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[1] O'Dell, B. L., Regan, W. O., and Beach, T. J., *Univ. Missouri Res. Bull.*, **702**, 1 (1959).
[2] Byers, J. H., and Broquist, H. P., *J. Dairy Sci.*, **43**, 873 (1960).
[3] Byers, J. H., and Broquist, H. P., *J. Dairy Sci.*, **44**, 1179 (1961).
[4] Watson, D. F., Virginia Polytechnic Inst., Blacksburg, Va. (personal communication, 1961).
[5] Guss, S. B., Pennsylvania State Univ. (personal communication, 1961).
[6] Smalley, E. B., Nichols, R. E., Crump, M. H., and Henning, J. N., *Phytopath.*, **52**, 753 (1962).
[7] Crump, M. H., Smalley, E. B., Henning, J. N., and Nichols, R. E., *J. Amer. Vet. Med. Assoc.*, **143**, 990 (1963).
[8] Gregory, K. F., Allen, O. N., Riker, A. J., and Peterson, W. H., *Amer. J. Bot.*, **39**, 405 (1952).

Isolation of a Parasympathomimetic Alkaloid of Fungal Origin

Since 1947, reports have reached us of excessive salivation in ruminants after consuming certain forages. Frequently these forages were red clover hay, but other types of forages were involved. The cattle would consume from 1 to 3 feedings of such forage, salivate excessively, and then refuse further feed. The refusal of the cattle to consume 'slobber forage' represents an economic loss to the farmer, including a loss of milk production and the necessity of purchasing replacement forage.

Byers and Broquist[1] reported that slobbering in cattle and guinea-pigs could be also induced following the administration of a hot water extract of 'slobber forage'; certain properties of such extracts suggested that the salivation factor might be alkaloid in nature[1,2]. Smalley *et al.*[3] observed that all samples of 'slobber forage' were infested with a black fungus. They isolated the fungus in pure culture and identified it as *Rhizoctonia leguminicola;* guinea-pigs force-fed the mycelia salivated profusely. This report concerns the isolation of an alkaloid from this fungus having salivation factor activity, which thus appears to account for the early observations from this laboratory on 'slobber forages'.

Rhizoctonia leguminicola was obtained from Dr. J. W. Gerdemann of this University, who had isolated a pure culture of this fungus from red clover. The culture was maintained on potato–dextrose–agar slants and produced the salivation factor maximally when grown aerobically on a cold-water extract of second-cutting red clover hay. Medium was prepared by infusing 4 kg chopped hay with 20 l. cold water for 4 h and straining through cheesecloth. The medium was dispensed in 300-ml. quantities in 1-l. Roux bottles and autoclaved for 30 min at 120° C. The flasks were then inoculated from agar slants by the aseptic addition of *R. leguminicola* mycelium to the side of the glass bottles at the surface of the medium. The mould was grown in stationary culture at room temperature until the mycelial pad had covered the surface of the medium (generally about three weeks). Salivation factor activity was assayed qualitatively by injecting intraperitoneally guinea-pigs weighing 200–300 g with appropriate test materials and observing the degree of salivation at 15-min intervals. The degree of salivation was graded from 0 to 3; only sub-lethal doses were graded.

Approximately 2 kg of mycelia (wet wt.) was homogenized in a Waring blender with 95 per cent ethanol. The homogenate was filtered through Soxhlet extraction thimbles. The thimbles containing the homogenized mycelia were then placed in Soxhlet extractors and extracted with the ethanolic filtrate for 48 h. The extract was then concentrated *in vacuo* to remove the ethanol and water added to a volume of 1 l. This solution, made slightly acidic (hydrochloric acid), was repeatedly extracted with $CHCl_3$ until fresh $CHCl_3$ extracts were colourless. The organic layer was discarded and the aqueous layer was then brought to *p*H 10 (powdered Na_2CO_3) and extracted with three volumes of $CHCl_3$. The $CHCl_3$ fraction was retained, concentrated *in vacuo* to dryness, and the residue taken up in 100 ml. 0·01 N hydrochloric acid. The process of extracting the aqueous layer with $CHCl_3$ under acidic and then alkaline conditions was repeated. Following removal of the $CHCl_3$ from the latter step, the salivation factor concentrate was taken up in water and lyophilized, finally to yield 143 mg crude salivation factor which served as convenient source material for subsequent purification procedures.

When this material (50γ) was chromatographed on paper in a butanol : acetic acid : water (4 : 1 : 1) system, one major and two minor components were found which stained pink following spraying with Dragendorff's reagent, a reagent diagnostic for alkaloids[4]. The major component (R_F = 0·25) was accumulated by large-scale preparative paper chromatography and when eluted from the chromatogram elicited salivation factor activity.

Crude salivation factor was dissolved in dry $CHCl_3$ and hydrochloric acid gas passed through the solution. On standing overnight a reddish, semi-solid oil formed. Attempts were made to crystallize this material, thought to be salivation factor hydrochloride, from propanol–ethylacetate mixtures, but only amorphous precipitates were obtained. Such material was further purified by dissolving in water and then adding stepwise one volume, and then a second volume of Mayer's reagent[5]. The buff-coloured precipitates that resulted were combined and contained the bulk of salivation factor activity. Crystalline material was obtained, however, when the residual mother liquor was allowed to stand for several days at room temperature. The crystals melted with decomposition at 230°–235° C.

5·5 mg of such crystals was suspended in water and hydrogen sulphide passed through the suspension until no further precipitation occurred. HgS was removed by centrifugation and excess hydrogen sulphide removed by aeration. An aliquot of this solution when chromatographed on paper in the solvent system previously described gave a single Dragendorff positive spot, R_F = 0·25. A second aliquot of this solution, equivalent to 0·5 mg original crystalline material, gave a rating of 3 in the salivation factor assay in guinea-pigs.

The parasympathomimetic action of salivation factor suggests that it may be an anticholinesterase or an acetylcholine-like substance. Its action in this respect is very much like those of physostigmine or pilocarpine, and is reversed by atropine. Salivation factor can be distinguished from these latter alkaloids, however, by paper chromatography and other chemical criteria. *In vitro* experiments have failed to show anticholinesterase activity. Experiments are being carried out to determine the chemical nature of the salivation factor together with its mode of action.

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[1] Byers, J. H., and Broquist, H. P., *J. Dairy Sci.*, **43**, 873 (1960).
[2] Byers, J. H., and Broquist, H. P., *J. Dairy Sci.*, **44**, 1179 (1961).
[3] Smalley, E. B., Nichols, R. E., Crump, M. H., and Henning, J. N., *Phytopath.*, **52**, 753 (1962).
[4] Bregoff, H. M., Roberts, E., and Delwiche, C. C., *J. Biol. Chem.*, **205**, 565 (1953).
[5] Stephenson, Charles H., *Some Microchemical Tests for Alkaloids*, 110 (J. B. Lippincott, Philadelphia and London, 1921).