tween BP and different purines or pyrimidines (8, 9), and where a similar shift takes place, it might be inferred that also in this case the interaction of the two molecular species, *i.e.*, the hydrocarbon and the purine or pyrimidine, leads to a formation of a molecular complex.

The difference between the solubilization of BP in CCB or in simple detergent solution above the critical concentration would perhaps be explained with an analogous mechanism. In the first case, however, more directional and specific forces would act between the solubilized molecule and the solubilizing agent.

Furthermore, when CCB is the host molecule, the amount of BP dissolved shows a different trend corresponding to the transition previously detected with conductivity, potentiometry, and refractrometry measurements.

REFERENCES

(1) Dordoni, F., Giacomello, G., and Stein, M. L., *Ric. Sci.*, **21**, 70(1951).
(2) Beckett, H. A., and Woodward, R. J., *J. Pharm. Pharmacol.*, **15**, 422(1963).
(3) Botré, C., Crescenzi, V., and Mele, A., *J. Phys. Chem.*, **63**, 650(1959).
(4) Le Rosen, A. L., and Reid, C. E., *J. Chem. Phys.*, **20**, 233(1952).
(5) Riegelman, S., *et al.*, *J. Colloid Sci.*, **13**, 208(1958).
(6) Ekwall, P., Setala, K., and Sjoblom, L., *Acta Chem. Scand.*, **5**, 175(1951).
(7) Klevens, H. B., *J. Phys. Colloid Chem.*, **54**, 283(1950).
(8) Weil-Malherbe, H., *Brit. J. Cancer*, **1**, 423(1947); *Biochem. J.* **40**, 351(1946).
(9) Liquori, A. M., Ascoli, F., and Botré, C., *J. Mol. Biol.*, **5**, 521(1962).

Identification of α- and β-Amanitin by Thin-Layer Chromatography

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A thin-layer chromatographic method using Silica Gel G plates developed in methanol–methyl ethyl ketone (1 : 1) proved suitable for the rapid detection of α- and β-amanitin in mushroom extracts. Application of the procedure to *Lepiota cepaestipes* and *Lepiota clypeolaria* indicated the amanitins were not present in these species.

EXISTING paper chromatographic methods for the detection of amanita toxins are generally unsatisfactory. Two very similar procedures are described in the literature. In the original method of Wieland and Schmidt (1), a methanolic extract of the fungus was spotted on S&S 2043b filter paper and the chromatogram developed for about 2 hr. with the upper phase of a solvent mixture comprising methyl ethyl ketone–acetone–water (20:2:5). If additional separation was desired, the chromatogram was developed in a second direction with ethyl formate–acetone–water (20:29:8). Visualization of α- and β-amanitin and phalloidin was effected with a number of reagents, especially diazotized sulfanilic acid and cinnamaldehyde–hydrochloric acid.

A slight modification of this procedure was developed by Block *et al.* (2). A methanol extract of the fungus which had been repeatedly evaporated to dryness to coagulate polypeptides was applied to 1 × 14 in. filter paper strips (S&S 2043b or Whatman No. 1) and formed for 2 hr. with a solvent mixture made up of methyl ethyl ketone–acetone–water–*n*-butanol (20:6:5:1). Spraying with 1% cinnamaldehyde in methanol and exposure to hydrochloric acid vapor caused the amanita toxins to appear as violet- or blue-colored spots.

As has been previously pointed out (3), these methods have definite drawbacks, and their application to fungal extracts requires considerable experience and definite precautions to avoid erroneous interpretation of the results. The spotting of large amounts of concentrated methanol extracts, as recommended by Block *et al.* (2), often gives streaked and distorted chromatograms, probably due to the presence of accompanying lipids. The Wieland and Schmidt (1) solvent mixture forms an emulsion which must be centrifuged prior to use, and neither it nor the modified system of Block *et al.* gives reproducible results. R_f values of the amanitins vary appreciably with the quantity spotted, and freshly prepared solvent mixture gives different values than solvent aged several days prior to use.

It therefore seemed advantageous to develop a procedure which would permit rapid identification of α- and β-amanitin with greater certainty. Thin-layer chromatography appeared to present a suitable method, and its application to the problem was subsequently undertaken.

EXPERIMENTAL

Chromatographic Procedure.—Several solvents, such as ethanol, methanol, and methyl ethyl ketone, and a number of solvent mixtures including methyl ethyl ketone–acetone–water–*n*-butanol (20:6:5:1), *n*-butanol–ethanol—water (4:1:1), methanol–diethylamine (20:1), methanol–methyl ethyl ketone–diethylamine (various proportions), and methanol–methyl ethyl ketone (1:1) were tested for their ability to separate α- and β-amanitin on thin-layer plates. Best results were obtained with plates prepared with Silica Gel G and developed with a solvent system composed of methanol–methyl ethyl ketone (1:1).

The plates were prepared by vigorously shaking

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30 Gm. of Silica Gel G with 60 ml. of distilled water for 1 min. This slurry was then applied in a 200-μ layer to 8 × 8 in. glass plates. After standing for 5 min. at room temperature, the plates were activated by heating at 105° for 90 min.

Crude and purified methanolic extracts of *Amanita phalloides* (Fr.) Secr. and *Galerina marginata* (Fr.) Kühn., previously shown to contain α- and β-amanitin (3), were spotted on the plates. These then were placed in a chromatographic chamber and developed with a mixture of methanol–methyl ethyl ketone (1:1) for a distance of 10 cm. (approximately 20 to 25 min.).

The amanitins were visualized by spraying the plates with 1% cinnamaldehyde in methanol, drying, and exposing the plates to hydrochloric acid vapor for about 10 min. α-Amanitin and β-amanitin appeared as discrete violet spots at R_f 0.46 and 0.23, respectively. Alternatively, the compounds were detected by spraying with Pauly's reagent (4), revealing pink spots with the same R_f values. This reagent proved to be approximately 10 times more sensitive for the amanitins than cinnamaldehyde–HCl. The identity of α- and β-amanitin was verified by scraping the respective spots from the plates (prior to spraying), eluting the compounds from the silica gel by extraction with methanol in a Soxhlet apparatus, concentrating, and chromatographing the respective extracts by the method of Block *et al.* (2).

Methanol extracts of fungi prepared by the method of Block *et al.* (2) gave satisfactory results with this procedure, but the amanitins were accompanied by relatively large quantities of impurities. Improved separation and resolution were achieved when the extract was subjected to a preliminary purification as follows.

Four grams of *G. marginata* was exhaustively extracted with 100 ml. of methanol in a Soxhlet apparatus. The resulting extract was chilled at 10° and separated from the precipitated residue by centrifugation. The clear supernatant liquid was then concentrated to about 20 ml. in a flash evaporator, again chilled to 10°, centrifuged, the clear liquid decanted, and small volumes of water added to it until precipitation ceased. After centrifuging, the hydromethanolic supernatant liquid was evaporated to dryness and the residue redissolved in 4 ml. of methanol. Chromatograms prepared from such extracts (5 to 100 μl.) showed excellent separation of α- and β-amanitin with a minimum of interfering substances.

Application to Fungal Extracts.—Several species of mushrooms were investigated for the presence of amanitins utilizing this thin-layer chromatographic procedure. A purified extract of *G. marginata* was employed as a reference standard. Using their paper chromatographic method, Block *et al.* (5) had detected a compound with chromatographic and colorimetric properties resembling α-amanitin in *Lepiota cretacea* (Bull.) Morgan [*Lepiota cepaestipes* (Fr.) Kummer or *Leucocoprinus cepaestipes* (Fr.) Pat. sensu str.]. However, the small quantities of the mushroom available to them did not produce toxic symptoms in mice.

Availability of an authentic specimen of *L. cepaestipes*, collected in Bradley Park, Peoria, Ill., on June 16, 1958, prompted the authors to undertake a re-examination of this species, using both paper and thin-layer chromatographic methods. At the same time, another species of *Lepiota* [*L. clypeolaria* (Fr.) Quél.] also was tested on the basis of its resemblance to *Lepiota helveola* Bres. sensu Joss., a European species known to be poisonous (6). Samples (approximately 1 Gm., dry weight) of these mushrooms, together with *G. marginata*, were extracted and chromatographed by the method of Block *et al.* (2) and by the thin-layer chromatographic procedure. Both α- and β-amanitin were identified in *G. marginata* by both procedures, but neither could be detected in *L. cepaestipes* or *L. clypeolaria*.

The thin-layer chromatographic method herein described should prove useful for the rapid, unequivocal determination of α- and β-amanitin in mushrooms. Since accurate interpretation of results obtained with it does not require previous experience or special precautions, it should prove particularly useful in toxicological laboratories where determination of mushroom toxins is a necessary but infrequent analytical procedure.

REFERENCES

(1) Wieland, T., and Schmidt, G., *Ann. Chem.*, **577**, 215 (1952).
(2) Block, S. S., *et al.*, *Science*, **121**, 505(1955).
(3) Tyler, V. E., Jr., *et al.*, *Lloydia*, **26**, 154(1963).
(4) Block, R. J., Le Strange, R., and Zweig, G., "Paper Chromatography," Academic Press Inc., New York, N. Y., 1952, p. 64.
(5) Block, S. S., Stephens, R. L., and Murrill, W. A., *J. Agr. Food Chem.*, **3**, 584(1955).
(6) Smith, A. H., "Mushrooms in their Natural Habitats," Sawyer's Inc., Portland, Oreg., 1949, p. 438.