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The Annelid Phosphagens

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After the work of Eggleton & Eggleton (1929) and of Meyerhof (1928) it was generally assumed that the creatine phosphate characteristic of vertebrate muscle is always replaced by arginine phosphate in the muscle of invertebrates. A broad comparative study of invertebrate animals by Needham, Needham, Baldwin & Yudkin (1932) did not reveal the occurrence of any phosphagen other than arginine phosphate, with certain notable exceptions among the echinoderms. Greenwald (1946), however, had shown that the reproductive organs of certain marine annelids contain a material that is strongly chromogenic in the Jaffe reaction and thus might be creatine. Baldwin & Yudkin (1950) described the presence of an arginine-like phosphagen in annelid and geophyean worms and showed that a second phosphagen, possibly creatine phosphate, is present in certain annelids. The work reported here, carried out on annelids at Roscoff in 1951, demonstrates that the creatine-like substance described by Baldwin & Yudkin (1950) in annelids is in fact creatine phosphate and extends the examination to a number of annelid species not previously studied.

By the use of paper chromatography during the period 1951-4, Roche and his co-workers have shown the presence of new guanidine derivatives in annelids. Thoai, Roche, Robin & Thiem (1953a) have isolated taurocyamine and glycocyamine from annelids and glycocyamine from the nemertine *Lineus gesserensis* (Thoai, Roche, Robin & Thiem (1953b)). They postulated that these bases occur as phosphates and constitute the arginine-like phosphagen described by Baldwin & Yudkin (1950). Some of this work has been confirmed.

METHODS

Preparation of tissue extracts. Freshly collected animals were cooled in sea water to 0° before use. The larger species were gutted before being extracted; the smaller were extracted whole. The chilled material was ground with sand and extracted in an ice-cold mortar with ice-cold 8% (w/v) trichloroacetic acid, usually in the proportion of 5 ml./g. of tissue. The extract was centrifuged at about 600 g at 0°. The supernatant was neutralized (pink to phenolphthalein), and the $\text{Ca}(\text{OH})_2$ - CaCl_2 reagent of Fiske & Subbarow (1929) was added to precipitate the inorganic phosphate. After standing 10 min. in ice the tubes were centrifuged. Phosphagens were estimated in the supernatant by the method described by Baldwin & Yudkin (1950).

For chromatographic analysis, the neutralized extract prepared as described above was placed on Whatman no. 1 paper. In some cases the phosphagens were first concentrated by precipitation at 0° with 3 vol. of ice-cold acetone and purified by dissolving in the minimum quantity of water and reprecipitating with acetone.

The solvent suggested by Hanes & Isherwood (1949), *n*-propanol-ammonia (sp.gr. 0.880)-water (60:30:10), was used and was allowed to flow down the paper. Up to 0.05 ml. of the extract was put on the paper, the diameter of the spot always being kept below 0.7 cm. The chromatograms were run at room temperature (22°) usually for 6 hr.; during this time the solvent front moved about 25 cm. An alternative solvent system of Thoai & Robin (1954) [isopentanol-pyridine-water (40:80:70)] was also used.

Creatine phosphate. This was prepared by the method of Ennor & Stocken (1948).

Taurocyamine phosphate and glycocyamine phosphate were prepared enzymically as described by Hobson (1955).

RESULTS

In 1951 species hitherto unexamined were analysed for the creatine-like and arginine-like phosphagens by a hydrolysis method identical in all essentials

Table 1. *Distribution of arginine-like and creatine-like phosphagens in Annelida*

Results are expressed in mg. of P/100 g. of muscle. Analysis was carried out on trichloroacetic acid extracts of whole animal unless otherwise stated. Phosphate analysis and Walpole and Sakaguchi tests were according to the method described by Baldwin & Yudkin (1950).

Animal	Inorganic phosphate	'Creatine' phosphate	'Arginine' phosphate	Total phosphagen phosphate	Walpole test	Sakaguchi test
<i>Nereis diversicolor</i>	18.8	2.1	7.8	9.9	Negative	Positive*
<i>Nephtys hombergii</i>	104	—	31.2	31.2	Negative	Positive
<i>Glycera gigantea</i> (body wall)	150	14.6	16.4	31.0	Positive	Very weakly positive
<i>G. convoluta</i>	20.6	4.2	—	4.2	Positive	Negative
<i>Eunice harrassi</i>	39.6	8.0	—	8.0	Positive	Negative
<i>Leiochone clypeata</i>	15.0	—	6.7	6.7	Negative	Positive
<i>Scholoplos armiger</i>	11.5	12.0	—	12.0	Positive	Negative
<i>Polymnia nebulosa</i> (1)	7.9	—	7.0	7.0	Negative	Positive
<i>Polymnia nebulosa</i> (2)	9.0	—	6.2	6.2	Negative	Positive
<i>Halosydna gelatinosa</i>	11.1	2.6	7.4	10.0	Very weakly positive	Positive
<i>Eulalia viridis</i>	40.5	0.8	6.3	7.1	Positive	Positive
<i>Lumbriconereis impatiens</i>	35.3	4.4	—	4.4	Positive	Negative
<i>Travisia forbesii</i>	15.8	4.4	6.4	10.8	Positive	Negative
<i>Myxicola infundibulum</i>	18.4	0.85	7.65	8.5	Negative	Positive*
<i>Amphitrite edwardsi</i>	14.1	—	1.9	1.9	Negative	Positive
<i>Hermione hystrix</i>	65.0	6.3	—	6.3	Positive	Negative
<i>Ophelia bicornis</i>	5.95	—	1.25	1.25	Positive	Orange-like* colour not definitely positive

* Colour reaction does not agree with colour given by arginine in Sakaguchi reaction.

with that used by Baldwin & Yudkin (1950). The results are presented in Table 1, and confirm Baldwin & Yudkin's conclusion that the distribution of these substances is altogether erratic and not in any way correlated with the taxonomy, habits or habitat of the species concerned.

Certain species, e.g. *Glycera gigantea* and *Ophelia bicornis* (Table 1), were found to contain a phosphagen showing behaviour on hydrolysis similar to that of arginine phosphate. After hydrolysis, muscle extracts from these animals failed to give a Sakaguchi reaction or, if they did, the reaction was far weaker than would be expected from the amounts of arginine calculated from estimates of the labile phosphagen originally present.

For extracts containing creatine-like phosphagen according to the phosphagen-hydrolysis method, the test for creatine described by Walpole (1911) was positive (Table 1). It was not possible to obtain a positive Walpole reaction with *Nereis diversicolor*, but the level of the creatine-like phosphagens present was extremely low and was not always detectable by the hydrolysis method. To obtain more information on the nature of the substance detected by the latter method, it was decided in 1954 to investigate muscle extracts by chromatography.

Chromatographic investigations

Hydrolysed extracts of *Glycera convoluta* and *G. gigantea* were run with creatine as a marker and the

chromatograms were dried in the laboratory. They were sprayed first with a fresh solution of 5% (w/v) α -naphthol in 2.5N-NaOH, and then, after drying, with a 0.1% fresh aqueous solution of diacetyl. The slow appearance of a claret-red colour after 1–2 min. indicated the presence of creatine. Both *G. gigantea* and *G. convoluta* extracts were found to contain creatine (Table 2), as shown by the colour and R_f value compared with a creatine standard.

The chromatographic position of phosphagens run in non-hydrolysed extracts was determined by spraying the chromatograms with the reagent described by Hanes & Isherwood (1949) (5 ml. of 60%, w/v, perchloric acid, 25 ml. of 4%, w/v, ammonium molybdate solution, 10 ml. of N hydrochloric acid and water to 100 ml.). This was followed by heating the chromatograms at 85° for 10 min. After 1 min., but before the appearance of blue coloration, the creatine phosphate could be seen as a yellow spot owing to the formation of a phosphomolybdic acid; this aided the later interpretation of the chromatograms. After heating, the papers were left in an atmosphere of hydrogen sulphide for about 30 min. and finally exposed in the laboratory. Extracts of *G. gigantea* gave a spot with the same R_f value as the synthetic creatine phosphate.

Detection of the arginine-like bases. The chromatograms were dipped in a solution of 8-hydroxyquinoline (oxine) in acetone, 0.1%, then dried in

Table 2. *Chromatography of guanidine bases and phosphagens*

Method of detection of substances described in the text.

Nature of tissue extract or marker substance	Method of detection	R_f value in		Comment
		<i>n</i> -Propanol- NH ₃ -H ₂ O	<i>iso</i> Pentanol- pyridine-H ₂ O	
Creatine	Walpole reagent	0.49	0.28	—
Arginine	Sakaguchi reagent	0.40	0.20	—
Taurocyamine	Sakaguchi reagent	0.54	0.46	—
Glycocyamine	Sakaguchi reagent	0.42	0.33	—
Creatine phosphate (synthetic)	Phosphate test	0.29	—	—
Arginine phosphate	Phosphate test	0.37	—	—
Taurocyamine phosphate	Phosphate test	0.24	0.18	—
Glycocyamine phosphate	Phosphate test	0.21	0.08	—
<i>Maia squinado</i> (muscle extract)				
Hydrolysed	Sakaguchi reagent	0.39	—	Arginine
Non-hydrolysed	Phosphate test	0.37	—	Arginine phosphate
<i>Glycera gigantea</i> (body wall)				
Hydrolysed extract	Walpole reagent	0.52	—	Creatine
Non-hydrolysed extract	Phosphate test	0.30	—	Creatine phosphate
<i>Glycera convoluta</i>				
Hydrolysed extract	Walpole reagent	0.50	—	Creatine
Non-hydrolysed extract	Phosphate test	0.31	—	Creatine phosphate
<i>Arenicola marina</i>				
Hydrolysed extract	Sakaguchi reagent	0.54	0.45	Taurocyamine
Non-hydrolysed extract	Phosphate test	0.25	0.19	Taurocyamine phosphate
<i>Nereis diversicolor</i>				
Hydrolysed extract	Sakaguchi reagent	0.43	0.33	Glycocyamine
Non-hydrolysed extract	Phosphate test	0.22	0.08	Glycocyamine phosphate

air, and lightly sprayed with a freshly prepared solution of alkaline bromine (0.2 ml. of bromine in 100 ml. of 0.5N sodium hydroxide solution). Arginine gives a brilliant orange-red area which is stabilized by spraying with an excess of 40% (w/v) aqueous urea solution to counteract the excess of hypobromite. As has been described by Roche, Felix, Robin & Thoai (1951), the substituted-guanidine bases taurocyamine and glycocyamine gave only a transient colour. Such areas were observed in hydrolysed extracts of *Nereis diversicolor* and *Arenicola marina*.

Two methods were employed to identify the phosphagens of *N. diversicolor* and *A. marina*: a series of chromatograms were run of extracts of these animals (pH 7.0–7.4) and tested for phosphates. Duplicate chromatograms were sprayed with 0.1N hydrochloric acid, dried at 85° and treated with Sakaguchi reagents. These chromatograms showed the development of a transient spot in an area occupied by a phosphate ester on the chromatograms developed for phosphates. Similar runs were made with extracts of the muscle of *Maia squinado*, where arginine and arginine phosphate were detected. With the enzymic preparation of glycocyamine phosphate and taurocyamine

phosphate it was also possible to identify the phosphagens of *N. diversicolor* and *A. marina* by comparative R_f values (Table 2).

DISCUSSION

From an examination of the distribution of the phosphagens in the annelid there is no correlation with taxonomy and habitat. This is strongly brought out by the discovery that in the single genus *Glycera* one species, *G. gigantea*, contained both phosphagens, whereas *G. convoluta*, like *G. dibranchiata*, examined by Baldwin & Yudkin (1950), contained only creatine phosphate. Roche *et al.* (1951) considered that a correlation of the distribution of the phosphagens with habitat existed in the annelid. However, more recent studies have shown that this is not the case (Roche, private communication).

Chromatographic studies reported in this paper show the arginine-like phosphate detected by Baldwin & Yudkin (1950) and described in this paper to be the phosphates of taurocyamine and glycocyamine. Hobson (1955) has now demonstrated that the annelids contain phosphokinases, which will phosphorylate taurocyamine and glycocyamine at the expense of ATP.

The presence of creatine phosphate in the annelid can hardly suggest any close relationship between this phylum and the Chordata, as formerly postulated by Dohrn (1875) and Semper (1875, 1876). Developmental, embryological and anatomical evidence is against such a relationship and the occurrence of creatine is presumably due to convergent chemical evolution.

SUMMARY

1. Analysis of the phosphagen content of a number of species of annelids is reported.
2. Creatine and creatine phosphate have been shown to be present in muscles of certain annelids.
3. A preliminary investigation by chromatography of the monosubstituted guanidine phosphates is described.

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The Incorporation of Labelled Phosphate into the Phosphoglycerides of Intact Rat Liver

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Speculation has often occurred as to whether any of the individual phosphoglycerides present in animal tissues are formed from other phospholipids in the same tissue. Popják & Muir (1950), for example, found some evidence for the interconversion of phosphoglycerides in rabbit liver after the injection of ^{32}P -labelled phospholipid emulsions. Another method of investigating this problem is to compare the specific radioactivities of the individual liver phosphoglycerides after labelled-phosphate administration. Previous attempts to measure the specific radioactivities of the various liver phospholipids have relied upon solvent fractionation methods (e.g. Hahn & Tyrén, 1946) or adsorption of the non-choline-containing phospholipids on magnesium oxide (e.g. Zilversmit, Entenman & Chaikoff, 1948), both of which yield fractions which are extremely heterogeneous in their base distribu-

tion. It is not possible therefore to reach any reliable conclusions about phospholipid interconversion from the results of such experiments.

The development of a new method of determining the specific radioactivities of the phospholipids contained in a ^{32}P -labelled tissue sample (Dawson, 1954) has presented an opportunity for gaining information about any such phospholipid interconversion. By extending observations obtained in other investigations (Dawson, 1955) it has been possible to compare the specific radioactivities of the individual liver phosphoglycerides of the rat at various times after the injection of labelled phosphate. The results, while showing (in contrast to those of many other workers) that marked differences can occur in the specific radioactivities of liver phosphoglycerides, give no evidence of any type of phospholipid interconversion.