THE PHOSPHAGEN OF PROTOZOA

by

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It is generally assumed that phosphocreatine serves as the phosphagen of vertebrate tissue and is replaced by arginine phosphate in invertebrate tissue. BALDWIN AND YUDKIN[1] however, have found an apparently new arginine-like phosphate to be present in some annelid worms. This compound (hereafter referred to as annelid phosphagen) although resembling arginine phosphate is not identical with it. It therefore appeared of interest to attempt to characterize the phosphagen of Protozoa; especially since the only previous investigation with members of this phylum (NEEDHAM, ROBERTSON, NEEDHAM AND BALDWIN[2]) failed to demonstrate a phosphagen of any type.

METHODS

The typical free living ciliated protozoan, *Tetrahymena geleii S*, was cultured in two liter Erlenmeyer flasks containing one liter of 1% Difco proteose-peptone plus 0.2% Difco yeast extract. After 72 hours growth at 24–27° C the cells were harvested, washed, and concentrated as described previously (SEAMAN[3]).

Two volumes of ice-cold 10% trichloroacetic acid were added to the cell concentrate and the mixture was homogenized in a glass grinder. A small amount of powdered quartz was added to aid in the grinding procedure. The mixture was diluted to 5.0 ml with trichloroacetic acid, the protein removed by centrifugation at 0° C, and the preformed inorganic phosphorus was removed by the addition of 1/10 volume of ice-cold $Ca(OH)_2$ — $CaCl_2$ mixture (pH 8.3) to the neutralized supernatant.

Inorganic phosphorus was determined by the method of FISKE AND SUBBAROW[4]. Arginine was estimated by the ninhydrin method as described by PEREIRA AND SERRA[5] following ionophoretic separation on filter paper strips (DURRUM[6]).

RESULTS

Preliminary experiments indicated that in the trichloroacetic acid filtrate there was no rapidly hydrolyzable compound resembling phosphocreatine. However, when the filtrate was acidified with H_2SO_4 to a final concentration of 0.5 *N* and incubated at 37° C for 18 hours, the liberation of 0.106 mg of inorganic phosphate/g of dry weight of cells was observed. This indicates that the phosphagen present in *Tetrahymena* is, as would be expected, a slowly hydrolyzable ester, as is arginine phosphate and the annelid phosphagen.

As had been pointed out (BALDWIN AND YUDKIN[1]), the mere demonstration of the presence of arginine in the trichloroacetic acid filtrate is not evidence that the phosphagen in question is actually arginine phosphate. For example, in the present investigation it was to be anticipated that arginine could be recovered from the extract since

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the amino acid is a growth requirement of the organism (ELLIOTT[7] and KIDDER AND DEWEY[8]) and in addition, is metabolically active in the synthesis of urea by *T. geleii S* (SEAMAN[9]). However, if the protozoan phosphagen is arginine phosphate, the molar concentration of arginine after acid hydrolysis of the filtrate must be at least equal to the concentration of phosphorus liberated. This is not the case; the arginine content of the hydrolysate does not approach the phosphorus value (Table I).

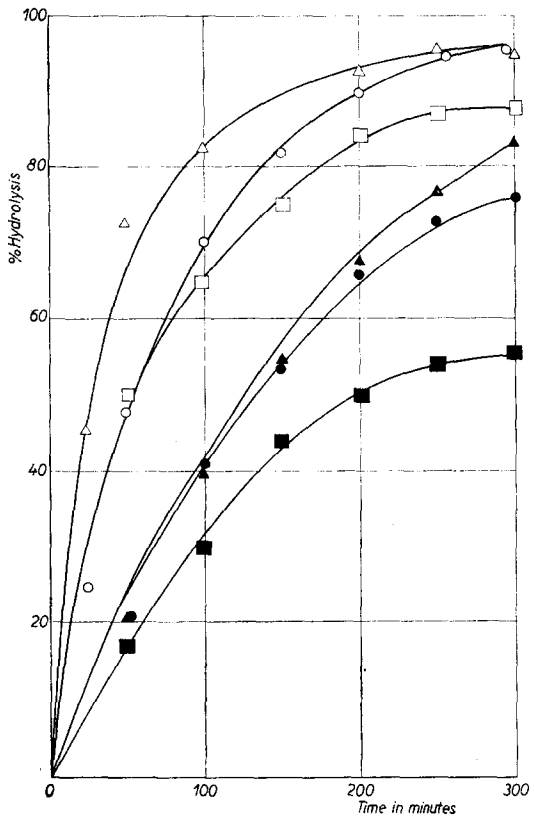

Fig. 1. Influence of molybdate on hydrolysis of invertebrate phosphogens. Molybdate concentration, when present, 0.25%; sulfuric acid concentration 0.5 *N* in all cases. Temperature 37° C. Each value is the mean of at least 7 determinations.

△ Arginine phosphate minus molybdate
▲ Arginine phosphate plus molybdate
○ Annelid phosphagen minus molybdate
● Annelid phosphagen plus molybdate
□ Protozoan phosphagen minus molybdate
■ Protozoan phosphagen plus molybdate

TABLE I

ACID LABILE P AND TOTAL ARGININE IN TRICHLOROACETIC ACID EXTRACTS OF *Tetrahymena* HYDROLYSIS CARRIED OUT IN 0.5 *N* H_2SO_4 FOR 18 HOURS AT 37° C. EACH VALUE IS THE MEAN OF AT LEAST DUPLICATE DETERMINATIONS

Extract	*Dry weight of cells used*	*Labile P*	*Arginine*
$4a_1$	74	0.245	0.012
$4a_2$	51	0.182	0.009
$5b_1$	78	0.301	0.041
$9a_2$	53	0.216	0.011

Hydrolysis of both arginine phosphate and of the annelid phosphagen is retarded in the presence of molybdate (BALDWIN AND YUDKIN[1], MEYERHOF AND LOHMANN[10], and NEEDHAM, BALDWIN AND YUDKIN[11]); the degree of retardation is much less with annelid phosphagen than with arginine phosphate. The behavior of the phosphagen on hydrolysis in the presence and in the absence of molybdate may thus be used to characterize the compounds. Rates of hydrolysis of the phosphagen of *T. geleii* ir the presence and absence of molybdate were therefore determined and the resulting data compared with those obtained with extracts of the claw muscle of the crab, *Callinectes sp**, which contains arginine phosphate (ARNOLD AND LUCK[12]), and with extracts of the body wall of the marine worm, *Phascalosoma gouldii**, which contains annelid phosphagen (BALDWIN AND YUDKIN[1]). Fig. 1 shows that in both the persence and absence of molybdate the hydrolysis of the protozoan phosphagen proceeds at a slower rate than does the hydrolysis of either arginine phosphate or the annelid phosphagen.

* Living crabs were obtained from a local market and living *Phascalosoma* from the Supply Department, Marine Biological Laboratory, Woods Hole, Mass.

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DISCUSSION

The phosphagen of protozoans is similar to arginine phosphate and the annelid phosphagen in that it is a slowly hydrolyzable ester and that the rate of acid hydrolysis of the compound is inhibited by the presence of molybdate. However, since the concentration of arginine after hydrolysis does not approach the concentration of phosphorus liberated (Table I) it cannot be identical with arginine phosphate. Likewise, since hydrolysis curves, both in the presence and in the absence of molybdate, of the protozoan phosphagen are quite different from those of arginine phosphate and of annelid phosphagen, it is apparent that the protozoan phosphagen is not identical with either of these previously described phosphagens.

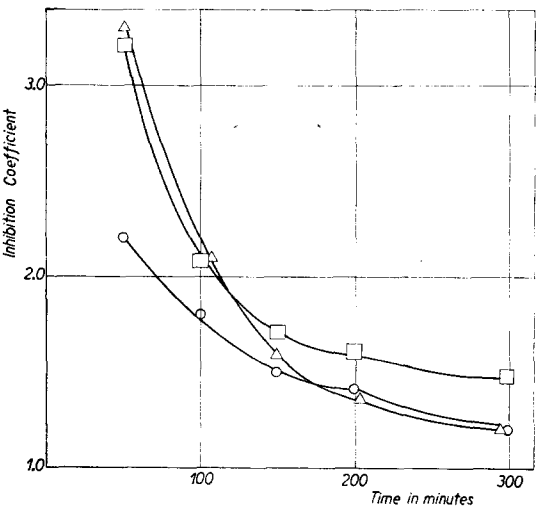

Fig. 2. Inhibition coefficients of molybdate effect on acid hydrolysis of invertebrate phosphogens in relation to time. Values calculated from data in Fig. 1.

Coefficient = $\frac{\%\text{ hydrolysis in absence of molybdate}}{\%\text{ hydrolysis in presence of molybdate}}$

□ Protozoan phosphagen
○ Annelid phosphagen
△ Arginine phosphate

The results shown in Fig. 1 seem to indicate that the inhibition effected by molybdate is quite different for each of the three phosphagens. The retardation factor* is 4.1, 3.4, and 2.4 for arginine phosphate, protozoan phosphagen, and annelid phosphagen, respectively. However, if the degree of inhibition is plotted as hydrolysis proceeds, quite a different situation exists. Fig. 2 shows that the inhibition coefficient of molybdate for the protozoan phosphagen is almost identical with that for arginine phosphate. But, as is indicated above, the protozoan phosphagen is not arginine phosphate. This, then, seems to indicate that chemically the protozoan phosphagen is probably very similar to, but not identical with, arginine phosphate.

SUMMARY

The phosphagen of Protozoa is not identical with creatine phosphate, arginine phosphate, or the annelid phosphagen.

Studies of molybdate inhibition of hydrolysis indicate that the new phosphagen is probably chemically more similar to arginine phosphate than to the other previously described phosphagens.

RÉSUMÉ

Le phosphagène des protozoaires n'est identique ni au phosphate de créatine, ni au phosphate d'arginine, ni au phosphagène des annélides.

Les études concernant l'inhibition par le molybdate de l'hydrolyse du nouveau phosphagène font penser que ce dernier ressemble probablement d'avantage au phosphate d'arginine qu'à tous les autres phosphagènes décrits précédemment.

* Ratio of time for 50% hydrolysis in the presence and in the absence of molybdate (MEYERHOF AND LOHMANN[10]).

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ZUSAMMENFASSUNG

Das Phosphagen der Protozoen ist weder mit Creatinphosphat, noch mit Argininphosphat, noch mit dem Phosphagen der Ringwürmer identisch.

Eine Untersuchung der Molybdat-Hemmung der Hydrolyse dieses neuen Phosphagens weist darauf hin, dass dieses wahrscheinlich dem Argininphosphat chemisch ähnlicher ist, als allen anderen bisher beschriebenen Phosphagenen.

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Short Communications and Preliminary Notes

CUPRIC ION INHIBITION OF ASCORBIC ACID OXIDASE

by

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In a study of the mechanism of activation of ascorbic acid oxidase (AAO) by thyroxine and related substances[1,2], it was observed that low concentrations of cyanide ion could also activate the AAO system. Higher concentrations of cyanide ion inhibited the enzyme as previously reported[3,4]. The relationship between the logarithm of the cyanide concentration and ascorbic acid oxidase activity is shown in Fig. 1. Maximum activation of the AAO** employed in these experiments occurs at $2\text{–}3 \cdot 10^{-5}$ *M* cyanide with virtually complete inhibition at $1 \cdot 10^{-3}$ *M* cyanide. The fact that cyanide concentrations below 10^{-4} *M* are required to show activation may explain why the activating effect of cyanide has not been noted earlier. The presence of extraneous protein which might activate AAO could also mask cyanide activation.

The activation by cyanide suggested the possibility of metal ion inactivation of AAO. That the enzyme is extremely sensitive to cupric ion is indicated in Fig. 2. Complete inactivation of approximately $2 \cdot 10^{-8}$ *M* AAO occurs with about $5 \cdot 10^{-7}$ *M* cupric ion. The residual activity is ap-

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** Ascorbic acid oxidase, Lot No. E521C, purchased from the Reheis Co., Berkeley Heights, New Jersey, had 0.24% Cu, 1000 Lovett-Janison units/mg; this preparation is estimated to be approximately 50% AAO based on the homogeneous preparation of DUNN AND DAWSON[6].