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THE FLUORIMETRIC DETECTION OF AMATOXINS, PHALLOTOXINS, AND OTHER PEPTIDES IN *AMANITA SUBALLIACEA*

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ABSTRACT.—A method has been developed for the detection and quantification of the cyclic peptides, alpha-amanitin and phalloidin, which is based upon reaction with fluorescamine before and after acid hydrolysis. This method was applied to fractionated extracts of the toxic mushroom *Amanita suballiacea* and resulted in quantitative detection of these and other toxins with negligible interference by other compounds. At least four additional peptides have been found by this approach. With a limit of detection of 0.1 nmole peptide, this approach should be very useful in identifying cyclic peptides, independent of biological activity, in a number of systems.

The early work of T. Wieland, H. Faulstich, and co-workers on the lethal constituents of European *Amanita phalloides* has culminated in the detailed characterization of amatoxins and phallotoxins as the major classes of bicyclic peptide toxins (1). Recently, virotoxins have been isolated and characterized as a class of monocyclic peptide toxins from *Amanita virosa* (2). The toxicity of the amatoxins resides in their specific and potent inhibition of RNA polymerase II, while the phallotoxins and virotoxins perturb the dynamic equilibrium between F and G actin via stabilization of the F form. Amatoxins and phallotoxins have been quantified in American mushrooms including the locally abundant species *Amanita suballiacea* (3-7).

Surveys on the levels of these compounds in taxonomically related species of *Amanita* showed that only a few species produce them in amounts greater than 100 μg/g dry weight (1, 3-5). The levels of individual toxins in *A. phalloides* and *A. virosa* have been well documented (6, 8). Only in the case of *A. phalloides* has the presence of cyclic peptides, *e.g.*, antamanide (7) and cycloamanides (9), been detected, which lack the biological activities characteristic of the amatoxins, phallotoxins, and virotoxins. In order to identify and quantitate toxic and nontoxic cyclic peptides in extracts of *A. suballiacea* carpophores, an assay was developed to detect peptides, which relies solely upon their unique chemical nature and which is independent of their biological activity. The optimal conditions for this method, based upon the reaction of fluorescamine with free amino groups before and after acid hydrolysis, are described here along with the application of this method for the quantitative determination of MeOH soluble peptides in *A. suballiacea*.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS.—All solutions were prepared in distilled deionized H_2O, except for fluorescamine (Fluram) which was prepared in Me_2CO (0.3 mg/ml). Reagents were ACS grade or better, except for MeOH and Me_2CO, which were spectral- or hplc-grade solvents. Gramicidin S, bacitracin, Fluram, and amino acids were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Phalloidin and α-amanitin were purified from *A. suballiacea* by sequential chromatography on Sephadex LH 20 (Pharmacia) with 50% aqueous MeOH, Biogel P-2 (BioRad Laboratories, 200-400 mesh) with H_2O, and again on Sephadex LH 20 (H_2O) as described (3, 10, 11), with purity established by tlc and uv spectrophotometry.

INSTRUMENTATION.—Fluorimetric intensities were measured using an Aminco Fluoro-Colorimeter equipped with a Corning 7-51 primary filter and Wratten #4 secondary filter and F4T4 blue-light lamp. An adaptor was fitted to accommodate 10 × 75 mm borosilicate tubes in which the intensities were recorded. A Gilford 2400 Spectrophotometer was used for the calibration of toxins, using $^{304}\epsilon = 15,400$ for amatoxins (12) and $^{300}\epsilon = 10,100$ for phallotoxins (1), and for monitoring absorbance of column fractions.

A Waters M6000A Liquid Chromatograph fitted with a 0.9 × 25 cm Zorbax (Dupont) C-18 column was used for isocratic reverse phase resolution of peptides using 22% acetonitrile in H_2O.

MUSHROOM IDENTIFICATION.—Specimens used for this study were identified as *A. suballiacea* Murrill by microscopic and gross morphological properties as described (3), and vouchers have been accessioned in the Mycological Herbarium, University of Florida, as F51921. All specimens analyzed were collected during June, July, and August of 1981 within five miles of Gainesville, FL.

PEPTIDE EXTRACTION AND FRACTIONATION.—Fresh specimens (8 kg) were chopped, extracted with two volumes of MeOH with continuous shaking on a New Brunswick G-10 gyrotary shaker at 100 rpm for 24 h at 25° and filtered through Whatman No. 1 paper. After concentration to a small volume (less than 100 ml), 9 volumes of ice-cold MeOH were added, followed again by filtration. The filtrate was concentrated to 50 ml, extracted three times with 3 volumes of anhydrous Et_2O, and evaporated *in vacuo* to remove residual Et_2O.

The aqueous solution was brought to 50% with respect to MeOH and chromatographed in 50% aqueous MeOH on a preparative (4.9 × 92 cm) Sephadex LH 20 column. The fractions eluting between 0.63-0.75 column volumes were concentrated to dryness and redissolved in H_2O, and an aliquot was applied (approximately 5 mg total peptide) to an analytical (0.5 × 25 cm) Biogel P-2 column. Peptides were eluted with H_2O and detected by spectrophotometric, fluorimetric, and biological analyses.

BIOASSAYS.—Amatoxins were quantified by inhibition of calf thymus RNA polymerase II as previously described (4), and phallotoxins were quantified by their interaction with F-actin as described by Mullersman and Preston (10).

FLUORESCAMINE ASSAY.—In assessing optimal detection conditions, duplicate portions of pure peptide or amino acid solutions in MeOH were flash evaporated to dryness in screw-cap culture tubes. To the portion to be hydrolyzed, 0.5 ml 6 N HCl was added, the tube was flushed with N_2 and sealed with a teflon lined cap. Following hydrolysis at 110° for 20 h (determined to be adequate for maximal amino acid release, data not shown), the samples were again flash evaporated to rapidly remove HCl. To both the hydrolyzed and nonhydrolyzed samples, 1.0 ml of 1% Me_2CO in H_2O was added to assist solvation of lipophilic components. Duplicate aliquots (0.01-0.10 ml) were transferred to 10 × 75 mm culture tubes for reaction with Fluram.

The volume of sample in each 10 × 75 mm tube was brought to 1.85 ml with 0.05 M borate buffer, and the fluorescent intensity was recorded. To the same tube, 0.3 mg/ml Fluram in Me_2CO was added while vortexing, using the sequential method of Chen *et al.* (13) to provide a final Fluram concentration of 240 μM, and the fluorescence was again recorded. Tubes were used once and then discarded.

RESULTS

OPTIMAL CONDITIONS FOR FLURAM REACTIVITY.—Several workers have described pH optima for reactions involving Fluram and primary amines. However, because the optimal pH for maximal fluorescent intensity resulting from fluorescamine reaction with different amino acids may range from 7 to 10 (14-16), we chose to reexamine the influence of pH on fluorescamine reactivity of hydrolyzates of the toxins we wished to ultimately detect. The optimal fluorescence occurs at pH 8.5 for both α-amanitin and phalloidin, as well as their constituent amino acids L-isoleucine and D-threonine (data not shown). This condition was, therefore, adopted in all subsequent experiments.

Figure 1 describes the enhancing effect of acid hydrolysis on the fluorescent yield obtained with peptides devoid of reactive amino groups prior to hydrolysis, *i.e.*, α-amanitin and phalloidin, as well as with peptides containing one or more amino groups prior to hydrolysis, *i.e.*, bacitracin and gramicidin S. In all cases, the enhancement observed after hydrolysis is significant, while it is apparent that the peptides lacking amino groups are unreactive prior to hydrolysis. The sensitivity of the assay for detecting amanitin, phalloidin, gramicidin, and bacitracin establishes the lower limit of 0.1 nmole for cyclic peptides lacking an amino group (Table 1).

FRACTIONATION OF PEPTIDES ON BIOGEL P-2.—When the toxin fraction (representing 0.32 g dry weight mushroom) from the preparative fractionation on Sephadex LH-20 (50% aqueous MeOH) is further resolved on Biogel P-2, the elution profile shown in Figure 2A is obtained. Subsequent examination of tube contents representing

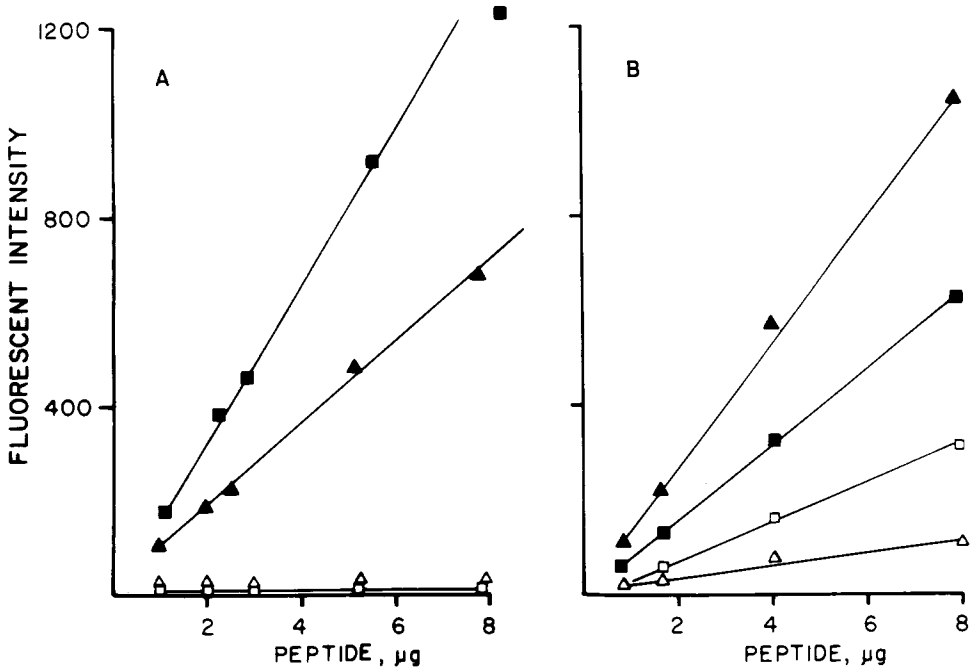

FIGURE 1. Linearity of detection response and the requirement for hydrolysis for cyclic peptide detection by Fluram. Cyclic peptides were reacted with Fluram before (open symbols) or after (closed symbols) acid hydrolysis.
A. The response given by α-amanitin (squares) or phalloidin (triangles).
B. Response of bacitracin (triangles) or gramicidin S (squares).

TABLE 1. Limits of Detection of Peptides Via Fluram Following Acid Hydrolysis

Peptide	Molecular Weight	I/µg	Limits of Detection[a]	
			µg	nmole
α-Amanitin	918	145	0.08	0.09
Phalloidin	789	108	0.09	0.11
Gramicidin-S . . .	1141	190	0.05	0.04
Bacitracin	1411	375	0.03	0.02

[a]Based upon fluorimeter setting of 10×.

peak fractions (data not shown) by amino acid analysis, tlc (cinnamaldehyde reactivity) or QAE-Sephadex binding (12) identified the following: tube 19: β-amanitin and acidic phallotoxins; tube 30: phalloidin; and tube 38: α-amanitin.

The fluorescent intensity profile generated by these same fractions can be seen in Figure 2B. There is a striking correlation between the absorbance profile (Fig. 2A) and that of fluorescent intensity (following acid hydrolysis) that suggests the presence of large amounts of chromophore-containing peptides throughout the fractions. The fluorescent detection of both the α-amanitin and phalloidin fractions shows the requirement for hydrolysis as observed before (Fig. 1A), with the other fractions appearing only slightly reactive without such treatment. The bioassay profile in Figure 2C depicts the levels of components capable of mediating phallotoxin- or amatoxin-like biological properties that are distributed throughout these fractions and further establishes the identities of the phalloidin and α-amanitin fractions.

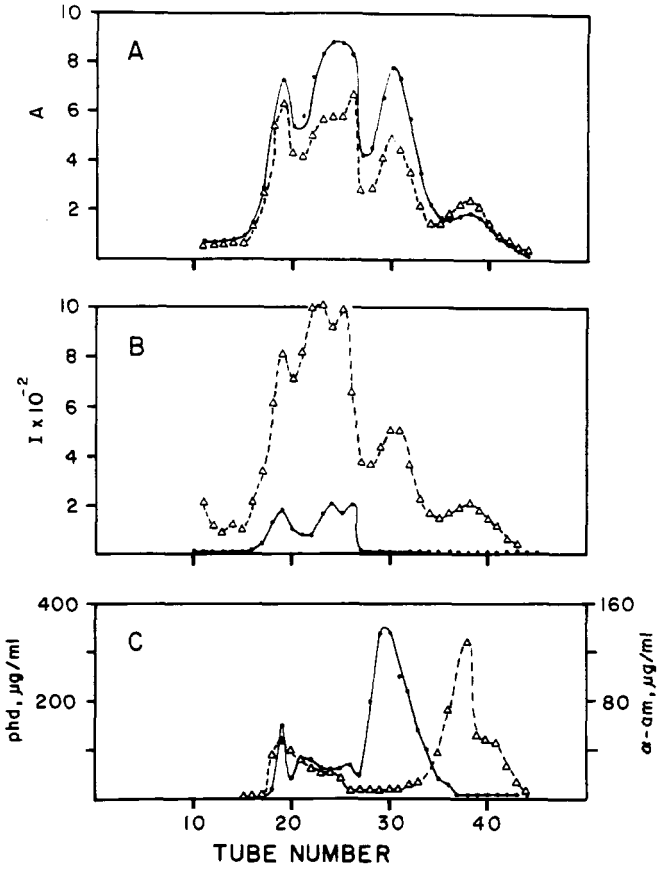

FIGURE 2. Fractionation of *A. suballiacea* extract on Biogel P-2. Peptides derived from a Sephadex LH20 column were further resolved on a Biogel P-2 column (0.5 × 25 cm) eluting with H_2O (5.6 ml/h) and collecting 0.7 ml fractions.
A. The uv absorbance for individual fractions using the maxima for α-amanitin at 304 nm (open triangles) or phalloidin at 292 nm (closed circles).
B. Fluorescent intensity (I) for these same fractions reacted with Fluram prior to (closed circles) or after (open triangles) acid hydrolysis.
C. The distribution of amatoxins as α-amanitin equivalents (open triangles) or phallotoxins as phalloidin equivalents (closed circles) determined by bioassay as described in the text.

A comparison between the bioassay curves and the fluorescamine-mediated fluorescence profile obtained after acid hydrolysis is given in Table 2. Tubes 30 and 38, which contain phalloidin and α-amanitin, respectively, show fluorescent yields close to those expected, based on the bioassays. Other peak fractions, *i.e.*, those represented by tubes 23 and 25, show fluorescent yields more than tenfold greater than was expected, based on the levels of amatoxins and phallotoxins estimated by the bioassays.

RESOLUTION OF BIOGEL P-2 FRACTIONS BY HPLC.—The fractions eluting from Biogel P-2 represented by tubes 23-25, which showed the high fluorescamine-dependent fluorescent yields after acid hydrolysis and relatively low levels of phallotoxins and amatoxins with bioassays, were pooled and fractionated by reverse-phase hplc. As shown in Figure 3, this procedure resulted in the resolution of four major peaks, desig-

TABLE 2. Comparison of Expected vs Observed Fluorescence Values For Biogel P-2 Fractions After Acid Hydrolysis and Reaction With Fluorescamine

Tube No.	Toxin by Bioassay (μg/ml)		Expected I[c]	Observed I[d]
	Phtx[a]	Ama[b]		
19	150	48	232	800
23	60	22	96	1000
25	55	25	95	1000
30	350	5	385	510
38	0	128	187	200

[a]Phallotoxin quantified by release of DNase I inhibition by actin.

[b]Amatoxin quantified by inhibition of ^{3}H-UMP incorporation by calf thymus RNA Polymerase II.

[c]Total expected fluorescence after acid hydrolysis (per 10 μl fraction) based on toxin levels (μg/ml) and fluorescence values (I/μg, Table 1) for pure α-amanitin or phalloidin.

[d]Observed relative fluorescence per 10 μl fraction after acid hydrolysis.

nated A, B, C, and D. All of these peaks were nonfluorescent with (Table 3) or without Fluram treatment (data not shown). After acid hydrolysis, significant Fluram-mediated fluorescence was detected, suggesting these peaks contained cyclic or *N*-blocked peptides.

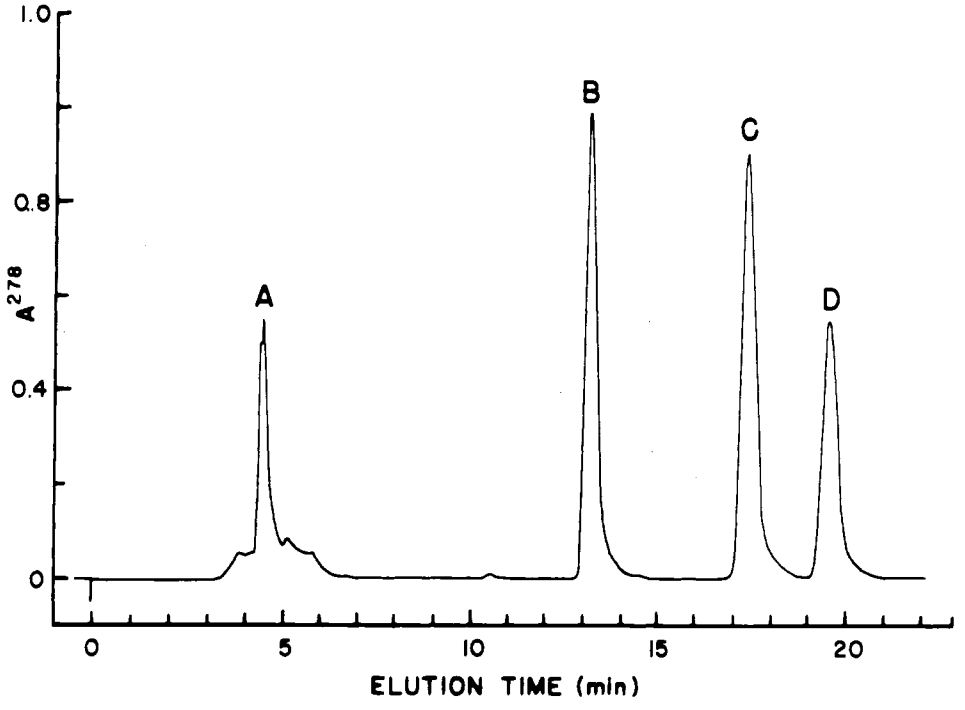

FIGURE 3. Reverse phase hplc of the peptide-rich P-2 fractions from *A. suballiacea*. Tubes (23-25) from the Biogel P-2 column that yielded high fluorescent intensity (Table 2) were pooled and resolved by hplc as described in the text. Twenty μl of peaks A, B, C, D derived from a 0.2-ml injection were further evaluated for peptides by Fluram reactivity (Table 3).

TABLE 3. Fluram Reactivity of hplc Fractions Before and After Acid Hydrolysis

Fraction[a]	I[b] Before	I[b] After
A	0	106
B	0	129
C	0	120
D	0	87

[a]Fractions were collected by hand to select absorbance peaks eluting from the column profile of Fig. 3 and assayed as described in the text.

[b]Average of duplicates per 20 μl fraction assayed as described in the text.

DISCUSSION

Using tryptic digests of RNase, Nakai, *et al.* (16) have demonstrated enhanced sensitivity in the detection of linear peptides with Fluram after hydrolysis. We show here such hydrolysis is an absolute requirement for cyclic peptides lacking primary amino groups. This observation contrasts with that of Becker, *et al.* (17), who reported the tlc detection of α-amanitin by direct reaction with Fluram on tlc plates. However, a direct comparison between these observations is not valid because the reactivity in the latter case was observed on moist silica gel plates containing concentrated NH_4OH and cinnamaldehyde, and the chemistry in this case is therefore unclear.

For each of the major peaks resolved on Biogel P-2 (Fig. 2), a comparison was made between the fluorescence expected in a given peak (based upon the fluorescent yield/μg for pure toxin) quantified by bioassay with fluorescence observed under the assay conditions (Table 2). Based upon the spectral and bioassays, fractions represented by tubes 30 and 38 contain phalloidin and α-amanitin, respectively, and their detection appears to be nearly quantitative. Although they are not necessarily pure at this fractionation step, these are the singular peptides comprising these peaks; we may conclude, then, that quenching by other components in the fraction does not contribute to a major loss in sensitivity.

Based upon bioassay analyses, an interesting contrast between expected and observed fluorescence levels is seen in the other three peaks represented by tubes 19, 23, and 25. Here, the observed levels based upon Fluram reactivity after acid hydrolysis are fourfold to tenfold greater than what is expected by bioassay and this suggests that other peptides lacking the tested biological activities may be present. Further resolution by hplc of combined Biogel P-2 fractions 23-25 (Fig. 3) demonstrates at least four peptides (A, B, C, D) present that account for the Fluram reactivity after acid hydrolysis. Examination by tlc and uv spectroscopy (data not shown) shows A to be polar, nonreactive toward cinnamaldehyde-HCl, and to possess a λ max (H_2O) of 260 nm; fractions B, C, and D are viroisin, an isomer of viroidin, and viroidin, respectively, and will be addressed in a subsequent publication (18).

The average yield of toxins on a mg/g dry weight basis may be calculated by summing the areas under the bioassay curve for individual toxins (Fig. 2C), given that these fractions were derived from 0.32 g dry weight of tissue. These levels (normalized for 1 g dry weight) have been estimated as: neutral amatoxins, 1.22 mg; acidic amatoxins, 0.16 mg; neutral phallotoxins, 3.38 mg; and acidic phallotoxins, 0.94 mg. Preparative fractionation (data not shown) in this laboratory of these groups, coupled with tlc and amino acid analysis, have demonstrated that the neutral amatoxins include mainly

α-amanitin with traces of γ-amanitin; acidic amatoxins include mainly β-amanitin with traces of ε-amanitin and amanullinic acid; neutral phallotoxins are exclusively phalloidin, while phallacidin and phallisacin comprise the acidic phallotoxin fraction.

In comparison with European and American *A. phalloides*, in which acidic toxins prevail (1), *A. suballiacea* is remarkable for its high levels of neutral toxins, with phalloidin present in amounts almost threefold greater than α-amanitin. Although the toxin profiles of *A. virosa* have demonstrated some variability (6), the presence of virotoxins and absence of β-, γ-, and ε-amanitin seem to distinguish this species (2). Thus, the unique chemotaxonomic niche occupied by *A. suballiacea* is manifest by its potential for synthesis of those toxins lacking in *A. virosa*, high levels of neutral toxins, and virotoxins (18).

In conclusion, we feel this approach could have a useful application in the detection of low levels of peptides, cyclic or *N*-blocked, irrespective of any biological activity they possess. The ability to quantitatively detect α-amanitin and phalloidin in low levels reflects its sensitivity, but a more redeeming feature is that it has demonstrated the presence of other peptides in this species. It is expected that a number of cyclic peptides with unknown biological activities may be detected and quantified with this approach.

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LITERATURE CITED

1. T. Wieland and H. Faulstich, *Crt. Rev. Biochem.*, **5**, 185 (1978).
2. H. Faulstich, A. Buku, H. Bodenmuller, and T. Wieland, *Biochemistry* **19**, 3334 (1980).
3. J.F. Preston, B.E.C. Johnson, M. Little, T. Romeo, H.J. Stark, and J.E. Mullersman, "Investigations on the Function of Amatoxins in *Amanita* Species: A Case for Amatoxins as Potential Regulators of Transcription," in: "Peptide Antibiotics-Biosynthesis and Function." Ed. by H. Kleinkauf and H. von Dohren, de Gruyter and Co., Berlin, 1982, pp. 399-426.
4. J.F. Preston, H.J. Stark, and J.W. Kimbrough, *Lloydia*, **38**, 153 (1975).
5. P.A. Horgen and J.F. Ammirati, *Lloydia*, **39**, 368 (1976).
6. R.R.Yocum and D.M. Simons, *Lloydia*, **40**, 178 (1977).
7. T. Wieland, G. Luben, H. Ottenheym, J. Faesel, J.X. de Vries, A. Prox, and J. Schmid, *Angew, Chem. Internat. Edit.*, **7**, 204 (1968).
8. H. Faulstich, D. Georgopoulos, and M. Bloching, *J. Chromatog.*, **79**, 257 (1973).
9. A. Gauhe and T. Wieland, *Liebigs Ann. Chem.*, 859 (1977).
10. J.E. Mullersman and J.F. Preston, *Anal. Biochem.*, **119**, 266 (1982).
11. B. Johnson and J.F. Preston, *Biochim. Biophys. Acta*, **607**, 102 (1980).
12. R.R. Yocum, *Biochemistry*, **17**, 3786 (1978).
13. R.F. Chen, P.D. Smith, and M. Maly, *Arch. Biochem. Biophys.*, **189**, 241 (1978).
14. S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele, *Science*, **17**, 871 (1972).
15. S. deBernardo, M. Weigele, V. Toome, K. Manhart, and W. Leimgruber, *Arch. Biochem. Biophys.*, **163**, 390 (1974).
16. N. Nakai, C.Y. Lai, and B.L. Horecker, *Anal. Biochem.*, **58**, 563 (1974).
17. C. E. Becker, T.G. Tong, U. Boerner, R.L. Roe, R.A. Scott, M.B. MacQuarrie, and F. Bartter, *West J. Med.*, **125**, 100 (1976).
18. M.C. Little, J.F. Preston, R.W. King, and L.C. Taylor. Manuscript in preparation.

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