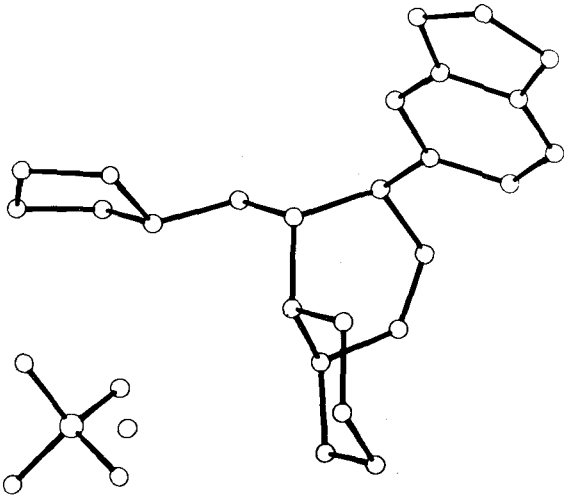
Acid hydrolysis of (II) gave pyrrolidine and catalytic reduction afforded the dihydro derivative m.p. 163°. The presence of a disubstituted double bond was confirmed by osmylation to the diol m.p. 167° which on cleavage with periodate gave the dialdehyde m.p. 119°. Cyclostachine A on reduction with $LiAlH_4$ formed the amine (III) isolated as its sulphate, m.p. 110°, the structure of which has been determined by X-ray crystal analysis. $[(C_{22}H_{30}NO_2)^+ (HSO_4)^- H_2O$; monoclinic, a = 15.874, b = 6.584, c = 21.729 Å; β = 98.33°, Z = 4; space group $P2_1/a$ (which confirms the racemic nature of these



compounds); refined to R 0.046 for 2958 reflexions]. The figure depicting molecule III as found in the crystal shows that atoms C (7–10, 15) in the unsaturated ring are coplanar and C(1), C(14) and C(17) are all axially substituted. This is in contrast to NMR results which show that II in solution has these three substituent atoms equatorial – a rather crowded conformation.

The difference is presumably due to the greater volume required by the non-planar amine ring compared with the amide form, and the need to proximate the sulphate ion to the nitrogen atom for the ionic and hydrogen bonded linkage.

The structure of cyclostachine A has been independently confirmed by synthesis. Intramolecular Diels-Alder reaction of the ester (IV)[3] yielded a mixture of (V) m.p. 137° and (VI) m.p. 88°. Hydrolysis of (V) gave the acid (VII), m.p. 141–142°, the chloride of which was condensed with pyrrolidine to yield (II) identical in all respects with the natural sample[4].

Summary. A novel alkaloid designated cyclostachine A has been proved to have the structure (II) on the basis of spectral and degradative data. The structure has been confirmed by synthesis, and by X-ray analysis of the derived amine sulphate (III).

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The Identification of Dansyl Sarcosine and its Occurrence in Molluscs

A convenient, sensitive procedure for the analysis of tissue amines and amino acids, involving the two-dimensional chromatography of their dansyl derivatives, has been developed and optimised by Neuhoff et al.[1,2]. This technique has been used to screen for the existence and distribution of putative neurotransmitters in nervous tissue[3–6]. To facilitate interpretation of the resulting chromatographs, several reference maps have been published[1,2,7], based on the migration of standards. However, there are several unknowns among the frequently observed chromatographic spots. In trying to identify the transmitter at the squid giant synapse (G. A. Cottrell, unpublished observations), we encountered an intriguing unknown that appeared to be localized to the post-synaptic axon but absent from the pre-synaptic axon. Purification and characterization of the compound suggest it is the N-dansylated derivative of sarcosine (N-methyl glycine). This report gives the evidence for the identification and some preliminary results of the distribution in molluscan nervous systems.

Materials and methods. Specimens of *Eledone cirrhosa* were obtained locally and maintained in aquaria. *Helix pomatia* were obtained from Gerrard and Haig (Surrey, England), and *Loligo* from Naples, Italy.

Dansylation and subsequent chromatography were executed as described elsewhere[2,7].

Fluorimetry. Comparisons of the unknown and standards were performed in 0.17 ml quartz microcuvettes on an Aminco-Bowman spectrophotofluorometer. Since cephalopod brain was a rich source of the unknown, homogenates of octopus (*Eledone cirrhosa*) brain were dansylated and chromatographed. Pooled unknown spots were extracted with absolute ethanol[2] and compared to standard dansylsarcosine in terms of their excitation spectra (emission set at 522 nm) and emission spectra (excitation set at

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340 nm). No fluorescence was observed in control ethanolic extracts of blank polyamide.

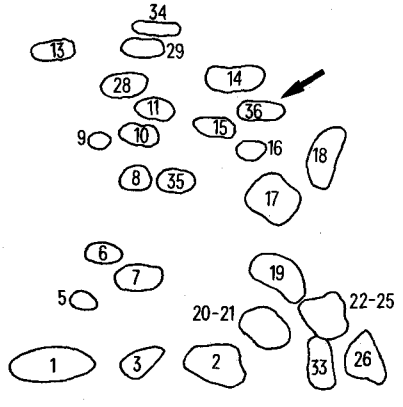
Mass spectrometry. 10 *Eledone* brains were homogenized and dansylated in bulk[8]. The alkaline dansylation solution was extracted with chloroform: tert-butanol (6:4) after reaction. This quantitatively extracted the unknown, and partially extracted the dansyl derivatives of leucine, isoleucine, valine, proline, tyrosine, phenylalanine, and alanine. The unknown was purified by preparative thin-layer chromatography on Silica Gel G with development in chloroform: benzyl alcohol: acetic acid (70:30:3). The chromatographically pure unknown and standard were extracted from the gel with methanol, taken to dryness in vacuo, and dissolved in a small volume of acetone. Aliquots of this were introduced with the direct inlet probe into a A.E.I. Type MS902 mass spectrometer. Probe temperature was 150 °C., electron source temperature 250 °C, and the beam energy was 70 eV.

Results. In the reference map of dansyl derivatives (Figure), unknown spot 36 was identified as dansyl sarcosine based on: 1. co-migration in different solvent systems (Table). 2. coincidence of their excitation and emission spectra, and 3. coincidence of their fragmentation patterns[9]. The position of dansyl sarcosine, using the common 2 solvent systems[1,2,7], was clearly bracketed by the derivatives of proline, valine, and GABA. This has been previously designated as 'spot 13'[10], 'unknown U_5'[11], 'spot 31'[3], and 'spot 36'[12].

Mobilities of unknown spot 36 compared to standard dansyl-sarcosine

Solvent system	Unknown Rf	Dans-sarcosine Rf
1	0.78	0.79
2	0.17	0.167
3	0.385	0.385
4	0.84	0.835
5	0.95	0.95
6	0.55	0.56

Values were obtained on polyamide thin layers (Carl Schleicher & Schull F1700) and Silica Gel G (Merck) using different solvent systems; 1. ethyl acetate:methanol:acetic acid (20:1:1); 2. benzene: triethylamine (5:1); 3. carbon tetrachloride:methanol (15:1); 4. benzene: pyridine: acetic acid (80:20:1); 5. chloroform:tert-butanol: acetic acid (6:3:1), and 6. *n*-butanol:*n*-heptane:acetic acid (3:3:1).



Standard chromatogram[8] to show the relative positions of substances reacted with dansyl chloride. Two-dimensional development was in water/formic acid (100:3 v/v) in the horizontal direction and benzene/acetic acid (9:1 v/v) in the vertical direction. Spot 1 is the origin and the dansyl products are 2-taurine, 3-dansyl-OH, 5-tryptophan, 6-lysine, 7-ornithine, 8-phenylalanine, 9-*bis*-histidine, 10-leucine, 11-isoleucine, 13-5-OH indole, 14-proline, 15-valine, 16-GABA, 17-alanine, dansyl-NH_2, 18-ethanolamine, 19-glycine, 20/21-aspartic and glutamic acids, 22-25-glutamine, asparagine, serine, threonine, citrulline, 26- ε-lysine, arginine, 28-putrescine, 29-cadaverine, 33-unknown, 34-unknown, 35-methionine, 36-sarcosine.

The reaction proceeded quantitatively over a range of dansyl chloride and sarcosine concentrations, with a faster rate than for glycine or alanine. The N-methyl substitution raises the pK of the amino group from 9.8 to 10.2, making it a better base and more reactive nucleophile for dansylation. Thus, sarcosine can be quantitatively estimated from the yield of its dansyl derivative.

Samples of the pre- and post-synaptic axons and cell bodies were taken from the squid giant synapse (a junction between second- and third-order giant fibres in *Loligo* stellate ganglion)[13] and analyzed by the dansyl chromatography technique. Preliminary results indicated sarcosine was found only in postsynaptic tissue. A more extensive examination in another cephalopod, *Eledone cirrhosa*, revealed that sarcosine was asymmetrically distributed throughout brain regions, but was uniformly higher in brain than in other tissues. Its levels were highest in the median superior frontal lobes and the buccal lobes, and it was absent from the connective tissue and protective cranial jelly surrounding the brain.

In *Helix pomatia*, sarcosine was highest in the circumoesophageal and buccal ganglia, although there were detectable levels in the blood. It was not found in other tissues. Studies on individual neurones showed it to be absent from the giant buccal cells[14] and from the metacerebral giant neurones, but present in suboesophageal neurone 'E'[3]. Sarcosine was tested on individual *Helix* neurones during intracellular recording and on the isolated clam heart without effect.

Discussion. In considering the significance of fluctuations in sacrosine levels, it is important to know its metabolic origin. Sarcosine can arise by either the catabolism of choline through betaine, or the direct N-methylation of glycine. High levels of both potential precursors[15,16] in the mollusc tissues we examined obscures clear interpretation. Consequently, sarcosine may reflect metabolism of choline and/or glycine (both compounds of neurochemical interest).

The predominant localization of sarcosine to nervous tissue, and its nonuniform distribution therein, suggest it is the product of a largely neurochemical pathway. It may in fact have an activity of its own, but efforts to detect a pharmacological activity have not been successful. In this regard, it is interesting to note that glycine N-methyltransferase has been proposed as a 'regulatory enzyme' in aging vertebrate tissues[17]. In brain, N-methylation

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may contribute to terminating the action of the putative neurotransmitter glycine. The significance of the postsynaptic localization of sarcosine in the squid giant synapse is unknown.

Examination of previously published dansyl chromatograms show sarcosine is restricted to the brain in *Helix*[12] tissues and present in the blood[10]. It is unaffected by electrical stimulation of the brain[18], or by treatment with LSD-25[11]. Our own and earlier comparative studies show it to be the highest in crustaceans and cephalopods[19]; but absent from the leech segmental nerve chain[20], dorsal root ganglia[6], and the optic pathway[2,5].

Summary. An unknown dansyl derivative was identified as dansyl sarcosine. In molluscs, sarcosine was found to be largely localized to the nervous system. Examination of individual snail neurones, regions of the octopus brain and the squid giant synapse showed dramatic variations in sarcosine levels.

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Effect of Some Phosphodiesterase Inhibitors on Two Different Preparations of Adenosine 3′,5′-Monophosphate Phosphodiesterase

Many cellular functions are regulated by adenosine 3′,5′-cyclic monophosphate (cyclic AMP). The intracellular concentration of cyclic AMP is generally under hormonal control. Therefore it seems of special interest to study the effect of chemicals, especially therapeutic agents, on the regulation of cyclic AMP in in vitro systems and in intact cells.

Cyclic AMP phosphodiesterase (PDE), the enzyme which degrades cyclic AMP to 5′-AMP, represents a possible target for influencing the intracellular concentration of cyclic AMP by chemical means. When compounds are tested for their activity as inhibitors of PDE, often the concentration of the compound necessary to inhibit PDE activity by 50% (I_{50}) is determined[1-3].

PDE seems to exist in a variety of isoenzymic forms which may occur simultaneously in a particular tissue[4-6]. To evaluate the potency of a compound as PDE inhibitor in a specific tissue, it seems desirable to measure its PDE inhibiting activity on more than one preparation of PDE. Such a study, using two different PDE preparations, is presented in this paper.

Materials and methods. Two different PDE preparations from rat brain cortex were used. One was prepared according to KAKIUCHI[7] which included Sephadex G-200 column chromatography of a supernatant obtained after centrifugation of the homogenate of rat brain cortex. The Sephadex G-200 column chromatography yielded 3 fractions with PDE activity. One fraction showed enzymatic activity only in the presence of modulator protein and calcium ions. This fraction was used further and will be designated PDE I. Modulator protein was prepared by heating a hog brain cortex homogenate for 5 min at 95 °C in a water bath[7].

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I_{50} values of some PDE inhibitors using two different PDE preparations and two different substrate concentrations

Substrate	Concentration (μM)	IBMX I_{50} (μM)	Papaverin I_{50} (μM)	SQ66-007 I_{50} (μM)	SQ20-009 I_{50} (μM)	n
PDE I	500	9.25 ± 4.3	48.5 ± 19	35.1 ± 9.7	28.5 ± 4.6	4
PDE I	0.25–2	24.6 ± 5.3	94.4 ± 3.7	64 ± 10.8	n.d.	4
PDE II	500	67.75 ± 6.1[b]	52.87 ± 5.5 n.s.	56.5 ± 10.1[a]	n.d.	5
PDE II	0.25–2	44.2 ± 5.4[b]	33.7 ± 19.6[b]	19.1 ± 4.6[b]	n.d.	5
PDE I	500	$2p < 0.01$[c]	$2p < 0.01$[c]	$2p < 0.01$[c]		
PDE I	0.25–2					
PDE II	500	$2p < 0.001$[c]	n.s.[c]	$2p < 0.001$[c]		
PDE II	0.25–2					

The incubation mixture contained in a total volume of 1.0 ml; 0.2 mM dithiothreitol; 1 mM 5′-AMP; 3 mM $MgSO_4$; 0.5 mM ^{3}H 3′,5′-cyclic AMP (60 nCi); 0.1 mM $CaCl_2$; varying concentrations of inhibitor and 36 µg of PDE I plus 71 µg modulator protein or 420 µg PDE II. The reaction was stopped by addition of 0.2 ml $ZnSO_4$ (0.2 mM) and 0.2 ml $Ba(OH)_2$ (0.2 mM). After centrifugation, radioactivity of the supernatant was determined. [a] < 0.05, [b] < 0.001 as compared to PDE I. n.s. not significant, [c] Iso values at high substrate concentration compared to those at low substrate concentration: n.d., not determined. IBMX: Isobutylmethylxantine. All values represent mean ± S.D.