INCORPORATION AND METABOLISM OF CYSTEINE IN THE
HAEMOLYMPH AND SALIVA OF A PLANT BUG

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Summary

When injected into the haemolymph of a pentatomid plant bug, cysteine labelled with $^{35}$S was transferred to the salivary glands and was there incorporated as cysteine-cystine into the precursors of the salivary "stylet sheath". Cysteine did not appear to be converted into methionine either in the haemolymph or in the salivary glands. Some of the injected cysteine was incorporated into soluble protein or peptide in the haemolymph and salivary secretions, some was excreted as cysteine and cystine, and some was deaminated in the haemolymph and was also found in this form in the salivary glands and excreta.

I. INTRODUCTION

The reported formation of disulphide bonds in the salivary "stylet sheath" of the Heteroptera: Pentatomorpha and of the Homoptera (Miles 1964, 1965, 1967) would seem to be anomalous on the basis of present knowledge of the physiology of the structural proteins of insects. The sheath material, which gels rapidly when discharged by the insect, contains protein and lipid and is mixed with a polyphenol oxidase and diphenolic substrate—circumstances similar to those accompanying the formation by insects of structures containing tanned proteins (Brunet 1963); and sulphur-containing compounds are thought to be either absent from the tanned proteins of insects (Hackman and Goldberg 1960; Mercer 1961), or present in small quantities only (Hackman and Goldberg 1968).

Evidence that the polyphenol oxidase is unnecessary for the formation of the sheath has been reported (Miles 1967). Claims by the same author that the gelling of the sheath is accompanied by the formation of disulphide bonds are based on histochemical tests for sulphydryl groups and on the solubility of the material in reagents: neither class of evidence can be considered definitive, and Sogawa (1967) was unable to find evidence for the presence of free sulphydryl groups in the salivary glands of two species of Homoptera. If disulphide bonds occur it should be possible to provide more convincing evidence for their existence by labelling them with $^{35}$S, and this paper records the results of attempts to demonstrate the incorporation of $^{35}$S-cysteine into the salivary secretions of Eumecopus punctiventris Stål (Heteroptera: Pentatomidae). At the same time, the opportunity was taken to determine whether any of the labelled cysteine was metabolized to methionine, in view of the possibility that some insects, unlike many other animals, are able to replace dietary methionine with cysteine (Gilmour 1965).

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II. Materials and Methods

(a) Source of the Insects

Fifth-instar larvae of *E. punctiventris* were collected from under the bark of sugar gums (*Eucalyptus cladocalyx* F. Muell.) for immediate use in experiments.

(b) Reagents

The “salt solution” was that of Martignoni and Scallion (1961). The borate buffer (pH 10) was that of Hackman and Lazarus (1956); when reducing conditions were required, 0·1 mg/ml of Cleland’s reagent (dithiothreitol) was added. All reagents were made up from analytical grade chemicals.

(c) Injections

Solutions were injected as described by Miles (1967). They were: (1) DL-3-[14C]cysteine hydrochloride, 0·5 mCi/ml salt solution, 5 μl at a time; and (2) L-[35S]cysteine hydrochloride (30 mCi/m mole), 1·0 mCi/ml salt solution, 10 μl at a time.

(d) Salivary Secretions

Attempts to obtain watery saliva (Miles 1967) immediately after injection failed in these experiments. Sheath material was obtained by leaving the insects overnight in a glass dish. Flanges of sheath material (termed “collars” in previous publications) could then be scraped off the sides of the dishes (Miles 1964). For autoradiography, the flanges were left overnight in a large volume of borate buffer (pH 10) to extract any soluble contaminants. They were then rinsed three times in distilled water, dried, and mounted on a microscope slide with a clear, quick-drying cement. The same material was later dissolved off the slide in acetone, washed in three changes of acetone, and hydrolysed for chromatography as described in Section II(e).

(e) Salivary Glands

The glands were sampled at 2, 5, and 10 hr after the injections. The insects were held dorsum down in a drop of salt solution under a dissecting microscope. The neck membrane was cut on each side, and the head was gripped with forceps and carefully pulled away from the body so as to bring the salivary glands with it. Unruptured glands were quickly removed by cutting the principal salivary duct and the accessory duct.

(f) Gelled Contents of Glands

The salivary glands have two lobes, and each lobe has a lumen that collects the secretions of that lobe. The contents of the anterior lobe gel irreversibly in glycerol and are considered to be the precursors of the sheath material (Miles 1964). Twenty glands (for each time of sampling) were rinsed twice in large volumes of salt solution and transferred to glycerol overnight. The glands were then passed rapidly through three 1-ml volumes of distilled water and, in the last rinse, the cellular walls of the gland were separated from the sheath precursors and discarded. The gelled precursors (about 5 mg from each batch of 20 glands) were finally placed in a large volume of borate buffer for 3 days to extract soluble compounds and to remove adherent tissue; they were then washed in three changes of distilled water and dried.

(g) Soluble Contents of Glands

The 1-ml aliquots of distilled water in which the glands were rinsed and dissected after immersion in glycerol contained the soluble contents of the glands. The aliquots were combined, evaporated to dryness, and the residue was dissolved in 10 μl buffer containing Cleland’s reagent.

(h) Hydrolysis

Washed and dried sheath material, or gelled material from the glands, was hydrolysed in 6N HCl (about 0·2 ml/mg) in sealed glass ampoules at 60°C for 3 days. One-half of the hydrolysates
of the gelled material were set aside for oxidation as indicated in Section II(j). The remaining solutions, and the whole of the hydrolysate of about 30 flanges of sheath material (original weight < 0.2 mg) were evaporated to dryness, the residues dissolved in water, evaporated three times, and finally dissolved in 5 μl borate buffer containing Cleland’s reagent.

(i) Chromatography

Thin layers of silica gel G (Merek) were loaded with 1-μl aliquots of standards and test solutions. Two solvent systems were used: (1) 64% (v/v) aqueous isopropanol, containing 0.01% (w/v) Cleland’s reagent; and (2) 74% (w/w) aqueous phenol (colourless) in borate buffer, containing 0.01% (w/w) Cleland’s reagent.

Thin-layer chromatograms were sprayed with 0.1% ninhydrin in ethanol-collidine-acetic acid (50 : 2 : 15 v/v) and then dried at 100°C for 15–20 min.

(j) Oxidation for Chromatography

To assist identification of compounds on chromatograms, the hydrolysates and washings and some standards were run in two forms: (1) in their original form as solutions in borate buffer; (2) after oxidation with hydrogen peroxide. The hydrolysates of the precursors were oxidized in acid solution as described by Hackman and Lazarus (1956). This procedure was not convenient for the other preparations, however. Part of these solutions (in buffer) was applied to chromatograms; to the remainder was added an equal volume of “100 vol.” hydrogen peroxide, the solution was allowed to stand for 3 hr at 25°C, and evaporated to dryness at 60°C. The residue was dissolved in a volume of distilled water equal to that of the original solution, and this solution was applied to chromatograms as the oxidized preparation.

(k) Use of Standards

Standards were used as 20 μg of compound per millilitre of borate buffer containing Cleland’s reagent. Cysteine in solution oxidizes readily to cystine, and hydrogen peroxide converts it to cysteic acid. Chromatograms of the cysteine standard showed one large spot of the compound and a variable “tail” that was recognized as cystine by reference to standards of the latter. (The colours of spots as well as their positions facilitated this identification.) After cysteine had been oxidized with peroxide, whether in acid or alkaline solution, chromatograms showed a large spot of cysteic acid, and sometimes traces of cysteine and cystine. Inclusion of Cleland’s reagent in all solvents greatly helped reduce unwanted oxidation of cysteine and did not appear to interfere with subsequent oxidation by peroxide.

Methionine runs above cysteine on silica gel using 64% isopropanol as solvent. Oxidation in acid solution converts it mainly to methionine sulphone, which runs behind cysteic acid. Oxidation in alkaline solution, on the other hand, caused most of the methionine to disappear, leaving only a small amount of the sulphone.

(l) Autoradiography

After development (and before treatment with ninhydrin) chromatograms were dried in an oven at 100°C, and left for 2 days in a stream of warm, dry air. The chromatograms and the preparation of mounted flanges of sheath material were covered with Melinex 25-S (I.C.I.) and pressed against X-ray film in a light-tight, lead-lined box for 1 month.

III. Results

(a) Incorporation of Labelled Cysteine into the Sheath Material

Sheath material became radioactive after the insects had been injected with [14C]cysteine or [35S]cysteine. In the experiments with 14C, insufficient radioactivity remained in preparations of the salivary glands at the time of sampling for the
analyses of the glands to be useful. The experiment is mentioned only because it provided insight into the excretion of injected cysteine—as indicated in Section III(c).

After injection of $^{[35]S}$cysteine, the gelled precursor became increasingly radioactive from 2 to 5 hr later. The content of radiotracer was greatly diminished by 10 hr, however, presumably due to its replacement with fresh secretion.

Chromatography and autoradiography of the chromatograms of the hydrolysates of both sheath material and the precursor provided good evidence that the labelled moiety was cysteine–cystine. In all the autoradiographs, some of the radioactivity remained at the origin and presumably represented incompletely hydrolysed protein or peptides. Radioactivity also appeared in positions corresponding to cysteine and cystine on the chromatograms of unoxidized hydrolysates, and in a position corresponding to cysteic acid in chromatograms of oxidized hydrolysates. Chromatograms of the hydrolysate of the secreted sheath material, using either isopropanol or phenol as solvent, corroborated these identifications.

Ninhydrin revealed the presence of amino compounds at positions of radioactivity on the chromatograms of hydrolysates. No quantitative determinations were made, but from the relative sizes of radioactive spots and chemically revealed spots it was estimated that the amount of cysteine–cystine in the protein of the sheath material probably lay between 1 and 10%. It is not known whether this protein is variable in composition and therefore to what extent the injection of cysteine into the haemolymph would be likely to affect the composition of the sheath material. In these experiments, each insect was injected with 40 $\mu$g of labelled and carrier cysteine. About 500 $\mu$g of gelled sheath precursors was subsequently obtained from each pair of salivary glands, and hence the total quantity of cysteine–cystine that was present in the gelled precursors probably lay between 5 and 50 $\mu$g. At the same time, only a part of the $^{35}$S from the injected cysteine was transferred to the sheath precursors [cf. Section III(b)].

(b) Cysteine in the Haemolymph and Soluble Contents of the Salivary Glands

Chromatograms were also run of haemolymph from undissected insects 5 hr after injection with labelled cysteine. The haemolymph contained much material that reacted with ninhydrin. The radioactivity present was mostly concentrated at the origin, however, and presumably indicated soluble proteins or peptides of the haemolymph into which the labelled cysteine had been incorporated. A faint indication was sometimes obtained of the presence of labelled free cysteine and cystine in the haemolymph, but not of labelled methionine. On the other hand, there appeared a distinct radioactive spot that ran ahead of all the amino compounds on the chromatogram.

The chromatogram of the soluble contents of the salivary glands removed 5 hr after injection of the insect with labelled cysteine showed only small quantities of compounds that reacted with ninhydrin, but contained much more radioactive material than chromatograms of the haemolymph. Sites of radioactivity tended to coincide on chromatograms of the haemolymph and of the soluble contents of the glands and, in particular, both chromatograms showed the presence of the radioactive, apparently non-amino compound. This compound was absent from chromatograms of the hydrolysates of the sheath precursors, indicating that the preparation of the
precursors was effectively free from contamination from either haemolymph or the soluble contents of the glands.

(c) Excretion of the Injected Cysteine

An insect that had been injected with $^{14}$C-cysteine deposited a drop of radioactive excreta on the filter paper lining its overnight container. An ad hoc investigation of the excreta was carried out to determine whether any metabolism of the cysteine had occurred during the process of its transfer from haemolymph to excreta.

A 1-μl drop of a standard solution containing 10 mg/ml each of (unlabelled) cysteine hydrochloride and methionine in borate buffer with Cleland's reagent was placed at the centre of the drop of excreta. The filter paper (Whatman No. 1) was shaped and treated as a two-dimensional chromatogram. Other papers, loaded only with standards, were treated similarly. The first run was in 64% isopropanol; and the second in butanol–acetic acid–water (77 : 6 : 17 v/v). The developed papers were autoradiographed, and later dipped in 1% ninyhdrin in ethanol containing 0·25% triethylamine, and heated to 100°C to reveal amino compounds. This treatment separated cysteine, cystine, and methionine standards; and cysteine and cystine were additionally recognizable because of the brown and reddish brown colours produced by them respectively.

Radioactive material in the excreta coincided well with the positions taken by the added cysteine, but was absent from the area occupied by methionine. A radioactive area that did not react with ninyhdrin was also present [cf. Section III(b)].

IV. Discussion

The results reported above are consistent with the transfer to the salivary glands of injected cysteine, and its incorporation there into the precursor of the sheath material. Evidence was also provided for the presence of cysteine–cystine in the secreted sheath material.

Although these experiments do not themselves indicate that disulphide bonds are present in the stylist sheath, the identification of quantities of cysteine–cystine in hydrolysates strongly corroborates previous evidence (Miles 1964).

No evidence was obtained for the conversion of cysteine to methionine, either in the haemolymph or salivary glands. Some of the injected cysteine appeared to have been deaminated in the haemolymph, however, to a compound that retained its sulphur label. If this was the same radioactive, non-amino compound excreted by the insect after injection of $^{14}$C-cysteine, the metabolite must also have retained at least the labelled methylene group adjacent to the sulphur atom.

In one insect at least, much of the injected cysteine appeared to have been excreted, and any that remained in the haemolymph of these insects seemed mostly to be incorporated into soluble protein or peptide. Moreover, although a preparation (of unknown dilution) of the soluble contents of the salivary glands was relatively poor in amino acids compared with the undiluted haemolymph, a larger quantity of soluble derivatives of the injected cysteine appeared in the preparation from the salivary glands. It is evident, therefore, that only low concentrations of free cysteine–cystine are maintained in the haemolymph of this insect, whether due to excretion, incorporation into protein, or their active uptake by the salivary glands.
V. References


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