PHOSPHOPROTEIN PHOSPHATASE, A NEW ENZYME FROM THE FROG EGG

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In the nutrition of the young, the rôle of phosphoproteins is, without doubt, an important one. As casein in milk, or as the yellow yolk of many vertebrate and invertebrate eggs, phosphoprotein occurs in substantial quantities. In the frog egg, for example, the yolk platelets occupy almost half the volume of the cell. According to McClendon (5), the yolk of the frog egg consists of phosphoprotein bound to lipide; thus the platelets are a ready source of protein, lipide, and phosphorus. And yet, in spite of the obvious importance of phosphoprotein, little information is available about the manner of its utilization by the developing embryo. It has been assumed that ordinary proteolytic enzymes degrade the protein, and that ordinary phosphomonoesterases split the phosphate from phosphopeptone or from phosphoserine.

In the course of an investigation of the distribution of enzymes in the frog egg, a new enzyme (or enzymes) was discovered which is apparently capable of splitting inorganic phosphate from the intact protein molecule. It is the purpose of this paper to present the experiments which led to this conclusion.

EXPERIMENTAL

Methods—Ovarian eggs of the common leopard-frog, *Rana pipiens*, or, in a few cases, those of the bullfrog, *Rana catesbiana*, were used. The ovaries were removed, soaked for half an hour or more in 0.1 M sodium citrate, blotted rapidly on filter paper, and weighed to the nearest cg. They were then suspended in fresh citrate solution[1] and ground with a homogenizer patterned after that of Potter and Elvehjem (6) but modified in that the

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[1] For the general purpose of the investigation sodium citrate appeared to be the best suspension medium for the homogenate, since the cytoplasmic granules of the egg protoplasm appeared microscopically to be in good condition, and these granules could be isolated in an essentially quantitative manner by differential centrifuging. Brei prepared in sodium chloride or potassium chloride was apparently not homogeneous and could not be fractionated by differential centrifuging in a satisfactory manner.

rotating pestle was smooth and fitted loosely in the surrounding test-tube. This modification was found to be particularly suitable for homogenizing frog ovary, since the large, fully developed eggs are readily ruptured, while the connective tissue sheet and the small cells embedded in it remain intact. This sheet becomes wrapped about the stem of the pestle, is later removed, and its weight subtracted from that of the whole ovary.

In most of the experiments reported here, the homogenate was incubated in citrate buffer for a suitable time and then treated with trichloroacetic acid to precipitate proteins. Unfortunately, certain substances of the frog

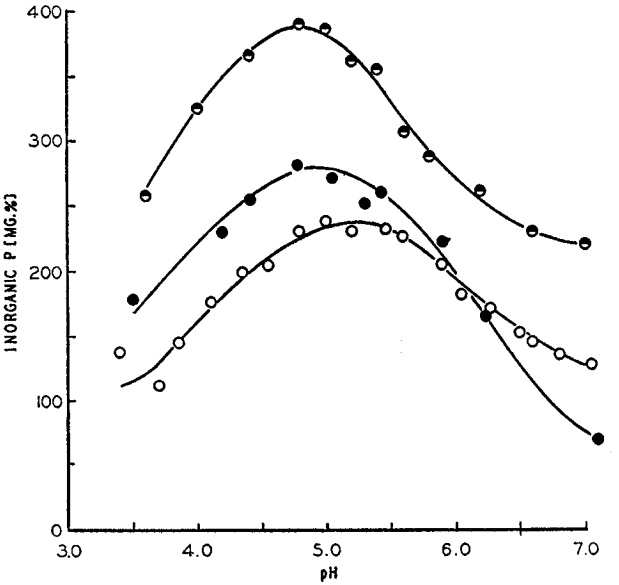

FIG. 1. Inorganic phosphate in autolyzed ovarian homogenate. Half circles, *Rana pipiens*, 60 minutes, 37°; solid circles, *Rana pipiens*, 5 minutes, 23°; open circles, *Rana catesbiana*, 60 minutes, 37°.

egg are not precipitated readily, and to obtain a water-clear filtrate it was often necessary to allow precipitation to continue overnight before filtering or centrifuging. Undesirable as this procedure may be, no way of avoiding it was found.

Phosphate was determined according to the method of Fiske and Subbarow (2). Although citrate may, in sufficient concentration, delay the development of color in this method, no difficulties were experienced in the experiments reported here. Phosphate is expressed as mg. of phosphorus per 100 gm. of wet weight.

General Characteristics of Reaction—In an attempt to demonstrate in

the frog egg homogenate a phosphomonoesterase hydrolyzing sodium β-glycerophosphate, extremely high values of inorganic phosphate were consistently found in the brei incubated without added substrate as a control. As much as 500 mg. per cent of inorganic phosphate were found after an incubation period of 1 hour. Moreover, there was a close dependence upon pH, maximum phosphate being found in the neighborhood of pH 5.0. Fig. 1 illustrates typical data obtained in three independent experiments. The high value of phosphorus shown in the uppermost

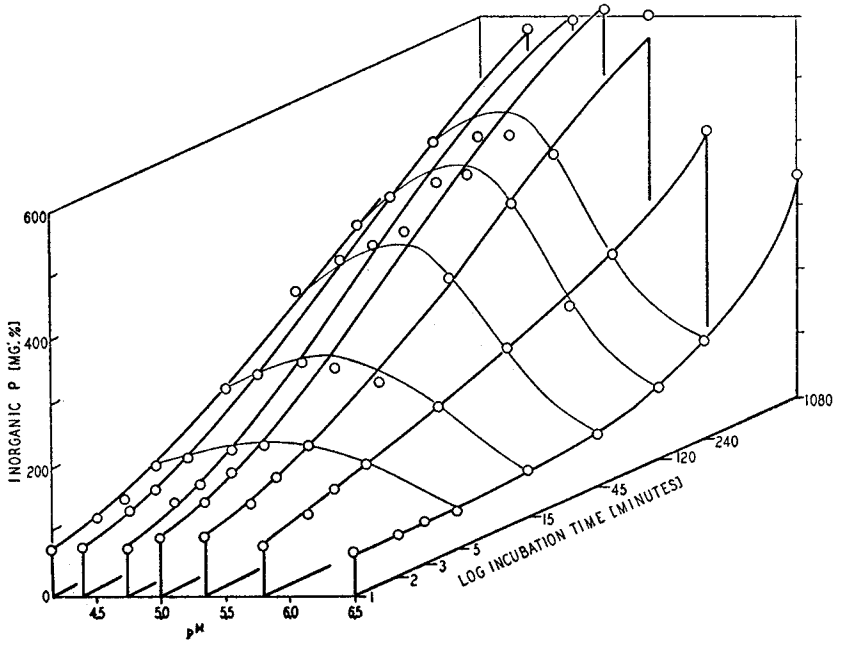

FIG. 2. Liberation of inorganic phosphate in autolyzing ovarian homogenate. A logarithmic time scale is used for convenience; the curves are empirical.

curve at pH 7.0 may be erroneous, since the reaction is very sensitive to pH in this region and pH was calculated rather than determined in this experiment. There is virtually no hydrolysis in alkaline solutions. Since all of these experiments were carried out on the crude autolyzing brei, considerable variability is to be expected, but the general characteristics are reproducible.

Fig. 2 illustrates a more complete experiment, in which the course of the reaction was followed for a considerable period of time at the different pH values indicated. For convenience a logarithmic time scale is used, since after the first 15 to 20 minutes the reaction proceeds very slowly.

These data strongly suggest that some naturally occurring phosphorus

compound, presumably an ester, is hydrolyzed. That this is an enzymatic hydrolysis rather than a simple acid hydrolysis is indicated by the dependence upon pH, the fact that the reaction is stopped by the addition of trichloroacetic acid, and by the great speed of the reaction. Indeed, as Fig. 1 demonstrates, as much as 250 mg. per cent of inorganic phosphorus can be liberated in 5 minutes at room temperature. It has also been shown that the reaction can be prevented by heating the brei.

Nature of Substrate—Assuming the reaction to be an enzymatic hydrolysis of a naturally occurring phosphate ester, attempts were made to identify the substrate. No significant changes could be demonstrated in the acid-soluble esters hydrolyzable by 1 N hydrochloric acid. A general fractionation of the brei was therefore made as follows. Aliquots of the autolyzing brei were pipetted into trichloroacetic acid at suitable time intervals. Inorganic phosphorus was determined and, after ashing with perchloric acid, total acid-soluble phosphorus was determined. The difference between these values was taken to be organic acid-soluble phosphorus. Since the trichloroacetic acid filtrate was perfectly clear, it was assumed that all the lipide was carried down with the precipitated protein.[2] The precipitate was extracted two or three times with hot alcohol-ether, and the residue considered to be protein. After all traces of alcohol and ether were driven off on a steam bath, the lipide and protein fractions were ashed with perchloric acid and phosphorus was determined.

A representative experiment carried out at pH 5.0 is shown in Fig. 3. The obvious rise in inorganic phosphorus is completely accounted for by the fall in protein phosphorus, while there is no change in the lipide fraction, and but a slight increase in the organic acid-soluble phosphorus. This increase has not been extensively investigated. There seems little reason to doubt that protein phosphorus is the source of the inorganic phosphate released in the autolyzing brei. It will be seen from Fig. 3 that part of the protein phosphorus is resistant to hydrolysis. The interpretation of this will be discussed below.

There are in the frog egg two proteins which contain substantial amounts of phosphorus, nucleoprotein, and phosphoprotein. Either might be the substrate for this enzyme. To distinguish between these two, whole brei was incubated alone, with added casein, or with added ribose nucleic acid. Fig. 4 shows the phosphorus liberated in 30 minutes from the added substrates as well as from the natural substrate present in the autolyzing brei. A full interpretation of these data cannot be given at present, but

[2]This assumption is doubtless correct, since a good deal of lipide is bound to protein and thus would be precipitated directly. The free fat droplets appear to be covered by a film of protein, and might be precipitated directly, but would in any case be carried down mechanically with the bulky protein precipitate.

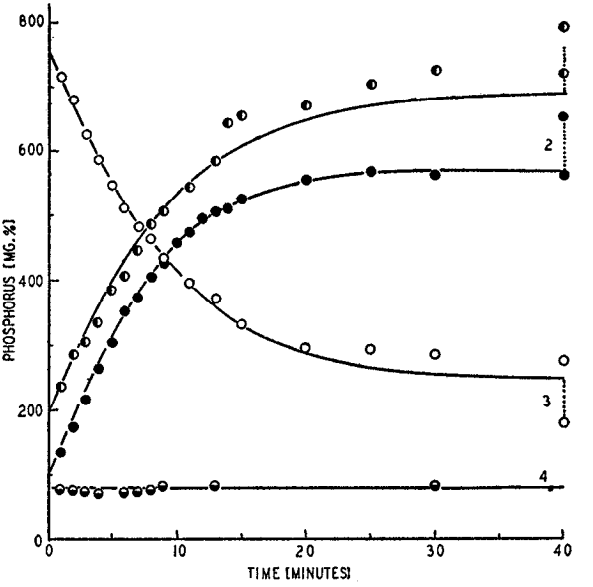

FIG. 3. Change in distribution of phosphorus in homogenate incubated at 23°, pH 5.0. Curve 1, total acid-soluble phosphorus; Curve 2, inorganic phosphorus; Curve 3, protein phosphorus; Curve 4, lipoid phosphorus. The points at the end of the dotted lines are the values at 24 hours. The curves are empirical.

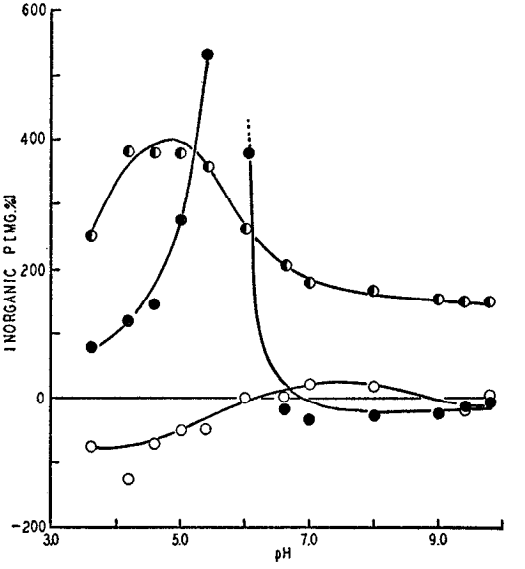

FIG. 4. Effect of the enzyme on various substrates. Half circles, natural substrate; solid circles, casein; open circles, yeast nucleic acid. Incubation time, 30 minutes; the curves are empirical.

there are several obvious features. The autolysis of the naturally occurring substrate is well illustrated but the high level of inorganic phosphate obtained from pH 7.0 to 10.0 is probably an artifact due to the rapid hydrolysis occurring during homogenization. Obviously there is a tremendous increase in inorganic phosphate when casein is added as a substrate, and the highest point on the curve may not be shown, since the reaction was not carried out at close enough intervals near the pH optimum. The general form of the casein curve seems essentially that of the autolytic curve, although the optimum is somewhat shifted, possibly due to the relative insolubility of casein at pH 5.0. Clearly, casein can serve as a substrate for this enzyme, which may therefore be called a phosphoprotein phosphatase. This observation has been repeatedly confirmed. Casein has been used routinely as a substrate with absolutely consistent results. The natural substrate in the egg is therefore phosphoprotein and this is present in large amounts in the yolk platelets.

Nucleic acid is evidently not hydrolyzed; as a matter of fact, less phosphate seems to be liberated from the yolk when nucleic acid is present. Presumably, the unhydrolyzable protein phosphate noted in Fig. 3 is largely nucleoprotein phosphate.

Localization of Enzyme—Since the work thus far reported was part of a general study of the distribution of enzymes within the frog egg, a few preliminary experiments were carried out to see whether the enzyme was bound to the yolk platelets, obviously the natural substrate. A typical experiment will illustrate the results obtained. Whole brei was centrifuged and the yolk-free brei was decanted from the sedimented yolk. The yolk was then washed three times by centrifugation in 10 volumes of fresh sodium citrate, so that any enzyme not bound to the yolk particles would be washed away. Finally the yolk was resuspended in citrate solution. Samples of the yolk suspension and of the yolk-free brei were set aside and the balance of each heated to 80° for 30 minutes to inactivate the enzyme. Aliquots of the heated and unheated yolk and yolk-free brei were then incubated at 37° for 30 minutes at pH 5.0 in the presence of the following substrates: none, heated yolk suspension, vitellin prepared from hen's eggs, according to the method of Calvery and White (1), sodium β-glycerophosphate, and disodium phenyl phosphate. Table I shows the amount of phosphate liberated from the added substrate by the action of the enzyme. These figures were obtained by subtracting from the total inorganic phosphate found in the digest the inorganic phosphate found in the substrate solution after incubation, and the inorganic phosphate liberated by autolysis in the enzyme solution. The latter figure is given in the first column of Table I.

A number of conclusions are clearly indicated by these data. (1) The

activity of the enzyme is destroyed by heating. The deviations from zero when heated enzyme solutions were used are not significant. (2) The enzyme is bound in part to the yolk platelets. The alternative explanation, that the enzyme is adsorbed during the course of preparation, is not very probable. The dilution of the egg protoplasm which takes place during preparation of the brei and the washing of the yolk are conditions which would tend to remove the enzyme rather than cause it to be adsorbed. In the absence of kinetic data, it is not possible to determine how much enzyme remains adsorbed to the yolk particles. Furthermore, the amount eluted during the course of preparation is indeterminate, so that a qualitative demonstration of the physical association of the enzyme and substrate is all that is possible at the present time. (3) The enzyme attacks all three phosphoproteins tested with considerable vigor, but has relatively little activity on either glycerophosphate or phenyl phosphate.

TABLE I

Localization of Enzyme and Effect on Various Substrates

Enzyme source	P liberated (mg. per 100 gm.) in 30 minutes, 37°, pH 5.0					
	Substrate					
	None	Yolk (heated)	Casein	Vitellin	Glycero-phos-phate	Phenyl phos-phate
Yolk-free brei	45	83	359	108	0	14
Same (heated)	5	1	23	−11	0	−8
Yolk	86	94	219	99	3	−11
" (heated)	0	−5	29	−16	−7	−21

Kinetics—A number of experiments have provided data which are suitable for a preliminary analysis of the kinetics of the autolytic reaction. Chemically, one would expect a pseudomonomolecular reaction, since the reaction catalyzed is presumably a simple hydrolysis of serine phosphate, which according to Lipmann (4) is the only phosphorus compound present in casein. One may follow the disappearance of the substrate ($A - x$) directly, by analysis of protein phosphorus, with suitable correction for unhydrolyzable phosphorus, or indirectly through the appearance of inorganic phosphate (x). To the extent that organic acid-soluble phosphorus is formed from phosphoprotein, the results of these two methods will differ. However, the general results obtained by either approach are the same, as Fig. 5 demonstrates.

This figure is a plot of $\log A - x$ against time for four independent experiments. The solid circles represent data obtained by a direct analysis of protein phosphorus, while all the other experiments are based on the

appearance of inorganic phosphate. The reaction appears to follow monomolecular kinetics quite exactly for 15 to 20 minutes, but there is apparently a sudden break in the rate of the reaction which then proceeds more slowly than would be predicted by theory. Sufficient data about the later stages of the reaction are not available to justify any particular theoretical interpretation. Hence the straight line following the break should be regarded as empirical. However, considering the complicated physical conditions which prevail in the crude brei, with much of the substrate solid and part of the enzyme bound, it is surprising, indeed, that the reaction has predictable kinetics at all.

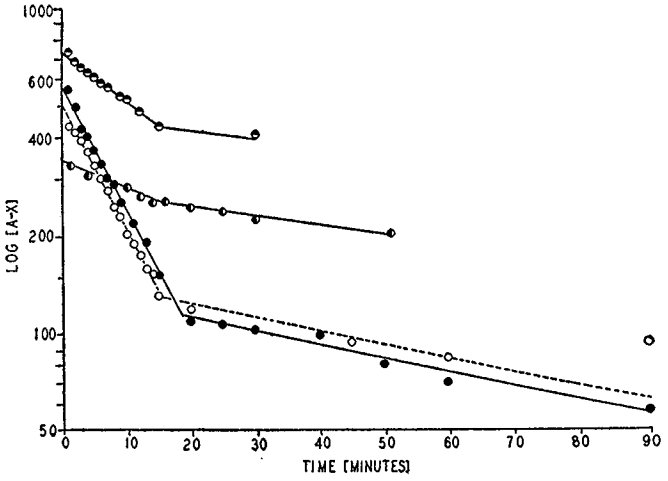

FIG. 5. Kinetics of the autolytic reaction. The solid circles are based on the direct analysis of protein phosphorus; other data on the appearance of inorganic phosphorus. The initial straight line is based on the equation; $kt = \log(A/A-x)$; the straight line following the break is empirical.

These kinetic results lend support to the idea, suggested by the speed of the reaction, that the hydrolysis of phosphorus from phosphoprotein is a direct one without the intervention of preliminary proteolytic activity. Direct support for this idea is provided by the fact that no increase in acid-soluble nitrogen has been detected.

DISCUSSION

The above data seem to justify the conclusion that the ovarian eggs of the leopard-frog contain an active enzyme which attacks phosphoprotein with the liberation of inorganic phosphate. The eggs of the bullfrog likewise contain this enzyme, but in a single experiment it was not demonstrated in the hen's egg. It is to be expected that the enzyme may occur

in other eggs of vertebrates and invertebrates which contain large amounts of phosphoprotein.

Detailed studies of the specificity of this enzyme are still needed, but the phosphatases described in the literature do not show the properties displayed by the frog egg enzyme. Most authors report that phosphatases have no action on casein. Kay (3) and Rimington and Kay (8) state that bone and kidney phosphatases do not split phosphate from casein, although the phosphopeptone resulting from tryptic hydrolysis is attacked by both enzymes. Taka-phosphoesterase causes weak hydrolysis of phosphopeptone at pH 5.6, according to Sadanitzu (9) and Sorimati (11). Travia and Veronese (12) report that an alkaline phosphatase from ox spleen does attack casein. Intestinal phosphatase has no action on casein, according to Schmidt and Thannhauser (10). Thus it would seem that most phosphatases have little or no action on casein. Certainly no phosphatase previously described attacks phosphoprotein more readily than glycerophosphate.

No exact study of the kinetics of this reaction or of the influence of cytological structure upon it can be made until the enzyme has been purified. It seems remarkable, however, that the enzyme is able to liberate phosphate with such speed when it is considered that two proteins are involved, and particularly when it is realized that much of the substrate is not in solution. The fact that the reaction appears to proceed in two stages is reminiscent of the observations of Rimington and Kay (8) and Rimington (7), who noted that only part of the phosphate could be split from phosphopeptone by bone phosphatase or alkali, whereas all could be released by kidney phosphatase.

This enzyme may prove to be a useful tool in studying the chemical and physical properties of phosphoproteins. Thus, information as to the mode of linkage of phosphorus in casein might be obtained. Studies on dephosphorized proteins might be facilitated by the specific dephosphorylation method in place of the alkali treatment heretofore used.

The autolytic reaction which occurs so dramatically when the frog egg is ruptured must somehow be held in check in the intact cell, for the eggs remain in a relatively static state throughout the entire winter. After fertilization of the egg, small quantities of phosphate must presumably be liberated gradually or from time to time to meet the metabolic needs of the embryo. The mechanism by which the enzyme is held in check or controlled is uncertain. A favorite explanation in such cases is that enzyme and substrate are not in contact. This seems not to be true here, for a considerable proportion of the enzyme appears to be bound directly to the yolk particles. Other possible interpretations include inhibitors within the cell, addition of activators on homogenization, or other altera-

tions in the physical and chemical conditions. At present, it would seem that the pH of the cell may be adverse, since near neutrality the activity of the enzyme is profoundly affected by small changes in pH. On fertilization perhaps enough acidity develops to activate the enzyme.

I am grateful for helpful discussions with the late Dr. Carl L. A. Schmidt and Dr. D. M. Greenberg, and for the cooperation of Miss Mary Cregar who assisted me in some of these experiments.

SUMMARY

1. A rapid, heat-labile autolytic reaction has been demonstrated in homogenates of ovarian frog eggs, resulting in the liberation of large amounts of inorganic phosphate.

2. This reaction occurs over a broad range in acid solutions with an optimum near pH 5.0.

3. There is a decrease in protein phosphorus which completely accounts for the increase in inorganic phosphate, while organic acid-soluble phosphorus and lipoid phosphorus remain essentially unchanged.

4. Phosphate is split readily from added casein, vitellin of hen's eggs, and yolk of frog eggs, while nucleic acid, glycerophosphate, and phenyl phosphate are attacked slowly, if at all.

5. It is concluded that the reaction is enzymatic and that the enzyme is best characterized as a phosphoprotein phosphatase. A survey of the literature indicates that an enzyme with this specificity has not previously been described.

6. Preliminary data on the kinetics of the reaction are presented.

7. Part of the enzyme seems to be bound directly to the yolk platelets which contain the natural substrate.

8. The rôle of the enzyme in metabolism is briefly discussed.

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