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A Stable Phosphorus Compound in the Body-wall Muscle of *Ascaris lumbricoides*

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Abstract. Acid-soluble phosphorus compounds of the muscle of *Ascaris lumbricoides* were fractionated according to their stability to acid hydrolysis and the solubility of their barium salts. While phosphoarginine was found to be absent or present in negligible amounts only, the extracts contained a large amount of acid-stable phosphorus compounds which in diluted solutions are not precipitated by barium and alcohol. This "barium soluble — alcohol soluble" fraction is composed mostly or entirely of a compound which closely resembles propanediolphosphate in stability, solubility of barium salts and mobility in paper ionophoresis (salicylate buffer of pH 3.9). It is, however, readily hydrolysed by heating with periodic acid while synthetic propanediol-1-phosphate remains stable under these conditions.

The energy-yielding biochemical processes in *Ascaris lumbricoides* have been studied to considerable details. As early as 1901 WEINLAND demonstrated the anaerobic conversion of glycogen to carbon dioxide, lower fatty acids and a small amount of lactic acid. His results were corroborated by many other authors (VON BRAND 1934) and led to the conclusion that these worms depend largely on anaerobic reactions for covering their energy needs. Although ADAM (1932) found a rather considerable oxygen consumption in *Ascaris* in aerobic conditions, it was shown later by VON BRAND (1934) that there is little if any Pasteur effect in these animals; thus their oxidative metabolism cannot be regarded as very effective. Enzymological studies substantiated these assumptions: no cytochrome oxidase can be detected in the *Ascaris* tissues (BUEDING 1952) with the exception of eggs which appear to have a complete oxidative metabolism based on the citric acid cycle (CASTELLO and BROWN 1962, CASTELLO, OYA and SMITH 1963).

In adult worms, energy seems to be derived from the reactions of the Embden—Meyerhof pathway leading from glycogen to pyruvate and, in addition, from the hydrogen transfer of the NAD to the succinate in a process representing in fact only a small part of the oxidative phosphorylation (SEIDMAN and ENTNER 1961).

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This may be the reason for accumulating succinate and its metabolic products, α-methylvalerate and α-methylbutyrate (SAZ and WEIL 1960, 1962).

Contrary to the great interest in metabolic end products little attention has been paid to the metabolism of the phosphorus compounds important for energy transfer (adenosin phosphates, phosphagen) and as glycolytic intermediates.

In this paper, some characteristic features of the overall pattern of the distribution of the acid-soluble phosphorus compounds in the *Ascaris* muscle are given with special attention to the unusually abundant fraction of stable phosphates.

METHODS

Animals. Living *Ascaris lumbricoides* var. *suis* were obtained from freshly slaughtered pigs and transferred into a Krebs—Ringer phosphate solution heated to 37 °C. In this solution they were put in a Dewar flask and kept in the laboratory at 37 °C in oxygenated Krebs—Ringer phosphate. Within six hours after killing the pigs the worms were taken for an analysis; only living, spontaneously moving worms were used.

Homogenisation and extraction. For homogenisation, freezing of the tissue in liquid nitrogen and powdering of the tissue in a frozen state appeared most satisfactory for securing a better and faster desintegration than any of other methods tried. The worms were dissected longitudinally, the digestive tract and reproductive organs removed, the body-wall washed with physiological saline, dried with filter paper, weighed and frozen. The frozen tissue was powdered in a porcelain dish, extracted immediately with 3 per cent perchloric acid, transferred quantitatively to a tube and centrifuged. The protein precipitate was again extracted with perchloric acid, centrifuged, the supernatants collected and neutralised with potassium hydroxide; the precipitated potassium perchlorate was centrifuged off; glycogen was removed overnight from the supernatant by precipitation with 1.2 volumes of alcohol and centrifugation. The neutral glycogen-free extract was taken for phosphorus analysis or for barium fractionation. During these procedures the samples were continuously cooled with ice.

Fractionation of phosphorus compounds according to their stability. The value P_0 (inorganic phosphate) was obtained by direct determination of the phosphate in the neutral extract. P_1 was obtained by heating the sample for 1 min with 0.06 sulphuric acid. The values P_7 and P_{180} were measured in samples hydrolysed in 1N sulphuric acid at 100 °C for 7 and 180 min respectively. P_t (total acid-soluble phosphorus) was determined in a sample mineralised with sulphuric acid and hydrogen peroxide.

Fractionation by precipitation with barium ions. In principle, the procedure described by SACKS (1949) was followed. Approximately 0.4 ml of saturated barium acetate to 1 g of fresh tissue was given to the glycogen-free extract, adding 1.3 volumes of alcohol; the reaction was adjusted with potassium hydroxide until the phenolphtalein attained a pinkish colour. After 30 min the precipitate was centrifuged off. Both the supernatant and the precipitate were analysed. Further fractionation of the precipitated barium salts was not performed. For the phosphorus determination, the precipitate was decomposed by N sulphuric acid and the resulting barium sulphate washed twice with N sulphuric acid. The same procedure was used for removing Ba^{2+} ions from the supernatant to be analysed for phosphorus.

Paper ionophoresis. For the paper ionophoresis we used the supernatant remaining after the barium precipitation. Alcohol was evaporated in a water bath and barium ions were removed by means of the cation-exchanger Dowex 50. Acetic acid remaining from the barium acetate was extracted repeatedly with ethyl ether. Then the sample was concentrated by lyophilisation, dissolved in a small amount of water and neutralised with ammonia. We used Whatman No. 3 paper previously washed with 0.5 per cent sodium ethylendiaminotetraacetate (pH 8.5) and redistilled water. The

pH 3.9 of the buffer was similar as the butyrate buffer of WADE and MORGAN (1955). To avoid working with butyric acid, a 0.1 M salicylate buffer was used. The voltage used was 1200 V with paper strips 50 cm long. The paper was cooled with carbon tetrachloride saturated with salicylic acid to prevent a change of the pH due to the extraction of salicylic acid (the buffer is also saturated with respect to salicylic acid). In these conditions, the optimal duration of the ionophoresis was 135 minutes. Relative mobilities of several compounds examined (β-glycerophosphate, propanediol-1-phosphate, adenosine triphosphate, adenosine monophosphate) with respect to inorganic phosphate were observed to be practically identical with those found in butyrate buffer by WADE and MORGAN (1955). The spots of the phosphorus compounds were detected by spraying with 0.1 per cent ferric chloride in 0.1 N hydrochloric acid in 80 per cent alcohol and subsequently with 1 per cent sulphosalicylic acid in 75 per cent alcohol (WADE and MORGAN 1955). Phosphorus compounds appeared as white spots on a brownish background. The detection is not connected with the decomposition of the phosphorus compounds which may be eluted and analysed further. Details on this method will be published separately.

Paper chromatography. Various systems were used: acetone-formic acid-water, acetone-monochloracetic acid-water (BURROWS, GRYLLS, HARRISON 1952), propanol-ammonia-water (HANES and ISHERWOOD 1949). Descending chromatography was used in all cases. For detection, the ferric chloride-sulphosalicylic acid method of WADE and MORGAN (1955) was applied.

Treatment with periodic acid. To determine the stability of phosphorus compounds towards periodic acid the procedure of BURMASTER (1946) for the glycerophosphate analysis was followed.

Synthesis of propanediol phosphate. Propanediol phosphate was synthetised from propylen oxide and potassium hydrogen phosphate after LAMPSON and LARDY (1949) in the modification of RUDNEY (1954); it was isolated from the reaction mixture by precipitation of its barium salt with alcohol and ether.

Colorimetric determination of phosphate. We used a modification of the butanol extraction method as described by WEIL—MALHERBE and GREEN (1951). In samples treated previously with periodic acid the excess of the reagent was decomposed by titration with 20 per cent sodium sulphite; before adding alcohol and stannous chloride the butanolic extract was washed with an acidified solution of sodium sulphite to remove any trace of oxidating material.

RESULTS

1. *Distribution of phosphorus compounds of different stability*

The pattern of distribution of acid-soluble phosphorus compounds from the *Ascaris* muscle with respect to their stability to acid hydrolysis is given in Table 1. The value P_1 is not mentioned because of the insignificant difference from the value P_0 (the presence of some phosphagen is rather doubtful). Thus the values $P_7—P_0$ may be considered to represent the sum of the hydrolysable phosphorus of adenosine triphosphate and diphosphate. It appears that the level of the adenosine triphosphate does not exceed the value 3.4 μ moles/g (if adenosine diphosphate is not taken into account).

A further very characteristic feature of the *Ascaris* muscle appears to be the high value of the acid-stable fraction ($P_t—P_{180}$). This is a rare phenomenon as in most muscles the main components of the acid-soluble fraction are inorganic phosphate, phosphagen and adenosine phosphates. We than directed our further work towards characterising the compound (or compounds) present in the stable fraction.

Table 1. Distribution of labile and stable acid-soluble phosphorus compounds in the body-wall of *Ascaris lumbricoides*

Fraction	No of determinations	Content	
		μ moles/g fresh weight	per cent of the total P content
P_0	9	4.2 ± 1.5	13.5 ± 4.9
$P_7 - P_0$	8	$6.8 + 3.6$	21.0 ± 8.4
$P_{180} - P_7$	4	0.82 ± 0.31	3.2 ± 1.7
$P_T - P_{180}$	20	20.2 ± 4.2	$64.0 + 7.3$
P_T	20	32.0 ± 7.1	—

2. *Fractionation of the barium salts*

Precipitation of the barium salts of the phosphorus compounds was chosen to find out whether the compound was present in the "barium-insoluble" or "barium-soluble — alcohol-insoluble" fraction. Contrary than expected, in all experiments a considerable amount of phosphorus compounds remained in the supernatant after precipitation with barium and alcohol. This "barium-soluble — alcohol-soluble" fraction contains practically only the most stable compounds (Table 2). It appears

Table 2. Distribution of hydrolysable and stable phosphorus compounds in the extracts of Ascaris muscles, in the precipitate, obtained by adding barium acetate and alcohol, and in the remaining supernatant

Final concentration of the extract with respect to the tissue mg fresh weight/ml	Acid-soluble phosphates — μmoles/g								
	Before Ba^{2+} precipitation			After Ba^{2+} precipitation					
				Ba^{2+} precipitate			Supernatant		
	P_{180}	P_T	$P_T - P_{180}$	P_{180}	P_T	$P_T - P_{180}$	P_{180}	P_T	$P_T - P_{180}$
50	16.7	43.3	26.6	10.6	17.1	6.5		—	—
40	4.7	21.2	16.5	4.9	6.7	1.8	—	—	—
27	14.4	38.6	24.2	11.8	14.8	3.0	0.49	17.5	17.0
35	9.4	32.4	23.0	—		—	0.37	9.9	9.5*
40	6.8	26.4	19.6	5.6	10.0	4.4	0.25	14.7	14.5
140	21.2	40.0	18.8	—	—		0.7	5.2	4.5

*) Purified for ionophoresis.

that the stable compounds of the *Ascaris* muscle form barium salts which are more soluble in 80 per cent alcohol than most of the major tissue phosphates. The yield of the stable compound in the supernatant is variable. A closer inspection of the data reveals that it is in indirect proportion to the concentration of the extract: from more concentrated solutions relatively more is precipitated with barium. It seems that fractionation is carried out at the limits of the solubility of the corresponding barium salt. This was substantiated by further experiments using the same extracts in a different dilution (Table 3). In more concentrated extracts less than one half of all acid-stable compounds was recovered in the supernatant.

The precipitation in more diluted extracts (all other conditions including the amount of barium acetate being the same) increased considerably the relative yield in the supernatant. At the same time this is evidence that barium ions are present in abundance and therefore the presence of the phosphate in the supernatant cannot be explained by their shortage (which may have been caused by the presence of some unknown compound in the extract forming a precipitate or some complexes with barium ions).

Table 3. Relation between the concentration of the extract and the amount of the stable phosphorus compounds remaining in the supernatant after precipitation by barium acetate in 82 per cent alcohol at slightly alcaline pH (Sacks 1949)

No of the extract	Final concentration mg fresh weight/ml	Stable phosphate		
		Total μmoles/g fresh weight	In supernatant μ moles/g fresh weight	Per cent of the total
1	70.0	18.2	6.24	34.4
	7.0	18.2	13.60	75.0
2	65.0	20.7	7.70	37.0
	6.5	20.7	14.50	70.0
3	60.0	18.5	6.70	36.2
	6.0	18.5	13.40	72.0

To make sure that the precipitation from a tissue extract of the same concentration range can be quantitative if compounds are present which are supposed to form insoluble salts, the precipitation was repeated with an extract from an anoxic insect muscle which is rich in glycerophosphate; there was some indication of similarity of the stable compound from the *Ascaris* to the glycerophosphate. The results were entirely different: from the extract of the final concentration corresponding to 16 mg fresh tissue/ml (total phosphate 47.5 μmoles/g, fresh weight, stable phosphate 23.7 μmoles/g,) 98 per cent of the total phosphate was precipitated and only 2 per cent (corresponding to 4 per cent of the stable phosphate) remained in the supernatant.

Thus it seems to be proved that the bulk of the acid-stable fraction of the phosphorus compounds in the *Ascaris* muscle is formed by some compounds yielding rather alcohol-soluble barium salts. In the further identification of these compounds this fact made it possible to continue in the work with a supernatant deprived of almost all other phosphorus compounds.

3. *Paper ionophoresis and chromatography*

To obtain further information about the acid-stable fraction, barium acetate and alcohol were removed from the supernatant and the concentrated neutralised solution subjected to paper ionophoresis. After 135 minutes of separation on paper we obtained a single spot with a mobility of 0.825 ± 0.01 with respect to the

inorganic phosphate. In control experiments, the mobility value of the β-glycerophosphate was 0.80±0.01; the value of the propanediol phosphate synthetised in the laboratory was 0.825. The latter values are in agreement with the value 0.81 and 0.83 respectively, given by WADE and MORGAN for the mobilities in the butyrate buffer of the same pH. These results indicated the most probable presence of a single compound in the supernatant. Its stability, high solubility of its barium salt in alcohol and mobility in paper ionophoresis suggested that it might be propanediol phosphate.

In earlier papers this compound was designated as "barium-soluble — alcohol-soluble" (SACKS 1949, LE PAGE 1949). Later RUDNEY (1954) found that a considerable quantity of propanediol phosphate may be precipitated by barium and alcohol. The difference may probably be ascribed to the different concentrations used by the authors. RUDNEY (1954) worked with the isotope dilution technique and added a considerable quantity of labelled propanediol phosphate to the extract thus increasing its concentration several times. It may be assumed that by exceeding the solubility of the barium salt he obtained the compound in the precipitate. As shown in Table 2, the stable compound from the *Ascaris* has a similar behaviour: it is partly precipitated from more concentrate solutions, but in diluted extracts it remains in the supernatant. The mobility of the compound in ionophoresis also suggests its identity with propanediol phosphate.

The attempt to confirm the identity of the extracted compound with propanediol phosphate by means of paper chromatography led to equivocal results. For chromatography, material eluted from ionophoretic spots was used; cations were removed by treatment with Dowex 50 and salicylic acid extracted with ether. From all chromatographic systems tried, a satisfactory separation of the compound from inorganic phosphate was only achieved by using the propanol-ammonia-water mixture of HANES and ISHERWOOD (1949). In this system the extract and synthetic propanediol phosphate formed the principal spots of different R_F with respect to the inorganic phosphate (1.28 and 1.57 respectively). Sometimes a weak spot appeared in the synthetic compound the R_F of which was very near to that of the main spot of the extract; as the synthetic procedure used should lead to a mixture of two isomers (propanediol-1 and 2-phosphates), this second spot might be due to the presence of propanediol-2-phosphate which should appear in much smaller amounts than the second isomer (RUDNEY 1954). According to these results it seems improbable that the extracted compound is identical with the main component of the synthetic product, propanediol-1-phosphate; the identity with the other isomer still remains an open question.

4. *Treatment with periodic acid*

According to BURMASTER (1946) treatment with periodic acid leads to the decomposition of the phosphates containing neighbouring free hydroxyl groups, e.g. glycerophosphate. Propanediol phosphate should not decompose in these conditions. This was confirmed by experiments (Table 4). In the same test the stable compound

from the *Ascaris* is readily hydrolysed to phosphate. This is a further evidence that the stable compound from the *Ascaris* muscle is not propanediol-1-phosphate.

Table 4. Liberation of phosphate from glycerophosphate, propanediol phosphate and the stable compounds from the *Ascaris* muscle by boiling with periodic acid (Burmaster 1946)

Substance	Total P content μ moles	Duration of treatment (minutes)	Phosphate liberated		
			μ moles	Per cent of total phosphate	Without periodic acid (heated with H_2SO_4 only) per cent
Glycerophosphate (20 % α – and 80 % β-glycerophosphate)	0.299	15	0.286	96.0	
	0.299	30	0.275	92.0	–
	0.299	60	0.284	95.0	25
	0.516	90	0.531	103.0	–
Propanediol phosphate*)	0.301	60	0.026	8.5	9.1
	0.382	90	0.029	8.7	8.7
Stable compounds from Ascaris. Supernant after barium precipitation	0.206	15	0.206	100.0	–
	0.206	30	0.190	92.0	–
	0.206	60	0.210	102.0	
	0.344	90	–	–	6.8
Eluted from ionophorogram	0.194	60	0.194	100.0	1.0
Stable compounds from the whole extract ($P_o - P_{180}$)	0.292	60	0.274	94.0	–
	0.292	240	0.279	95.0	–
	0.202	60	0.190	94.0	12.4

*) Synthetic product composed mostly of propane-1,2-diol-1-phosphate.

5. *Stability to acid hydrolysis.*

The substance eluted from the ionophoretic spot was heated with 5 N sulphuric acid in a boiling water-bath and the time course of the hydrolysis was followed (Fig. 1). The compound was found to be very stable: after 16 hours only 25 per cent of it were hydrolysed.

DISCUSSION

Although the stable compound occurring in an unusual quantity in the muscle of the *Ascaris* could not yet be indentified with any of the known major constituents of animal tissue, the results presented offer a certain information on its nature. With its high resistance to acid hydrolysis it resembles typical ester phosphates. The electrophoretic mobility in acidic pH places it into the group of C_3 compounds

and makes the presence of a positively charged group in the molecule highly improbable. With respect to a rather high solubility of its barium salt in alcohol the substance seems to be more similar to the compounds with only one aditional polar group in the molecule, e.g. propanediol phosphate or phosphorylethanolamine (CARDINI, LELOIR 1957). From the latter compound, however, it differs in the electrophoretic mobility, from propanediol-l-phosphate in its lability in periodic acid test. The possibility, however, remains that it may be propanediol-2-phosphate. It would be premature to speculate on the possible physiological meaning of the unusual accumulation of stable phosphorus compounds in the *Ascaris* muscle.

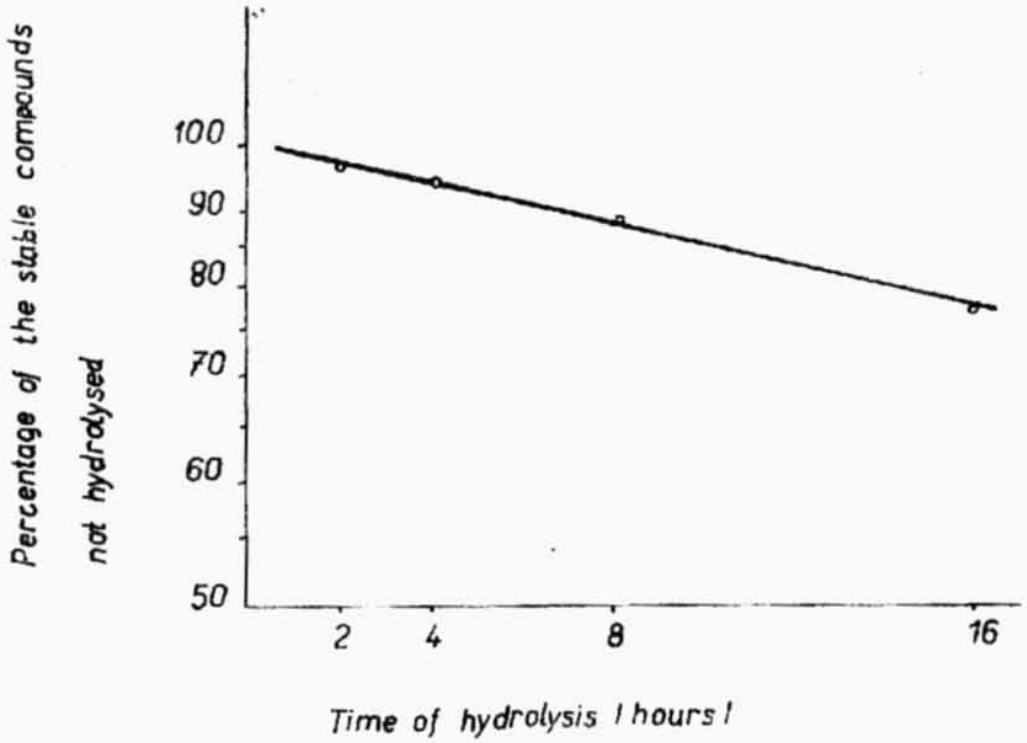

Fig. 1. Hydrolysis in 5N HSO_4 of the stable compounds (eluted from the ionophoretic spot)

One conspicuous fact, however, deserves to bee pointed out: the unstable (energy rich) phosphates are present in very low quantities, the total phosphate content, however, reaches quite a respectable value. It seems as if the stable compounds were compensating for the lack of phosphagen and the low adenosine triphosphate content. As phosphorus compounds play also an important role as impermeant anions in maintaining the osmotic balance of the cell, such a substitution may be substantiated. Further work for the final identification of the compound is in progress; it is hoped that it might contribute to the better understanding of the metabolic specifities of these parasitic helminths.

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