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THE ORIGIN OF PHENOLIC TSETSE ATTRACTANTS FROM HOST URINE: STUDIES ON THE PRO-ATTRACTANTS AND MICROBES INVOLVED

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Abstract—Phenolic tsetse attractants in host urine were shown to form gradually from pro-attractants identified as a mixture of glucuronates and sulphates. The breakdown of pro-attractants is predominantly due to microbial activity. Of the eight bacteria isolated from buffalo urine three have demonstrated varying ability to give rise to free phenols the formation of which was monitored by observing the build-up of 4-cresol by means of gas chromatography. These observations may provide a useful biotechnological model for controlled release of these semiochemicals in the field.

Key Words: Glossina pallidipes, tsetse, urine, phenols, 4-cresol, 3-*n*-propylphenol, kairomones, glucuronides, sulphates, pro-attractants, controlled-release, *Aerococcus viridans*

Résumé—Il été montre que les substances phénoliques attractives du tsetse, qui sont contenues dans les urines de l'hôte, se forment de façon graduelle à partir de substances pro-attractives identifiés comme étant un mèlange de glucuronates et de sulfates. La decomposition de ces substances pro-attractives est principalement due a une activité microbienne.

Trois bacteries sur les huit isolées des urines du bufle ont montré des capacités variées à engendrer des phénols libres. La formation de cephenols libres a été enregistrée en observant le dévélopement du 4-cresol a l'aide de la chromotographie gazeuse.

Ces observations peuvent constituer un modèle biotechnologique utile pour la liberation controlée de produits semi-chimiques sur le terrain.

Mots Cléfs: Glossina pallidipes, tsetse, urines, phenols, 4-cresol, 3-*n*-propylphenol, kairomones, glucuronides, sulfates, substances pro-attractives, liberation controlée, *Aerococcus viridans*

INTRODUCTION

Considerable progress has recently been made in the identification of kairomones that mediate host-seeking behaviour of tsetse. These compounds form part of the excretory products of the host animals and include breath volatiles like carbon dioxide, acetone, butanone and 1-octen-3-ol (Vale, 1980; Hall et al., 1984; Vale and Hall, 1985a,b) and urine phenols comprising the parent phenol, 3- and 4-cresol, 3- and 4-ethylphenols, and 3- and 4-*n*-propylphenols of which 4-cresol and 3-*n*-propylphenol have been found to be the crucial components (Hassanali et al., 1986; Owaga et al., 1988; Vale et al., 1988). Recent field utilization of these kairomones have taken two major forms: Zimbabwean workers have favoured the use of blends of synthetic 1-octen-3-ol, 4-cresol and 3-*n*-

propylphenol delivered from polythene sachets, while ICIPE workers have favoured the use of acetone and cow urine (the latter as a source of phenols) dispensed separately from the vicinity of the traps.

In view of the employment of host urines in field studies, a basic understanding of the origin and kinetics of emission of the phenols is clearly warranted. Moreover, the performance of samples of buffalo urine in the field and in the laboratory suggested that there was an in-built mechanism for gradual formation of these phenols, which might be subject to manipulation and optimization and a possible model for controlled-release of semiochemicals. The present study was motivated by these considerations and here-in we report our findings to date.

MATERIALS AND METHODS

Buffalo urine

Urine used in the study was collected from two male buffaloes (*Syncerus caffer*) kept at the Langata Orphanage, Nairobi, Kenya.

Demonstration of formation of phenols with storage

A freshly collected sample of buffalo urine was divided into two equal parts. One part was kept at -20°C to suspend any chemical transformation and the other part left at room temperature and allowed to age. After 16 (arbitrarily chosen) days each part was extracted with dichloromethane (25 ml x 3) and the solvent removed *in vacuo* at 25°C. The residues were dissolved in ethyl acetate and the solutions examined by gas chromatography (Packard model 438 chromatograph; column: 50m Sil 5 fused silica; temp programme: 50°C (isothermal for 0.5 min) to 180°C at 10°C/min).

Demonstration of involvement of microbes

A freshly collected sample of buffalo urine was divided into four equal parts (A–D) and treated as follows:

(A) Stored at room temperature without any treatment;

(B) Autoclaved at 120°C, cooled to room temperature and stored under sterile conditions;

(C) Filter-sterilized through a 0.22 μm millipore filter paper and then stored at room temperature under sterile conditions;

(D) Stored at -20°C.

After 16 days equal volumes of the above samples (A–D) were extracted with dichloromethane (20 ml x 3) and solvent removed *in vacuo* at 25°C. The residues were dissolved in methanol and the solutions examined by High Performance Liquid Chromatography (HPLC) (Varian model 5000; column: Zorbax ODS reverse phase 25 x 0.46 cm; eluent: 60% aqueous methanol; detector: UV at 254nm).

Isolation and identification of the pro-attractants

Fresh buffalo urine was freeze-dried and fractionated on the sephadex G-10. The fractions obtained were examined by uv spectroscopy. The fraction giving the spectrum of a phenolic ether was collected and appropriate portions successively chromatographed on a Varian semi-preparative column (50 x 0.8 cm) and the fraction corresponding to the major component was pooled together. This was rechromatographed on the same column to give a homogeneous sample for which H^1 NMR spectrum in D_2O was obtained at 200 MHz.

Isolation of bacteria

Portions of buffalo urine, allowed to age under ambient conditions in the laboratory, were inoculated onto buffalo urine agar (BUA) and tryptone soya agar (TSA) plates. Colonies resulting from the growth of microoganisms were transferred onto new plates. Pure cultures selected from single colonies were preserved for screening.

Screening isolates for their ability to biodegrade the pro-attractants in the buffalo urine

Fresh urine was obtained and filter-sterilized using 0.22 μm millipore filters. Aliquots of sterilized urine (200 ml) were placed into sterile 500 ml Erlenmeyer flasks. These flasks were inoculated with a loopful of 24-hr-old cultures of isolated organisms and incubated at room temperature. After 24 hr incubation and every 2 days intervals, 10 ml samples were taken from test-flasks and extracted with dichloromethane in the usual way. The purity of the organism in each flask was confirmed on each extraction day by inoculating a portion onto BUA plates and inspecting these after a period of growth. The extracts were examined by gas chromatography and/or HPLC as described earlier and the peak corresponding to 4-cresol, the major phenolic component of buffalo urine, was checked for in the chromatograms.

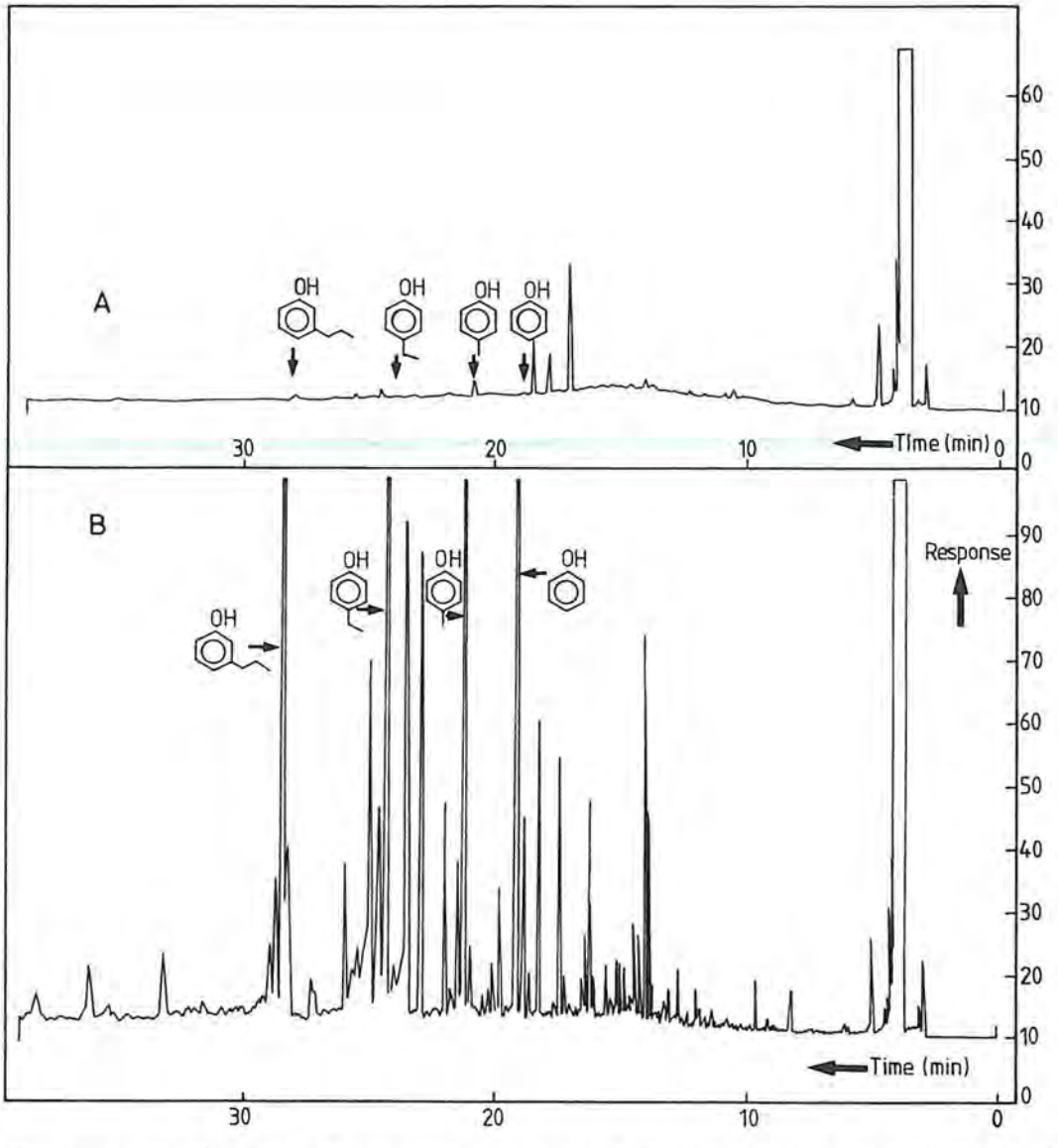

Fig. 1. Gas chromatographic profiles of dichloromethane extracts of buffalo urine portions kept at -20°C soon after collection (A); at room temperature for 16 days (B) (Column: 50 CP Sil fused silica; temperature programme: 50°C (0.5 min) to 180°C at 10°C/min.

Identification of organism "A"

The identity of "A" was established by screening it against substrates in API 20 Strep, a commercially available kit for the identification of streptococci.

RESULTS

Figure 1 shows gas chromatographic profiles of dichloromethane extractable materials from the two portions of a sample of buffalo urine one of which was stored at -20°C and the other at room temperature for 16 days. There is a marked difference between the two with virtually no phenolic components present in the frozen half.

Figure 2 shows HPLC elution profiles of dichloromethane extractable materials from the four portions (A–D) of a sample of buffalo urine treated as described in the Materials and Methods section. Only the portion (A) which had been left at ambient temperature unsterilized showed evidence of significant accumulation of degradation products including phenolic components thus

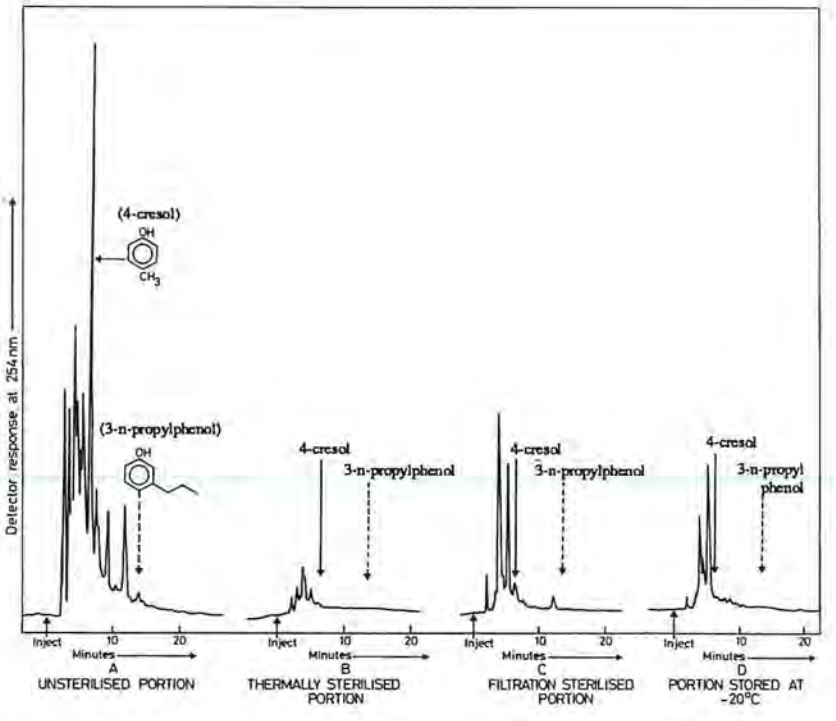

Fig. 2. HPLC elution profiles of dichloromethane extracts of four portions of buffalo urine representing unsterilized (A), thermally sterilized (B), filtration sterilized (C) and frozen (D) parts (column: Zorbax ODS reverse-phase 25 x 0.46 cm column; eluent: 60% aqueous methanol; detector: UV at 254nm).

establishing conclusively that the formation of the tsetse kairomones from pro-kairomones is predominantly due to microbial activity.

Chemical nature of pro-kairomones

A 200 MHz H^1 NMR spectrum of the major fraction obtained after two cycles of preparative HPLC (Fig. 3) showed features which corresponded closely to those of an amalgam of 4-cresol and glucuronic acid. However, a weak AB pattern in the aromatic region suggested the presence of an additional minor component. Integration of the signals due to glucuronide moiety and those due to the 4-cresol moiety indicated a slight excess of protons due to the latter suggesting that the minor component might be an inorganic ester of 4-cresol. Acid hydrolysis of a portion of the HPLC fraction followed by treatment with a solution of barium chloride gave a white precipitate showing that a sulphate is present in the fraction.

Isolation, screening and identification of bacteria

Eight organisms repeatedly encountered in relatively fresh urine were isolated and cultured. Of these only three were found to last in the urine for sufficiently long periods to be considered as candidate agents for the hydrolyis of pro-attractants to the phenols. These comprised a Gram-positive streptococci (referred to as "A") and two Gram-positive bacilli (referred to as "L" and "M" respectively). The three showed varying degree of hydrolytic ability with respect to the pro-attractants, with "A" being the most effective and "M" the least. Whereas "A" and "M" continued to persist indefinitely in urine samples "L" disappeared within 6 days. "A" was identified as *Aerococcus viridans* on the basis of results obtained with the substrates of API 20 Strep.

DISCUSSION

Although it has long been recognized that the urine of many mammals contains phenolic compounds, it has been generally assumed that these occur both in the free state and as conjugates (Folin and Denis, 1915; Williams, 1959; Bakke, 1969). Our results with buffalo urine show that the former are present at most in trace quantities. The conjugates act as the reservoir for the phenols and their gradual hydrolysis constitutes the basis for controlled release of these phenols.

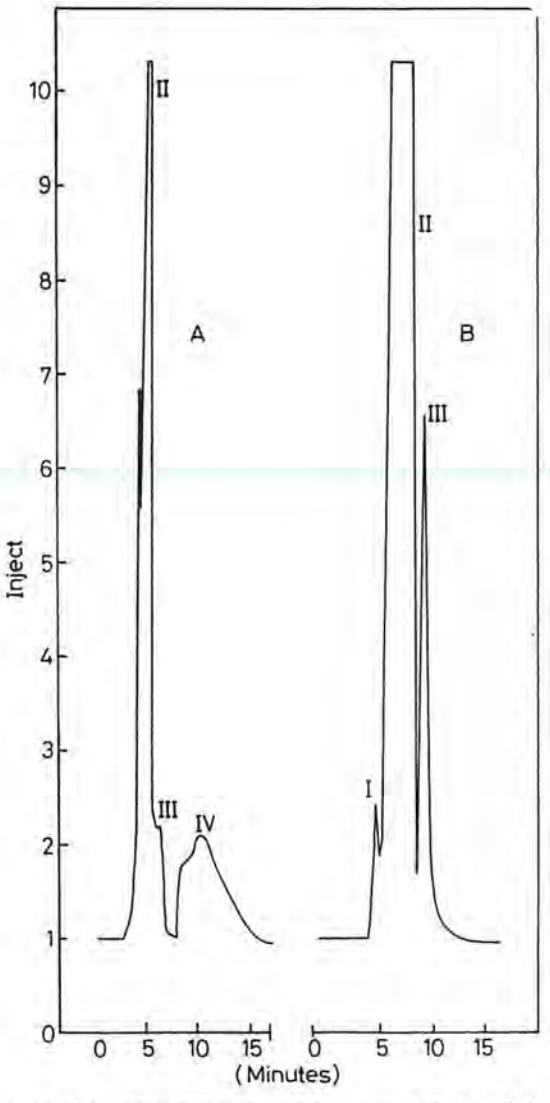

Fig. 3. Chromatograms showing two cycles of HPLC separation of phenolic conjugates from buffalo urine on a Varian semi-preparative reverse-phase column, 50 cm x 8 mm. Fraction IIB was examined by NMR spectroscopy.

Previous attempts to characterize phenolic conjugates from mammalian urine had been based on hydrolysis with either acids or semi-purified samples of enzymes followed by identification of the products in the hydrolysates by comparison with known compounds (Folin and Denis, 1915; Grant, 1948). These studies have led to the inference that phenolic conjugates in mammalian urine occur as glucuronates or sulphates (Folin and Denis, 1915; Williams, 1959; Grant, 1948). Although the origin and excretion of phenols in mammals have received considerable attention in biomedical research, to our knowledge the present work based on direct spectral examination of a chromatographically purified material, represents the first confirmation of the existence of urine phenols as glucuronates and sulphates. Our NMR data suggest that in buffalo urine these conjugates occur in about 4:1 ratio.

Of special interest is the finding that hydrolytic breakdown of these conjugates occurs predominantly through the growth of appropriate microbes in the urine. We had earlier surmised that the hydrolysis of the conjugates could occur with increasing rapidity through self-catalysis resulting from the two accumulating acidic products (glucuronic and sulphuric acids). However, neither sterile samples of urine nor those inoculated with inappropriate microbes showed any evidence of the formation of phenols. Moreover, buffalo urine was found to be somewhat alkaline (pH 9–10) and this did not change significantly during its fermentation, showing that the two acidic products are either metabolised or precipitated out of the solution as metallic salts.

It is clear that a fermenting sample of urine with accumulating phenolic compounds represents a rather harsh environment for most microbes. Thus, most bacteria encountered early in the process disappear very rapidly. *Aerococcus viridans*, the most effective organism found in urine samples so far, commonly occurs in air, but it has also been found as a human pathogen in the urinary tract (Parker and Ball, 1976). Identification of the other two organisms that are able to hydrolyse phenolic conjugates is in hand. Cultures of these organisms will permit studies to be undertaken on their growth characteristics in cow and buffalo urine under different laboratory and field conditions, and on the kinetics of emission of phenolic attractants. In the longer term, manipulation of the conjugate structures through analogue synthesis may allow an optimization of the rate of emission of phenolic kairomones. If successful, this could provide a useful biotechnological model for the controlled release of other semiochemicals in the field.

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