(5) Shubik, P., Della Porta, G., and Spencer, K., *Acta Unio. Intern. Contra Cancrum*, **15**, 232(1959).
(6) Merenmies, L., *Acta Pathol. Microbiol. Scand. Suppl.*, **130**, 1(1959).
(7) Della Porta, G., Terracini, B., Dammert, K., and Shubik, P., *J. Natl. Cancer Inst.*, **25**, 573(1960).
(8) Della Porta, G., Shubik, P., Dammert, K., and Terracini, B., *ibid.*, **25**, 607(1960).
(9) Shubik, P. A., *Acta Unio. Intern. Contra Cancrum*, **17**, 22(1961).
(10) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," 4th ed., Burgess Publishing Co., Minneapolis, Minn., 1964.
(11) Blank, H., Rosenberg, E. W., and Sarkany, I., *J. Invest. Dermatol.*, **34**, 307(1961).
(12) Wilmsmann, H., and Marks, A., *Fette, Seifen, Anstrichmittel*, **61**, 965(1959).
(13) Wilmsmann, H., *Am. Perfumer Cosmet.*, **78**, 21(1963).
(14) Polgár, L., *Acta Physiol. Acad. Sci. Hung.*, **21**, 313(1962).
(15) Jambor, J. J., and Suskind, R. R., *J. Invest. Dermatol.*, **24**, 379(1955).
(16) Jambor, J. J., *ibid.*, **24**, 387(1955).
(17) Ferguson, E. H., and Rothman, S., *Arch. Dermatol.*, **80**, 300(1959).
(18) Suskind, R. R., Meister, M. M., Scheen, S. R., and Rebello, D. J. A., *ibid.*, **88**, 117(1963).
(19) Van Scott, E. J., and Lyon, J. B., *J. Invest. Dermatol.*, **21**, 199(1953).
(20) Blank, I. H., *ibid.*, **25**, 391(1955).
(21) Szakall, A., *Arch. Klin. Exptl. Dermatol.*, **201**, 311(1955).
(22) Paschound, J. M., and Schmidli, B., *Dermatologica*, **110**, 323(1955).
(23) Flesch, P., and Esoda, E. C. J., *Nature (London)*, **178**, 211(1956).

Occurrence of Amanita Toxins in American Collections of Deadly Amanitas

By V. E. TYLER, JR., R. G. BENEDICT, L. R. BRADY, and J. E. ROBBERS*

Chromatographic examination of *Amanita phalloides* carpophores collected in the Pacific Northwest revealed the presence of relatively large amounts (1.5–1.9 mg./Gm. dry wt.) of β-amanitin but lesser concentrations (trace to 0.93 mg./Gm.) of α-amanitin. It was concluded that these represent a distinct chemical race of *A. phalloides*, apparently restricted in its occurrence to the states of Washington, Oregon, and California. Other species investigated included *Amanita bisporigera*, the most toxic yet found in the United States, containing 2.25–5.0 mg. of α- and β-amanitins per Gm. *Amanita verna* specimens contained variable amounts of the two toxins (0 to 1.7 mg./Gm.), but very small quantities (0 to <0.1 mg./Gm.) of α-amanitin only were detected in samples of *Amanita virosa*.

CONFUSION ABOUNDS in the literature pertaining to the identity and toxicity of *Amanita* species. In the United States this is particularly true of *Amanita phalloides* (Fr.) Secr. and related species (so-called deadly amanitas); in fact, all literature prior to the last decade or two must be carefully evaluated to determine, if possible, the identity of the mushroom which was actually studied under a particular designation.

Until 1918, all species of deadly amanitas occurring in the U. S. were generally referred to *A. phalloides*, which was considered to represent a single polymorphic species. For example, Murrill (1), referring to *A. phalloides* in 1916, wrote of "The variety of colors assumed by this species—white, yellow, green, gray, brown, blackish . . ." In 1918, Atkinson (2) recognized that the most common *Amanita* species in the eastern U. S., usually interpreted as a dark brown form of *A. phalloides*, was actually a different species. He subsequently described it and assigned the name *Amanita brunnescens* Atk. The various color forms were gradually sorted out with the passing years, the white forms being identified as *Amanita verna* (Fr.) Vitt. s. Boud., *Amanita virosa* Secr., or *Amanita bisporigera* Atk., the yellow or green as *Amanita citrina* S. F. Gray, and the blackish or gray as *Amanita porphyria* (Fr.) Secr. Finally, none remained which could actually be designated *A. phalloides*.

Changes in nomenclature are generally accepted with reluctance; thus, *A. phalloides* is still frequently referred to in the popular press and even in scientific writings. As late as 1955, the term "brown *A. phalloides*" was used to designate *A. brunnescens* (3). Disregarding this use of antiquated nomenclature, mycologists began to assume that authentic *A. phalloides* did not occur in the U. S. (4).

In 1958, Smith (5) reported that the species did occur rarely in California, but details were not presented. A year later, specimens were found in Ashland, Ore., which greatly resembled *A. phalloides*, and analysis of them revealed the presence of β-amanitin (6) as well as a smaller amount of α-amanitin (7). A fatal case of mushroom poisoning with symptoms identical to those

Received March 10, 1966, from the Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, and the *College of Pharmacy, University of Houston, Houston, Tex.
Accepted for publication April 1, 1966.
Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
This investigation was supported in part by grant GM 07515-06 from the U. S. Public Health Service, Bethesda, Md.

Fig. 1.—*Amanita phalloides* carpophore collected in Seattle, Wash.

produced by amanita toxins was reported from California in 1965 (8). The species responsible was identified as *A. phalloides*, but botanical details were not presented. In October 1965, Mrs. Betty Dorland collected specimens on Mercer Island, Seattle, Wash., which were identical to the 1959 collection from Oregon. The carpophores (Fig. 1), found growing in sandy grass-covered soil under old birch trees, were identified as *A. phalloides*[1] (Table I).

This discovery of specimens of deadly amanitas in the Pacific Northwest prompted the present study with its dual objectives: (*a*) to determine if the specimens of *A. phalloides* collected in the U. S. were similar in their toxin content to specimens of European origin and (*b*) to evaluate the quality and quantity of toxins in other American collections of deadly amanitas.

EXPERIMENTAL

Analysis of Washington A. phalloides.—Dried carpophore tissue (550 mg.) was exhaustively extracted with methanol in a Soxhlet apparatus and the resulting extract (*A*) concentrated to a volume of 2.0 ml. To insure complete extraction, the dried marc was subsequently re-extracted with 70% ethanol, and this extract (*B*) was evaporated to dryness, and the residue redissolved in 1.0 ml. of 70% ethanol.

Quantities of these extracts ranging from 1–10 μl. were spotted on thin-layer plates prepared with Silica Gel G and were chromatographed in a solvent mixture composed of methanol–methyl ethyl ketone (1:1), as described by Sullivan *et al.* (9). For reference, α- and β-amanitin[2] were chromatographed concurrently and in admixture with the extracts. Spraying the chromatograms with 1% cinnamaldehyde in methanol followed by exposure to hydrochloric acid vapor revealed the amanita toxins as violet-colored spots. As little as 0.3 mcg. of pure α- or β-amanitin could be detected with certainty by this procedure. The results, including average R_f values and approximate quantities determined by visual comparison of the size and intensity of the spots with those of the reference standards, are summarized in Table II.

Quantities (< 20 μl.) of both extracts *A* and *B* and of the reference compounds were also chromatographed on Silica Gel G plates with *n*-butanol–acetic acid–water (4:1:1) as described by Benedict *et al.* (10) and on Whatman No. 1 and No. 3 filter papers with methyl ethyl ketone–acetone–water–*n*-butanol (20:6:5:1) as recommended by Block *et al.* (3). In all of these systems extract *A* yielded relatively large amounts of β-amanitin; extract *B* contained a very small amount of β-amanitin and a trace only of α-amanitin.

Analysis of Other Deadly Amanitas.—Additional *Amanita* species which have been suspected of containing amanita toxins include *A. bisporigera*, *A. tenuifolia* Murr., *A. verna*, and *A. virosa* (11). Of these, samples of *A. tenuifolia* and *A. verna* were examined chromatographically by Block *et al.* (3); the former was found to contain only β-amanitin but the latter both α- and β-amanitin. However, several specimens identified as *A. verna* contained no amanita toxins.

For their studies the authors were able to obtain several samples of *A. bisporigera*,[3] *A. phalloides* (European origin), *A. phalloides* (Oregon origin), *A. verna*,[3] and *A. virosa*. Details of these collections are presented in Table I. One-hundred-milligram samples of *A. bisporigera* and 500-mg. samples of *A. phalloides*, *A. verna*, and *A. virosa* were extracted with methanol in a Soxhlet apparatus and quantities of the concentrated extracts were spotted and chromatographed in the methanol–methyl ethyl ketone system as previously described. The amanitins were detected with cinnamaldehyde spray and verified, in doubtful cases, with Pauly's reagent (12). R_f values and estimated quantities of toxins detected are described in Table II.

RESULTS AND DISCUSSION

Chromatographic examination of extracts of the specimen of *Amanita* collected in the fall of 1965 in Seattle, Wash., revealed the presence of amanita toxins. The results were similar but not identical to those obtained by analysis of a similar specimen collected in Oregon in 1959 (6). β-Amanitin predominated in both samples, but only traces of α-amanitin could be detected with difficulty in the Seattle specimen following a second extraction of the carpophore with aqueous ethanol. A somewhat larger amount of α-amanitin was present in the Oregon mushroom. Although the total concentrations of these two amanitins (1.9–2.43 mg./Gm.)

[1] The authors are indebted to Prof. D. E. Stuntz, Department of Botany, University of Washington, Seattle, for authoritative identification of all Pacific Northwest and Texas specimens utilized in this investigation.

[2] Supplied through the courtesy of Prof. Dr. Theodor Wieland, Institut für Organische Chemie, der Universität Frankfurt am Main, Frankfurt am Main, West Germany.

[3] Two collections of *A. bisporigera* (No. 27518 and 27653) and four of the collections of *A. verna* (No. 5362, 22065, 27055, 25698) were supplied through the courtesy of Dr. L. R. Hesler, University of Tennessee, Knoxville.

TABLE I.—TIME AND PLACE OF DEADLY *Amanita* COLLECTIONS

Collection No.	Species	Collection Site	Date
27518	*A. bisporigera* Atk.	Toxaway Gorge, N. C.	7/29/1961
B2	*A. bisporigera*	Cades Cove, Tenn.	7/27/1963
27653	*A. bisporigera*	Gatlinburg, Tenn.	8/6/1963
E1	*A. phalloides* (Fr.) Secr.	Harz Mountains, Germany	9/11/1963
O1	*A. phalloides*	Ashland, Ore.	Fall 1959
W1	*A. phalloides*	Seattle, Wash.	10/24/1965
5362	*A. verna* (Fr.) Vitt. s. Boud.	Knoxville, Tenn.	8/19/1934
22065	*A. verna*	Highlands, N. C.	8/24/1955
27055	*A. verna*	Bergen, N. Y.	10/11/1962
25698	*A. verna*	Cades Cove, Tenn.	7/27/1963
B1	*A. verna*	Fall City Falls, Tenn.	8/3/1963
Ta	*A. verna*	Huntsville State Park, Tex.	11/8/1964
Tb	*A. verna*	Huntsville State Park, Tex.	11/8/1964
T2a	*A. verna*	Huntsville State Park, Tex.	11/14/1965
T2b	*A. verna*	Huntsville State Park, Tex.	11/14/1965
T2c	*A. verna*	Huntsville State Park, Tex.	11/14/1965
T1a	*A. virosa* Secr.	Huntsville State Park, Tex.	11/8/1964
T1b	*A. virosa*	Huntsville State Park, Tex.	11/8/1964
T4	*A. virosa*	Huntsville State Park, Tex.	11/14/1965

TABLE II.—R_f VALUES AND ESTIMATED CONCENTRATIONS OF AMANITA TOXINS DETECTED IN DEADLY *Amanita* SPECIES

Species Investigated	Av. R_f Values (MEK:MeOH)		Estimated Quantity, mg./Gm.	
	α-Amanitin	β-Amanitin	α-Amanitin	β-Amanitin
A. bisporigera No. 27518	0.45	0.21	1.75	0.5
A. bisporigera No. B2	0.47	0.24	3.5	1.5
A. bisporigera No. 27653	0.46	0.23	2.63	0.75
A. phalloides No. E1	0.47	0.24	1.2	0.8
A. phalloides No. O1	0.44	0.21	0.93	1.5
A. phalloides No. W1				
Ext. A	...	0.24	} tr.	1.9
Ext. B	0.47	0.23		
A. verna No. 5362	...	...	0	0
A. verna No. 22065	0.45	0.22	0.28	tr.
A. verna No. 27055	0.46	...	<0.1	0
A. verna No. 25698	0.45	...	<0.1	0
A. verna No. B1	...	...	0	0
A. verna No. Ta	0.46	0.22	1.4	0.3
A. verna No. Tb	0.46	0.23	0.7	0.3
A. verna No. T2a	0.47	0.24	0.58	0.25
A. verna No. T2b	0.46	0.22	0.72	tr.
A. verna No. T2c	0.47	0.23	1.4	0.3
A. virosa No. T1a	...	...	0	0
A. virosa No. T1b	0.44	...	<0.1	0
A. virosa No. T4	0.45	...	<0.1	0
Reference amanitins	0.46	0.23		

approximated that detected in a typical European collection of *A. phalloides* (2.0 mg./Gm.), the unusually high proportions of β- to α-amanitin (1.9/trace, 1.5/0.93) are quite different from that detected in European specimens (about 2/3).

However, since morphological differences between the Pacific Northwest collections and European specimens of *A. phalloides* are essentially nonexistent, it appears most useful to describe the former as members of a chemical race of *A. phalloides* in which β-amanitin is the predominant amanitin. Until large numbers of single carpophores of the European species can be investigated for their content of both α- and β-amanitin, it is not possible to determine if this chemical race was transferred relatively recently to the Pacific coast together with the various introduced trees (*Betula, Populus*), under which it has been found and with which it probably forms mycorrhizal associations, or if it is a race originating in the Pacific Northwest and restricted there in its occurrence. In any event, the evidence indicates conclusively the existence of a chemical race of *A. phalloides* distinguished by a high proportion of β-amanitin, specimens of which are rare in Washington, Oregon, and presumably California.

It is apparent from the data in Table II that *A. bisporigera* is the most toxic American mushroom yet examined. Its total content of α- and β-amanitins was found to range from 2.25–5.0 mg./Gm. in contrast to 1.9–2.43 mg./Gm. for the native *A. phalloides*, 0–1.7 mg./Gm. for *A. verna*, and 0–<0.1 mg./Gm. for *A. virosa*. Singer (13) does not classify *A. bisporigera* in section 6, *Euamanita*, of subgenus II, *Euamanita*, together with other amanita toxin-containing species, but instead lists it under section 5, *Amidellae*. On the basis of its high concentration of α- and β-amanitin, as well as its botanical affinities to *A. virosa* (14), it would seem that the species should properly be classified in section 6, *Euamanita*.

The finding of variable concentrations of toxins in *A. verna* was not unexpected. Although the complete absence of toxins from collection No. 5362 may

be attributed to its excessive age and prolonged storage (31 years) preceding analysis, failure to detect toxins in the more recent collection No. B1 must be due to natural variablility. Block *et al.* (3) report similar results from their study of toxin-containing *Amanita* species in which several specimens identified as *A. verna* produced no toxic symptoms in mice nor could toxins be detected in them chromatographically. It is not clear whether the differences observed by Block *et al.* and by the authors are due to environmental conditions, ontogenetic considerations, or genetic factors, but it must be concluded that the amanita-toxin content of *A. verna* carpophores is extremely variable and, in general, appreciably less than that of *A. bisporigera* or *A. phalloides*.

No amanitins were detected in one sample of *A. virosa* (Tla), but another carpophore (Tlb) collected at the same time from the same site contained a very small amount of α-amanitin (<0.1 mg./Gm.), as did another, more recent collection (T4). Although standard reference works (15, 16) all refer to *A. virosa* as a deadly poisonous species, apparently the only previous experimental work on the subject was that of Ford (17). He found an extract to be toxic to guinea pigs and concluded that the toxins of the species were identical to those of *A. phalloides*. Identification of small amounts of α-amanitin in two carpophores from different collections confirms this earlier finding. However, as in the case of *A. verna*, the effects of environment, ontogenesis, and genetics must all be investigated before a definite explanation can be given for the irregular low-level occurrence of toxins in *A. virosa*.

REFERENCES

(1) Murrill, W. A., *Mycologia*, **8**, 231(1916).
(2) Atkinson, G. F., *Proc. Am. Phil. Soc.*, **57**, 354(1918).
(3) Block, S. S., Stephens, R. L., and Murrill, W. A., *J. Agr. Food Chem.*, **3**, 584(1955).
(4) Gilbert, E. J., *Iconographia Mycol. (Suppl. 1)*, **27**, 316(1941).
(5) Smith, A. H., "The Mushroom Hunter's Field Guide," University of Michigan Press, Ann Arbor, Mich., 1958, p. 138.
(6) Isaacs, B. F., and Tyler, V. E., Jr., *Mycologia*, **55**, 124 (1963).
(7) Tyler, V. E., Jr., Brady, L. R., Benedict, R. G., Khanna, J. M., and Malone, M. H., *Lloydia*, **26**, 154(1963).
(8) Harrison, D. C., Coggins, C. H., Welland, F. H., and Nelson, S., *Am. J. Med.*, **38**, 787(1965).
(9) Sullivan, G., Brady, L. R., and Tyler, V. E., Jr., *J. Pharm. Sci.*, **54**, 921(1965).
(10) Benedict, R. G., Tyler, V. E., Jr., Brady, L. R., and Weber, L. J., *J. Bacteriol.*, **91**, 1380(1966).
(11) Tyler, V. E., Jr., *Progr. Chem. Toxicol.*, **1**, 339(1963).
(12) Block, R. J., Le Strange, R., and Zweig, G., "Paper Chromatography," Academic Press Inc., New York, N. Y., 1952, p. 64.
(13) Singer, R., "The Agaricales in Modern Taxonomy," J. Cramer, Weinheim, West Germany, 1962, p. 428.
(14) Gilbert, E. J., *Iconographia Mycol. (Suppl. 1)*, **27**, 327 (1941).
(15) Hennig, B., "Michael/Hennig Handbuch für Pilzfreunde," vol. 1, Gustav Fischer Verlag, Jena, East Germany, 1958, p. 131.
(16) Heim, R., "Les Champignons Toxiques et Hallucinogènes," Editions N. Boubée & Cie, Paris, France, 1963, p. 79.
(17) Ford, W. W., *J. Pharmacol. Exptl. Therap.*, **1**, 277 (1909).

Kinetics and Mechanisms of Action of Antibiotics on Microorganisms V

Chloramphenicol and Tetracycline Affected *Escherichia coli* Generation Rates

By EDWARD R. GARRETT, GEORGE H. MILLER, and M. R. W. BROWN*

Total and viable count methods were used to study *Escherichia coli* generation rates at various temperatures and tetracycline and chloramphenicol concentrations. The negative dependence of apparent first-order generation rate constants on concentrations of these antibiotics was determined for all temperatures. The coincidence of the heat of activation for *E. coli* growth in antibiotic-free media and for the inhibitory rate constants was observed. The facile reversibility of antibiotic effects to predictable rates of *E. coli* generation in the subinhibitory concentration ranges of these antibiotics was demonstrated. A quantitative model consistent with the observed concentration dependencies and observed reversibilities is proposed which relates antibiotic partitioning from the media, a critical value for protein synthesis that results in microbial generation, and the present concept that these antibiotics compete for ribosomal binding sites and so inhibit the function of messenger RNA in protein synthesis.

THE CLINICAL importance of chloramphenicol and tetracycline has resulted in many investigations of their biochemical mode of action (1–4) and is generally ascribed to the inhibition of protein synthesis which has been principally observed under conditions of complete inhibition of growth. A more complete understanding of the action of these antibiotics could be realized if the kinetics of bacterial generation was more completely elucidated in antibiotic concentrations less than those that result in complete growth inhibition, *i.e.*, subinhibitory concentrations.

Received February 28, 1966, from the College of Pharmacy, University of Florida, Gainesville.
Accepted for publication April 5, 1966.
Presented in part to the Scientific Section, A.PH.A., New York City meeting, August 1964.
Abstracted in part from the prize-winning manuscript submitted by George H. Miller to the Southern Region, Lunsford-Richardson Pharmacy Award Competition, 1964.
This investigation was supported in part by research grant GM-09864-01,-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and in part by a grant from the University of Florida Computing Center, Gainesville.
The technical assistance of George L. Perry and rewarding discussions with Professor Arthur L. Koch are acknowledged.
* Present address: School of Pharmacy, Bristol College of Science and Technology, Ashley Down, Bristol, England.