CHEMICAL INVESTIGATIONS OF THE GIANT NERVE FIBERS OF THE SQUID

III. IDENTIFICATION AND QUANTITATIVE ESTIMATION OF FREE ORGANIC NINHYDRIN-NEGATIVE CONSTITUENTS

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SUMMARY

In the dialyzable portion of axoplasm of *Loligo pealii* and *Dosidicus gigas* were identified and quantitatively estimated: glycocoll-betaine, glycerol and myo-inositol in the free state. The total of these three components, described for the first time in the axoplasm of both species of squid, amounts to approximately 18 % and 23 % respectively, on the dry weight basis of the material investigated.

Hypoxanthine and carbohydrates, are present in small quantities. It is surprising to find sucrose among the carbohydrates.

INTRODUCTION

In a program having as its aim an exhaustive chemical investigation of the constituents of the axoplasm of the giant nerve fiber of the squid we have thus far reported on the free amino acids[1] and on a substance previously not described in biological material, cysteic acid amide (β-sulfoalaninamide)[2,50]. The latter substance represents a third sulfonate found in relatively high concentrations in the axoplasm of *Loligo pealii* but in rather small amounts in *Dosidicus gigas* axoplasm, the other two being isethionic acid (2-hydroxyethane sulfonic acid) and taurine. Despite the presence of these sulfonates and of high concentrations of free amino acids, cysteine and cystine appear to be absent in the free state. Ornithine is present instead of histidine.

From the analytical results reported on the dialyzable constituents of squid axoplasm it appears that, in addition to certain constituents such as peptides (present in very low concentrations) ninhydrin-positive constituents represent about 30 % in *Loligo* and 25 % in *Dosidicus*. From the data of others[3-5], inorganic ions represent 22 % in *Loligo*. This leaves a deficit, made up of ninhydrin-negative substances, of 48 % in *Loligo* and probably somewhat higher in *Dosidicus* (51 %, assuming the same concentration of inorganic ions as in *Loligo*).

In the analytical investigation to be described in this paper the following substances have been identified for the first time in squid axoplasm, and some of them

have been shown to be present in substantial concentrations: glycocoll-betaine, glycerol, myo-inositol, muscle adenylic acid (5'-AMP), homarine, hypoxanthine, sucrose, glucose and fructose*. These substances add up to 22 % of the dialyzable constituents of the axoplasm of *Loligo* and 26 % of that of *Dosidicus*. This reduces the unidentified material to less than 10 % as will be shown in the balance sheet given in the following paper[6].

All results pertain to lyophilized axoplasm which had been dialyzed against distilled water in the cold as previously described[1].

EXPERIMENTAL

Material and methods

The axoplasm used in these analyses was part of the same lyophilized and dialyzed material used for the estimation of free amino acids[1].

Separation into 11 fractions was accomplished by means of preparative paper electrophoresis at pH 3.9, using an acetic acid–pyridine buffer[7]. The resulting neutral fraction ($N.F._{3.9}$) was fractionated at pH 2.4 with 0.5 N acetic acid as electrolyte, yielding 5 fractions, one neutral ($N.F._{2.4}$), and four which were deflected with different mobilities toward the cathode. The methods of fractionation, visual demonstration and partial identification of the constituents were given previously[1]. The next step in the electrophoretic fractionation of $N.F._{2.4}$ at pH 10.2 (using a tri-ethylamine–acetic acid buffer), mainly into a neutral migrating fraction ($N.F._{10.2}$) and a fraction strongly deflected toward the anode, is described below.

RESULTS

With the exception of muscle adenylic acid, all the components herein described are contained in $N.F._{3.9}$. By electrophoretic separation of this fraction at pH 2.4 betaine and hypoxanthine were isolated. Further electrophoretic separation of $N.F._{2.4}$ at pH 10.2 freed this fraction of taurine and cysteic acid amide, which at this pH are deflected toward the anode, leaving the neutral constituents glycerol, myo-inositol, homarine and carbohydrates. Muscle adenylic acid is found in the region of glutamic acid after the electrophoresis at pH 3.9.

Diagram I shows the steps of electrophoretic separation; the substances in brackets are not considered in this article.

Table I represents the proportion by weight of the neutral fractions obtained by electrophoresis at pH 3.9, 2.4 and 10.2, respectively, based on dry weight.

The electrophoretic behavior of the components at different pH values together with their weights indicated some chemical characteristics of these substances and also reflected the degree of purity of the fractions. The use of easily volatilizable buffers for the electrophoresis is a prerequisite for a weight determination of such

* The dialyzable organic constituents described thus far by us in squid axoplasm are, in the main, not unique for nerve but occur in varying concentrations also in squid blood. This was discovered by one of us (G.G.J.D.) soon after the initiation of a program of investigation of the constituents of squid blood. These constituents are present at a lower level of total concentration than in axoplasm. Among the free organic substances present in axoplasm but not yet demonstrated in squid blood are cysteic acid amide, isethionic acid and peptides[1,2]. The results of the detailed analyses will be communicated in due course. They are referred to here primarily because of their bearing on the possible physiological role of the dialyzable organic substances thus far demonstrated to occur in axoplasm.

DIAGRAM I

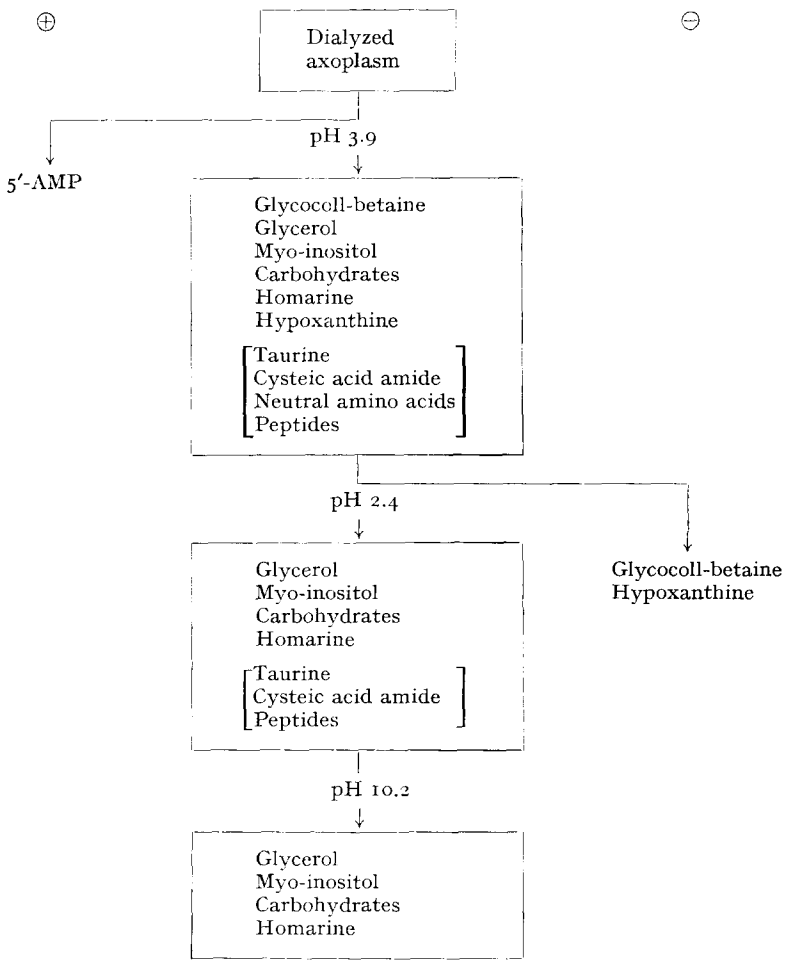


TABLE I

DISTRIBUTION BY WEIGHT OF THE ELECTROPHORETICALLY NEUTRAL FRACTIONS OF THE DIALYZED AXOPLASM

Fraction	% *dry weight*	
	Loligo	*Dosidicus*
N.F.$_{3.9}$	37.96	32.42
N.F.$_{2.4}$	25.34	14.82
N.F.$_{10.2}$	12.07	10.57

electrophoretically separated fractions. The final identification and quantitative estimation of their individual components was achieved by paper chromatography, specific color reactions on paper and in solution, observation of the behavior under u.v. light of the components being studied, and titration methods.

Identification and quantitative estimation

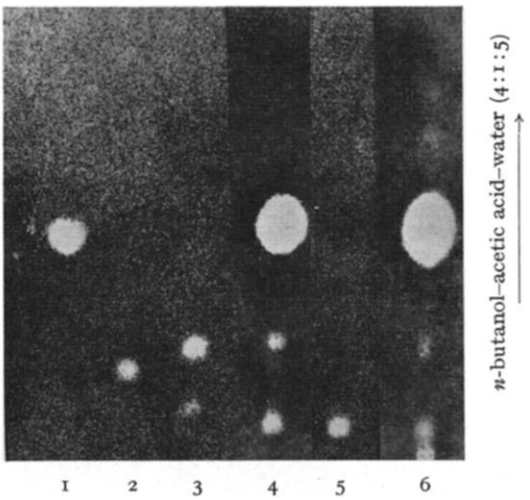
Based on earlier results, the portion of ninhydrin-positive substances in N.F.$_{3.9}$ is calculated as 16 % for *Loligo*, but only 6 % for *Dosidicus*. Comparison of these figures with the total weight of the corresponding neutral fractions shown in Table I emphasizes the quantitative significance of these fractions. The loss of weight which N.F.$_{3.9}$ undergoes after electrophoretic separation at pH 2.4 is substantially compensated by a single, moderately cathode-deflected fraction. The substance present in high concentration in this fraction, providing the color reaction of a quaternary ammonium compound, was previously demonstrated and given the tentative designation X_1. This compound has now been identified as glycocoll-betaine. A u.v.-absorbent substance showing approximately the same cathodal deflection, designated X_2 at the same time, has now been identified as hypoxanthine. A principal component of N.F.$_{2.4}$ designated as X_3 has been identified as glycerol.

The identification of the various constituents was accomplished in the following manner.

Glycerol, myo-inositol, carbohydrates and muscle adenylic acid: A striking physical characteristic of the three neutral fractions, N.F.$_{3.9}$, N.F.$_{2.4}$, and N.F.$_{10.2}$, in that order, is their gradual decrease in viscosity. Concerning the component previously designated as X_3, indications pointed to a polyvalent alcohol, more specifically glycerol, based on the facts that: it remained in a liquid state and behaved consistently neutral in electrophoresis under different pH conditions, was not susceptible to coloration on the paper chromatogram, and showed no u.v. absorption. Furthermore, the possible presence of a polyhydroxyderivative of cyclohexane, in all probability myo-inositol, had to be considered. The micro-reaction with vanadium oxinate for primary, secondary, and tertiary alcohols[8,9] was positive and showed the same shade of purple as that given by one of the reference substances, glycerol. The presence of a polyhydroxyderivative of cyclohexane was confirmed by means of an extremely sensitive color reaction specific to this class of compounds, a reaction based on the formation of red water-insoluble alkaline earth salts with certain keto acids[10]. In the case of inositol the particular keto acid is leuconic acid, which is the main oxidation product of this alcohol by nitric acid. A further proof of these substances was found in paper chromatography with corresponding reference substances. As solvents we used *n*-butanol–acetic acid–water (4:1:5) and phenol–*o*-cresol (1:1), water-saturated. At 25 ± 2° the R_F values are, in the former solvent, 0.46 for glycerol and 0.07 for myo-inositol; in phenol–*o*-cresol the values are 0.70 and 0.07, respectively. Since in nature there occur two further polyhydroxyderivatives of cyclohexane, mytilyte and scyllite, whose paper chromatographic behavior on a short run is similar to that of myo-inositol, several sheet lengths were run using water-saturated phenol as solvent. Here too, complete identity of the natural product with myo-inositol as the reference substance was found. For displaying the separated substances on the paper chromatogram the method of KARRER *et al.*[11] was used. The thoroughly dried chromatogram was sprayed with a 0.5 % solution of sodium periodate, allowed to react for 5 min at room temperature, then sprayed with a 0.5 % benzidine solution (0.5 g of benzidine in 20 ml acetic acid and 80 ml ethanol). White spots appear on a deep blue background indicating the location of the oxidized substances (Fig. 1).

The carbohydrates contained in the neutral fractions were also affected by this procedure (see Fig. 2). The presence of hexoses in the dialysed axoplasm of both

Loligo and *Dosidicus* was indicated by the pure green solution obtained with anthrone[12,13]. However, if the dialyzable portion of the axoplasm is permitted to react with orcine, an olive-green color is obtained. Hexoses which are ketoses react



1: Glycerol (Reference Subst.)
2: Glucose
3: Fructose
↕
Sucrose

4: L.M.P.$_{D\ g.}$, Neutral Fract. at pH 10.2
5: Inositol
6: L.M.P.$_{L.g.}$, Neutral Fract. at pH 10.2

Fig. 1. The appearance of glycerol, myo-inositol and part of the carbohydrates using the periodate method after paper chromatographic separation from the corresponding neutral fractions. *Loligo*, and sometimes *Dosidicus*, too, leave a stain at the starting point; this stain shows the typical color given by phosphate in reaction with molybdic acid after reduction, and its paper chromatographic behavior under the conditions mentioned also agree with a carbohydrate phosphate. Inferred is the presence of a loose addition complex in order to explain the neutral behavior of this substance during electrophoresis.

with orcine[14,15] to produce a reddish-brown color, whereas pentoses and pentose derivatives yield a deep green color (Bial's reaction). These reactions are not inhibited by the large excess of other components in axoplasm as shown by the admixture of small known quantities of the carbohydrates in question to the material. Since the reaction of a reference mixture of fructose and ribose with orcine also led to an olive-green solution (mixture of reddish-brown and green), the presence of at least one ketose and one pentose was certain. To test for free sugars, which at all pH values of the buffer system used behave electrically neutral, reactions were carried out with anthrone and orcine on fractions N.F.$_{3.9}$, N.F.$_{2.4}$, and N.F.$_{10.2}$ from both species. With anthrone, green coloring was obtained; with orcine, reddish-brown, which in the neutral fraction clearly indicates the presence of the aldohexose class and the ketohexose class.

The anionic fractions in the region of the glutamic acid ⟷ phosphate fraction were tested for the presence of carbohydrate phosphoric acid esters, using anthrone as reagent. These tests in general turned out negative, while the use of orcine in the region of the glutamic acid fraction produced intensive green coloring after only brief

boiling. With the electrophoretic deflection and the curious fact that muscle adenylic acid reacts faster and 1.3 times more strongly with orcine than does free ribose[15], the conclusion was that 5'-AMP must be involved, especially since its presence was indicated theoretically. Of the three reference substances, 5'-AMP, ADP, and ATP, only the first showed the same rate of deflection in electrophoresis as the substance being sought. In contradistinction to yeast adenylic acid, muscle adenylic acid is, by its constitution, capable of giving a positive periodate reaction. Therefore this reaction was carried out on the paper chromatogram, giving the expected positive results; 5'-AMP, ADP and ATP, and yeast adenylic acid were included in the same run as reference substances. U.V. absorption served to determine the location of the spots. The absorption maximum of the glutamic acid fraction, which carried the muscle adenylic acid, was at 260 mμ, which was in exact accordance with the absorption maximum of muscle adenylic acid, used as a reference substance.

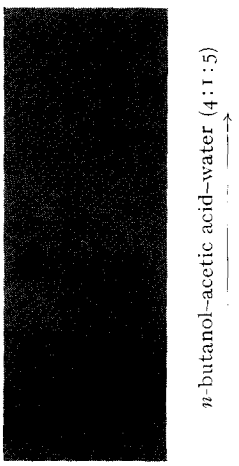


Fig. 2. The paper chromatographic identification of sucrose and fructose with anthrone as coloring reagent.

The identification of individual carbohydrates was achieved by comparing their R_F values with the corresponding reference substances on the paper chromatogram, using various solvent systems. Aliquots of N.F.$_{2.4}$ and N.F.$_{10.2}$ from both species were investigated in this manner, using as spray reagents aniline phthalate[16] for reducing carbohydrates and naphthoresorcine[17,18] for non-reducing carbohydrates. In regard to the colorings with ketoses, those made with anthrone were especially satisfactory[19]. Glucose and fructose were identified without difficulty, and the presence of sucrose was also quickly established (Fig. 2). The proof that it actually was sucrose was established by mild hydrolysis in hydrochloric acid (0.1 N HCl, 6 h, 100°); after this hydrolysis this substance could no longer be found; the color of the glucose and fructose spots became more intense, but no additional spot could be observed. The free glucose and fructose found are not products of the hydrolysis of sucrose; the

experimental conditions under which the entire separation and processing were carried out precluded such a possibility.

It is probable that considerable proportions of fructose and glucose occur in the form of their phosphoric acid esters in the axoplasm of the living animal. After the death of the animal there takes place a very rapid enzymic cleavage of the phosphoric acid esters. The weak and not always reproducible reactions with anthrone in the region of the glutamic acid—and aspartic acid—fractions, obtained from axoplasm material collected at different times, seem to indicate a scattered presence of such phosphate esters. However, the formation of loose addition complexes must be considered, such as those thought to occur in axoplasm. These present problems in the isolation of pure components.

For the quantitative estimation of glycerol and inositol the method of HIRST[20] was employed, in which the formic acid, obtained by oxidation of those substances with periodate, is determined by titration. First, aliquots of N.F.$_{2.4}$ and N.F.$_{10.2}$ of both species were used, then aliquots of glycerol and inositol which had been separated by paper chromatography using the solvent system *n*-butanol–ethanol–water (4:5:1) and then eluted with water. In the latter case a reference mixture of glycerol and myo-inositol was the basis for calculations; this mixture was separated by paper chromatography under identical conditions. Eluates of substance-free paper strips served as blanks. Since in the former procedure the carbohydrates are also involved, it became necessary to ascertain the accurate number of moles of the liberated formic acid of each participant in the reaction (see ref. 20).

It was necessary to know the individual amounts of carbohydrates, the sum total of which was determined with anthrone by the difference procedure (extinction of maximum absorption at 625 mμ minus extinction of minimum absorption at 700 mμ). To find the concentrations, reference was made to standard solutions of glucose. The individual values were calculated from the size of the spots and the color intensity of the paper-chromatically-separated substances by comparison with the reference substances in various concentrations, run concurrently on the chromatogram. The sum total of the concentrations obtained showed good agreement with the total concentration obtained with anthrone in solution.

Periodate oxidation in solution for determination of glycerol and inositol proceeded as follows: Aliquots of N.F.$_{2.4}$ and N.F.$_{10.2}$ containing 1 to 3 mg polyalcohols (plus carbohydrates), the respective eluates of glycerol and inositol from the paper chromatogram (the substances were applied as a linear streak 30 cm in length), were dissolved in 30 ml of water and treated with 1 ml 0.25 *M* sodium periodate solution. The mixture was boiled for 20 min in the waterbath, cooled, and 0.2 ml ethylene glycol added to destroy the excess periodate. A drop of methylene red was added and the formic acid which had formed was titrated with 0.01 *N* NaOH. As a basis for the quantitative calculation, the results from titrated standard solutions of the two polyalcohols and the carbohydrates respectively were used. Each experiment was accompanied by a blank. The total values obtained from N.F.$_{2.4}$ and N.F.$_{10.2}$ were in good agreement with one another and, after the deduction of the very small values calculated for the carbohydrates, in good agreement with the individual values obtained for glycerol and inositol by the eluating method. The concentrations found for glycerol, inositol and carbohydrates are summarized in Table II.

The quantitative estimation of the 5′-AMP was carried out in solution with orcine

on the total axoplasm dialysate as well as on the glutamic acid fraction. Standard solutions of 5′-AMP in various concentrations which were added to the D.P. showed that the reaction was not influenced by any of the components of the dialyzable portion. It was somewhat surprising that only traces could be found of 5′-AMP in the glutamic acid fraction of the dialyzable portion of *Loligo* axoplasm. However,

TABLE II

Component	*Loligo*		*Dosidicus*	
	μmoles/100 mg D.P. (dry weight)	*Weight percent*	*μmoles/100 mg D.P. (dry weight)*	*Weight percent*
Glycocoll–betaine·H_2O	68.00	9.20	113.00	15.40
Glycerol	81.60	7.50	58.00	5.30
Myo-inositol·$2H_2O$	7.00	1.50	9.00	1.98
Glucose·H_2O	0.22	0.04	0.22	0.04
Fructose	0.22	0.04	0.22	0.04
Sucrose	0.23	0.08	0.23	0.08
Homarine·H_2O	18.80	2.92	20.30	3.14
Hypoxanthine	0.70	0.10	0.70	0.10
5′-AMP	—	—	0.45	0.17

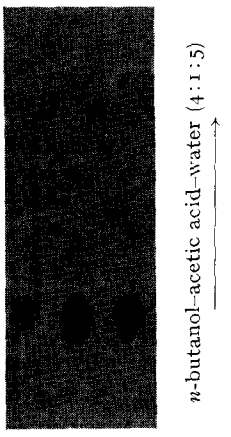
the explanation is simple: an enzymic cleavage, freeing pyrophosphoric acid from ATP, *post mortem*, is to a considerable extent suppressed due to the fact that, for the dissection and preparation of the much smaller *Loligo*, the Institute laboratories afford more favorable conditions. The ATP should be found in the region of the phosphate fraction of *Loligo*, but since there is an insufficient quantity of this fraction, an estimation of ATP could not be made from this material. The concentrations of the substances in *Dosidicus* axoplasm dialysate are given in Table II.

Homarine: KOECHLIN[21] has already mentioned this component of squid axoplasm. An aromatic betaine, homarine behaves strictly neutral under every condition of electrophoresis; its absorption maximum lies at 273 mμ; with Dragendorff's reagent[22] it yields the color of a quaternary ammonium compound, though only faintly. The substance was positively identified by paper chromatography. As reference substance the synthetic product was used, according to the directions of HOPPE-SEYLER[23].

In the course of the quantitative evaluation of the components of the N.F.$_{10.2}$ of *Loligo* and *Dosidicus*, we repeated the estimation for homarine and arrived at somewhat higher values than those given in our first paper[1]. Measurements were made with the Beckman spectrophotometer (Model DU) at 273 mμ and 290 mμ of the extinctions of aliquot portions of *Loligo* and *Dosidicus* dialysates and of the two neutral fractions N.F.$_{10.2}$; in parallel we measured an aqueous standard solution of the synthetic substance. The concentrations are given in Table II.

Glycocoll-betaine and hypoxanthine: The test for betaine was made by fusion with metallic potassium which distinctly showed the escape of trimethylamine. The paper chromatogram of the elctrophoretic fraction in question had already shown as main component a quaternary ammonium compound by the positive reaction with Dragendorff's reagent. Since the reaction was delayed, the presence of a carboxyl group was likely. The lack of u.v. absorption pointed to the presence of an aliphatic compound. All these criteria agreed well with the presence of glycocoll-betaine, which gave the

same behavior on the paper chromatogram as the substance being sought (Fig. 3). When conditions during electrophoresis were optimal, it was possible to separate betaine from X_2, whereby the latter substance showed a somewhat stronger deflection toward the cathode. For the quantitative determination of these substances we used in each case 200 mg of dialyzed axoplasm from both species of squid, transformed the



1: Glycocoll–Betaine·HCl (Ref.-Subst.) 2: L.M.P.$_{D.g.}$, Fract. II at pH 2.4
3: L.M.P.$_{L.p.}$, Fract. II at pH 2.4

Fig. 3. Paper chromatographic separation of the betaine- and hypoxanthine fraction after spraying with Dragendorff's reagent. It accords with our earlier investigations showing that the difference in concentration of these new-found components in the D.P. of the axoplasm of both squid species may be quite considerable between otherwise identical molecules. This difference, however, is by and large restricted to the molecules which remain neutral at pH 7.

isolated betaine to its hydrochloride and weighed it. Corresponding concentrations of the betaine, which crystallizes with one molecule of water of crystallization, are shown in Table II.

The u.v. absorbing substance X_2 could not be trigonelline, the isomer of homarine, primarily because trigonelline shows a distinctly different R_F value (0.5) in the butanol–acetic acid system. Neither could it be picolinic acid because, although its R_F value agreed very well with that of picolinic acid in the solvent mentioned, the reaction with Fe^{II} salts was negative, whereas it is characteristically positive in the case of pyridine-2-carbonic acids. Nevertheless, based on the cathodic deflection at pH 2.4, the presence of a basic group had to be assumed. The solution to the problem was finally found in the compound's susceptibility to coloration with eosine on the paper chromatogram; coloration is positive for the purine derivatives adenine, guanine, and hypoxanthine[24]. The pyrimidine derivatives uracil and thymine, which are also u.v.-absorbent, do not respond to this reaction which furnishes purple-red stains and is very sensitive. Proof was found in the identical R_F values of X_2 and the reference substance hypoxanthine ($R_F = 0.42$ in *n*-butanol–acetic acid). The concentrations of both substances are given in Table II.

The quantitative analysis consisted in calculating the u.v. absorption at the maximum of 251 mμ with reference to a standard solution of synthetic hypoxanthine.

DISCUSSION

Of the free ninhydrin-negative substances discussed here, glycocoll-betaine, glycerol, myo-inositol, hypoxanthine and the carbohydrates glucose, fructose and sucrose had not previously been identified in the dialyzed squid axoplasm. KOECHLIN[21] has already drawn attention to homarine, which was also recognized early in our own analyses. He has also determined the amount of total carbohydrates in the non-dialyzable part of axoplasm, but did not identify the individual carbohydrates.

Taurine and glycocoll-betaine occur in the axoplasm of both species of squid and in high concentrations. It is interesting to note that these two substances, together with glycine, are found also in high concentrations in the muscle tissue of other marine invertebrates, as has been shown by the extensive research of ACKERMANN *et al.*[25–27], and other authors[28–31]. GASTEIGER[32] thinks that the widespread occurrence of homarine in marine invertebrates[25,33–37], in contradistinction to fresh water invertebrates, is possibly connected with an osmosis-regulating function.

It is interesting that mytilite, the monomethyl derivative of inositol, as well as hypoxanthine, were isolated early from marine invertebrates[25,26,38–40].

It is quite possible that quaternary ammonium compounds, such as those already known from extracts of lower marine animals[26,35,37], will also be found among the traces of substances which can be rendered visible with Dragendorff's reagent from dialyzed axoplasm after paper chromatographic separation of enriched basic electrophoretic fractions.

No suggestion can be made as to the function of the high concentrations of glycocoll-betaine in the axoplasm. The same must be said of the considerable amounts of free glycerol, which is found only in traces in the brain. The presence of free myo-inositol in the axoplasm is not too surprising. Its presence in free form has been detected in the brain and various other organs[41,42], in the blood plasma[41–44], and in the thyroid gland of various species[45–47]. Its concentrations in the thyroid are considerable and partly exceed those in the axoplasm of the giant nerve fiber. It has been shown that certain strains of human cells need myo-inositol for their propagation[43,45–47].

Recently the discovery of hyaluronic acid in the axoplasm and neurilemma of amphibian and mammalian peripheral nerves was announced[48], and we have a recent description of the myo-inositol metabolism in the rat which proceeds by way of D-glucoronic acid[49]. KOECHLIN[21] found glucosamine in the non-dialyzable portion of squid axoplasm, and we confirmed his findings.

The possible role of sucrose in the chemical economy of squid nerve remains to be determined*.

ACKNOWLEDGEMENTS

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* Since the preparation of this manuscript, our attention has been called to the paper by Y. KOJIMA AND H. KUSAKABE, Isolation of Natural Substances by Ion Exchange Resins, VI[50].

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