OXIDATIVE PHOSPHORYLATION IN INFUSORIA

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The present investigation was undertaken for the purpose of studying the influence of the phytoncides of garlic on coupled oxidative phosphorylation in infusoria; however, the actual process of phosphorylation in infusoria has been little studied. Assimilation of orthophosphate and synthesis of adenosinetriphosphate (ATP) has not been traced; moreover, there is in general no information on the presence of the latter in bodies of infusoria. This communication is devoted to elucidation of these questions.

Method of Investigation

Glaucoma scintillans served as the test object. They were cultivated on hay infusion with the addition of Krebs-Ringer phosphate in the ratio of 1:10. In the experiment, cultures were not taken before 2 weeks, and not after 2 months. Before the experiment, the infusoria were washed free from the culture fluid and bacteria by means of repeated centrifugation in a hand centrifuge with water or Krebs-Ringer phosphate, diluted 10 times. As will be shown later, we succeeded in establishing in the infusoria bodies the presence of adenosinetriphosphate. It was quantitatively determined in the suspensions of the infusoria by determining the acid-labile phosphorus (P^{16}) in the mercury precipitates of the nucleotides. The phosphates were determined by the colorimetric method according to Fiske-Subbaroy. We estimated the sythesis of adenosinetriphosphate by the inclusion in it of the orthophosphate fraction, marked by the radioactive phosphorus (P^{32}). In order to measure the degree of inclusion of P^{32}, the specific activity of the adenosinetriphosphate fraction was determined. For this purpose, the latter was separated from the trichloroacetic extract of the infusoria suspensions in the form of preparations freed from the orthophosphate mixture, according to the adsorption method worked out in the Department of Biochemistry of our Institute by S. A. Neifakh and E. P. Zdrodovska. The calculation was made in impulses per minute per 1 g of phosphorus. The P^{32} was added in an amount calculated so that the activity of the samples upon incubation equalled 2000-3000 imp/min/mL. The activity of the samples was calculated with a Geiger-Müller counter in conditions of 12-14 imp/min.

EXPERIMENTAL RESULTS

On addition to the neutralized trichloroacetic extract (pH 8.2, phenolphthalein as indicator) from the infusoria suspension of a 20% mercury acetate solution in the cold, a precipitate of nucleotides was obtained.

The residue was subjected to hydrolysis in a 1N HCl solution, on a boiling water bath for 5, 10, 30 and 90 minutes; the orthophosphate content was determined in the solution. The findings obtained show that complete mineralization of the phosphorus of the nucleotides took place during a 10 minute hydrolysis. The hydrolysis curve for the mercurial residue had the same character as the hydrolysis curve for the barium salt formed from adenosinetriphosphate [1]. This served as a first indication of the identity of the detected nucleotides and the adenosinetriphosphate.

The content of the nucleotides amounted to 290 mg% of the inorganic phosphorus to the dry weight of the infusoria (Fig. 1).

For further identification it was necessary to clarify whether the adenine nucleotide found by us was subject to metabolic transformations, i. e., whether a rapid renewal of the phosphate groups takes place within it.

At first, the experiments were conducted with introduction of P^{32} into the ATP of the infusoria in Warburg vessels. The infusoria were incubated in Krebs-phosphate, diluted 10 times, at 21°C for 2 hours. In such conditions absorption of oxygen occurred, but absorption of P^{32} was not observed. As a result, the method was changed in order to approximate the conditions of the experiment to the conditions of the normal existence of infusoria.

To the beaker with the hay infusion was added P^{32} in such a concentration that the final activity amounted to 3000 imp/min/ml. Some time later, on the surface of the liquid there formed a bacterial layer (the bacteria which formed in it equalled the content of P^{32} in the medium, and thus were "marked"). After this, the infusoria were introduced into the beaker. Within 24 hours the infusoria were extracted from the beaker, washed free of bacteria, and treated in the cold with trichloroacetic acid. The adenine nucleotides, passing into the trichloroacetic extract, were separated by the absorption method (see above) in the form of preparations freed from the orthophosphate mixture, after which their specific radioactivity was determined: in addition the content and radioactivity of mineral phosphate in the culture liquid was determined.

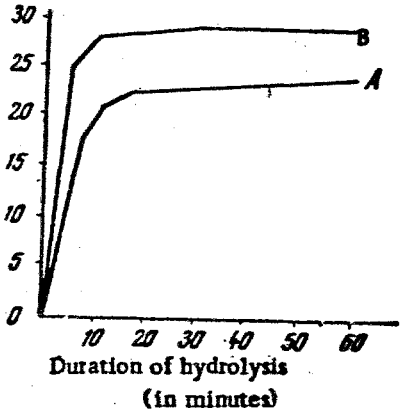

Fig. 1. Curves of hydrolysis of the salts of the nucleotides.
A) Barium salts formed from adenosinetriphosphate (phosphorus content expressed in Gammas per 0.1 g brain tissue; B) Mercurial salt of the nucleotide found by us (phosphorus content expressed in Gammas per 0.1 g dried weight of infusoria). Phosphorus content in Gammas along vertical axis.

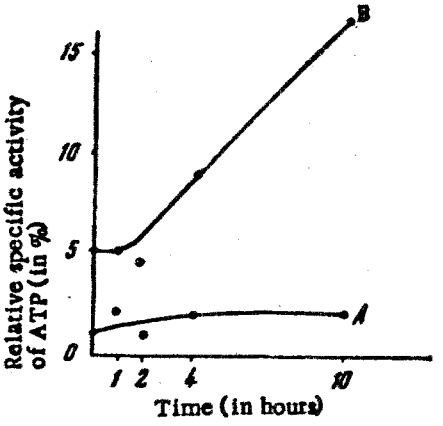

Fig. 2. Speed of renewal of adenosinetriphosphate in the infusoria upon incubation in the presence and absence of food.
A) Radioactivity of the adenosinetriphosphate (ATP) fraction in the infusoria, incubated in a sterile hay infusion containing P^{32}; B) The same in the infusoria, incubated together with "marked" bacteria.

Analysis showed that the specific activity of the adenine nucleotides was 30 imp/mg P, the orthophosphate content in the culture liquid 58 mg/ml and its specific activity 33 impulses per 1 μg of phosphorus. Thus, the relative specific activity of the adenine nucleotide was equal to 90.9%, i.e., during 24 hours in the infusoria, there took place an almost complete renewal of its labile phosphate groups. This observation led to identification of the adenine nucleotide found in the bodies of the infusoria as an adenosinetriphosphate.

In this connection there arises the question as to the reason for the failure of the attempts to record incorporation of P^{32} in the adenosinetriphosphate of the infusoria in the first series of experiments — was it only by the transience of the incubation or also by reason of the fact that the infusoria were incapable of directly assimilating mineral phosphate from the medium, and that assimilation of the phosphate takes place through the nutrient consumed by infusoria (bacterial bodies)? It was necessary to trace the process of inclusion of P^{32} in the adenosinetriphosphate of the infusoria in shorter incubation periods, and to compare the intensity of this process upon incubation of the infusoria in the presence and absence of food.

We prepared a sterile hay infusion with the addition of P^{32}. Bacteria were planted in one section of the infusion (wild flora, isolated from the beaker), leaving a sterile second section. Both parts of the infusion were placed in a thermostat for 24 hours at 28°C, after which the infusion was poured into 50 ml conical flasks; one series of 5 flasks each had 8 ml of the sterile infusion; the second series of 5 flasks each had 8 ml of the infusion containing the marked bacteria. In each flask, was added 2 ml of a thick suspension of the infusoria, so calculated that to each sample there were 300,000 protozoan bodies. The duration of the exposure was 0.1, 2, 4 and 10 hours. On expiration of the fixed time , corresponding samples were poured into the centrifuge tubes, and centrifuged at 30 g for one minute.* The precipitated liquid was poured off and the mineral phosphate content was determined as was its specific activity; in the precipitate the specific activity of the adenosinetriphosphate was determined. The results are presented in Figure 2.

As is apparent from Figure 2, absorption of P^{32} in the adenosinetriphosphate of the infusoria took place only in the presence of nutrients and some time (2-3 hours) passed before the ingested** P^{32} was included in the adenosinetriphosphate.***

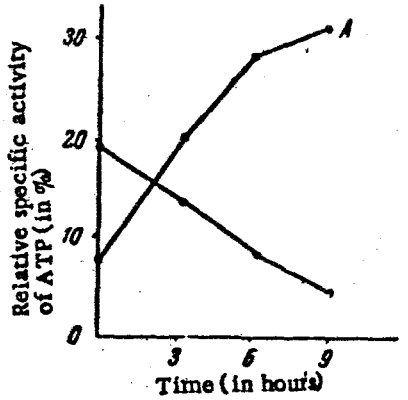

Fig. 3. Speed of renewal of adenosinetriphosphate (ATP) in samples containing infusoria and bacteria. A) Radioactivity of ATP of fraction infusoria and bacteria; B) Radioactivity of ATP of fraction bacteria.

The findings presented, it would seem, give convincing proof of the renewal of the phosphate groupings in the adenosinetriphosphate in the infusoria. However, the objection may be raised that the increase in the activity of the adenosinetriphosphate is caused by the increase in the activity of ATP in the bacteria precipitating together with the infusoria.

In order to demonstrate conclusively the law-governed growth of the activity of adenosinetriphosphorate in the infusoria, a further series of experiments were arranged in which we endeavored to compare the changes in the specific activity of the samples containing the infusoria and bacteria, with the changes in the specific activity of the samples containing only bacteria.

In arranging the experiments the following changes were introduced: 1) together with the samples containing the infusoria and bacteria, samples were arranged containing only bacteria; 2) centrifugation after incubation was so calculated that all the bacteria were precipitated; 3) the incubation of the infusoria with "marked" bacteria was conducted not in a hay infusion, but in water, for which purpose the infusoria were cleansed from the culture liquid with water the previous night and left for the night in the form of a not too thick suspension. Before the experiment this suspension was somewhat thickened. The bacteria directly before the experiment were precipitated from the hay infusion by centrifugation and introduced in the infusoria samples in the form of a thick suspension. Thus, the infusoria were in that medium to which they had already adapted themselves and the bacteria were in a more dilute solution.

We noted a fall (in the course of incubation) of the specific radioactivity of the adenosinetriphosphate of the samples containing only bacteria with a rise in the specific radioactivity of the samples containing the infusoria and bacteria.

The results of a typical experiment are given in Figure 3.

These findings indisputably testify to the growth of the relative specific activity of adenosinetriphosphate in the infusoria.

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* Such a small unit (g) and short period of centrifugation were used in order to precipitate as far as possible, all the infusoria, and as few bacteria as possible; however, with this, a certain proportion of the bacteria were nevertheless precipitated.

** As our observations showed, ingestion of the bacteria began immediately. We judged this upon vital microscopy and by the growth of radioactivity of the trichloroacetic acid residue.

*** After carrying out these experiments, we became acquainted with the work of Evans and Pendleton [2], in which the authors reached the conclusion that P^{32} enters the body of the infusoria only in the presence of food.

Thus, the presence of adenosinetriphosphate was established by its in the bodies of the infusoria and the path of renewal of its labile phosphate groupings traced; we also showed that the phosphate from the external medium enters the adenosinetriphosphorate of the infusoria only in the presence of microbial food.

I should like to thank S. A. Neifakh for valuable guidance in the carrying out of this work.

LITERATURE CITED

[1] Gromova, K. G., Metabolism of Phosphoric Compounds in the Brain in Cerebral Anemia, Author's abstract dissertation* (1954).

[2] Evans, F. R., and Pendleton, R. C., Biol. Bull. Vol. 103, 2, pp. 190-194 (1952).

* In Russian.