CHEMICAL INVESTIGATIONS OF THE GIANT NERVE FIBERS OF THE SQUID

II. DETECTION AND IDENTIFICATION OF CYSTEIC ACID AMIDE (β-SULFOALANINAMIDE) IN SQUID NERVE AXOPLASM

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SUMMARY

Cysteic acid amide (β-sulfoalaninamide), a new biological amide, was found and its concn. determined quantitatively in the dialysable portion of the axoplasm of the squid *Loligo pealii* and *Dosidicus gigas*. The identification was accomplished in the following manner:

1. The correspondence of the paper chromatographic behavior of the natural product and its methyl ester with the synthetic substances.

2. The correspondence of the rate of acid saponification of the carbon-amide-nitrogen bond in the natural product, and in the synthetic substance.

INTRODUCTION

In a previous communication[1], we have described, among other materials, a ninhydrin-positive component in the dialyzable portion of the nerve axoplasm of *Loligo pealii* and *Dosidicus gigas*, which, after acid hydrolysis, yields cysteic acid but no cysteine or cystine. After fractionation of the dialyzable part by means of preparative paper electrophoresis at pH 3.9, this compound is found in the neutral fraction. From this fraction, which is still quite complex, the neutral amino acids and other substances can be separated by electrophoresis at pH 2.4 when they migrate to the cathode. In the neutral fraction at pH 2.4, there are found as ninhydrin-positive substances the cysteic acid derivative, large amounts of taurine, and, in much smaller concns., peptides of unknown structure. The ninhydrin-negative components of the neutral fraction at pH 3.9 will be described in a future communication.

By means of paper chromatography, it is possible to further separate the ninhydrin-positive components of the fraction which is neutral at pH 2.4* into a "taurine area" and a "peptide area"[1].

After elution of the "taurine area" from the chromatogram and after acid hydrolysis, one obtains, by renewed paper chromatography or paper electrophoresis, large amounts of taurine and some amino acids, derived from another peptide hidden

* Hereinafter to be designated the "neutral fraction".

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in the taurine spot. Elution and hydrolysis of the "peptide area" yields cysteic acid in an amount equal to 40 % of the hydrolysate and, as expected, a number of free amino acids. The relatively high concn. of cysteic acid and the fact that its appearance in peptide linkage has never been observed, leads to the conclusion that the substance in question must be a derivative of cysteic acid in which the carboxyl group is neutralized. Such an assumption would be in agreement with the electrophoretic behavior of the substance at pH 3.9 and 2.4. Our early supposition that this substance may be β-sulfoalaninamide has in the meantime been substantiated.

Because of its neutral behavior at pH 3.9 and 2.4 and the relatively low concns. in which it occurs, it is difficult to isolate the substance. However, even without isolation it was possible to prove with certainty that this component is cysteic acid amide. For this identification we employed the following experimental criteria and have shown that the natural product agrees in properties with the synthetic substance:

1. The paper chromatographic behavior in different solvent-systems and, in addition, using a color reaction which is typical for the substance.

2. The ability to be easily converted to its carboxylic acid ester and the paper chromatographic behavior of the methylester.

3. The rate of acid saponification of the acid amide group.

It was impossible to make use of the classical identification reaction for carboxylic acid amides and carboxylic acid esters, namely their transformation by hydroxylamine into the corresponding hydroxamic acids and their demonstration with $FeCl_3$, because the possible presence of glutamine or asparagine in the peptides in this fraction would have interfered.

EXPERIMENTAL

Preparation of cysteic acid amide

So far as we are aware, the preparation of β-sulfoalaninamide has been reported only by DUVIGNEAUD *et al.*[2], in connection with their studies of the structure of oxytocin. We have made use of one of the older and simpler methods of preparation of aliphatic carboxylic acid amides and have synthesized the substance in the following way: 1 gram L-cysteic acid·H_2O (Mann Research Laboratories, New York N.Y.) was esterified in the usual way with waterfree methanol–HCl, and the product of the reaction was freed from the solvent by vacuum distillation. The last traces of the hydrogen chloride were removed by evaporation in the desiccator. The ester was then allowed to stand, with occasional shaking, for a number of days in an excess of concentrated ammonia, and then the ammonia was removed. Because the cysteic acid amide proved to be unexpectedly sparingly soluble in cold water, its final purification was accomplished by thrice recrystallization from hot water. The elementary analysis gave the following results*.

	N	S
Calculated:	16.66	19.05
Found:	16.77	19.07

The m.p., determined with the Kofler apparatus, was 270° (under decomposition). The yield was 18 %.

* Carried out by Dr. W. MANSER, E.T.H., Zürich, Switzerland.

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Preparation of cysteic acid methyl ester from the natural product and from the synthetic substance

Especially mild conditions using 0.1 *N* methanolic hydrochloric acid, were chosen for the preparation of the methyl ester. About 10 mg of the neutral fraction (containing about 250 μg of cysteic acid amide) were used. The neutral fraction and the reference substance were treated with 0.1 *N* methanolic HCl and the further preparation followed the lines of the procedure mentioned above, until the stage of the ester preparation was reached. As indicated by the paper chromatogram, the ester was obtained as the main product in both preparations, and, in addition to the unaltered amide, a small amount of cysteic acid also resulted. The quantitative photometric evaluation of the paper chromatographically separated esterification products of the synthetic cysteic acid amide gave the following data: 62.5 % cysteic acid methyl ester, 25 % cysteic acid amide and 12.5 % cysteic acid.

Rate of acid saponification of the carbon amide-nitrogen bond

Synthetic cysteic acid amide and asparagine were hydrolyzed with 0.1 *N* HCl at 100° over a period of 24 h, and the rate of the reaction was followed. The rate of saponification of the asparagine under these conditions was estimated to be about thrice that of cysteic acid amide. In the case of the natural product, we adopted the procedure of using 4 small covered test tubes, each containing 0.4 ml of 0.1 % solution of natural cysteic acid amide in the neutral fraction in 0.1 *N* HCl. These were kept in the drying oven at 100° and the tubes were taken out at intervals of 1, 3, 5 and 8 h. After cooling in the refrigerator and quantitative transfer of the material in a microvessel, the HCl was removed in a vacuum desiccator in about 1 h. The last procedure prevented further recognizable splitting of the amide bond, making neutralization of the samples unnecessary. The same method was employed with the synthetic cysteic acid amide. After simultaneous separation of the reaction products and those of the synthetic substance by horizontal electrophoresis at pH 3.9, the amount of cysteic acid produced was determined photometrically, using ninhydrin as the staining reagent.

Paper chromatography

For the paper chromatographic separation and identification, we found the following solvent systems most useful: (1) *n*-butanol–acetic acid–water (4:1:5); (2) phenol–*o*-cresol (1:1), saturated with water; (3) *n*-butanol–ethanol–water (4:1:5); (4) *n*-butanol–methanol–water (4:5:1). For 2-dimensional chromatography, a combination of (1) and (2) was used. Only the ascending method was employed using Whatman paper No. 1 at a temperature of 28° ± 2. The spray reagents used have been ninhydrin reagent, according to BRUEGGEMANN AND DREPPER[3], and the "copper-reagent" of KAWERAU AND WIELAND[4].

The identification of the cysteic acid amide on the chromatogram was facilitated by a color reaction which is not given by asparagine, glutamine, the amino acids, cysteic acid or its methyl ester. If one sprays with ninhydrin, the amide appears at first as a yellow spot which, within about 1 h, gradually changes to the blue-violet color, characteristic of most amino acids. This property the amide shares with the ester. If, several hours later, one sprays with the copper reagent, one obtains the familiar luminous red copper complex. In contrast to the color stability of this complex

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in the case of the amino acids, glutamine, asparagine and the cysteic acid methyl ester, the color of the cysteic acid amide changes in time from orange to yellow; sometimes it remains at the "orange stage" for reasons unknown. This behavior appears to be independent of the nature of the solvent used in paper chromatography.

Quantitative determination

The quantitative determination of the cysteic acid amide from the dialysable portion of *Loligo* axoplasm was made by photometric evaluation (at 546 mμ) of the ninhydrin-stained substance which had been separated by paper chromatography in solvent (3). The precision of the determination was somewhat affected by the large amounts of taurine which are present. As reference substances, the synthetic amide and taurine were used. The peptides, which are also present in the neutral fraction, do not interfere because they have a somewhat lower R_F value, and, in the concn. in which they occur, they are hardly stainable. The limit of error of the determination under these conditions was of the order of a few percent. Because of the appreciably lower concn. of the cysteic acid amide in the axoplasm of *Dosidicus gigas*, and because of the even more unfavorable quantitative relationship of the amide to taurine in the axoplasm of this species, photometric determination was inaccurate with *Disidicus* material. Therefore this determination was made by visually comparing the amide spot (revealed by ninhydrin) from the neutral fraction with a series of concns. of the reference substance, run on the same 1-dimensional chromatogram.

RESULTS

By 2-dimensional paper chromatography of the neutral fraction on the one hand, and the mixture of the synthetic cysteic acid amide and taurine on the other hand, one obtains in solvent systems (1) and (2) identical chromatograms (see Figs. 1 and 5 in

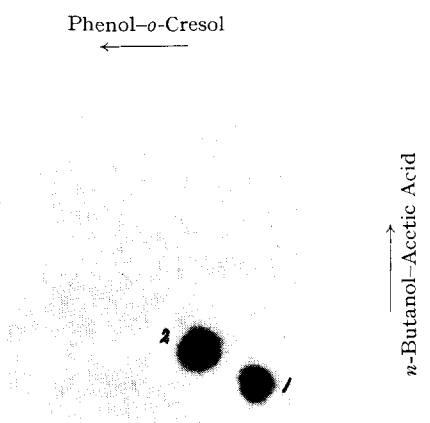

Fig. 1. 1. Cysteic acid amide. 2. Taurine.

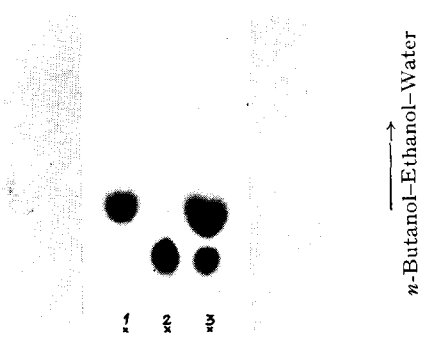

Fig. 2. 1. Taurine (reference substance)
2. Cysteic acid amide (ref. subst.).
3. "Neutral fraction"

ref.[1]). Fig. 2 shows (from right to left) the separation of the neutral fraction and the reference substances, the synthetic amide and taurine. In the original, the presence

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of the peptides which migrate with the lowest R_F value, is detected as a weak spot. Essentially similar separations were obtained with solvent (4) and the correspondence of the reference substance and the natural product is also thereby substantiated. If one uses solvent (1) for the chromatography of the esterified neutral fraction and employs for this purpose a mixture of cysteic acid, cysteic acid amide, taurine and cysteic acid methyl ester as reference substances, one achieves a separation which suffices for the identification of these 4 substances. Both the ester and the acid can now be demonstrated in the neutral fraction. The R_F values (in the sequence of the substances named) were 0.029, 0.066, 0.10 and 0.19. The separation of the 3 first named substances can be improved by repeating the chromatography after drying the paper, or by allowing the solvent to run several sheet lengths. In the latter case, the enhanced separation is partially obscured by the "tailing" effect. In all of these expts., the amide and the ester show agreement with the reference substances in shade of staining after spraying.

The ability of the natural product to form an ester is an important clue in the identification of the substance. That we are dealing with a primary amide, in which neither of the 2 hydrogen atoms of the amide nitrogen are substituted, could be proved by the agreement of its R_F values on the paper chromatograms with the reference substance in all of the solvent systems which we have tried. The introduction of the lipophilic methyl group into the molecule increases the R_F value substantially in the organic solvents. This is seen in the case of the cysteic acid methyl ester.

TABLE I

RATE OF OCCURRENCE OF CYSTEIC ACID DURING THE SAPONIFICATION OF SYNTHETIC AND NATURAL CYSTEIC ACID AMIDE

Saponified with 0.1 *N* HCl at 100°.

Time of hydrolysis *h*	*Delivery of cysteic acid from reference substance* %	*Delivery of cysteic acid from "neutral fraction"* %
1	3	5
3	13	14
5	30	32
8	76	76

Also, as shown in Table I, the rate of saponification of the carbon-nitrogen bond of the acid amide agrees well with that of the reference substance. In this connection, the acid amide structure occupies an intermediate position between the structurally related peptide linkage and the easily split ester bond.

In the quantitative determination, we find the concentration of cysteic acid amide in 100 mg dry wt. of the dialysable part of the axoplasm of *Loligo pealii* to be 4.4 μmoles, and that of *Dosidicus gigas* to be 0.4 μmole.

DISCUSSION

So far as we are aware the only reference to β-sulfoalininamide (cysteic acid amide) in the literature is that of DUVIGNEAUD *et al.*[2]. The present communication is the first in which its presence is reported in biological material. It constitutes the fourth

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sulfur-containing substance to be demonstrated in squid axoplasm. It was previously shown that isethionic acid is the major anion of squid axoplasm and that taurine is present in fairly high concn.; methionine is also present but in quite low concn. Among the ninhydrin-positive substances that exist in the free state, cysteic acid amide is the sixth most abundant in the axoplasm of *Loligo pealii*. Its concn. in the axoplasm of *Dosidicus gigas* is an order of magnitude less, corresponding perhaps to the fact that the concn. of most of the neutral amino acids and also that of taurine is much lower in *Dosidicus* axoplasm. To what extent these differences in the relative concns. of substances reflects true differences between the 2 genera of squid or may perhaps be due to post-mortem alterations that may have occurred during the processes of procurement and processing in Chile remains to be determined.

There is as yet no evidence concerning the pathways leading to the biosynthesis of cysteic acid amide. Cysteic acid, cysteine and cystine are absent in the dialysable part of axoplasm of both types of squid. Along purely formal lines the amide might be supposed to arise by attachment of urea onto isethionic acid with the splitting out of water or by the attachment of formamide onto taurine with the removal of two hydrogen atoms.

Comparative data concerning the occurrence of cysteic acid amide, and for that matter other substances discovered in the present analytical investigations of axoplasm, in vertebrate nerves are as yet unavailable. It is therefore impossible to assess the possibility that this compound, or others discovered in squid axoplasm, serves a unique role in nerve function, particularly in impulse propagation. Isethionic acid, present in high concn. in squid axoplasm, is absent, or present only in low concns. in crustacean and vertebrate nerve and may therefore be associated with the chemical metabolism characteristic of squid, or perhaps of molluscs, but not of nerve generally. This may in fact be true of all the sulfonates, including cysteic acid amide, which are found in relative abundance in squid axoplasm.

Cysteic acid amide and taurine lose their zwitterion properties and become strong acids at pH's above 7.6 and 8.6, respectively. If local changes in pH in this range can occur in or near the excitable membrane, in connection with trans-membrane movement of base, these sulfonates would then be capable of binding cations. Even if such reactions would occur, the deciding question of selective specificity of cation binding would remain unanswered.

Since free cysteic acid appears to be absent in axoplasm, it is improbable that the amide might react analogously to the case of glutamic acid $\rightleftharpoons$ glutamine, which is known to occur in mammalian brain. Moreover, to ascribe equivalence to cysteic acid amide with glutamine (or asparagine) is inappropriate since, in the case of β-sulfoalaninamide, the acid amide group is linked to the α-carbon atom. The reduction of saponification rate of its acid amide group as compared with those of glutamine and asparagine is apparent from consideration of the structure of the compounds.

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SPECTROPHOTOMETRIC INVESTIGATIONS ON ENZYME SYSTEMS IN LIVING OBJECTS

IV. KINETICS OF THE STEADY STATES

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SUMMARY

A spectrophotometric technique was developed by means of which changes in the steady states of the respiratory enzymes after removal or addition of oxygen, could be accurately recorded. The results point to the existence of a spatial organization of the respiratory system, in which the individual enzymes form multimolecular units.

INTRODUCTION

According to current opinions the main cytochromes act in an "electron ladder", *e.g.*

$$\rightharpoonup b \rightharpoonup c \rightharpoonup a_3 \rightharpoonup O_2,$$

the letters representing single molecules. Recent observations point to more complicated couplings[1-4] and an organization in multimolecular entities or groups, which interact only at special points or surfaces[5,6]. The fact that a specific time order of reduction of the steady states is observed in two very different materials, wheat roots and baker's yeast[6], indicates not only the existence of a common scheme of spatial organization in the respiratory system but also a uniform access to oxygen in the state of normal aeration.

Observations on baker's yeast[5] have shown that the steady states of the cytochromes a_3 and c vary approximately synchronously and the same is true for cytochrome b and flavoprotein (FP). The latter begin to be reduced only after the cyto-

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