N, 7.0%. This demonstrated that the reaction involves a 1:1 complexation of picric acid and chlormadinone as well as alkaline hydrolysis of the steroidal 17-acetoxy entity. This is consistent with the mechanism (4) proposed for the anionic complexation of picric acid and α,β-unsaturated lactones.

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Enzymatic Methylation of Nicotinamide by *Claviceps purpurea*

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Soluble supernatant fractions from two strains of *Claviceps purpurea* were tested for the presence of enzymes capable of methylating tryptamine, 5-hydroxytryptamine, tyramine, and nicotinamide. The latter compound was the only one of the substrates tested in which methylation took place.

SEVERAL PSYCHOTOMIMETIC indole substances (1), such as bufotenin and psilocybin, which are found in certain fungi are *N*-methylated tryptamine derivatives. Lysergic acid, a derivative of which is the most potent psychotogen known, also contains an *N*-methyl group, and a residue of this compound occurs in the alkaloids of ergot.

It has been reported (2) that the *N*-methyl group in the ergot alkaloids is probably derived from a transmethylation reaction from methionine.

Cantoni (3) first reported that *S*-adenosylmethionine was an active methyl donor and has studied in great detail the methylation of nicotinamide. Axelrod (4) has isolated an enzyme from rabbit lung that is capable of methylating a number of naturally occurring compounds to psychotomimetic derivatives utilizing *S*-adenosylmethionine as the methyl donor.

The literature is replete with information concerning the biosynthesis of the ergot alkaloids from *C. purpurea*, but relatively little information is available concerning the activity of the enzymes found in this organism. The present investigation has been conducted in an attempt to determine if a cell-free extract of the fungus possesses an enzyme system which can methylate tryptamine, 5-hydroxytryptamine (5-HT), tyramine, and nicotinamide, since their methylated derivatives have been associated with psychotomimetic activity.

EXPERIMENTAL

Cultures.—Two strains of *C. purpurea* (Fries) Tulasne were used in this investigation. One of the strains, designated CPM, was isolated from sclerotia obtained from the Department of Plant Pathology, University of Minnesota, Minneapolis. The other, an alkaloid producing strain, designated 47A, was obtained as a slant culture from the College of Pharmacy, University of Washington, Seattle.

The fungi were cultured in 50 ml. of Abe's medium (5) in cotton-stoppered 250-ml. conical flasks grown as still cultures in the dark at room temperature. The cultures were harvested for extraction at periods of from 21 to 27 days.

Preparation of Enzyme Fraction.—The mycelial tissue of both CPM and 47A cultures was separated from the culture medium by centrifugation, washed three times with distilled water, recentrifuged, and finally pressed between sheets of filter paper.

The enzyme fraction was prepared from 6–7 Gm. (fresh weight) of mycelial tissue, which was homogenized with 15 ml. of phosphate buffer (0.1 *M* K_2HPO_4, pH 7.9) in a VirTis 45 homogenizer for 10 minutes in a chilled vessel in an ice bath. After the homogenate was obtained, it was centrifuged at 2500 r.p.m. (755 × *g*) for 10 minutes and the resultant supernatant fraction recentrifuged at 16,000 r.p.m. (31,000 × *g*) for 20 minutes in a refrigerated Servall centrifuge at 0–3°. The soluble supernatant fraction was then separated and employed as the enzyme preparation.

In addition to the above method, three other means were tested for extraction of the mycelium: (*a*) homogenizing the mycelium with buffer as previously described, then subjecting the homogenate to ultrasonic vibrations, (*b*) grinding the mycelium in buffer solution with sand, and (*c*) grinding with alumina. After several preliminary trials, it was decided to use homogenization only as the extraction method for further investigations since this method proved most successful.

Enzymatic Activity of Soluble Fractions.—Nicotinamide,[1] tryptamine hydrochloride,[1] tyramine hydrochloride,[1] and 5-hydroxytryptamine creatinine sulfate[1] were tested with the soluble supernatant fraction using *S*-adenosylmethionine[2] as a methyl donor to determine if the soluble fraction contained an enzyme(s) capable of methylating these physiologically active naturally occurring substances.

Nicotinamide—The incubation mixture consisted of 0.005 mole of *S*-adenosylmethionine (0.2 ml.), 0.01 *M* nicotinamide (0.3 ml.), and an amount of the soluble supernatant fraction equivalent to 6

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[1] Nicotinamide, tryptamine hydrochloride, tyramine hydrochloride, and 5-hydroxytryptamine creatinine sulfate were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.
[2] *S*-Adenosylmethionine was obtained from Calbiochem, Los Angeles, Calif.

mg. of nitrogen, all in a volume of 5 ml., using 0.1 *M* K_2HPO_4 (pH 7.9) as the diluent. This mixture was incubated in a 15-ml. glass-stoppered centrifuge tube in a shaking water bath at 25° for 1 hour. Two other tubes—one containing a similar mixture less the soluble supernatant fraction and the second tube containing the soluble supernatant fraction only—served as controls. The controls were brought to a volume of 5 ml. using the phosphate buffer described above as the diluent. An equal volume of 10% trichloroacetic acid was added to each of the tubes at the end of the incubation period to stop any enzymatic action, and the vessels were left to stand at room temperature for 30 minutes. The resultant precipitate was removed by centrifugation.

The method used for detecting the formation of *N*-methylnicotinamide in the reaction mixture was similar to that of Huff and Perlzweig (6). Glacial acetic acid (0.1 ml.) was added to 1 ml. of the reaction mixture and to the corresponding controls followed by 1.0 ml. of methyl ethyl ketone and 1.0 ml. of 6 *N* NaOH. This mixture was immediately shaken and allowed to stand for 5 minutes at room temperature, after which 6 *N* HCl was added to a pH of 3.0. The reaction vessels were placed in boiling water for 5 minutes, cooled, then 1 ml. of 20% KH_2PO_4 was added. The fluorescence of the solutions was measured in an Aminco-Bowman spectrophotofluorometer at an activation maximum of 365 mμ and a fluorescence maximum of 440 mμ.

It was determined by this method that *N*-methylnicotinamide was present in the incubation mixture, but none could be detected in either of the controls employed. A reference sample of *N*-methylnicotinamide was used in the same extraction procedure to ascertain the validity of this method. The results obtained were identical.

Tyramine, Tryptamine, and 5-Hydroxytryptamine.—The incubation mixture was composed of 0.01 *M* (0.5 ml.) of the substrate to be tested, 0.005 mole (0.2 ml.) of *S*-adenosylmethionine, and a volume of the soluble supernatant fraction containing 6 mg. of nitrogen. This was brought to a volume of 5 ml. using phosphate buffer (pH 7.9). These mixtures along with controls, consisting of the soluble supernatant fraction alone and the substrate and methyl donor without the enzyme fraction, were incubated in 15-ml. glass-stoppered centrifuge tubes on a shaking machine at 25° for 90 minutes. At the end of this period, the pH was adjusted to 10 using borate buffer. The contents were frozen and lyophilized for 36 hours, and the resulting residue then shaken with isoamyl alcohol (tryptamine and 5-HT) or *n*-butanol (tyramine). After filtration, the extract was concentrated *in vacuo* at 50° to dryness, then reconstituted to 1–2 ml. with the same solvent. All extracts, reference samples of the substrates used, and methylated derivatives of these substances were chromatographed. The materials were spotted on Whatman No. 1 paper and the papers equilibrated for 4 hours, then allowed to develop for 18 hours using a solvent system of butanol–acetic acid–water (4:1:1), using the descending method. After the papers were removed from the glass chambers and dried, they were sprayed with 2% paradimethylaminobenzaldehyde (Ehrlich's reagent) in equal parts of ethanol and 6 *N* HCl. After development, the spots produced in the extract samples were compared with those appearing in the corresponding controls and with the reference compounds. The spots found in the extract samples corresponded to those found in the controls; no spots corresponding to the methylated derivatives could be detected. The 5-hydroxytryptamine spots had an R_f value of 0.45, were blue, later changing to green; the tryptamine spots had an R_f value of 0.66 and at first were a purple-pink, later changing to green. The tyramine spots had an R_f value of 0.52 and turned yellow.

SUMMARY

Soluble supernatant fractions were prepared by homogenization and centrifugation from 21–27-day-old cultures of two strains of *C. purpurea*, one of which produced ergot alkaloids in saprophytic culture.

Tryptamine, tyramine, 5-hydroxytryptamine, and nicotinamide were incubated with these extracts using *S*-adenosylmethionine as a methyl donor in order to determine if methylated derivatives of these derivatives could be detected.

N-Methylnicotinamide was formed from nicotinamide using extracts of both strains of the fungi, but no methylated derivatives of the other substrates employed could be detected.

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