CHEMICAL INVESTIGATIONS OF THE GIANT NERVE FIBERS OF THE SQUID

I. FRACTIONATION OF DIALYZABLE CONSTITUENTS OF AXOPLASM AND QUANTITATIVE DETERMINATION OF THE FREE AMINO ACIDS

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SUMMARY

By means of preparative paper electrophoresis, the dialyzable portion of the axoplasm of the squid (*Loligo pealii* and *Dosidicus gigas*) have been separated into eleven fractions at pH 3.9. The fraction that behaves neutral under these conditions has been further fractionated by electrophoresis at pH 2.4. The resulting fractions were investigated with respect to their content of free amino acids which were determined quantitatively.

Arginine, lysine, ornithine were demonstrated, together with a number of neutral amino acids which thus far have not been described in this material. Striking is the fact that the neutral amino acids are present in the axon of the squid *Dosidicus gigas* in an order of magnitude lower than that of *Loligo pealii*; its content of taurine is also demonstrably lower.

In the fraction that behaves neutral at pH 2.4, peptides of medium chain lengths and of similar amino acid constitution were demonstrated. As yet, relatively little is known about the nature of the individual peptides although it is clear that their presence is not caused by a post-mortem splitting off from proteins.

The "neutral" fraction carries with it also a component which, after hydrolysis, yields cysteic acid (but no cysteine or cystine). The substance probably exists in axoplasm as the amide. Its possible significance as an ionogenic substance is pointed out.

Of three other components that have been detected for the first time, and whose total concentration is very substantial, one belongs in the class of the quaternary ammonium compounds but little can be said as yet about the nature of the two other components.

INTRODUCTION

The giant nerve fiber of the squid is very favorable for the investigation of the chemical constituents of axoplasm and the satellite cell sheath. Because of its large diameter (0.5 to 1.0 mm), it is possible, by extrusion, to obtain axoplasm uncontaminated by sheath or extra-axonic material. Early investigations[1-4] were concerned primarily with the determination of the inorganic constituents, particularly Na and K, which

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are intimately concerned with the bioelectric properties of the fiber. These analyses confirmed a supposition arrived at by indirect evidence on vertebrate and invertebrate nerves much earlier, namely that a large fraction of the anions of axoplasm must be organic. A surprisingly high concentration of free amino acids was found[5–7] in squid axoplasm and in lobster nerves but, since only a fraction of these was dicarboxylic, they accounted for only a relatively small part of the anion deficit.

With the purpose of determining the nature of the remaining anions and, if possible, of establishing the acid–base balance in squid axoplasm, KOECHLIN[8,9] isolated and identified the substance responsible for the largest fraction of the organic anions. This proved to be a derivative of taurine not previously described in biological material, namely 2-hydroxyethane sulfonic acid, or isethionic acid. KOECHLIN also obtained evidence for the existence of another organic acid, possibly a polycarboxylic acid related to the Krebs cycle acids, having a molecular weight in the range of 150–200 and which he designated "axonic acid". From KOECHLIN's and other previous work, the acid–base balance in squid axoplasm was established to the extent that a deficit of anions of only about 7 % (on the wet weight basis) and of cations of about 16 % still remained to be explained. For a review of the situation up to 1957, see the paper of SCHMITT AND GESCHWIND[10].

The present series of investigations, begun in the Spring of 1957, had as their goal not only the identification of the remaining substances which contribute materially to the acid–base balance, but also the initiation of a systematic investigation of as many as possible of the organic constituents of axoplasm, non-electrolytes as well as electrolytes.

Although this analytical program is a long range one and is meant to be systematic and exhaustive in its execution, nevertheless as substances are identified which may be possible candidates for crucial roles in physiological function of nerve, they may be made the subject of special investigations. Of particular significance in this connection will be substances which may be ionogenic and which may react specifically with basic ions, particularly sodium and potassium. Thus, as a result of the analysis so far, several substances have been singled out for special investigation.

In the initial experiments, the axoplasm was obtained from the New England squid, *Loligo pealii* or *Loligo omistraphes*. Later on, axoplasm from the large squid *Dosidicus gigas*, which abound in the Humboldt current off the coast of Chile, became available. This was valuable not only because of the large quantities of material that could be obtained, but because it permitted comparative studies of the axoplasm of the two types of squid.

In the present communication, we shall describe the general methods of preparation and analysis employed in the systematic investigation of axoplasmic constituents. We shall also give definitive data concerning the content of free amino acids in the two types of squid axoplasm. In subsequent communications will be given the results of the analysis of other types of constituents.

EXPERIMENTAL

A. Materials

1. *Preparation of axoplasm*

The transport of live squid to the laboratory in Cambridge, the dissection of the

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fibers, and the extrusion of axoplasm followed closely that described by MAXFIELD[11] and KOECHLIN[9]. However, care was taken not to wash the isolated nerve fibers with salt-free water. The freshly extruded axoplasm, without dilution, was chilled to dry ice temperature immediately after removal from the sheath. In this way, a stockpile of material was obtained during the summer of 1956 and was preserved at —20° C. The two nerves of each individual 25 cm squid furnished an average of about 1 mg of axoplasm on the ash free basis (according to the data of KOECHLIN). The large South American squid, *Dosidicus gigas*, with a mantle length of about a meter, contains at least 35 times as much axoplasm (according to the estimates of LUXORO) as does *Loligo*; but, with rapid, routine methods of dissection, the average figure is probably nearer to 25 times the amount obtained by routine methods from smaller squid. The large squid were obtained at the Chilean Marine Biological Station at Montemar (a suburb of Valparaiso, Chile, about 60 miles north of Santiago) by technicians specially trained for this work.

The dissected and ligated giant nerve fibers were first placed in chilled, filtered sea water. After removal of most of the surrounding connective tissue, the axoplasm was extruded. Axoplasm was always kept close to its freezing point. To prevent bacterial contamination, a droplet of toluol was added to the suspension of axoplasm in water before lyophilization. The material so prepared was transported to the Cambridge laboratories from the Chilean Unit in glass stoppered bottles. The starting material for the present investigations was the dialyzable portion of this material, containing the relatively low molecular weight constituents. Analytical results obtained from a number of different samples of dialyzed axoplasm led to reproducible results.

2. *Separation of the relatively low molecular weight material by dialysis*

Quadruple change of the dialyzing fluid with a relationship of 50:1 between the dialyzing water and the suspension of axoplasm led, under most favorable circumstances, within 48 hours to complete separation of the low molecular weight material. It is necessary, also, to add a droplet of toluol to the dialyzing fluid kept at a temperature of 4° C or less. The concentration of the dialysate is increased to any desirable extent and transferred quantitatively to an appropriate measuring cylinder. The solid content was determined on an aliquot of the solution placed in a desiccator under P_2O_5 until achieving constant weight. The concentrated solution of the dialysate was frozen at —20° C, the high molecular weight lyophilized portion was also weighed out and kept in a desiccator. In *Loligo* axoplasm the dialyzable low molecular weight fraction equals 71.2 % of the total axoplasm; the equivalent figure for the large South American squid, *Dosidicus*, is 68.5 %. In the following text the abbreviation DP_{Lp} will represent the dialyzable portion of *Loligo pealii* axoplasm; DP_{Dg} will represent the dialyzable portion of the *Dosidicus gigas* axoplasm.

3. *Preparation of sheaths and entire nerve fibers*

For experiments on the entire nerve fibers and on the squid sheath preparations, the fibers were freed as much as possible of surrounding connective tissue and smaller nerve fibers. They were then washed in filtered sea water and, for removal of adhering salt, very briefly washed three times in distilled water, after which they were blotted on a hard filter paper. The nylon thread with which the ends were ligated after

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dissection, served to hang the fiber individually to a glass rod while the other nylon thread held the fiber extended during the period of drying. After the fibers were thoroughly dried and after cutting off the threads, approximately 6 cm of fiber remained, on the average. The individual predried fibers were then placed in a desiccator under P_2O_5. To determine the weight of the sheath and of the entire nerve, the average of 50 fibers was taken. The proportion of the dry weight of the axoplasm was then obtained by difference.

B. Methods

For the analysis of amino acids, the method of GRASSMANN, HANNIG AND PLÖCKL[12] was employed. This consists of a combination of continuous preparative paper electrophoresis[13] and paper chromatography. These methods of separation supplement each other in ideal fashion.

After electrophoresis at pH 3.9, one obtains a separation into groups (acid, neutral, and basic) and this subdivision provides important preliminary information concerning the composition of the material to be analyzed. The use of preparative electrophoresis as an effective procedure for preliminary separation is satisfactory guarantee against the uncertainty as to whether *all* components in this complex mixture have been quantitatively separated, a question which, in our case, could not be answered with certainty by means of ion exchange chromatography. Also, the high ratio of the substances passed through per unit of elution volume, together with the simplicity of the procedure, argues strongly for the use of electrophoresis. By the use of volatile buffers, there is achieved a desalting of the material from the most rapidly migrating strong inorganic anions and cations which are separated off at the sides of the electrode chambers and are carried away in the electrode fluid. This circumstance is very important for the transition to paper chromatography, which is used in the further separation of material. Since the inorganic cations, which play a crucial role in nerve axoplasm, can be determined with precision by flame spectrometry, their removal in the electrophoresis apparatus as mentioned above is no handicap. However, one can obtain these ions also electrophoretically and quantitatively if one brings the substance for analysis close to the anode. This holds also for the strongly deflected anions. The fractions we have obtained in this way at pH values of 3.9 and 2.4 are still complex, and we have proceeded, with paper chromatography, to make further separation. For the discovery and characterization of the components in the various fractions, staining methods, and appearance in ultraviolet light were used.

C. Evaluation of methods

1. *Electrophoretic separation of groups of substances*

The dialyzable portion, containing the relatively low molecular weight materials, was obtained from axoplasm which had been kept in frozen condition for five to eight months in 1956 and also from axoplasm freshly obtained in 1957. From the former material there were two determinations of the free amino acids. In the later material, only one determination was made. For the investigation of the dialyzable portion of *Dosidicus gigas* axoplasm, several samples from Chile were available; of these we selected two sent in 1957 and one in 1958. Since the systematic separation of fractions

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from the dialyzable portions is favorable for the separation of amino acids, it was possible to detect and characterize substances even though they may be present in very small concentrations. This is the case, for example, with most of the free neutral amino acids in the axoplasm of *Dosidicus gigas*. For carrying out exact amino acid analysis with at least two parallel determinations, it is necessary after the preliminary separation of substances by electrophoresis, that each individual substance be present in concentrations of between 0.05 and 0.2 μmoles to permit the subsequent carrying out of the colorimetric determination on the paper strips made transparent by the method of GRASSMANN AND HANNIG[14]. If one exceeds this concentration range, one reduces the precision of separation; if the concentration is significantly below this range, the relative error of the determination becomes large. For these reasons the electrophoretically separated fractions which contain the free amino acids were taken from experiments in which at least 100 mg of the dialyzable portion of *Loligo pealii* or 400 mg of dialyzable portion of *Dosidicus gigas* on the average were used.

To achieve an optimum in the sharpness of separation for all of the substances in the dialyzable fraction, about 1 mg of total amino acids per hour were added to the paper curtain which had been kept under the conditions of the experiment for six hours prior to the run to wash soluble substances from the paper. A concentration of 2–4 % of the dissolved substance in the appropriate buffer was chosen for analysis. For the preliminary separation we found it appropriate to make use of the pyridine acetate buffer[15], pH 3.9, with a driving voltage of from 280 to 300 volts and a current of 12–15 mA. The temperature was maintained between 13 and 15° C. After completion of the inflow of sample, the current flow was maintained for at least 15 hours to assure a quantitative transfer of the fractions into the collecting tubes. The testing of the amino acids was carried out by depositing a 0.01 ml droplet on chromatogram strips which were numbered according to the test tube row; these were then developed with ninhydrin. Four cleanly separated fractions thus became visible: those containing aspartic acid, glutamic acid, the neutral fraction, and the basic amino acid fraction. The contents of each tube belonging to a particular group was then quantitatively collected and the eluate in each tube immediately to the left and to the right of the fraction was added thereto in order to assure completeness of collection. The volume of fractions so united were then reduced in volume by microdistillation and were quantitatively transferred into a small heart-shaped vessel whose content, after pre-cooling, was placed in a desiccator over P_2O_5 and KOH until a constant weight was achieved. The remaining ninhydrin-negative tubes were also brought together according to the experience which was obtained in the preliminary investigation. Since the relatively very large quantities of taurine, as well as the high concentrations of glycine, and alanine interfere with the further carrying out of the amino acid analysis, the fraction called "neutral" obtained by electrophoresis at pH 3.9 was subjected to a further electrophoresis at pH 2.4. Under these conditions the aliphatic monoamino monocarboxylic amino acids move with characteristic velocity to the cathode, while the migration of the aromatic amino acids still permits the separation from taurine which, even under these conditions, reacts neutral. As an electrolyte for these separations, 0.5 *M* acetic acid was used, with a voltage of 600 to 700 V and a current of 10 mA. The working up of the eluate followed essentially as described above. This separation at pH 2.4 was carried out with larger amounts of the "neutral" fraction for reasons that will become clear below.

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In the further course of the analysis, we next determined the *absolute* amounts of the amino acids in the four fractions obtained from the separation at pH 3.9 and in the three primary fractions (containing taurine, alanine, and glycine) from the separation at pH 2.4. For this we made use of the ninhydrin reaction in solution according to the method of MOORE AND STEIN[16]. The results were calculated by the simple relationship of the extinction values with the known values of the reference mixture or reference substance. The extinction was measured at 578 mμ with the Beckman model DU spectrophotometer. Proline and hydroxyproline can not be determined at this wave length.

2. *Paper chromatographic separation and staining*

As the solvent system for the neutral amino acids, we used: (1) *n*-butanol–acetic acid–water (4:1:5); (2) phenol–*o*-cresol (1:1), water-saturated; (3) α-picoline–water (6:4). The basic amino acids were separated with solvent mixture (2) in KCN and in ammonia atmospheres. For the two-dimensional chromatography, solvent systems (1) and (2) and (1) and (3) were used. The chromatography was carried out using only the ascending method with Whatman No. 1 paper. All solvent mixtures were used in a state of purity required for chromatographic purposes. The phenol and *o*-cresol were purified by the method of DRAPER AND POLLARD[17]. Where one-dimensional paper chromatography was applied, aliquot parts of the fractions obtained by electrophoretic separation were brought upon the paper strips as 3 cm-long streaks in a concentration between 0.05 and 0.1 μmoles. During the run the temperature was kept at 25° $\pm$ 1°. The drying, spraying and developing of the chromatograms was carried out according to the directions of GRASSMANN *et al.*[12].

3. *Photometric evaluation of chromatograms and calculation of results*

Because of the instability of the ninhydrin color complex, the evaluation has to be made in a matter of a few hours. For this purpose the cut strips of paper, made transparent with oil[14] in a vacuum, were photometered with an "Elphor-Integraph"[18], at a wave length of 546 mμ. Proline was determined with the isatin reagent according to GRASSMANN AND VON ARNIM[19] as modified according to ACHER AND FROMAGEOT[20].

The results were expressed in μmoles and are summarized as a simple relationship of the average extinction values of the analyzed substances to the average extinction values of the known reference substances. The sum of the μmoles obtained with the paper chromatographic colorimetric determination should be in good agreement with the values of the groups determined in solution. For details, the reader is referred to the original literature[12].

RESULTS

Fig. 1 illustrates the electrophoretic separation of the dialyzable fraction at pH 3.9. The pictures of the separation for *Loligo* and for *Dosidicus* are identical. The method by which the 11 fractions were made visible was as follows: the pherogram which was dried in a stream of air at 80° appears blue-violet in ultraviolet light as a result of the residue of traces of pyridine. After spraying with a solution of umbelliferon[21] (7-hydroxycumarine), the dark violet bands of fractions 1 to 7 are made visible on a bright blue background. Of these fractions, numbers 1 and 2 contain inorganic anions;

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3, isethionic acid; 4, phosphate; 5, aspartic acid; 6, glutamic acid; 7, the neutral amino acids, taurine, homarine, and other substances, which will be discussed below. Fractions 8–11 appeared as white stripes with increasing brilliance in the order of the numbers. Fraction 8 represents the basic amino acids. In fraction 9 are found calcium and magnesium. Fractions 10 and 11 contain probably only the inorganic cations sodium and potassium. The ninhydrin-negative fractions 1, 2, 3, 4, 9, 10, and 11 were covered over with filter paper and the free area sprayed with ninhydrin to bring out separation of groups of amino acids. The inorganic cations are made visible by spraying with violuric acid[22] (nitrobarbituric acid), by which they are made to appear violet. Fraction 4, which contains a considerable amount of phosphate, is made visible by spraying with ammonium molybdate[23]. Since, after brief drying in air, a yellow coloration of this fraction occurs, it is possible to deduce the presence of free phosphate. This can be confirmed by paper chromatography. The phosphoric acid esters occur only after warming in a water atmosphere after seven minutes. Reduction with tin-chloride-containing HCl permits all of the phosphate to appear as a deep blue color (molybdenum blue).

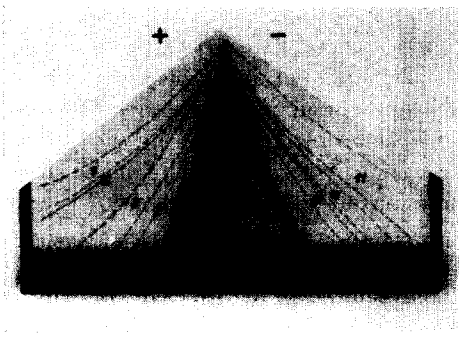

Fig. 1. Electrophoretic separation of dialyzable portion at pH 3.9.

Fig. 2 shows the paper chromatographic separation of the neutral fraction of *Loligo pealii* dialysate after electrophoresis at pH 2.4. In this picture the neutral migrating fraction (I) which contains taurine (but no other amino acid) is not shown. For simplicity all fractions (II–VI), together with the reference mixtures, are placed on one paper sheet. The separation occurs well in *n*-butanol–acetic acid and there seems to be no uncertainty. Two-dimensional paper chromatograms of the neutral fraction (obtained by electrophoresis at pH 3.9) which were carried out in various solvent systems, have reproducibly demonstrated the occurrence of spots which could not be ascribed to known amino acids. Because these substances are not to be seen in fraction I, they *must* be present in fractions II–VI. In the one-dimensional rechromatography in phenol–*o*-cresol or α-picoline–water, the agreement with the comparison substances in fractions II and III is not fulfilled in all details. But only in two-dimensional paper chromatography (Figs. 3 and 4) is shown clearly the presence of four further substances, of which one is known to be methionine. N_3 does not represent a contamination of the neutral fraction with glutamic acid because, in the electrophoresis after elution from the chromatogram, the substance reacts neutral. In Fraction II the intensity of the spot with the lowest R_F value may be almost

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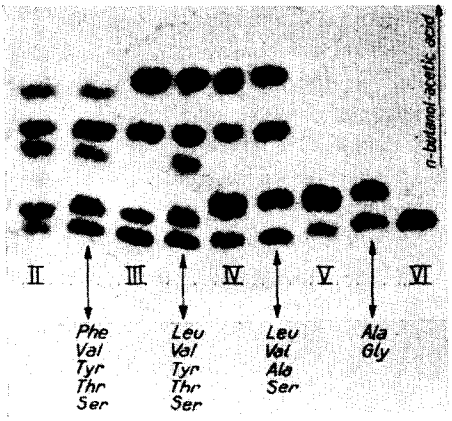

Fig. 2. Paper chromatographic separation of neutral amino acid fractions and of reference amino acid mixtures.

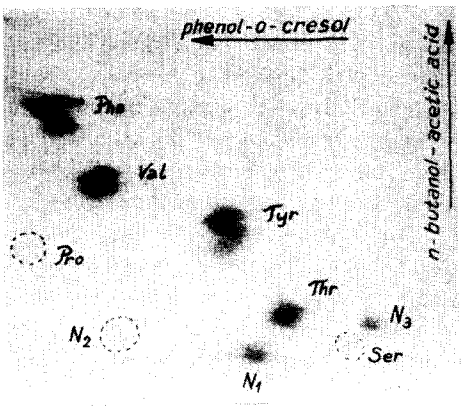

Fig. 3. Two-dimensional chromatography of fraction II.

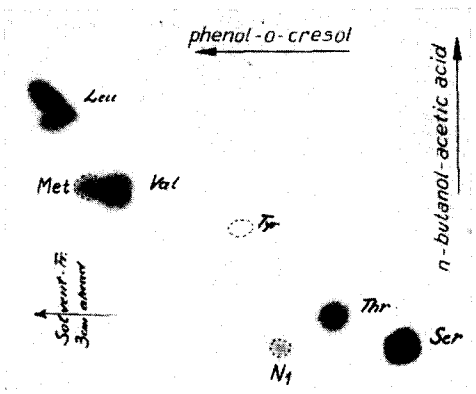

Fig. 4. Two-dimensional chromatography of fraction III.

completely ascribed to N_1 (see Fig. 3), while in fraction III this substance gives rise only to a small part of the intensity of the serine spot. N_3 is to be found only in fraction II and must there be distinguished from threonine. Finally in the colorimetry of Fraction II, N_2 (which is present only in small amount) increases the extinction values of N_1 and of threonine only a little. In order to obtain the true values for serine and threonine, one proceeds best, after staining the two-dimensional chromatograms of fraction II and III, by visual estimation of the intensity of the unknown spots. The values so calculated are deducted from those values obtained by the colorimetric procedure of the one-dimensional chromatograms.

In the dialyzable fraction of *Dosidicus* axoplasm, we have thus far not found N_1, N_2 or N_3, perhaps because they are present in too low a concentration. The further investigation of these substances, which are present only in very low concentrations and which might well be represented by peptides, we are unable to follow at the present time because of lack of sufficient material.

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Table I gives the results of the quantitative estimation of the free amino acids, including taurine. The values, which are calculated on the dry weight basis of the dialyzable material, can be converted into total axoplasm values, *i.e.* containing also the non-dialyzable portion, by multiplying by a factor of 0.712 (for *Loligo*) and by 0.685 (for *Dosidicus*). If the values in Table I are divided by 9.6 or 9.2, respectively, one obtains the concentration of substances in total axoplasm calculated on the wet weight basis; these stand in relation to the dry weight as 100:13.5

Table II gives the average value of nitrogen determinations in *Loligo* and *Dosidicus*. The portion represented by the sheath (about 140 gamma) represents about 1/7 of the weight of the whole nerve fiber (about 1 mg) in the case of *Loligo*, assuming an average length of 6 cm. These are the average values, determined by weighing 50 completely dried individual sheaths and fibers. Since we know the relation of the

TABLE I

CONCENTRATION OF FREE AMINO ACIDS IN THE DIALYZABLE PART OF SQUID AXOPLASM

Amino acid	*Loligo pealii*			*Dosidicus gigas*		
	N in percent of total N	*μmole/100 mg*	*Weight percent*	*N in percent of total N*	*μmole/100 mg*	*Weight percent*
Taurine, peptides and cysteic acid derivative	30.59	103.00	12.88	9.80	31.80	3.98
Aspartic acid	21.65	73.00	9.72	22.87	74.80	9.96
Glutamic acid	5.73	19.60	2.88	8.06	26.20	3.85
Glycine	3.18	10.70	0.80	3.27	10.40	0.78
Alanine	2.55	7.80	0.79	2.83	9.30	0.83
Serine	1.06	3.70	0.39	0.32	1.00	0.11
Leucine and isoleucine	0.85	2.70	0.35	0.06	0.20	0.026
Valine	0.64	2.22	0.26	0.15	0.50	0.059
Threonine	0.64	1.90	0.23	0.09	0.30	0.036
Proline	0.42	1.00	0.12	0.04	0.10	0.011
Tyrosine	0.21	0.70	0.13	0.08	0.30	0.054
Phenylalanine	0.19	0.60	0.10	0.04	0.15	0.025
Methionine	0.13	0.40	0.06	0.04	0.15	0.022
Arginine	4.24	3.20	0.56	5.88	4.10	0.71
Lysine	1.48	2.40	0.35	0.13	0.20	0.03
Ornithine	1.06	1.80	0.24	0.17	0.30	0.04
Total	74.62	234.70	29.86	53.83	159.80	20.52

TABLE II

NITROGEN CONTENT IN PERCENTAGE OF THE DRY WEIGHT

Loligo pealii					*Dosidicus gigas*				
Sheath	*Whole fiber*	*Dialyzable part*	*Non-dialyzable part*	*Axoplasm*	*Sheath*	*Whole fiber*	*Dialyzable part*	*Non-dialyzable part*	*Axoplasm*
11.34	7.99 7.71*	4.71	13.53	7.11	—	—	4.59	13.46	7.25 7.38*

* Calculated values assuming that the sheath is 1/7 of the weight of the whole nerve fiber, the dialyzable part of *Loligo* = 71.2%, the dialyzable part of *Dosidicus* = 68.5% of the axoplasm.

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weights of the entire fiber, axoplasm and sheath, and also the relation between the dialyzable and non-dialyzable portions of axoplasm, we can readily check our N-determinations. These gave very satisfactory agreement.

In Table I the values for taurine, the peptides and the cysteic acid derivative are given. These peptides, whose total concentration lies between 0.1 and 0.2 % of the dry weight of the axoplasm, are found in the "neutral" fraction which one obtains by electrophoresis at pH 2.4. Therefore, these substances should contain no free carboxyl group. That these substances did not arise by postmortem enzymic alteration of the non-dialyzable fraction, was shown by boiling some of the freshly extruded axoplasm and comparing the results with those obtained from untreated axoplasm. In the chromatography of this "neutral" fraction, in the solvent system *n*-butanol–acetic acid/phenol–*o*-cresol, one obtains a separation into two spots as is shown in Fig. 5.

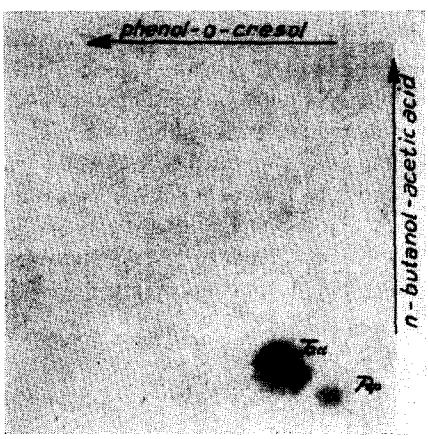

Fig. 5. Two-dimensional chromatography of the "neutral" fraction (pH 2.4).

After elution of the "taurine area" and the "peptide area", hydrolysis in hydrochloric acid in a nitrogen atmosphere is carried out. It can be shown that, after electrophoretic and paper chromatographic separation, underneath the intense spot of taurine still one peptide must be present. The chromatography of the HCl hydrolysis products of the peptide area yields, in addition to a series of amino acids which are not fully understood in all their details, and a still unidentified spot, cysteic acid, in a quantity of about 40 % of the total hydrolysate. No cysteine or cystine can be detected. By means of paper chromatography, using the solvent α-picoline–water, one can separate, though incompletely, the eluate from the peptide area into three or four components. The substance with the highest R_F value, after elution and hydrolysis, yields chiefly cysteic acid. One can conclude, as a result of investigations made especially upon the point, that the original substance exists as the amide of the cysteic acid. The paper chromatogram which one obtains from the breakdown products of other components are perhaps similar but by no means identical. The number of components varies between 6 and 10, and the chain lengths of the peptides may very well be longer. Since the paper chromatographic separation and the elution of the components of these peptide mixtures are not in themselves very illuminating, and

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the purity of the substances is questionable when one remembers the large excess of taurine, it seems desirable to be cautious for the present concerning the nature of the materials. With improved methods we shall continue these investigations as soon as a sufficient quantity of the neutral fraction has been obtained by electrophoresis of much larger quantities of axoplasm that are needed for the purpose.

Also in other connections, the fraction that migrates neutral at pH 2.4 is of interest. Aside from homarine[24], which is found in a concentration of 17.1 mole % in the dialyzable part of *Loligo* and 18.6 mole % in that of *Dosidicus*, we could detect a further substance by paper chromatography. This substance, which we shall give the tentative designation X_3, has an R_F value (in *n*-butanol–acetic acid) of 0.46 and shows no u.v. absorption. Its demonstration has thus far depended on spraying with weakly alkaline indicator solution, by which means the contrast between the spot and the background of the paper is intensified. This staining behavior of the substance could be explained by its "buffering" against the contribution of the acetic acid to the pH of the paper. After deduction of the taurine and the strictly neutral substances discussed above from the weight of the whole fraction, there remains a significant amount of material. Therefore, X_3 must be present in considerable amount.

Two other unknown ninhydrin negative substances, X_1 and X_2, can be separated by electrophoresis at pH 2.4 from the neutral migrating fraction at pH 3.9. These substances are weakly deflected to the cathode. X_2 shows u.v. absorption: its maximum lies at about 250 mμ. X_1 shows the staining reaction of a quaternary ammonium compound[25] and possesses a carboxyl group. The R_F value of X_1 (in *n*-butanol acetic acid) is 0.3, that of X_2 is 0.5. Their total concentration seems larger in *Dosidicus* than in *Loligo* dialyzable part.

DISCUSSION

A comparison of the free amino acids in the axoplasm of *Loligo* and of *Dosidicus*, particularly in the case of some of the neutral amino acids, shows a difference in concentration which may be an order of magnitude in amount. Striking also is the relatively small amount of taurine in the axon of *Dosidicus gigas*. On the other hand, the acid amino acids in *Dosidicus gigas* are more prominent, while the distribution of the basic amino acids in the axon of both animals is essentially the same and, compared with the organic anions, is extremely small.

The fact that axoplasm contains an extraordinarily high concentration of the two sulfonic acids, isethionic acid and taurine, but not a trace of cysteine or cystine is surprising and possibly significant. In detailed studies CHAPEVILLE AND FROMAGEOT[27] have demonstrated the formation of taurine in chicken embryos. According to them, the biosynthesis of taurine proceeds in three stages: (1) reduction of sulfate to sulfite; (2) coupling of sulfite with desulphydrylated cysteine leading to the formation of cysteic acid; (3) decarboxylation of cysteic acid to form taurine. Such a synthesis of taurine would not be expected to occur in the axon, which contains no cysteine in the dialyzable or non-dialyzable portion, before or after hydrolysis (though small quantities of cystine are found). However, the possibility remains that taurine is synthesized in the nerve cell body and then diffuses down the axon. Cysteine is not the only source of the synthesis. ELDJARN[28] and CAVALLINI[29] have shown that cystamine (the terminal component of coenzyme A) can be converted into cysteic acid. The possibility has

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been suggested[30,31] that the synthesis may also pursue an alternative pathway. It is improbable that the synthesis is extracellularly with subsequent diffusion into the cell across the plasma membrane.

The transport of sulfite on α-amino acrylic acid, suggested by CHAPEVILLE AND FROMAGEOT, which might also occur by dehydration from serine, seems rather attractive in the case of axoplasm because serine, along with alanine (and glycine), occur the only free neutral amino acid in substantial concentration in *Dosidicus* axoplasm. However, the authors have adduced experimental evidence that serine, alanine, and pyruvic acid inhibit the formation of cysteic acid and only cysteine provides the necessary condition for the reaction.

Noteworthy is the appearance of ornithine, which was demonstrated by the highly specific method of CHINARD's[26] color reaction. Citrulline, however, could not be demonstrated.

When one considers the substantial cation deficit, which was left unexplained according to the acid–base balance of KOECHLIN, together with our findings that the nitrogen content of the dialyzable portion of *Loligo* corresponds to three quarters and that in *Dosidicus* to only half the *total* nitrogen content, one suspects that this deficit must be made up by an organic base whose nature is at present completely unknown. We have sought diligently to discover such a cation. Although our experiments in this direction are not completed, we believe that we are justified in saying that such a substance, which to explain the acid–base balance of KOECHLIN would have to be present in a concentration roughly equal to that of aspartic acid, could not have been overlooked by us. We are inclined to assume that the X_1 and X_2 components may very well account for the nitrogen deficit. Further investigations of this matter are currently underway.

Because of the possible physiological significance of other substances in the "neutral" fraction, particularly the cysteic acid derivative and the peptides, special attention is now being devoted to these substances.

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NOTE ADDED IN PROOF

Since submission of this manuscript, the compounds designated as X_1 and X_3 have been identified as glycocol betaine and glycerol, respectively. Both are present in the free form and in substantial amounts in axoplasm. Free inositol is also present. The details of the identifications will be published in due course.

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