# CHEMICAL INVESTIGATIONS OF THE GIANT NERVE FIBERS OF THE SQUID

## IV. ACID–BASE BALANCE IN AXOPLASM

GOTTFRIED G. J. DEFFNER AND REIMAR E. HAFTER
*Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. (U.S.A.)*
(Received November 17th, 1959)

SUMMARY

Acid–base balance is substantially established for the dialyzable portion of the axoplasm of the giant nerve fiber of *Loligo pealii* and *Dosidicus gigas*. All the free components of this axoplasm which have so far been identified and quantitatively estimated are listed. For *Loligo pealii* this total portion is 93 %, for *Dosidicus gigas* 95 % of the weight of solutes present.

## INTRODUCTION

With the discovery of large amounts of free dicarboxylic amino acids[1–6] and of isethionic acid[5] in squid axoplasm the identity of the large amounts of organic anions needed to balance the inorganic cations seemed to have been substantially determined. KOECHLIN's analyses suggested a cation deficit amounting to some 80 μequiv. (out of 520 μequiv.) and suggested that this may be composed of unidentified organic bases.

The analysis in these investigations of the dialyzable portion of squid axoplasm has identified about 95 % of its constituents. The only organic cations found were arginine, lysine and ornithine in relatively low concentration. The presence of organic bases, such as quaternary ammonium compounds, in amounts totaling 80 μequiv./g was thereby excluded. No evidence has been obtained for other organic acids, such as "axonic" acid, "sulfonate x", fumaric and succinic acid, thought by KOECHLIN to occur in the peripheral nerves of the lobster, crab and rat, as well as in squid.

Because of the key role of isethionic acid in the acid–base balance in squid axoplasm an independent determination of its concentration was made for comparison with KOECHLIN's value. The result was substantially lower: 19 % as compared with the 28 % found by KOECHLIN. This reduced correspondingly the necessity for organic base whose existence seemed to have been excluded by analyses already completed. In these estimates we assumed the values of inorganic base as determined in *Loligo* axoplasm independently by these groups of investigators[9–11].

## EXPERIMENTAL

### *Material and methods*

The dialyzed axoplasm used in the investigations came from the same sample used in all previously reported analyses[6–8]. The methods of electrophoretic fractionation were given in detail in earlier papers[6–8].

If, at pH 3.9 in an acetic acid–pyridine buffer, one feeds the dialyzed axoplasm not at the center, but near the cathode of the paper curtain, thereby sacrificing the collection of the basic components, one obtains under optimal conditions in descending order of their migration rates: chloride, sulfate, isethionic acid and phosphate as pure fractions. The quantitative collection of each separate fraction is facilitated if, after termination of the separation, a control experiment is carried out under identical conditions and the result observed under u.v. light.

*Estimation of isethionic acid*

Direct weighing of the fractions, even when thoroughly dried, is not practicable, because the strong acid retains tenaciously some pyridine from the buffer. Moreover, the free acid is syrupy and strongly hygroscopic. The quantitative estimation was gravimetric and titrimetric, with control experiments run concurrently. The gravimetric method consisted of oxidation with nitric acid in the sealed tube and precipitation of the resulting sulfate in the form of barium sulfate[12]. It was investigated with the pure fractions as well as with the total dialyzable portion; in the former case 20–40 mg were used, in the latter approx. 100 mg. The relatively small amounts of *Loligo* material available allowed only one estimation each. This procedure also involved the sulfur-containing components already quantitatively determined with ninhydrin, *i.e.*, taurine, cysteic acid amide, methionine, and methionine-sulfoxide, the latter recently found by us. Consequently, we were able to determine, after deduction for the values of the above substances, whether there were any sulfur-containing substances in substantial concentrations present in free form in the axoplasm. The results were unequivocally negative.

In the titrimetric method, the pyridine was first expelled by adding 10 ml of 0.01 *N* NaOH to aliquots of isethionic acid (approximately 5 mg in each case) and boiling for 5 min; the odor of the escaping pyridine was distinctly perceptible. Subsequently the added NaOH was neutralized with 0.01 *N* HCl, then the sulfonate titrated with 0.01 *N* NaOH, using phenolphtaleine as indicator. The results given in Table I represent the mean values obtained from the gravimetric and titrimetric estimations.

*Estimation of chloride, sulfate, and phosphate*

Chloride and sulfate were estimated gravimetrically as AgCl and $BaSO_4$, respectively. Phosphate was determined from the fraction and from the total dialysate by spectrophotometry at 830 m$\mu$ following the somewhat modified method of BOLTZ AND MELLON[13]. The fractions, individually collected after the electrophoretic separation, were freed of buffer in the desiccator, an excess of 0.1 *N* NaOH having been added previously to the chloride fraction.

*Estimation of potassium*

For this the directions of PIPER[14] were followed. The method is based on the precipitation of the potassium in the form of the practically insoluble potassium-sodium-cobalt$^{III}$-nitrite and subsequent oxidation of the nitrite into nitrate by means of permanganate. Three reference tests conducted with potassium chloride led in each case to values which were somewhat too high, with an average error of about 3 %. The inorganic cations of aliquots of dialyzed *Dosidicus* axoplasm (approx.

TABLE I

ACID-BASE BALANCE IN SQUID NERVE AXOPLASM (pH 7.0)

*Neutral constituents*

| | *Loligo* | | | *Dosidicus* | | |
|---|---|---|---|---|---|---|
| | *μmoles/ 100 mg D.P.* | *Weight percent* | *N in percent of total N* | *μmoles/ 100 mg D.P.* | *Weight percent* | *N in percent of total N* |
| Glycine | 10.70 | 0.80 | 3.18 | 10.40 | 0.78 | 3.27 |
| Alanine | 7.80 | 0.79 | 2.55 | 9.30 | 0.83 | 2.83 |
| Serine | 3.70 | 0.39 | 1.06 | 1.00 | 0.11 | 0.32 |
| Leucine + Isoleucine | 2.70 | 0.35 | 0.85 | 0.20 | 0.03 | 0.06 |
| Valine | 2.22 | 0.26 | 0.64 | 0.50 | 0.06 | 0.15 |
| Threonine | 1.90 | 0.23 | 0.64 | 0.30 | 0.04 | 0.09 |
| Proline | 1.00 | 0.12 | 0.42 | 0.10 | 0.01 | 0.04 |
| Tyrosine | 0.70 | 0.13 | 0.21 | 0.30 | 0.06 | 0.08 |
| Phenylalanine | 0.60 | 0.10 | 0.19 | 0.15 | 0.03 | 0.04 |
| Methionine | 0.50 | 0.08 | 0.16 | 0.30 | 0.04 | 0.08 |
| Methioninesulfoxide** | 0.30 | 0.05 | 0.09 | 0.20 | 0.03 | 0.07 |
| Citrulline** | 0.40 | 0.07 | 0.36 | 0.15 | 0.03 | 0.16 |
| $\alpha$-$NH_2$-butyric acid | | | | 0.05 | 0.01 | 0.02 |
| Cysteic acid amide | 4.50 | 0.74 | 2.65 | 0.04 | 0.07 | 0.25 |
| Taurine | 98.50 | 12.33 | 29.32 | 31.40 | 3.93 | 9.60 |
| Glycocoll-betaine·$H_2O$ | 68.00 | 9.20 | 20.20 | 113.00 | 15.40 | 34.75 |
| Homarine·$H_2O$ | 18.80 | 2.92 | 5.55 | 20.30 | 3.14 | 6.05 |
| 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 | |
| Hypoxanthine | 0.70 | 0.10 | 0.87 | 0.70 | 0.10 | 0.89 |
| Total neutral | 311.99 | 37.82 | 68.94 | 256.42 | 32.04 | 58.75 |

*Anions*

| | *Loligo* | | | | *Dosidicus* | | | |
|---|---|---|---|---|---|---|---|---|
| | *μequiv./ 100 mg D.P.* | *μmoles/ 100 mg D.P.* | *Weight percent* | *N in percent of total N* | *μequiv./ 100 mg D.P.* | *μmoles/ 100 mg D.P.* | *Weight percent* | *N in percent of total N* |
| Bicarbonate* | | | | | | | | |
| Chloride | 140.00 | 140.00 | 4.96 | | 142.00 | 142.00 | 5.02 | |
| Sulfate | | | | | 14.20 | 7.10 | 0.68 | |
| Phosphate $HPO_4''$–$H_2PO_4'$ 61.4/38.6 | 26.60 | 16.50 | 1.57 | | 22.75 | 14.10 | 1.34 | |
| 5′-AMP | | | | | 0.90 | 0.45 | 0.17 | 0.72 |
| Isethionic acid | 152.00 | 152.00 | 19.10 | | 157.00 | 157.00 | 19.80 | |
| Aspartic acid | 73.00 | 73.00 | 9.72 | 21.65 | 105.60*** | 105.60 | 14.05 | 31.42 |
| Glutamic acid | 19.60 | 19.60 | 2.88 | 5.81 | 26.20 | 26.20 | 3.85 | 8.06 |
| Total acid | 411.20 | 401.10 | 38.23 | 27.46 | 468.65 | 452.45 | 44.91 | 40.20 |

* Not determined.

** Methioninesulfoxide and Citrulline were given in an earlier paper the tentative designation $N_2$ and $N_1$, respectively[6].

*** Value given in the first paper was found to be too low[6].

*Continued* TABLE I

| | *Cations* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Loligo* | | | | *Dosidicus* | | | |
| | *μequiv./ 100 mg D.P.* | *μmoles/ 100 mg D.P.* | *Weight percent* | *N in percent of total N* | *μequiv./ 100 mg D.P.* | *μmoles/ 100 mg D.P.* | *Weight percent* | *N in percent of total N* |
| Potassium | 344.00 | 344.00 | 13.40 | | 388.00 | 388.00 | 15.18 | |
| Sodium | 65.00 | 65.00 | 1.50 | | 77.00 | 77.00 | 1.77 | |
| Calcium | 7.00 | 3.50 | 0.14 | | 7.00 | 3.50 | 0.14 | |
| Magnesium | 20.00 | 10.00 | 0.24 | | 20.00 | 10.00 | 0.24 | |
| Arginine | 3.20 | 3.20 | 0.56 | 4.24 | 4.10 | 4.10 | 0.71 | 5.88 |
| Lysine | 2.40 | 2.40 | 0.35 | 1.48 | 0.20 | 0.20 | 0.03 | 0.13 |
| Ornithine | 1.80 | 1.80 | 0.24 | 1.06 | 0.30 | 0.30 | 0.04 | 0.17 |
| Total base | 443.40 | 429.90 | 16.43 | 6.78 | 496.60 | 483.10 | 18.11 | 6.18 |
| | | | *Weight percent* | *N in percent of total N* | | | *Weight percent* | *N in percent of total N* |
| TOTAL | | | 92.48 | 103.18 | | | 95.06 | 105.13 |

50 mg, dried to constant weight) were converted to sulfates by means of concentrated sulfuric acid and concentrated nitric acid, the organic components being destroyed. The sulfates were then weighed. The small concentration of the phosphate in the dialysate interfered very little, as phosphate and sulfate have almost the same molecular weight. The potassium, precipitated from its sulfate with potassium-sodium-cobalt$^{III}$-nitrite as complex salt, was filtered off and washed in accordance with the conditions of the gravimetric analysis. The precipitate was dissolved with a measured excess of a sulphuric acid–permanganate solution under heat, and after addition of a known quantity of oxalic acid retitrated with permanganate.

The values for calcium and magnesium found by VALLEE[11] for *Loligo* axoplasm were used in the acid–base estimate for *Dosidicus* axoplasm. In view of the very low concentrations of these cations this procedure seemed justified. The value given by us for sodium was estimated from the difference and is therefore less reliable.

*Tests for polycarboxylic acids and oxo-carboxylic acids*

Through preliminary tests, information was obtained about the electrophoretic deflection at pH 3.9 and the paper chromatographic behavior of those acids which are members of the citric acid cycle. These substances were made visible on paper and determined in solution[15,16]. The information thus gained was applied to the appropriate fractions of the axoplasm of both species of squid. No acid belonging to this class of compounds was found despite KOECHLIN's statement that succinic and fumaric acids occur in *Loligo* axoplasm. To eliminate all doubt the following procedure was adopted: we subjected to further electrophoretic separation at pH 2.4 those anionic fractions which since the beginning of our investigations of *Dosidicus* axoplasm have become enriched through numerous electrophoretic separations at pH 3.9 and within whose area the two acids mentioned ought to be found. At pH 2.4 the acid amino acids are weakly deflected toward the cathode, whereas the polybasic acids run neutral. However, the paper chromatographic examination of this neutral

fraction (collected on a broader base) with MUNIER's method[15], which allows sharp separations, did not yield any results, nor did the Baeyer test which is sensitive for olefinic double bonds.

## ANALYTICAL RESULTS

The correction of the value for isethionic acid, the fact that we were unable to find the two polycarboxylic acids mentioned by KOECHLIN, and the fact that we see no indication of the presence of further acids, lead to the result, contrary to KOECHLIN's investigations, that there is now a deficit of approximately 30 μequiv. on the anion side of the acid–base balance of the squid axoplasm.

Theoretically, the balance might be established on the basis of the following considerations: for the brain, plasma, and cerebrospinal fluid of man, HARRISON[17] and MANERY[18] have found bicarbonate concentrations of 12, 27, and 18 equiv./g or ml, respectively. Since all these values are based on wet weight, their conversion to dry weight would increase the anion side of the acid–base balance of the axoplasm on the average, by about 15 μequiv. Furthermore, if we put into the acid–base balance of the axoplasm of *Loligo* the sulfate concentration, which in *Dosidicus* amounted to 15 μequiv., we would then find the balance re-established. The small excess of cations in the balance which would follow from this explanation might possibly be attributed to a slight overestimation of the potassium value.

In Table I are shown all free components of the dialyzable part of the axoplasm found to date. The portion of the *Loligo* axoplasm identified at the present date amounts to 93 %, that for *Dosidicus* to 95 %. But it is to be assumed that the proportion of the known substances in the dialyzed axoplasm of both species is actually somewhat higher, since experience has shown that the dialyzable portion of the axoplasm even after thorough drying in the desiccator, still tenaciously retains water which can be temporarily freed in the drying oven. This retention of water is primarily due to the isethionic acid, already mentioned. In the investigation of such complex material a somewhat larger margin of error must be expected in the quantitative estimations.

Since the theoretically possible value for total nitrogen is already exceeded by a few percent, additional nitrogenous compounds (for the presence of which we have proof) can be expected to be found only in small concentrations.

## DISCUSSION

From analyses of the nerves of several invertebrate types (see ref. 19), aspartic acid is found to be prominent in all. Its function in nerve is probably not restricted to the maintenance of the ion balance alone. Possibly significant in this connection is the fact that the fibrous protein of the axon[20], which has been under study in these laboratories for some time, shows a high total concentration of mono-amino-dicarboxylic acids, with glutamic acid as the principal component, and also a high percentage of amide nitrogen[21].

Since there is no appreciable concentration of organic base in the dialyzable portion of squid axoplasm, such substances would appear to be excluded as carriers of the bioelectric stimulating current.

ACKNOWLEDGEMENTS

These investigations are part of a continuing program of biophysical and biochemical research on nerve under the direction of Professor F. O. SCHMITT to whom we wish to express our appreciation for advice and assistance.

These studies were aided by a research grant (B-24) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, U.S. Public Health Service; by a contract between the Office of Naval Research, Department of the Navy, and the Massachusetts Institute of Technology (NR-119-100); and by grants from the Trustees under the Wills of Charles A. and Marjorie King, and from the Lou and Gene Marron Foundation.

REFERENCES

1 R. S. BEAR AND F. O. SCHMITT, *J. Cellular Comp. Physiol.*, 14 (1939) 205.
2 F. O. SCHMITT, R. S. BEAR AND R. H. SILBER, *J. Cellular Comp. Physiol.*, 14 (1939) 351.
3 R. H. SILBER AND F. O. SCHMITT, *J. Cellular Comp. Physiol.*, 16 (1940) 247.
4 R. H. SILBER, *J. Cellular Comp. Physiol.*, 18 (1941) 21.
5 E. A. KOECHLIN, *J. Biophys. Biochem. Cytol.*, 1 (1955) 511.
6 G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 32 (1959) 362.
7 G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 35 (1959) 334.
8 G. G. J. DEFFNER AND R. E. HAFTER, *Biochim. Biophys. Acta*, 42 (1960) 189.
9 H. B. STEINBACH AND S. SPIEGELMAN, *J. Cellular Comp. Physiol.*, 22 (1943) 187.
10 R. D. KEYNES AND P. R. LEWIS, *J. Physiol., (London)*. 114 (1951) 151.
11 B. L. VALLEE, see B. A. KOECHLIN, *J. Biophys. Biochem. Cytol.*, 1 (1955) 511.
12 O. HOENIGSCHMID AND E. ZINTL, in *Anleitung zur quantitativen Gewichtsanalyse*, München, 1921, Chem. Lab. d. Staates.
13 D. F. BOLTZ AND M. G. MELLON, *Anal. Chem.*, 19 (1947) 873.
14 C. S. PIPER, *Handbuch der Analytischen Chemie*, 3. Teil, Band Ia, Berlin, Springer Verlag, 1940.
15 R. MUNIER, *Bull. soc. chim. France*, (1952) 852.
16 Z. DISCHE, R. WEIL AND E. LANDSBERG, *J. Biol. Chem.*, 208 (1954) 23.
17 G. A. HARRISON, in *Chemical Methods in Clinical Medicine*, 3rd Ed., London, Churchill, 1947.
18 I. F. MANERY, in *The Biology of Mental Health and Disease*, London, Casell, 1952.
19 F. O. SCHMITT AND N. GESCHWIND, *Progr. in Biophys.*, 8 (1957) 165.
20 F. O. SCHMITT, *J. Cellular Comp. Physiol.*, 49 (1957) 165.
21 P. F. DAVISON AND E. W. TAYLOR, *J. Gen. Physiol.*, 43 (1960) 801.