were comparatively smaller presumably due to further oxidative degradation of the five-membered heterocyclic ring carrying the epoxy group.

Ecgonine and Cocaine—Ecgonine is a carboxy derivative of tropanol, while cocaine is a carbomethoxy derivative of tropanyl benzoate. Although these compounds would be expected to yield the same products of degradation, the yield of six-membered heterocyclic compounds was in fact, very poor (Fig. 1,E and F). Presumably these compounds do not decarboxylate easily and are retained by the basic support.

CONCLUSIONS

The experimental data reported above demonstrate that the described technique should prove of value in establishing the identity of tropane alkaloids as well as in recognizing the tropane carbon skeleton in alkaloids of unknown structure.

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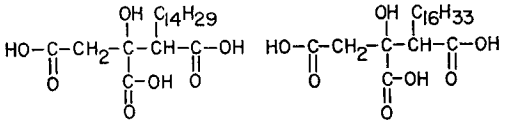
Pharmacologic Study of Norcaperatic and Agaricic Acids

By RICHARD A. CARRANO* and MARVIN H. MALONE

In rats norcaperatic acid produced delayed onset, dose-related effects of mydriasis, skeletal muscle weakness, and central nervous system depression similar to those induced by its homolog, agaricic acid. Agaricic acid was a nonspecific potentiator of furtrethonium in isolated rat jejunum. Both acids produced a slow onset of leiomyotonic effects on isolated guinea pig ileum. This effect could be blocked by papaverine but was not affected by high concentrations of atropine, pyribenzamine, or by a depolarized muscle. Tonic activity could also be produced in a K^+ deficient muscle. Both norcaperatic acid and agaricic acid were competitive inhibitors of the enzyme aconitase with norcaperatic acid being slightly more potent. The potentially toxic properties of *Cantharellus floccosus* due to the presence of norcaperatic acid may be related to the structural chemical relationships of this substance to citric acid.

CANTHARELLUS FLOCCOSUS Schw. has been reported to cause serious delayed gastrointestinal disturbances in individuals (1). Miyata *et al.* (2) isolated and characterized the active principle as norcaperatic or α-tetradecylcitric acid ($C_{20}H_{36}O_7$). Agaricic acid ($C_{22}H_{40}O_7$), an isolate from the mushroom *Polyporus officinalis* Fr., is a homolog of norcaperatic acid and has been included in this study (I and II).



NORCAPERATIC ACID I

AGARICIC ACID II

The isolation of norcaperatic acid from *C. floccosus* marked its first reported occurrence in nature, although agaricic acid had been isolated and studied by various investigators and classified as an anhydrotic and parasympatholytic agent (3–5). The literature reports do not satisfactorily settle whether the mechanism of action on smooth muscle is musculotropic or neurotropic. This study hoped to clarify this problem and to study the possible mechanisms by which these agents could induce mushroom poisoning.

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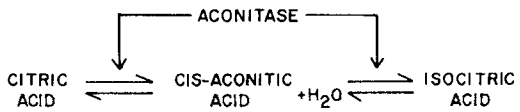
EXPERIMENTAL

In Vivo Hippocratic Screening—Nonfasted albino rats of either sex (Wistar strain, 150–250 Gm.) were injected intraperitoneally with various log-doses of the test drugs suspended in 0.25% agar according to the gross observational method of Malone and Robichaud (6). All required observations were made using the standard worksheet at +5, 10, 15, 30, and 60 min. postinjection, +2, 4, and 24 hr. postinjection, and +2, 4, and 7 days postinjection.

Isolated Rat Jejunum—Using the methods of Ariëns (7), van Rossum (8), and van Rossum and van den Brink (9), cumulative concentration-response curves were recorded with furtrethonium used as the reference agonist. Tyrode's solution oxygenated with 95% oxygen and 5% carbon dioxide and containing the calcium disodium salt of ethylenediaminetetraacetic acid (1×10^{-5} Gm./ml.) was the perfusion media. The rat jejunum section was mounted in a 50-ml. bath (37.5°) using a modified Magnus technique. All drug concentrations were calculated in terms of drug base.

Isolated Guinea Pig Ileum—The ileum wa mounted in the bath (37.5°) using a modified Magnus technique. In the protective studies a submaximal concentration of reference agonist was followed by: a specified concentration of test drug, a specified incubation period, and a repeat of the same concentration of reference agonist. In the antagonism studies a specified dose of experimental drug producing a suitable response was followed by: a specified concentration of prototype antagonist, a specified incubation period, and a repeat of the same concentration of experimental drug. In the K^+ deficient studies, KCl was omitted from the Tyrode's solution and replaced with an equivalent weight of NaCl. In the K^+ excess studies, KCl was added to the Tyrode's solution until the final concentration of potassium ion was 47 m*M*.

Enzymatic Studies—Aconitase was prepared from fresh pig hearts and diluted to a concentration giving workable activity (10). The reaction was studied in a Beckman DU spectrophotometer by following the change in light absorption (240 mμ and 25°) caused by the appearance or disappearance of *cis*-aconitic acid (Scheme I). The reaction was



Scheme I

started by the addition of substrate. Total cell volume was kept at 3.0 ml. by varying the amount of 0.05 *M* phosphate buffer. To assure initial reaction rates, the changes in extinction values (240 mμ at 25°) were recorded at intervals between start and +45 sec., checked for proper kinetics, and calculated as change in absorbance units/min. Experimental drugs were incubated with the enzyme preparation for 3–5 min. prior to activation with substrate. A control rate for a specific concentration of substrate was determined prior to testing the inhibitor's effect on that rate. All drug concentrations were calculated in terms of drug base and were made in 0.05 *M* phosphate buffer, pH 7.4 (10–12).

RESULTS AND DISCUSSION

In Vivo Hippocratic Screening—The whole powdered mushroom of *C. floccosus* and the 70% ethanol extractive obtained from the Drug Plant Laboratory, University of Washington, Seattle, were toxic and showed definite dose-related activity beginning at a dose of 50 mg./Kg. Mydriasis, skeletal muscle weakness, and central nervous system depression accompanied by hypothermia were among the symptoms. Although parasympatholytic activity appeared to be indicated, the presence of lacrimation without salivation, a delayed onset of all activity, and a profound and progressive loss in body weight suggested a more complicated mechanism of action. The extract, as expected, was considerably more toxic and showed more pronounced symptoms. The marc was also studied and was shown to contain residual amounts of activity indicating that the 70% ethanol extraction did not entirely exhaust the active components of the mushroom.

The hippocratic profiles for the samples of agaricic acid and norcaperatic acid were qualitatively identical to that of the mushroom preparations (CNS depression, hypothermia, mydriasis, lacrimation, *etc.*). Fine body tremors were also apparent as a delayed effect. In general, these gross *in vivo* studies suggested a parasympatholytic (hyoscine-like) mechanism. However, the complicating symptomatology, the delayed onset of all effects, and the progressive loss of body weight all suggested metabolic blockade.

Isolated Rat Jejunum—Figure 1 shows the cumulative log-concentration curves of furtrethonium obtained in the presence of agaricic acid. In general, a potentiating effect was seen. Longer incubation resulted in a greater effect. When agaricic acid was incubated with the tissue for 30 min. and washed for 10 min., a significant decrease in the intrinsic activity but not in the affinity of the furtrethonium to the receptor was seen. Apparently, while agaricic acid could be washed out, the muscle or one of its components had been physically changed by the drug. Agaricic acid alone increased both the tone and spontaneous activity of the jejunum. After incubation for about 5 min., a small contrac-

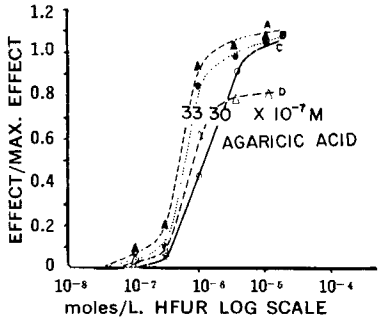


Fig. 1—Cumulative log-concentration curves for the acetylcholinomimetic furtrethonium (HFUR) in the presence of agaricic acid (3×10^{-7} M). Key: A, 30-min. incubation; B, 8-min. incubation; C, control with no agaricic acid; D, 30-min. incubation followed by three washes over a 10-min. period. Rat jejunum.

tion would result. A musculotropic (nonspecific) mechanism of action could explain these results.

Isolated Guinea Pig Ileum—Both agaricic and norcaperatic acids ($1–5 \times 10^{-5}M$) caused a delayed onset, slow, persistent contraction of the guinea pig ileum when the tissue was attached to a lightly loaded isotonic lever system. This leiomyotonic contraction had musculotropic rather than neurotropic characteristics in that it did not begin immediately upon introduction of the drugs but was seen only after about a 3-min. wait. The effect was also very slow (5–6 min. to reach maximum) and did not fade away as is the case with most neurotropic agents (*e.g.*, acetylcholine). The contraction could not be blocked by atropine ($1 \times 10^{-5}M$), pyribenzamine ($1 \times 10^{-5}M$), or by previously depolarizing the muscle by increasing the potassium ion concentration to 47 mM. It was still present in the absence of potassium ion in the perfusate. Papaverine, a nonspecific antagonist, could block the effect. These results indicated that the mechanism of action for the test drugs would have to be musculotropic in nature. Dose-related activity is one of the basic prerequisites for true drug activity. Figure 2 depicts the dose-response curve obtained when agaricic acid was accumulated in the bath.

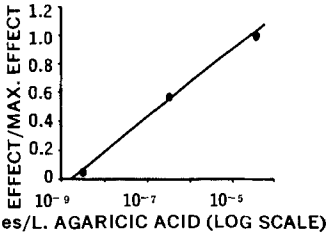


Fig. 2—Cumulative log-concentration curve of agaricic acid. Guinea pig ileum.

Enzymatic Studies—The musculotropic action on smooth muscle when considered along with the noted *in vivo* effects of loss of body weight and delayed onset of toxic activity strengthens the hippocratic diagnosis of metabolic blockade. The structural resemblance of these agents to citric acid initiated a study to determine the effect of these acids on the activity of the enzyme aconitase. Figures 3 and 4 show that agaricic acid and norcaperatic acid ($1 \times 10^{-4}M$) are competitive inhibitors of aconitase. In these experiments *cis*-aconitic acid was used as the substrate. As substrate concentration was increased, the inhibition caused by the test drugs was overcome. Finally, all of the enzyme surface was occupied by the substrate and the reaction velocity was only dependent on substrate concentration. The K_m for *cis*-aconitic acid was $3.3 \times 10^{-4}M$. The K_i for agaricic acid was $4.3 \times 10^{-4}M$ and for norcaperatic acid, $3.0 \times 10^{-4}M$.

Figures 5 and 6 show the results of a similar study in which *dl* + allo, trisodium isocitric acid was used as the substrate for aconitase. The test agents were confirmed to be competitive inhibitors of the enzyme. The K_m for *dl* + allo, trisodium isocitric acid is $6.9 \times 10^{-3}M$, while the K_i for agaricic and nor-

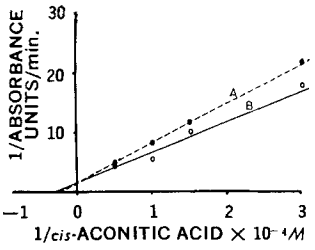


Fig. 3—Lineweaver-Burk plot of the reciprocal of reaction velocity versus *the reciprocal of the* cis-*aconitic acid concentration. Key:* A, *reaction in the presence of 1 × 10^{-4} M of agaricic acid;* B, *control.*

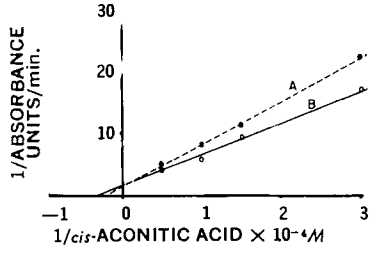


Fig. 4—Plot of the reciprocal of reaction velocity versus *the reciprocal of the* cis-*aconitic acid concentration. Key:* A, *reaction in the presence of 1 × 10^{-4}* M *of norcaperatic acid;* B, *control.*

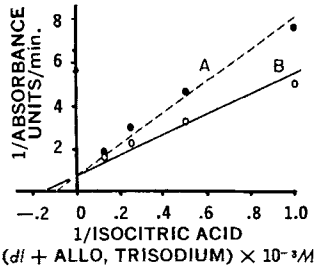


Fig. 5—Lineweaver-Burk plot of the reciprocal of reaction velocity versus *the reciprocal of the* dl + *allo, trisodium isocitric acid concentration. Key:* A, *reaction in the presence of 1 × 10^{-3}* M *of agaricic acid;* B, *control.*

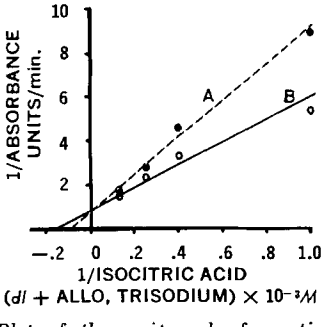


Fig. 6—Plot of the reciprocal of reaction velocity versus *the reciprocal of the* dl + *allo, trisodium isocitric acid concentration. Key:* A, *reaction in the presence of 1 × 10^{-3}* M *of norcaperatic acid;* B, *control.*

caperatic acids are 3.0×10^{-3} and $2.4 \times 10^{-3}M$, respectively. Norcaperatic acid seemed to have a greater affinity to the enzyme than either of the substrates or agaricic acid. The order of decreasing affinities to aconitase was: norcaperatic acid, *cis*-aconitic acid, agaricic acid, and *dl* + allo, trisodium isocitric acid.

A chemical-steric relationship exists between the test acids and fluorocitric acid. Peters (13) states that fluorocitric acid is a competitive inhibitor of aconitase. *In vivo* it produces effects similar to those seen with agaricic and norcaperatic acids.

The basic concepts of enzyme action and steric requirements have been discussed by Koshland (14) and Ogston (15, 16) and a three-point attachment of citric acid to aconitase has been postulated. The citric acid is the correct size and shape to bind to the enzyme surface at three points as the conformation of the enzyme changes to accommodate it. In this natural situation, the proper alignment of bonds needed for catalytic activity is formed. It may be that when the bulky molecule of norcaperatic acid (citric acid with a $C_{14}H_{29}$ substituted for a H atom) approaches and attaches to the enzyme surface, it is bound; but because of its size, the proper formation of the catalytic bonds is prevented (steric hindrance). Agaricic acid and fluorocitric acid could act in the same manner since a $C_{16}H_{33}$ group or a F atom is also sterically much larger than a H atom. Moreover norcaperatic, agaricic, and fluorocitric acids all contain two asymmetrical carbon atoms, while citric acid does not. This makes four stereoisomeric forms possible for each structure. These forms would each have varying abilities to form enzyme and/or receptor attachments (affinity). In conclusion, the two naturally occurring acids studied here may be useful biochemical tools—especially for seeking antidotes, since the gross symptoms of certain toxic mushrooms have been shown here to be due, at least in part, to subcellular activity.

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Effect of Tris(hydroxymethyl)aminomethane on Removal of Urea by Peritoneal Dialysis

By W. M. MCLEAN, D. M. POLAND, M. S. COHON, S. C. PENZOTTI, and A. M. MATTOCKS*

From earlier *in vitro* studies it was thought that tris(hydroxymethyl)aminomethane (tromethamine) might accelerate diffusion of substances across the peritoneal membrane by a mechanism other than causing ionization of a weak acid in the dialysis fluid. This concept was tested by measuring the rate of dialysis of urea in rabbits, urea being essentially nonionized at the pH of the tromethamine fluid. Blood and dialysate concentrations were measured following intravenous injection of tagged urea and following incorporation of tagged urea in the dialysis fluid (reverse dialysis). Dialysis rates were calculated. It was found that tromethamine increased the dialysis rate two to threefold. It is concluded that tromethamine accelerates dialysis by an unknown mechanism.

SEVERAL WORKERS have reported on the use of tris(hydroxymethyl)aminomethane (tromethamine)[1] in peritoneal dialysis (1–6). These have dealt with the use of tromethamine to remove salicylates, barbiturates, and urate, the theory being that the alkalinity of the dialysis fluid would cause ionization of the drug in the peritoneum and thus maintain a higher concentration gradient of unionized drug across the dialyzing membrane. It is important that the mechanism by which tromethamine exerts its effect on dialysis be understood in order that a search for other more effective substances be facilitated.

In a study conducted in these laboratories, it was found that both ionized and unionized pento-

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