparation of this compound follows that used for the preparation of glutathione monoethyl ester. The specific details for this preparation were as follows: Glutathione (10 g; 32.5 mmol) was added to a cold (4-6° C) solution of isopropanol (previously dried over a molecular sieve) containing hydrogen chloride (3 g; 65 mmol) in a volume of 127 ml in a 500 ml round bottom flask. The mixture was swirled occasionally and after 24 hours, cold diethyl ether was added to precipitate the product. The product was recovered by decantation of the supernatant solution and was dried over P_2O_5 and KOH under vacuum. The yield was 12.3 g (98%). Analysis on the Durrum model 500 amino acid analyzer showed that the product contained only 8% glutathione.

The effectiveness of the monoisopropyl ester of glutathione was examined in fasted mice pretreated (for 3 hours) with L-buthionine-SR-sulfoximine (BSO) (2 mmol/Kg). Two hours after intraperitoneal injection of glutathione monoisopropylester hydrochloride (dose, 10 mmol/Kg), the kidneys and livers were removed and analyzed for total glutathione by the automated DTNB-Reductase. The following results were obtained:

Treatment	GSH (μmol/g)	
	Kidney	Liver
BSO, alone	0.30 ± 0.02	0.58 ± 0.09
BSO, GSHisopropyl ester	1.63 ± 0.37	1.56 ± 0.23
BSO, GSHethyl ester	3.28 ± 0.34	1.44 ± 0.04

The findings indicate that the levels of glutathione in the liver and kidney were significantly increased by administration of glutathione isopropylester hydrochloride. However, the increase in the kidney was not as great as is found with glutathione ethyl ester hydrochloride. We have not yet carried out studies on the time course of the effect of this ester.

Synthesis of Glutathione Monoethyl Ester Sulfate

We have continued our efforts to obtain a more stable form of glutathione monoethyl ester. In the course of this work, we have prepared glutathione monoethyl ester sulfate (i.e., L-γ-glutamyl-L-cysteinylglycylethyl ester.0.5 H_2SO_4). The following procedure was used. Glutathione (2.5 g) was added to a 200 ml round bottom flask containing 0.59 ml of sulfuric acid and 25 ml of ethanol at 0° C. The flask was swirled occasionally and allowed to stand overnight. The unreacted glutathione was removed by filtration and the glutathione monoethyl ester sulfate was precipitated by addition of cold diethyl ether (about 200 ml). The product was dried over P_2O_5 in vacuum.

The biological effectiveness of glutathione monoethyl ester sulfate was examined in mice that had been fasted for 18 hours and previously treated with L-buthionine-SR-sulfoximine (2 mmol/Kg) four hours prior to sacrifice. The glutathione monoethyl ester sulfate was administered intraperitoneally at a dose of 10 mmol/Kg. Three hours later the mice were sacrificed and the kidneys and livers were removed and immediately homogenized in 5% (w/v) 5-sulfosalicylic acid. Total glutathione was determined by the automated DTNB reductase procedure. The following results were obtained:

Treatment	GSH, μmols	
	Kidney	Liver
BSO, alone	0.30 ± 0.02	0.58 ± 0.09
BSO, GSH	2.00 ± 0.59	1.04 ± 0.28
BSO, GSH ester sulfate	5.38 ± 0.83	1.57 ± 0.22

The findings indicate that glutathione monoethyl ester sulfate is effective in raising the glutathione levels of kidney and liver. The compound does not seem to be deliquescent and hopefully will prove to more stable on storage than the corresponding hydrogen chloride derivative.

Appendix 2 lists samples of glutathione esters sent to USAMRIID together with procedures for dissolving these compounds.

3c. γ-Glutamyl-Glutathione

In the course of our studies on the biliary excretion and metabolism of glutathione and its conjugates, we discovered a new metabolite of glutathione, namely γ-glutamyl glutathione. This tetrapeptide is formed by autotranspeptidation catalyzed by γ-glutamyl transpeptidase in a reaction involving 2 molecules of glutathione. The finding of γ-glutamyl glutathione in bile is most interesting because this is the first time that this autotranspeptidation product has been found in nature. In bile, γ-glutamyl glutathione may be present in concentrations that are greater than 300 μM. Notably, this level is much higher than that of most of the other amino acids normally found in bile. The chemical identity of this compound was established by showing that it yields 2 molecules of glutamate and 1 molecule of cysteinylglycine after exhaustive enzymatic hydrolysis by γ-glutamyl transpeptidase. The elution behavior of this compound on the amino acid analyzer has been determined. Whether γ-glutamyl glutathione can be transported into cells is not yet known. We plan to examine this point. We also need to know whether γ-glutamyl glutathione acts as a substrate for glutathione-S-transferase, γ-glutamyl cyclotransferase, and glutathione reductase. Further studies on the formation of γ-glutamyl glutathione by interaction of glutathione with γ-glutamyl transpeptidase are being done. We plan to carry out studies on the large scale chemical preparation of this compound.

3d. Aminoethanol Analog of Glutathione

Our research on the new analog of glutathione in which the glycine residue is replaced by an amino ethanol moiety has been continued. In the course of these studies, we discovered that some of the products obtained contained a new compound not previously detected. Some products contained about 10% of the new compound, whereas others contained as much as 50-70% of the new material. An effort is currently in progress in order to determine the nature of the new compound and the mechanism of its formation during borohydride reduction of γ-glutamylcysteinylglycyl ethyl ester.

In the course of this work an attempt was made to synthesize L-γ-glutamyl-L-cysteinylaminoethanol in the bioreactor system previously designed in this laboratory. In this approach, a strain of *E. coli* genetically engineered so as to contain high levels of γ-glutamylcysteine synthetase and glutathione synthetase is immobilized and rendered more permeable to low molecular weight compounds. This preparation of *E. coli* has been successfully used for the preparation of certain analogs of glutathione. Efforts to apply this method to the synthesis of γ-glutamylcysteinylaminoethanol were not fruitful, apparently because glutathione synthetase does not use aminoethanol in place of glycine.

3e. Studies on Enrichment Cultures

As part of our program to attempt isolation of enzymes that act upon cyanoginosin toxins, enrichment culture and related approaches are being

employed. Since instrument toxin was available at the initiation of this research, we were not able to carry out enrichment cultures with the toxin itself. However, since cyanoginosin-LA has been reported to contain a β-aspartyl linkage, we carried out preliminary enrichment cultures using the optical isomers of asparagine (aspartic acid-β-amide) as the substrate. This was done to explore the idea that an organism selected for its ability to cleave the β-amide of asparagine might also exhibit similar hydrolytic activity toward the β-aspartyl moiety of the toxin.

The procedure was essentially that described earlier for isolation of an organism containing large amounts of 5-oxoprolinase (13). The enrichment medium contained 40 mM L- or D-asparagine, 5.5 mM KH_2PO_4, 3.5 mM Na_2HPO_4, 0.80 mM $MgSO_4$, 0.20 mM $CaCl_2$, 15 μM $FeCl_3$, 15 μM $MnCl_2$, and 4.0 μM $Na_2M_oO_4$. The cultures were carried out at pH 7.0 at 26° C. Three pure cultures of bacterial were isolated by enrichment of L-asparagine and three pure cultures were isolated by enrichment on D-asparagine. These cells are being preserved and will be tested for activity against the toxins when sufficiently large samples of these materials become available to us.

3f. Studies on Sample of Cyanoginosin Toxin Supplied by USAMRIID (microcystin toxin 782001166C8).

A portion (approximately 2 micrograms) of this toxin was hydrolyzed in 6 M hydrochloric acid under nitrogen for 3 hours at 150° C. The hydrolysate was then derivatized with phenylisothiocyanate and the derivatized amino acids present were separated by HPLC. This analysis indicated the presence of the following amino acids: leucine, alanine, arginine, β-methylaspartic acid, and glutamic acid. These were present in approximately equimolar quantities (1:0.9:1:1:1). The assignments and relative ratios were based on the retention times and areas obtained with the corresponding authentic amino acid standards. β-Methylaspartic acid eluted as 2 peaks (2.2 and 2.6 minutes). The first peak coeluted with glutamic acid and the area for this amino acid peak was greater than that for the other amino acids. The areas corresponding to the authentic standards of β-methylaspartic acid and glutamic acid were found to be almost identical. Thus, the method of calculation of the relative ratios of these amino acids must be the sum of the areas of the peaks obtained at 2.6 and 2.2 minutes divided by a factor of 2. The findings indicate that the toxin supplied to us by USAMRIID is apparently equivalent to the cyanoginosin toxin designated as LR.

When we treated the toxin obtained from USAMRIID with phenylisothiocyanate, no derivative could be detected by HPLC. This is consistent with the absence of a free amino group as previously noted by others (14-17).

It is known that dehydropeptides can be converted to ammonia and the corresponding α-keto acid by enzymatic means and also by treatment with mineral acid. For example, glycyldehydroalanine is converted under such conditions to products that include ammonia and pyruvate. We have attempted to obtain evidence for the formation of pyruvic acid after treatment of cyanoginosin LR with HCl (1-2 M) at temperatures varying from 26° C to 100° C. Thus far no pyruvic acid has been found by sensitive enzymatic tests using lactate dehydrogenase and NADH; however, in control experiments with comparable amounts of authentic pyruvic acid and with standard dehydropeptide

preparations such as acetyldehydroalanine, we did not find pyruvate after acid treatment. Presumably any pyruvate formed from the toxin would have been destroyed under these conditions. This work is being continued.

We have also treated the USAMRIID toxin sample with sodium borohydride essentially by the procedure of Botes *et al* (18). A sample of the toxin was also subjected to pulse hydrolysis in 6 M hydrochloric acid under nitrogen at 100° C for 5 minutes. Total hydrolysis was carried out in 6 M hydrochloric acid under nitrogen at 150° C for 3 hours. After treatment of the toxin with sodium borohydride, no free amino acids were detected by the HPLC procedure. This finding suggests that the product of borohydride treatment is an intact cyclopeptide.

Toxicity studies were carried out on the untreated toxin, toxin subjected to pulse hydrolysis, toxin subjected to total hydrolysis, and toxin treated with sodium borohydride. In addition, a control was carried out in which the borohydride reaction mixture (lacking toxin) was examined for toxicity. Mice (male) weighing 20-25 g of the Swiss Webster strain (Taconic Farms) fed *ad libitum*, were used in these studies. A solution containing 4.6 μg/ml of toxin was employed and a dose of 0.046 μg/g of body weight was given. Eight animals were used in each group except for the borohydride control in which case four animals were used. The route of administration was intraperitoneal. No animals died when given toxin that had been subjected to pulse hydrolysis, total hydrolysis, toxin treated with borohydride, or the borohydride control. In the animals given untreated toxin, 7 out of 8 animals died within 3 hours. These findings indicate that the several treatments applied to the toxin result in loss of toxicity.

Botes *et al* (17) found that pulse hydrolysis of cyanoginosin LA gave a linear peptide apparently consisting of the following structure: ala-leu-β-methyl-aspartate-ala-adda(lacking a methoxy moiety)-glu. Our studies, as described above, indicate that pulse hydrolysis of cyanoginosin LR toxin, which would presumably lead to the formation of a comparable linear peptide except that arginine would replace the second alanine residue, leads to loss of toxicity. This finding could be interpreted to indicate that the dehydropeptide moiety is essential for toxicity. However, pulse hydrolysis apparently also leads to loss of a methoxy group so that toxicity might also require the intact adda structure. On the other hand, in our studies it was found that treatment of the LR toxin with sodium borohydride led to loss of toxicity. Since this reaction would not be expected to induce a loss of a methoxy moiety from the adda structure, the findings point to the dehydropeptide moiety as an essential structural feature required for toxicity. According to this interpretation, treatment of the toxin with borohydride converts the dehydropeptide linkage to a saturated peptide linkage and this change is associated with loss of toxicity. Since it appears that this is the only effect of borohydride treatment, we tentatively conclude that the dehydropeptide moiety of the toxin is essential for toxicity. This finding may be of considerable significance in relation to mechanisms for detoxication. Thus, it is known that dehydropeptides interact effectively with SH-containing compounds, and a number of SH-compounds that can be safely administered to animals are known (see, for example, below).

3g. Studies on the Interaction of Cyanoginosin Toxin with Glutathione

Since the toxin has now become available to us, it has been possible to initiate this work and to carry out some preliminary studies. This may be a promising pathway of research because our first studies have shown that mice that have approximately 50% depletion of hepatic glutathione are more sensitive to cyanoginosin toxin than are the control animals. In further work we plan to pursue the effect of buthionine sulfoximine on cyanoginosin toxicity because much lower levels of glutathione can be achieved through use of this drug. Various approaches previously developed in this laboratory will be applied to increase hepatic glutathione levels and to determine the effects of such metabolic modulation on toxicity.

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Appendix No. 1

Personnel

Alton Meister, Principal Investigator

Postdoctoral Associates

Mary E. Anderson	11/1/82-11/15/86
Andrew P. Seddon	11/15/85-11/15/86

Technical Assistants

Alice Erling	1/3/83-1/14/87
Betty Nulty	9/15/85-1/14/87
Melanie Szuba	2/10/86-9/16/86

Lab Helper

Danny Stewart	7/1/83-8/22/85

Appendix 2

Samples of glutathione esters, as follows, were sent to USAMRIID on May 19, 1986:

1 g Glutathione monoethyl ester hydrochloride (MW = 371.84)

1 g Glutathione mono-isopropyl ester.1/2 H_2SO_4 (MW = 398.44)

100 mg Glutathione monoethyl ester, free base (MW = 335.38)

In addition, a sample of 10.1 g of L-2-oxothiazolidine-4-carboxylic acid was also sent on May 19, 1986.

The following procedures are recommended for preparing solutions of these compounds for biological experiments:

1. 2-Oxothiazolidine-4-carboxylic acid is freely soluble (at least 1 M) and only needs to be pH adjusted to 7 with 1 equivalent of sodium hydroxide. As a solution it is stable.

2. Glutathione mono-isopropyl ester.1/2 H_2SO_4 and glutathione monoethyl ester free base are stable as powders, but should be kept desiccated to be completely safe. As with all esters, their solutions are not stable: Make them just before use. The glutathione monoethyl ester free base should require no or little sodium hydroxide to Ph adjust to 7. The glutathione monoethyl ester hydrochloride and sulfate may require about one equivalent of sodium hydroxide to pH adjust to 7.

Distilled and deionized water is recommended to reduce metal contamination, which can cause thiol oxidation.